POLYOLS: METHOD DEVELOPMENT AND THEIR APPLICATION TO THE MANAGEEMENT OF DIABETES

by

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SUMMARY

An enzymatic and a novel capillary gas-liquid chromatographic assay have been developed and compared for the analysis of red blood cell sorbitol concentrations. Factors affecting the performance of both assays were investigated and the analytical conditions optimised. The enzyme assay was found to be accurate and precise but lacked specificity with at least xylitol also acting as a substrate. The capillary GLC assay was accurate and precise with the advantage of increased specificity for polyol determinations.

The enzyme assay was used to determine the response of erythrocyte sorbitol content in a clinical trial of diabetics treated with oral Sorbinil; a novel aldose reductase inhibitor. The sorbitol content was reduced and this trend was reversed upon cessation of Sorbinil treatment. The capillary GLC method was used to determine the daily effect of oral Sorbinil upon erythrocyte sorbitol and myo-inositol concentrations in a clinical trial with diabetic subjects. It was found that Sorbinil therapy reduced the raised erythrocyte sorbitol by 60% within the first 24 hours of treatment at a lower dose than used in previous studies. The erythrocyte myo-inositol content of diabetic subjects was not altered by Sorbinil therapy. However, it was found, for the first time, that the myo-inositol level in diabetics was found to be significantly different to healthy, normal non-diabetic subjects.

The daily variation in erythrocyte sorbitol and myo-inositol in normal non-diabetic subjects was investigated. The levels were found to be very consistent both for individuals and as a group and were significantly different to the levels found in diabetic subjects.
The activity of erythrocyte aldose reductase was found to be greater in diabetics compared to normals. Capillary GLC was used to confirm the erythrocyte accumulation of sorbitol upon incubation with glucose. The concentration of other components in the erythrocyte were seen to change upon glucose incubation. The increase of one component was initially identified by its retention as glucosamine. An attempt to correlate the erythrocyte aldose reductase activity with the degree of severity of subjects with diabetic neuropathy was unsuccessful. Further work is required to optimise the assay conditions.

It is concluded that the erythrocyte sorbitol levels can be used in conjunction with other tests and clinical observations to manage the long term complications associated with diabetes. In particular, the enzyme assay may be of use as a screening test. The capillary GLC assay will be of most use in further studies to determine the polyol and carbohydrate changes and their role in the onset of neuropathy.
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CHAPTER 1

INTRODUCTION

1.1 General introduction to polyols

Polyols are polyhydric alcohols with the general formula $C_nH_{2n+2}O_n$ which form an homologous series starting from the two carbon diol, 1,2-ethanediol (ethylene glycol) and increasing in molecular weight with ascending carbon number. Polyols are commonly referred to as alditols, acyclic polyols or sugar alcohols. They are further subdivided on the basis of the number of carbon atoms (or hydroxyl groups) contained in the straight chain molecule into tetritol, pentitol, hexitol (which contain 4, 5 and 6 carbon atoms respectively) and so on. Polyols are highly polar, generally water soluble, hygroscopic materials that exhibit moderate viscosity in high aqueous concentrations. In general, the melting and boiling points and viscous properties of polyols increase with ascending molecular weight. Different polyols have a sweet taste ranging from a half to three-quarters that of sugars. Polyols are chemically closely related to carbohydrates from which they are naturally and synthetically derived. There is also a close biochemical relationship between sugars and polyols which has led the latter to be classed as reduced sugars. Xylitol and sorbitol (D-glucitol) are examples of the reduction of their respective sugar counterparts xylose and glucose.
Polyols like sugars contain chiral carbon centres and in solution display optical activity in a plane of polarized light. All polyols from the tetratslols upward contain more than one chiral carbon centre and exist as diastereomers. They follow the rule that the number of theoretically possible stereoisomers is given by $2^n$; where $n$ is the number of chiral carbon atoms present in the molecule. Some polyols, for example erythritol, xylitol and galactitol (dulcitol) are meso compounds and although they contain chiral carbon centres, do not display specific optical rotation. The melting point and the specific optical rotation, are the main physical properties used to identify individual polyols.

Polyols contain two primary and secondary alcohol groups and their chemical reactions include; esterification, ether formation, oxidation, reduction and cyclic anhydride formation. Examples of each reaction are; ester formation with acid anhydrides in the presence of an acid or base catalyst. Methyl ether formation from dimethyl sulphate in the presence of sodium hydroxide base. Oxidation with nitric acid, of the primary alcohol groups, producing dicarboxylic aldonic acids. Reduction by periodic acid, of adjacent hydroxyl groups, to two shorter chain aldose sugars. The elimination of a molecule of water, on heating with dilute sulphuric acid, to produce a cyclic anhydride. Many of the above reactions have important uses in the determination of the structures of polyols and in industry.

1.2 **The natural occurrence of polyols in the plant kingdom**

The most abundant naturally occurring polyol in the plant kingdom is the hexitol, D-mannitol, which was the first polyol to be isolated from manna of the ash tree in 1806. D-mannitol is the primary photosynthetic product in brown algae and has since
been found to be widely distributed in many other plant species, for example; green algae, fungi and the higher plants. By contrast L-mannitol is not known to occur naturally. Historically, after mannitol perseitol was the next polyol isolated, from avocado in 1831, galactitol from melampyrum in 1837 and sorbitol from sorbus in 1872 (Wallaart, 1980).

The natural sources of the hexitol sorbitol (D-glucitol) in the plant kingdom include red algae, the berries of the mountain ash and the fruit of the rosaceae such as apples, apricots, pears, cherries, peaches and plums (Dwivedi, 1977). All four pentitols occur naturally with ribitol as the major pentitol in plants, based on actual cell content and because all organisms require ribitol incorporation into riboflavin (vitamin B2). Ribitol is also a major component of some bacterial cell walls (Brimacombe and Webber, 1972). The five carbon pentitol xylitol occurs naturally in fruits, vegetables, cereals, mushrooms, lichens and seaweed (Dwivedi, 1977). The other two pentitols are D- and L-arabitol (D- and L-arabinitol, D- and L-lyxitol). The D- form has been reported to occur in fungi in varying amounts according to the conditions for growth. There are also reports of both D- and L-arabitol occurrence in yeasts and of an L-arabitol specific enzyme in tobacco, pea and wheat indicating that L-arabitol may be present in these plants as a minor component (Bieleski, 1982).

Of the three possible tetritols two occur naturally, erythritol occurs in algae, lichens and grasses and D-threitol occurs in an edible fungi. The remaining tetritol L-threitol has not been reported to occur naturally (Brimacombe and Webber, 1972). There are three naturally occurring heptitols (seven carbon polyols) and they are known by their trivial names as perseitol, volemitol and β-sedoheptitol and the avocado appears to be the main source for the first two. The avocado is also the natural source of the only
known octitol; D-erythro-D-galacto-octitol. To date there are no known naturally occurring nonitols or decitols (Brimacombe and Webber, 1972, Bieleski, 1982).

There are two further classes of polyols which occur naturally in the plant kingdom; they are derivatives of the straight chain sugar alcohols and the cyclitols. The main derivatives of the straight chain polyols are the two anhydro polyols, styracitol (1,5-anhydro-D-mannitol) and polygalitol (1,5-anhydro-D-glucitol), and several phosphorylated polyols. Examples of the latter are sorbitol-1-phosphate and sorbitol-6-phosphate, both of which have been identified in apricot leaves. However, little is known about the formation and function of the anhydro or phosphorylated polyols in plants (Bieleski, 1982).

The most commonly occurring cyclitol is inositol, which is the generic term used to describe, 1,2,3,4,5,6-cyclohexane hexol. There are 9 possible isomers of inositol of which 7 are meso and 2 form a chiral pair. Only four of the inositol isomers are known naturally, they are; myo-inositol, scyllo-inositol and the chiral pair D- and L-chiro-inositol. The most common natural isomer of inositol is myo-inositol which is present as the free cyclitol in virtually all plants (Loewus and Dickinson, 1982). The cyclisation of glucose-6-phosphate into 1L-myo-inositol-1-phosphate is the only known route for the biosynthesis of inositols in plants. In turn myo-inositol is a precursor to the synthesis of the other naturally occurring inositols, for example; the conversion of myo-inositol via D-pinitol to D-chiro-inositol. Myo-inositol exists as the glycoside ester glucinol (O-β-D-Glucopyranosyl-1,1-myo-inositol) in sugar cane and beet. Myo-inositol, incorporated into phosphatidic acid to produce phosphatidyl-inositol, plays an important role in the synthesis and structural integrity of plant membranes. The indole acetic acid ester of myo-inositol is believed to act as a plant growth hormone regulator;
the functions of which have still to be completely understood (Loewus and Dickinson, 1982).

Like the straight chain polyols inositol exists in various phosphorylated forms, of which the most common is the hexa-phosphate, phytic acid. The elements potassium, iron, magnesium and manganese are sequestered by the phosphate of phytic acid and are stored in seeds.

Research continues into the natural sources of polyols for three main reasons:

i) to further understand plant metabolism,

ii) as an aid to chemical plant taxonomy and

iii) to identify new sources of polyols of possible use to man.

Thus polyols occur widely in the plant kingdom and are ingested as such in the animal kingdom, in the diet of many living organisms, forming a food chain which can ultimately include man.

1.3 The natural occurrence of polyols in the animal kingdom

The natural occurrence of polyols in the animal kingdom appears to be limited. Polyols were first reported to occur, in mammalian physiology, in seminal vesicles and placenta (Hers, 1960). The normal occurrence of polyols in the animal kingdom has been summarized by Hayman and Kinoshita, (1965) and Touster, (1974) who reported the occurrence of sorbitol in sheep foetal blood. The polyols xylitol, galactitol and sorbitol were reported to be converted from their corresponding aldose sugars in the lens of the eye in experimental sugar cataracts (Kinoshita 1974). The enzyme Aldose Reductase, which was believed to be responsible for the conversion of allose sugars
to polyols, was first isolated from calf lens by Hayman and Kinoshita (1965). This early experimental work was concerned with the normal occurrence of polyols and the elucidation of any possible biochemical pathways involving them, rather than any pathological role of polyols.

There are several reports of the measurement of polyols in normal human serum, urine and cerebro-spinal fluid. The polyols, with their concentrations in parentheses, erythritol (0.45), threitol (0.20), ribitol (0.06), arabitol (0.37), xylitol (0.05), mannitol (0.41), galactitol (0.15) and sorbitol (0.16) μg/ml have been measured in the serum of normal subjects (Roboz et al., 1984). The same authors reported that arabitol is the only polyol produced in appreciable quantity by the fungus Candida and subjects infected with this fungus have correspondingly raised serum arabitol levels. The sorbitol content of plasma has been reported to vary with the carbohydrate content in diets given to healthy humans (Malaisse et al., 1981). Sorbitol is more slowly absorbed by the intestinal mucosa than glucose (Dwivedi, 1977). Therefore ingestion of fruits high in sorbitol content, along with the hygroscopic nature of sorbitol, account for their laxative effect due to intestinal inhibition of water absorption. The transitory laxative effects of sorbitol when given orally to humans has been studied by Koizumi et al., (1983). These authors estimated a maximum tolerable oral sorbitol dose of 0.15 g/kg body weight for males or 9.7 g for an average 64.4 kg man. By comparison the same authors found the maximum tolerable sorbitol dose in women to be 0.3 g/kg or 15.0 g for an average 50 kg female.

The metabolism of sorbitol and xylitol in man has been reviewed by Froesch and Jacob (1974). Upon absorption from the intestine sorbitol and xylitol are rapidly converted to glucose, mainly in the liver, in an insulin-independent manner (Forster 1974).
Sorbitol is metabolised via fructose in the liver, with the first step occurring as oxidation of sorbitol to fructose, by the cytosolic enzyme sorbitol dehydrogenase (SDH) and the cofactor nicotinic-adenine-dinucleotide (NAD). Xylitol is oxidized to D-xylulose, probably by the same or a closely related enzyme that initiates sorbitol metabolism and metabolised in the liver by the pentose phosphate shunt. The three carbon polyol glycerol, is a normal intermediate in mammalian fat metabolism and plasma glycerol levels can be used as an index of fat mobilization, Fenton and Aherne (1987).

The polyols, with their excretion concentrations in parentheses, erythritol (63), threitol (11), arabitol (53), ribitol (10), xylitol (22), myo-inositol (33), galactitol (6), sorbitol (23) and mannitol (26) µg/mg creatinine have been reported to occur in adult human urine (Pfaffenberger et al., 1975). A very extensive urinary profiling of polyols and other carbohydrates in humans receiving normal diets has been carried out by Jansen et al., (1986) who report decreases in the excretion of all polyols examined with increasing age from neonates to maturity. Blau (1972) has shown that galactitol and mannitol are excreted in urine in raised amounts (above normal) in hereditary galactosaemia. Also, Allen et al., (1981) have measured galactitol in amniotic fluid as a possible aid in the diagnosis of foetal galactosaemia.

The polyols ribitol, sorbitol and myo-inositol have been reported in normal human cerebrospinal fluid at respective concentrations of 3.4, 4.8 and 54 µg/ml, (Smith et al., 1984). The same authors reported the presence of 1,5-anhydro-sorbitol at a concentration of 37 µg/ml in cerebrospinal fluid which confirm the results of Pitkanen (1973). The origin of the anhydro-sorbitol is unclear but is probably derived from plant matter. Of interest is another closely related anhydro polyol, 2,5-anhydro-mannitol,
which has been shown to be an anti-metabolite, inhibiting glycolysis in bovine spermatozoa, carbohydrate stimulated protein synthesis in rat testis and glucose synthesis in rat hepatocytes (Dills et al., 1983). The 2,5-anhydro-mannitol has not been reported to occur naturally in the plant kingdom but the possibility that it is produced by plants in response to abnormal growth conditions, such as environmental pollution or hostile climates, has consequences for man which cannot be ignored.

The existence of phosphorylated straight-chain polyols is not known in man. This does not preclude the possibility that they are present in some tissues, as transient metabolic intermediates, at very low levels which have made their detection difficult.

The existence of the cyclitol myo-inositol, both in the free and phosphorylated form, in mammalian metabolism is well documented. Myo-inositol is an essential nutrient in the culture of endothelial cells (Lorenzi and Toledo, 1986) and has been shown to be important in the control of insulin release from cultured B-cells of the pancreas (Pace and Clements, 1981). Myo-inositol is an important precursor in the synthesis of phospholipids (Allen 1982) and plays an integral role in the generation of the intra-cellular second messenger (Berridge 1984, Berridge and Irvine 1984). Myo-inositol is important in the synthesis and metabolic regulation of phosphatidylinositol in nerve tissue (Berry et al., 1983). The maintenance of the cytosolic levels of free myo-inositol in the axon is believed to be important in the production of the action potential which generates nerve impulses (Simmons et al., 1982). The hexaphosphate of inositol, phytic acid, is a common mammalian dietary component and as such is probably another precursor source for free myo-inositol. The occurrence of polyols in nature is summarised in table 1. The structures of some of the naturally occurring polyols and their related sugars are shown in figure 1.
In general, there is a lack of data correlating polyol (or abnormal polyol) appearance in human tissues with the original amounts in different food sources. There is a need to investigate the absorption, distribution, metabolism and excretion of polyols in normal human nutrition before comparisons are made with abnormal and pathological conditions. The other major physiological significance of polyols in man has come from studies of pathological conditions; in particular diabetes which is discussed in more detail below (see 1.5).

Table 1 The Distribution of Polyols in Nature

<table>
<thead>
<tr>
<th>CARBON LENGTH</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>OTHER</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLANT KINGDOM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALGAE (Blue, Green Brown and Red)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LICHENS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FUNGI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>LIVERWORTS, MOSSES</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PRIMITIVE VASCULAR PLANTS</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>PLANTS</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>FERNS</td>
<td>-</td>
<td>-</td>
<td>?</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SEED-BEARING PLANTS</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FLOWERING PLANTS (Monocotyledonae)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(Dicotyledonae)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ANIMAL KINGDOM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACCESSORY REPRODUCTIVE TISSUES</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAMMALIAN PHYSIOLOGY</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>METABOLIC INTERMEDIATES</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
</tr>
</tbody>
</table>

The three carbon glycerol occurs widely in the plant and animal kingdoms. The most abundant six carbon (C6) polyol is D-mannitol. Other includes branched and longer chain C8, C9 and C10 polyols.

+ = Known to occur, ? = Questionable, - = Not yet established
Figure 1. Structures of Some Naturally Occurring Polyols and Their Related Sugars.
Six carbon

D-glucose

D-glucitol
(D-sorbitol)
(L-gulitol)

D-mannitol

L-iditol
(D-iditol does not occur naturally)

Allitol
(meso)

Galactitol
(meso)

Myo-Inositol

Scylo-Inositol

D-chiro (+)

L-chiro (-)

Figure 1. cont.
Seven carbon

\[
\begin{align*}
    &\text{D-glycero-D-galacto-Heptitol} \\
    &\text{(Perseitol)}
\end{align*}
\]

Eight carbon

\[
\begin{align*}
    &\text{D-erythro-D-galacto-octitol}
\end{align*}
\]

There are no known naturally occurring Nonitols or Decitolos

Figure 1. cont.
1.4 The industrial uses of polyols

The industrial uses of polyols is widespread and has increased with increased world production of raw sugar. Starch, raw cane or beet sugar acts as the starting material for the industrial production of some polyols. For example: pure glucose is produced from the hydrolysis of starch and known as dextrose, sorbitol is produced by catalytic hydrogenation of pure glucose (dextrose) using nickel catalyst and hydrogen at elevated temperatures and pressure. When pure fructose is similarly reduced equal quantities of sorbitol and mannitol are produced. Sucrose or cane sugar when hydrolyzed yields a mixture of glucose and fructose known as invert sugar and also acts as a raw source for the production of sorbitol and mannitol. In a similar manner xylitol is produced from the catalytic hydrogenation of xylose (wood sugar) which in turn is the hydrolysis product of the plant cell wall component, xylan. Historically, in the 60's, improvements to the industrial hydrogenation of glucose were made, initially on a pilot scale, by using Raney nickel catalyst (nickel purified from an aluminium-nickel alloy) with magnesium as a promoter. The author reported 100% yield of sorbitol from 2000 lb dextrose starting material (Phillips 1963).

Attempts have been made on the laboratory scale to produce mannitol in commercially acceptable yields from copra meal; a by-product of coconut oil extraction (Saittagaroon et al., 1985). This latter example is indicative of the commercial and economic pressure to discover and utilize alternative natural sources for polyol production as demand outpaces supply of the more usual raw starting material. In recent years molecular biology has made an impact on the industrial production of carbohydrates. One such example is the simultaneous production of sorbitol from fructose and gluconic acid.
from glucose, by immobilized bacterial cells that contain a specific enzyme complex capable of the conversion (Chun and Rogers, 1988). The authors demonstrated sustained production of sorbitol and gluconic acid of 7.6 and 7.2 g/l/hr respectively for a period of 125 hours on a laboratory scale. The advantages of the last example, over the catalytic hydrogenation approach to polyol production, are that the products require little further purification, there is no requirement for high temperatures, pressures or dangerous gases and that the whole process can be automated. It will be of interest to see how soon such a pilot scheme is converted into a large scale manufacturing process.

Sorbitol is used in the pharmaceutical and food industry as a sweetner, as a humectant in the cosmetic industry and in the printing, resin and glue industries (Phillips, 1963). The white crystalline solid sorbitol is used in the manufacture of ascorbic acid (vitamin C). Sorbitol is also used in the synthesis of the heart drug isosorbide-dinitrate, the clinical uses of which have been reviewed by Swedberg (1984). In 1974 Aminoff estimated the annual production of sorbitol to be 250,000 tons, of which approximately a third ends up in foods. By comparison the 1987 estimate for world sorbitol production was 330,000 tons with a world total sugar production in excess of 100 million tons (Anon, Nestlé; see reference under Nestec).

When an acid that contains more than one carboxylic acid group is reacted with an alcohol containing more than one hydroxyl group a polyester is produced; one such example is the reaction between ethylene glycol and terephthalic acid which produces Dacron. There is little doubt that the search for new polyols coincides with the advent of new polymers, in what is now a highly competitive and profitable industry.
Nitric acid esters of polyols have been used as explosives of which the best known example is nitroglycerine, the product of nitric acid and glycerol. As a drug nitroglycerine has been used for over a century in the treatment of angina and myocardial infarction and has been reviewed by Parratt (1979).

As already indicated polyols are used in the food and drink industry as additives, particularly sorbitol, mannitol and xylitol. Some of the properties of polyols, which include their similar calorific value to sugars, sweetness, insulin independent metabolism and non-toxicity, make them ideal candidates as alternatives to sugars in processed foods. Their use in the food industry has been reviewed by Griffin and Lynch (1972) and their toxicity by Salminen (1982).

The polyols erythritol, arabitol, xylitol, mannitol, sorbitol and inositol occur in sherry wines where their presence is believed to account for the smooth tasting texture. The concentrations of the polyols in sherry wines vary according to the storage conditions but are on average 0.1 g/l (Estrella et al., 1986). It is interesting that the effect of polyols in sherry, the smooth tasting texture, is the very reason why they are added in beverages.

More recently, xylitol has attracted much attention as a food additive because of its additional anti-caries property. The anti-caries action is believed to arise from the inability of the normal mouth bacterial enzymes to convert xylitol to an acid which attacks the teeth (Scheinin 1980). The use of xylitol as a food additive has been reviewed by Hyvonen et al., (1982) who estimated the total world production of polyols to be about 345,000 tons in 1978 of which approximately 6000 tons was as
xylitol and mannitol. It is interesting to compare the 1978 and 1987 estimates for industrial polyol production which appear for that decade to be relatively unchanged.

Thus the occurrence of polyols has had an enormous impact on man, from their many industrial uses to nutrition, health and disease. Conversely the impact that man has had on polyols is also considerable and can be illustrated as follows. Industrial pollution of the environment, for example in the form of acid rain, chemical smog or radioactive fallout, can have a direct harmful effect on plant photosynthesis (Levitt, 1972). Sorbitol is a major photosynthetic product in plum leaves and its fruit (Anderson et al., 1961) and as such can be affected by adverse environmental conditions, like those mentioned above. There becomes then an increased possibility that, under certain hostile environmental conditions, the plant produces abnormal polyol derivatives (like the anti-metabolite 2,5-anhydro-mannitol referred to earlier) in an effort to relieve the man induced stress. In this respect there are at least four research needs which arise out of the possible consequences of our actions. They are:

i) to identify polyol changes, especially abnormal polyols, in plants with man induced environmental stress,

ii) to determine whether any such changes are suitable for monitoring global pollution,

iii) to determine whether such abnormal polyols are of potential harm to man and

iv) to determine whether these polyols occur in man normally or are related to various disease conditions.

To date very little research in this area has been carried out. However, for (i) above, Villanueva et al., (1987) have demonstrated the abnormal accumulation of free fructose and glucose in the needles of pine trees damaged by air pollution. Therefore, as can
be seen in the examples above, polyols are a very diversely occurring group of compounds and their study is essential to our understanding of them.

1.5 The role of polyols in the long term complications of diabetes

In man carbohydrates constitute a major source of nutritional energy. Carbohydrates are broken down in the intestine to their monosaccharides of which the most important are the pentoses and hexoses. The physiologically most important pentoses are ribose and deoxyribose. Similarly the most important hexoses are glucose, galactose and fructose. These sugars are transported from the intestine, via the hepatic portal vein, to the liver in an insulin independent manner and are mostly metabolised by the hepatocytes.

Insulin is a hormone, produced by the beta cells of the pancreas in response to raised blood glucose levels (hyperglycaemia). Insulin induces the hepatic enzyme glucokinase and the hexokinase enzyme of other tissues in order to phosphorylate glucose to glucose-6-phosphate, the initial step of glucose metabolism in all cells. Insulin also stimulates hepatic enzymes to synthesise glycogen, the liver storage product of glucose. Any excess glucose, above the hepatic buffering capacity, is transformed into triglycerides by the liver and either stored there or in adipose tissue. Further systemic glucose is stored as glycogen in muscle or as triglycerides in adipose tissue. The entry of glucose into these two tissues is also insulin dependent. Therefore the liver, in conjunction with hormonal control, acts as a buffer for glucose and normalises the hyperglycaemia during ingestion of carbohydrates. This in turn regulates the blood glucose concentration to the rest of the body, especially the brain.
Glucose can be synthesised in the liver from triglycerides mobilised from adipose tissue. At low blood glucose levels (hypoglycaemia) this helps to maintain a regular blood glucose concentration. Therefore, even during the temporary hyperglycaemia after carbohydrate ingestion or when fasted, the brain under normal circumstances is assured of a fairly constant supply of glucose. This is a necessary requirement because glucose crosses the blood-brain barrier in a manner which is not dependent on insulin.

Physiologically, in all situations including extreme depletion of hepatic glucose such as excessive exercise, starvation or trauma, the brain takes priority in receiving alternative sources of energy derived from other tissues. When polyols are ingested in the diet they are metabolised as already described and will also contribute to an increase in glucose. In the case of poorly absorbed polyols, such as sorbitol, their contribution to systemic hyperglycaemia is small.

In diabetes mellitus there is an inability of the beta cells of the pancreas to produce appropriate or effective amounts of insulin in response to hyperglycaemia. The postprandial blood glucose levels are not maintained at an optimum and the result can be sustained hyperglycaemia. In the most severe diabetic case the consequence of prolonged hyperglycaemia can lead to coma and death. Fortunately diabetes can be treated (in descending order of severity) with replacement insulin by injection before eating, by oral therapy which stimulates the beta cells into insulin production or by diet control.

A characteristic of diabetes is, that once the condition is diagnosed, it is never resolved irrespective of the severity. The causes of diabetes are widespread and include
auto-immune attack of the beta cells, viral infections, alcoholism, environmental effects, genetic factors and various complex interactions which combine all or some of these together (Notkins 1979). In the United States diabetes has been estimated to affect 5% of the population and account for an economic cost of $5 billion per year (Notkins, 1979) and in the U.K. at between £260 to £600 million (Gerard et al., 1989).

Another characteristic of diabetes is the appearance of complications which have no particular pattern in terms of duration or severity of disease. The complications are known as long term because once diagnosed the prognosis is often poor. The long term complications of diabetes include retinopathy, cataract formation, nephropathy and neuropathy. Retinopathy has a 60-80% frequency in patients with 20-25 years duration of diabetes, but the incidence does not reach 100%. Cataract formation in the eye is common and often linked to the incidence of retinopathy. The chances of developing nephropathy decline after a duration of diabetes of 30 years. Neuropathy is widespread in incidence because of the different classifications used to describe the many symptoms. Approximately 25% of diabetics who survive indefinitely never develop any complications. Other patients develop more than one complication. In the case of nephropathy the complication progresses relentlessly to end-stage renal failure, often with other complications developing.

Retinopathy is now treated with laser photocoagulation, thereby stopping the microhaemorrhages in the eye. Corneal cataracts can be removed surgically under local anaesthetic. Nephropathy can be treated with renal dialysis and diabetic renal transplant accounts for one-quarter of all renal replacement therapy. Neuropathy, however, is an extremely painful and debilitating condition and a difficult complication
to manage medically. Until recently the only treatment was to control the hyperglycaemia in an attempt to relieve the worst symptoms. In the less severe cases of neuropathy, such as subjective observations by the patients of numbness or tingling sensations in the lower limbs, with improved glycaemic control the symptoms were often reduced. It was not surprising that neuropathy was associated with a predominantly hyperglycaemic state. As the normal glucose metabolic pathways are blocked or reduced in activity in diabetes, alternative pathways for the metabolism of excess glucose were sought as a possible biochemical explanation for the aetiology of the complications.

Evidence implicating polyols in the long term complications of diabetes has come from three main sources. They are:

i) changes in tissue polyol content

ii) tissue distribution of the enzyme aldose reductase (AR) and

iii) clinical trials of aldose reductase inhibitors.

1.5.1 Changes in Tissue Polyol Levels

The first report of polyols occurring in cataractous lens was by van Heyningen (1959a) who found that xylose, glucose and galactose, in rats fed these sugars in their diet, were converted to their corresponding polyols xylitol, sorbitol and galactitol. Polyols were estimated using a semi-quantitative paper electrophoretic method. Dialysed extracts of rat lens were found to reduce the sugars if the cofactor NADPH was present. This finding suggested that an enzyme was involved in the process. The change in polyol levels in the rat lens occurred under experimental conditions and it was not possible
to conclude that this effect was the cause of the cataract formation. Varma and Kinoshita (1974), using packed column gas liquid chromatography (GLC) to separate polyol trimethyl silyl ether (TMS) derivatives, showed accumulation of sorbitol in diabetic rat lens along with increased fructose. They demonstrated xylitol accumulation in isolated normal and diabetic rat lens incubated with 30 mM xylose. The xylitol accumulation was significantly higher in the diabetic rat lens than in the non-diabetic control.

Malone et al., (1984) demonstrated a high positive correlation between blood glucose levels and the sorbitol content in sciatic nerves, lens and erythrocyte of diabetic rats with varying degrees of glycaemia. Using an enzymatic assay they demonstrated a positive correlation between lens and sciatic nerve levels of sorbitol and the erythrocyte sorbitol content. Beyer-Mears et al., (1984) demonstrated that the glomerular sorbitol content of streptozotocin induced diabetic rats (after 6 weeks duration of diabetes) was significantly raised above normal non-diabetic control rats. In addition, they showed significant lowering of glomerular myo-inositol content compared to non-diabetic control after 9 weeks duration of diabetes. Polyols from the deprotienised glomerular samples were derivatised as the trifluoroacetates and analysed by GLC but no further assay details were given.

Willars et al., (1987) studied the sciatic nerve content of sugars and polyols in streptozotocin induced diabetic rats. The glucose, sorbitol and fructose levels in sciatic nerve, after 3 weeks duration of diabetes, were all significantly raised above non-diabetic normal rat. In addition, the myo-inositol content of sciatic nerve was significantly lowered in the diabetic group compared to normal control. The same
authors reported that in rats fed a diet supplemented with 30% galactose, for 5 days, the sciatic nerve galactitol content was significantly higher in diabetic rats compared to control. In this latter experiment the nerve fructose level was relatively unchanged but again the nerve myo-inositol level was depleted in the diabetic group.

Aloia (1973) showed elevated sorbitol levels in the serum of human diabetics; the mean serum levels (+ standard error) for normal and diabetics were 0.24 ± 0.01 and 0.59 ± 0.08 mg/100 ml respectively, (n=32). Samples were derivatised by TMS silylation and analysed by packed column GLC with flame ionisation detection (FID). Additionally, peracetate derivatives of extra samples were prepared to separate sorbitol and mannitol. Heath and Galton (1975) showed raised daily urinary excretion of glucose, sorbitol and myo-inositol in human diabetics. They reported a linear relationship between the 24 hour urinary excretion of glucose and the hexitols. They did not find however, increases in fructose, sorbitol or myo-inositol content of human diabetic lens. Trimethyl silyl ethers of polyols and sugars were separated isothermally at 160°C using a 7 foot narrow bore glass column coated with 3% S.E. 30. Using this method the authors reported the detection of an unidentified carbohydrate in adipose tissue, which does not appear to have been followed up. Clements et al., (1977) reported that urinary myo-inositol excretion was ten fold higher in untreated human diabetics compared to normal non-diabetic subjects. Insulin treatment normalised the diabetic myo-inositol excretion. Myo-inositol was analysed by a method similar to that of Heaf and Galton (1975).

Malone et al., (1980) using their enzyme assay showed that, after an 8 hour fast, there was a statistically significant difference between the erythrocyte sorbitol levels in 155
diabetic children compared to a normal non-diabetic group. The results in nmol/gm haemoglobin (+ standard deviation) were 28.0 ± 18.9 diabetic, (n=155) and 10.1 ± 7.2 normal (n=30). Mayhew et al., (1983), using packed column GLC of TMS polyol derivatives, showed significantly lower concentrations of free and lipid myo-inositol in human diabetic sciatic nerve, removed post-mortem, compared to non-diabetic control. The concentrations of glucose, fructose and sorbitol were higher in the diabetic group than control.

Popp-Snijders et al., (1984) demonstrated a difference in erythrocyte sorbitol content between diabetic and fasting normal non-diabetic human volunteers. They were the first to use capillary GLC to separate polyol acetate derivatives extracted from red blood cells. The results were in nmol/ml erythrocyte (mean ± standard error of the mean, SEM), diabetic 13.1 ± 0.9 (n=28), normal 5.2 ± 0.3 (n=30). The erythrocyte myo-inositol levels were unchanged between the two groups and not statistically different. Lapolla et al., (1985), using the enzymatic assay of Malone, obtained similar results to those of Popp-Snijders and again demonstrated significantly raised erythrocyte sorbitol concentrations in diabetic subjects compared to normal non-diabetics.

Finally, a recent report by Akanuma et al., (1988) found the presence of 1,5-anhydro-D-sorbitol in normal human plasma and its excretion in urine. This component was identified by them using GLC with mass spectrometric detection (GC-MS) of the acetate derivative but no further details were given. The authors found that diabetic patients had lower plasma levels of the anhydro-sorbitol than normal non-diabetics. They also showed a significant correlation between diabetic urinary
anhydro-sorbitol and glucose levels. This correlation was even more apparent during a 100g oral glucose tolerance test in diabetics and normals, where the urinary excretion of glucose and the anhydro-polyol were both raised. The significance of this finding is not clear, but emphasises the lack of knowledge about the relationship between hyperglycaemia and tissue formation and excretion of the less well known polyols.

There does, however, appear to be a distinct relationship between sorbitol and myo-inositol which can be summarised as follows. In rat and human diabetic tissues sorbitol is raised and myo-inositol is lowered in content compared to normal non-diabetic tissue. The tissues where these changes occur are, most notably those tissues affected by the long term complications of diabetes, namely the eye, kidney and nerve. These changes can be partly explained by a general biochemical hypothesis that has emerged for polyol formation and metabolism, which is as follows.

Aldose reducing sugars are reduced to their corresponding sugar alcohols by the enzyme Aldose Reductase (AR) in the presence of the reduced cofactor NADPH. In normal circumstances the polyols are further metabolised by the enzyme Polyol Dehydrogenase, referred to as Sorbitol Dehydrogenase (SDH), in the presence of oxidized NAD cofactor. The resulting sugar may then be further metabolised, as in the case of fructose, by phosphorylation and the glycolytic pathway. This series of events has become known as the "Polyol pathway" and is represented schematically below, with glucose as an example.
In addition, it has been postulated that tissues freely permeable to glucose (not dependent on insulin for transmembrane transport) contain AR which converts the glucose to sorbitol in an irreversible step. Evidence for this is supported by the observation that in diabetes the levels of NADPH and NADH are higher than normal with concomitantly lower levels of NADP and NAD, thus favouring sorbitol formation (Varma and Kinoshita 1974). In addition to these favourable conditions for sorbitol formation in diabetics the diffusion of this polyol through cell membranes is slow (Popp-Snijders et al., 1983). As a consequence of these events it has been postulated that sorbitol may undergo intracellular accumulation causing oedema which in turn affects the normal function of the cell (Gillon et al., 1986). However, this sequence of events does not take into account the role played by the enzyme SDH which further metabolises sorbitol to fructose. The activity of this enzyme would have to be diminished or non existent for sorbitol to accumulate in those cells that display abnormal function.

Three questions have emerged concerning the polyol pathway and the long term complications of diabetes:

i) is AR present in those tissues where the long term complications develop,
ii) what is the tissue distribution of SDH and is this step of polyol metabolism reduced in activity, thereby leading to accumulation of polyol and subsequent dysfunction of the tissue and

iii) what is the relationship between the polyol pathway and myo-inositol.

In the first instance several workers concentrated on determining the tissue distribution of the implicated enzymes AR and SDH and their results are reviewed in section 1.5.2.

1.5.2 Tissue Distribution of Aldose Reductase

Evidence for the reduced-triphosphopyridine nucleotide-dependent reduction of aldoses to polyols, in extracts of seminal vesicles and placenta, was first reported by Hers (1960) who named the enzyme responsible aldose reductase (AR). Hayman and Kinoshita (1965) were the first to isolate AR from calf lens. They purified AR by diethylaminoethyl-cellulose (DEAE) column chromatography and characterised the properties of the enzyme. They found only one enzyme which had a pH optimum of 5.5, was stimulated by sulphate ions, had a sulphydryl requirement, reduced aldoses and various aldehydes and was inhibited by various glycolytic intermediates; such as oxaloacetate, beta-hydroxybutyrate and acetoacetate. In addition, they found this enzyme to have the highest Michaelis constant ($K_m$) and therefore the lowest affinity for glucose. They also found that the enzyme was particularly sensitive to inhibition by organic anions, such as beta-ketoglutarate.

Gabbay and O'Sullivan (1968) isolated AR from normal and diabetic rat spinal cord and sciatic nerve. They found that the sciatic nerve AR had considerable polyol
forming ability compared to that from spinal cord. They were the first to report that AR was predominantly associated with sciatic nerve schwann cells and that SDH was associated with the axon. Clements et al., (1969) partially purified AR from the thoracic aorta wall of rabbit and human. They found that incubation of aortic AR with 5 mM glucose was stimulated approximately three fold by the addition of 5 µg/ml adrenalin, whereas nor-adrenalin had no effect. These authors also found SDH to be present in rabbit aorta.

Beutler and Guinto (1974) partially purified an enzyme from human erythrocytes which was capable of reducing glyceraldehyde to glycerol. They classed the enzyme as an L-hexonate dehydrogenase, rather than the AR obtained by Hayman and Kinoshita because of the different kinetic characteristics and elution pattern with DEAE chromatography. These authors suggested that AR was not present in the erythrocyte.

Ludvigson and Sorenson (1980) raised antibodies in rabbit to AR purified from rat seminal vesicles and used immuno-histochemical staining to locate AR in rat tissue. They found the presence of AR in numerous cell types of the eye including lens, cornea, iris, ciliary body and retina. Retinal capillary endothelium did not contain immunoreactive AR. They also found AR in the kidney predominantly in the inner medulla. It was also noted that the enzyme location applied to both diabetic and non-diabetic normal rat tissue. Srivastava et al., (1984) using immunochemical characterisation located AR in human aorta, brain, muscle and erythrocyte. They also demonstrated the presence of two other enzymes, aldehyde reductase I which was present in human kidney, liver and placenta and aldehyde reductase II present in those tissues as well as the human lung, brain and erythrocyte. Das and Srivastava (1985a)
carried out detailed in-vitro kinetic studies of purified human AR and found that the enzyme exhibited bi-phasic kinetics. This was explained by the finding that AR existed in the activated and inactivated (native) forms. The activated form arises when the enzyme is subjected in-vitro to NADPH, glucose and glucose-6-phosphate. It would appear that glucose-6-phosphate was an AR activator. The activated enzyme subsequently shows a strong affinity for glucose with a lower $K_m$ and less susceptibility to inhibition by glycolytic intermediates or AR inhibitors. Furthermore, the authors reported that AR purified from normal human lens displayed properties similar to the native (inactivated) form of AR, whereas AR purified from clear lens of severely hyperglycaemic diabetics displayed AR properties similar to the activated form.

Bagnasco et al., (1988) have shown the existence of AR in an in-vitro cell culture of an osmotically resistant cell line from rabbit papillary pelvic epithelium. Using SDS-PAGE and scanning densitometry they showed that a gradual decrease in AR activity was associated with a corresponding gradual decrease in AR enzyme protein. This was the first report that indicated that protein synthesis of AR may be a response related to the cellular glycaemic environment.

There is considerable controversy over exactly what form the AR enzyme takes, its kinetic characteristics and whether L-hexonate dehydrogenase is the same or a closely related enzyme. Nevertheless, the same tissue distribution of AR was observed when investigated by several distinctly different techniques. This made the evidence for the existence of AR more convincing. Thus AR would appear to be present in human tissues which are notably those tissues most affected by the long term consequences of diabetes. The AR tissue distribution, in conjunction with the polyol changes that
occur in those same tissues, support the argument that the long term side effects of diabetes may be biochemical in origin. As indicated above the biochemical explanation, namely the polyol pathway, only partly explains the polyol changes; which is most notably the conversion of glucose to sorbitol. The changes in myo-inositol levels observed in diabetes, the lowering of myo-inositol when sorbitol is raised, is not explained by the polyol pathway. The exact relationship between these two polyols (sorbitol and myo-inositol) has still to be determined.

There are less reports concerning the tissue distribution of SDH. Vaca et al., (1983 and 1984) showed SDH deficiency in several pig tissues. They also screened several animal species for erythrocyte SDH activity and found high activity in hamster, medium activity in mouse, guinea pig, rat and dog and low activity in rabbit, sheep, goat, cattle, horse and donkey. There was absence of erythrocyte SDH activity in pigs but in human erythrocytes the activity was reported to be 17.5 \( \mu \text{mol product formed per hour per g haemoglobin} \). The pattern of results are in general agreement with those of Agar (1979) who found high erythrocyte SDH activity in man, dog, guinea-pig and mouse. However the actual activity reported by Agar for human erythrocyte SDH is 5 times higher than that obtained by Vaca et al., while Varma and Kinoshita (1974) found the activity of SDH in diabetic rat lens to be decreased compared to control.

The role of AR in the development of the long term complications of diabetes has been reviewed by Kador and Kinoshita (1985), Greene and Lattimer (1985) and Gillon et al., (1986). Further evidence that AR and the associated polyol changes are involved in the biochemical aetiology of the complications of diabetes has come from studies of AR inhibitors in animal models and man.
1.5.3 **Studies Using Aldose Reductase Inhibitors**

The rationale behind the use of aldose reductase inhibitors came from the earlier observations of Hayman and Kinoshita (1965) that AR was inhibited by organic anions. There was, historically, a need to develop AR inhibitors for three reasons:

i) to use as probes in elucidating the complex biochemical interactions between polyols and AR,

ii) to determine the physiological changes that take place when AR is inhibited; such as motor nerve conduction velocity (MNCV),

iii) to determine the potential therapeutic use of AR inhibitors.

The belief was that if AR could be inhibited the formation of sorbitol from glucose would not occur. Therefore, the absence of accumulated sorbitol in certain tissues would inhibit the onset or continuance of complications. Most of the AR inhibitor studies have been carried out in rodents with experimental diabetes and clinical trials in man. The structures of the AR inhibitors most commonly under investigation are shown in figure 2.
Figure 2. Structures of Some Aldose Reductase Inhibitors
QUERCETIN, $X=\text{OH}$
QUERCITRIN, $X=\text{O-L-Rhamnose}$
(Ref Varma et al., 1975)

AY-27,773 "TOLRESTAT"
(Ref Hicks et al., 1984)

ONO-2235
(Ref Asano et al., 1986)

Figure 2 cont. Structures of Some Aldose Reductase Inhibitors
1.5.3.1 Animal AR Inhibition Studies

Peterson et al., (1979) showed inhibition in-vitro of calf lens AR by the Pfizer novel compound CP-45,634 (Sorbinil; d-6-fluoro-spiro[chroman-4,4'-imidazolidine]-2',5'dione). Using GLC analysis of TMS derivatives they found a lack of polyol formation in isolated rat sciatic nerve when incubated in-vitro with Sorbinil and 50 mM glucose. Furthermore, there was an 85% inhibition of sciatic nerve sorbitol accumulation in streptozotocin induced diabetic rats when given oral Sorbinil at 0.75 mg/kg. In a similar group of diabetic rats given Sorbinil orally, at a dose of 2.5 mg/kg for 1 week, the sorbitol accumulation in nerves and lens was reduced by 90%. At a daily Sorbinil dose of 5 mg/kg for 4 weeks galactitol formation was prevented in 90% of galactosaemic rats for up to 25 days. At 29 days 50% of the galactosaemic rats had developed lenticular opacities. At a Sorbinil dose of 10 mg/kg only 10% of the rats developed signs of cataract formation. It appeared that Sorbinil was a potent inhibitor of AR and that the effect was dose related.

Finegold et al., (1983) found that oral Sorbinil, dosed at 20 mg/kg daily for 2 weeks, completely prevented the fall in nerve myo-inositol and rise in nerve sorbitol in diabetic rats, compared to untreated control. Tissue myo-inositol concentrations were determined by GLC of the TMS derivative. Sorbinil, in this experiment, did not have an effect on diabetic plasma myo-inositol levels.

Gillon et al., (1983) obtained similar results, where Sorbinil was also shown to prevent an increase in nerve fructose content and reduced the extent to which motor nerve
conduction velocity (MNCV) was slowed. Serum myo-inositol (analysed by GLC of the TMS derivative) was raised in severely diabetic rats but normalised by treatment for 2 days with Sorbinil. Yue et al., (1984), using an enzymatic assay, again obtained similar results to the above in diabetic rats when treated with Sorbinil. They also showed a significant correlation between erythrocyte and nerve sorbitol content. Erythrocyte sorbitol was raised in the diabetic rats but was only partially normalised by Sorbinil treatment.

Greene and Lattimer (1984) studied the effect of Sorbinil treatment on sodium potassium adenosine-triphosphatase activity (Na/K ATPase) in sciatic nerve homogenate. They found the activity of this enzyme to be reduced by 25% in untreated diabetic rats and that Sorbinil treatment increased the activity towards normal. The authors found that Sorbinil prevented the depletion of myo-inositol content in diabetic rat sciatic nerve (analysis by GLC) and postulated that the restoration of nerve myo-inositol levels had a direct effect on the Na/K ATPase activity. Yeh et al., (1987) found that the transport activity of Na/K ATPase in lens from rats with a 2 week duration of diabetes was decreased. In these lenses the levels of sorbitol were ten fold higher than control with a very depleted myo-inositol content. The daily dose of Sorbinil required to restore the ATPase activity to normal was less than that required to restore the myo-inositol content to normal. The authors concluded that myo-inositol may not be directly linked with the restoration of ATPase activity. A similar result has been reported by Lambourne et al., (1988) who compared the restoration of ATPase activity with the inhibitors Sorbinil, statil and tolrestat.
Stribling et al., (1985) found that the novel ICI compound statil (ICI 128,436, (3-[4-bromo-2-fluorobenzyl]-4-oxo-3H-phthalazin-1-yl acetic acid) at a daily oral dose of 25 mg/kg completely prevented the development of cataracts in diabetic rats for up to 74 days. Statil was found to reduce the elevated sorbitol content in sciatic nerve, lens, retina and adrenal cortex of diabetic rats. In addition statil prevented galactitol accumulation in erythrocytes of rats receiving a galactose diet and restored the MNCV to normal. Tissue polyol levels were analysed by packed column GLC of the TMS derivatives.

Hotta et al., (1985) compared the novel Japanese compound ONO-2235, (E-3-Carboxymethyl-5-[((E)-2-methyl-3-phenylpropenylidene]rhodamine) with alrestatin (Ayerst) in streptozotocin diabetic rats fed a 72% fructose rich diet. They found a positive correlation between the sciatic nerve sorbitol content and impaired MNCV. Treatment with ONO-2235, at a daily dose of 50 mg/kg for 4 weeks, prevented both the nerve sorbitol accumulation and the impairment of MNCV. Using an enzymatic assay (similar to that of Malone et al., 1980) these authors found a positive correlation between the erythrocyte sorbitol content and the sorbitol content of sciatic nerve and retina. A further interesting observation was that diabetic rats receiving insulin treatment alone had normal MNCV.

Cameron et al., (1986) demonstrated similar results to those described above with oral Sorbinil, given at a daily dose of 25 mg/kg for 6 months, in diabetic rats. They also examined the morphology of nerves and found that in untreated rats the axon area was reduced by 14% compared to age matched Sorbinil treated rats. They suggested that the retarded axon growth could cause the defect in MNCV associated with diabetic
complications. This is an interesting observation when compared to the results of Gabbay and O’Sullivan who had associated AR mainly with the schwann cell and SDH predominantly with the axon. The inference from these observations are that schwann cell changes predominate over changes in the axon.

Other studies (Bareford et al., 1986, and Robey et al., 1987) have shown that erythrocyte deformability is decreased in diabetes and that Sorbinil can improve the ability of the erythrocyte to deform. This effect may be linked to the increased polyol content and swelling of the erythrocyte. The rheology of osmotically swollen erythrocytes suggests that the swollen red blood cell would not pass through the capillary vessel bed of tissues, as easily as normal blood cells, thereby decreasing the tissue oxygen supply. Lightman et al., (1987) found an increased permeability of sucrose across the blood-retinal barrier in long-term galactose fed rats. The permeability of sucrose across the blood-retinal barrier was reduced in galactose fed rats also given Sorbinil. The relationship between aldose reductase and increased sucrose permeability is unclear. However, there is an increase by 200% in the retinal capillary basement membrane thickness in the galactosaemic rats. This thickening is not seen in control or Sorbinil treated rats.

1.5.3.2 Clinical Observations of AR Inhibition in Man

One of the first studies in humans of an AR inhibitor was that of Gabbay et al., (1979). They reported their initial investigation of the Ayerst AR inhibitor alrestatin (1,3 dioxo-1H-benz(de)isoquinoline 2(3H) acetic acid) given orally to normal adult males and to four adult maturity onset diabetics. The normal adult males given
alrestatin orally at different doses provided valuable data on the pharmacokinetics of alrestatin. The four diabetic patients were characterised by the recent onset of clinically severe peripheral neuropathy, which was assessed by subjective observations and by decreased motor nerve conduction velocity. The diabetic subjects were given alrestatin orally at a dose of 1 g for 30 days. There was no improvement of the motor nerve conduction velocity at that dose. Two of the diabetic patients also received alrestatin by intravenous infusion. Two days after the infusion some objective benefit was reported but no improvement in the MNCV was observed. The authors also commented on the lack of a suitable in-vitro test to determine the effect of alrestatin on AR inhibition. Handelsman and Turtle (1981) reported no improvement in MNCV in nine insulin dependent diabetics with severe painful neuropathy when given alrestatin orally at individual daily doses ranging between 3-7 g. There was evidence, in the form of photosensitive skin rash, for a poor tolerance to alrestatin at the doses used.

Judzewitsch et al., (1983) studied the effect of oral Sorbinil on nerve conduction, at either a daily dose of 250 mg for 9 weeks or a 9 week placebo period, in 39 stable diabetic patients without signs of peripheral neuropathy. There was a small but statistically significant improvement in MNCV during the Sorbinil treatment period which declined upon cessation of Sorbinil treatment.

Popp-Snijders et al., (1984) found that oral Sorbinil, given at a daily dose of 200 mg for 12 weeks to 14 diabetic patients, reduced the erythrocyte sorbitol concentrations by 75% to within normal non-diabetic levels. A parallel placebo treated group of diabetics did not show any changes in erythrocyte sorbitol concentrations. There was no change in erythrocyte myo-inositol concentrations between placebo or Sorbinil.
treated diabetics. The study was conducted over 20 weeks with 4 week run-in and run-out placebo treated periods. Erythrocyte sorbitol and myo-inositol concentrations were determined once every 4 weeks using a modification of their capillary GLC assay.

Fagius et al., (1985) found a small but significant improvement in peripheral nerve function in 27 male diabetics given oral Sorbinil, 250 mg daily for 6 months, compared to matched placebo treated diabetics. Both placebo and Sorbinil treated groups comprised diabetics who had symptoms of symmetrical neuropathy for a least 1 year as evidenced by reduced nerve conduction in both legs. The authors noted that the Sorbinil treated diabetics who showed the most subjective improvement were the patients who had neuropathy symptoms of least duration.

Martyn et al., (1987) found no evidence of improvement in nerve function in 22 diabetics with abnormal nerve function when given Sorbinil at a daily dose of 125 mg for 6 months. Erythrocyte sorbitol was measured (using the method of Popp-Snijders) at 3 and 6 months and there was a noticeable 60% decrease to within and below the normal non-diabetic range. Guy et al., (1988) found no clinical or neurophysiological benefit, in diabetic patients with neuropathy, when treated with Sorbinil at a daily dose of 250 mg daily for 1 year.

The pattern that has emerged from studies of AR inhibitors in animals and man is as follows. In general, diabetic tissue levels of sorbitol and myo-inositol are respectively raised and lowered in content. Inhibition of AR tends to reverse the changes in polyol tissue content towards normal. This is especially evident from rodent studies. In man AR inhibition normalises the erythrocyte sorbitol content and is believed to be an
indicator of the same process in less accessible tissues: such as kidney and nerve. The
normalisation of erythrocyte sorbitol, upon AR inhibition, in rodents and man always
occurs irrespective of the degree of severity of diabetic complication. Most of the
erythrocyte sorbitol measurements, in the clinical trials, are made at least one week
apart. There is a need to establish in-vivo how quickly the erythrocyte sorbitol responds
to AR inhibition.

Erythrocyte myo-inositol levels do not appear to be affected by AR inhibitor therapy
in man. In this case the erythrocyte may not reflect the myo-inositol behaviour in less
accessible tissues. In the relatively simple erythrocyte, myo-inositol will be turned over
regularly with phosphatidylinositol in the membrane resulting in an average
intracellular myo-inositol concentration. In other tissues, for example nerve, the role
of myo-inositol is more specialised. In the nerve there are complicated biochemical
processes, such as the interaction between myo-inositol and the induction of the ATPase
protein, specifically designed to generate the action potential.

In diabetes the normalisation of MNCV appears to be dependent on the degree of
severity of neuropathy. There is noticeably much less benefit for patients treated with
AR inhibitors, when the degree of severity of neuropathy is increased. This observation
is supported both clinically (including subjective observations by the patient) and by
neurophysiological assessment. Patients with only a short duration of neuropathy
appear to respond best to AR inhibition therapy. This is especially evident from the
Sorbinil clinical trials in diabetics with varying degrees of complication. The
biochemical consequences of AR inhibition therapy must also not be overlooked.
Successful AR inhibition can contribute to hyperglycaemia, which in turn other
biochemical pathways will attempt to normalise, with subsequent and as yet unknown consequences.

However, the major and over-riding benefit of AR inhibition therapy is the relief of severe pain that results from diabetic polyneuropathy. Treatment early on at the first signs of complications developing may help to inhibit the onset of irreversible biochemical changes. To this end alone an in-vivo indication of the efficacy of AR inhibition can be obtained by measuring erythrocyte sorbitol levels before and during treatment with AR inhibitors. As yet there is no such simple test and it is one of the major aims of this thesis to try and establish one. The role of AR in diabetic complications of the eye has been reviewed by Kinoshita et al., (1979). Diabetic complications and AR inhibition has been reviewed by Clements (1985). Recent advances in AR therapy has been reviewed by Greene and Lattimer (1985). AR inhibition and nerve conduction has been reviewed by Pfeifer (1985). Sorbinil therapy in painful neuropathy has been reviewed by Jaspan et al., (1985).

From all the evidence above it is clear that the polyol pathway exists in animals and man. The physiological significance is unclear but the pathway probably exists for tissues that require fructose as an energy source. In tissues known to have this pathway normally, such as the seminal vesicles, the polyol flux through the activated pathway is high and only low levels of polyol are encountered. In some diabetic tissues there is a disturbance of the polyol flux through what is essentially a dormant pathway, which has been activated by a predominantly hyperglycaemic environment. The evidence that the pathway has been activated comes from the observation of changes in polyol content in tissues, of which the erythrocyte appears to partially model those changes.
in other tissues. From there on the exact sequence of pathophysiological events which cause diabetic complications has still to be determined.

In the case of diabetes the polyol pathway is not the only alternative route for excess glucose metabolism. Excess glucose can also undergo non-enzymatic glycosylation of proteins, of which the best known example is the glycosylation of haemoglobin, (Bunn et al., 1978). Also recent interest has centered around the non-enzymatic glycation by glucose of serum albumin and protein, which has become known by the term fructosamine, (Johnson et al., 1982). The clinical relevance to diabetes of fructosamine has been reviewed by Kennedy and Baynes (1984) and Armbuster (1987).

In many of the above studies the role of chemical analysis has been fundamental to the determination of the sometimes subtle polyol changes observed in various tissues. Therefore a reliable method for the determination of polyols in biological matrices is essential to our understanding of the role these compounds have in normal and disease conditions.

1.6 The Chemical Analysis of Polyols in Biological Samples

In general, the methods for the determination of polyols in biological samples falls into three categories; chemical, biochemical and chromatographic. Some of the chemical reactions of polyols can be used for their determination, especially where only one polyol is present in a pure solution. An example is the oxidative cleavage of sorbitol, by periodic acid, to formic acid and formaldehyde. The formaldehyde can be titrated against iodine and the titration value compared to sorbitol standards treated in the
same way. This sort of analysis is very unspecific when applied to biological samples containing other polyols, sugars or compounds which also react. Moreover, wet chemical techniques are limited by a lack of specificity when the desire is to measure a single component in a complex mixture. There is thus a requirement to separate the individual polyol components from other interfering compounds present in a biological or other complex mixture. However, after appropriate separation procedures, chemical reactions can still be employed to visualize the individual polyol and carbohydrate components.

An early separation technique for polyols, paper electrophoresis, was first suggested by Frahn and Mills (1956) who examined the migration of hexitols and their related hexoses in various electrolytes. The migrated hexitols were visualized as pale areas on a brown background when the paper was sprayed with potassium permanganate and chromium trioxide in sulphuric acid. A similar electrophoretic system was used by van Heyningen (1959b) for the semi-quantitative determination of polyols in cataractous rat lens. The major problem with the paper electrophoresis method was one of accurate quantitation, which is also a problem with paper chromatography. In order to overcome the lack of sensitivity, associated with these techniques, one alternative approach is the use of enzyme assays.

1.6.1 Enzymatic Methods for the Determination of Polyols

The advantage of enzyme methods for the determination of polyols over chemical, electrophoretic and paper chromatographic methods is speed of analysis and greater quantitative power. The principle action of an enzyme is as a biological catalyst in the
conversion of a substance (substrate) to a product. The substrate must specifically interact with the enzyme, often requiring a cofactor, to form a complex resulting in the formation of a product under suitable conditions of temperature and pH. Enzymatic assays are normally optimised so that the end point visualisation is dependent upon the substrate concentration.

Polyols are formed in nature by the biological reduction of their respective sugars by oxido-reductase enzymes (dehydrogenases) which require pyridine nucleotide cofactors; either the cofactor reduced nicotinamide-adenine dinucleotide (NADH₂) or the phosphorylated form of the latter reduced nicotinamide-adenine dinucleotide phosphate (NADPH₂). The respective oxidised version of these two cofactors is NAD⁺ and NADP⁺. The pyridine nucleotide cofactors have absorption spectra with maxima at 340 nm for the reduced form (NADH, NADPH) and 260 nm for the oxidized form (NAD⁺, NADP⁺). Oxidoreductive enzyme reactions can be followed by observing the change in absorbance, either an increase or a decrease, of the relevant reduced cofactor at 340 nm in a spectrophotometer. This fact is one of the most exploited spectral properties of the pyridine nucleotide dependent reactions forming the basis of many quantitative enzyme assays. The change in cofactor absorbance after a given period of time, or at end point of the reaction, is used for the determination of substrate concentration by interpolation from calibration standards. In this way many of the natural synthetic pathways for polyol formation, reduction of sugars by enzymes, can be exploited in the reverse direction, oxidation of polyols to sugars, for their rapid and quantitative determination.
There are different enzyme methods for the determination of a number of polyols. Polyols that have reported enzyme methods include ethylene glycol, glycerol, L-threitol, xylitol, ribitol, sorbitol, D-mannitol and myo-inositol (Bergmeyer, 1984). An early example of the determination of sorbitol using an enzyme method was reported by Clements et al., (1969) who used the enzyme sorbitol dehydrogenase (L-iditol: NAD oxidoreductase, EC 1.1.1.14) purified from sheep liver to determine the concentration of sorbitol in rabbit aorta. The concentration of sorbitol was also determined in-vitro by this enzyme method when sections of aorta were incubated with varying amounts of glucose. Malone et al., (1980) modified the previous method for sorbitol and used it to determine the erythrocyte sorbitol content. The standards in erythrocytes ranged from 0.2-9.0 μg/ml or 1.0-50.0 nmol/ml with a limit of quantitation of 0.06 μg/ml. The sorbitol was converted to fructose by sorbitol dehydrogenase (SDH) and the NADH produced detected fluorimetrically by excitation at 366 nm and emission at 452 nm. The fluorimetric detection of NADH adds to the sensitivity of the assay by being more specific for NADH than other compounds which increase the background absorbance at 340 nm. These authors gave no accuracy or precision data for the erythrocyte sorbitol assay but indicated that the enzyme preparation from sheep liver was highly specific for sorbitol. The specificity of the enzyme increases the sensitivity of the assay because the enzyme specifically converts only the substrate. Thus, highly sensitive and specific assays for individual polyols can be obtained by using purified specific enzymes along with fluorescence detection of the cofactor for enhanced detection.

The method of Beutler (1984a) for sorbitol determination has the advantage of increased specificity. Sorbitol is converted to fructose by SDH and the NADH produced is removed by lactate dehydrogenase with the concomitant conversion of
pyruvate to lactate. Fructose is converted by a series of coupled reactions to gluconate-6-phosphate with the production of NADPH. The enzyme phosphoglycerate isomerase that converts fructose-6-phosphate to its glucose counterpart is highly specific for fructose which is only produced by the conversion of sorbitol; therefore increasing the assay specificity for sorbitol. This assay was reported to be linear between 5 and 180 nmol/ml sorbitol per assay solution. The detection limit was about 3 nmol/ml which is higher than that reported by Malone et al (1980) and is mainly due to larger dilution factors used in the assay and detection of NADPH at 340 nm. The relative standard deviation of the assay was determined at two levels; 50 and 120 nmol/ml and were 0.6 and 1.0% respectively. When applied to human plasma the range of values obtained were 3.7-4.5 nmol/ml plasma in normal and less than 2.5 nmol/ml plasma in carbohydrate poor diets.

The hexitol mannitol has been determined in fungal spores and pharmaceutical preparations using the enzyme D-mannitol dehydrogenase purified from Aspergillus oryzae. The products of the enzyme reaction are fructose and NADPH and the assay was reported to have a standard deviation of 10% at the detection limit of 1 μmol per cuvette. However, the assay is non specific with glycerol, erythritol and galactitol reacting with the enzyme (Horikoshi 1984).

The hexahydroxycyclohexane myo-inositol can be determined using an inositol dehydrogenase enzyme purified from bacteria. The product of the reaction is scyllo-inosose (2,4,6/3,5 pentahydroxycyclohexanone) and NADPH. The method was reported to have a limit of detection of 0.01 μmol but no further assay data was available (Weissbach 1984). This method has been adapted by MacGregor and
Matschinsky (1984) for the determination of myo-inositol in serum and tissues. The enzyme myo-inositol dehydrogenase (myo-inositol: NAD⁺ oxidoreductase, EC 1.1.1.18) from the bacteria Enterobacter aerogenes converts myo-inositol to scyllo-inosose and the NADH produced is removed in a coupled reaction by the concomitant conversion of oxalacetate, by malate dehydrogenase, to malate which is determined fluorimetrically. This assay was shown to be highly specific for myo-inositol with no interference from other polyols, although excess glucose has to be removed to avoid interference. This method was reported to be extremely sensitive with detection limits as low as 1 pmol in tissues such as rat serum and hamster lens, cortex and pancreatic islets.

The three carbon polyol glycerol can be specifically determined by its conversion to L-glycerol-3-phosphate by glycerokinase and a coupled reaction that exclusively converts the phosphorylated glycerol to dihydroxyacetone phosphate and NADH; with the later conversion by the enzyme glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). Reference ranges in normal human serum are reported to be between 4-8 μg/ml with assay relative standard deviations between 1-4% (Wieland 1984).

The diol ethylene glycol can be determined by the enzyme alcohol dehydrogenase (ADH, EC 1.1.1.1) which in this reaction produces glycolaldehyde and NADH. Other volatile alcohols such as methanol and ethanol also react significantly but the polyols glycerol, sorbitol and mannitol do not interfere. The relative standard deviations of the assay were found to be 5.6 and 3% at levels of 10 and 183 μg/ml respectively (Eckfeldt and Light 1984).
Enzymatic methods for the determination of polyol derivatives include a method for 2,5-anhydro-mannitol (Dills et al., 1983) and sorbitol-6-phosphate (Supp 1984). In the case of 2,5-anhydro-mannitol it is phosphorylated by hexokinase and NADH is determined by a coupled enzyme reaction in a method that is relatively nonspecific, with fructose and other analogues of the anhydro-polyol also reacting. The presence of glucose which also interferes can be removed by pretreatment of the sample with the enzyme glucose oxidase. Validation of this method was carried out by recovery studies of anhydro-mannitol spiked into rat serum. It is of interest that no endogenous levels of anhydro-mannitol were found in rat serum, indicating that this compound is not normally present.

An enzymatic method for sorbitol-6-phosphate has been reported where the phosphate group is cleaved from the C6 position by the enzyme alkaline phosphatase and the free sorbitol is then converted to fructose by SDH with detection of NADH (Supp 1984). Using appropriate blanks any endogenous sorbitol can be accounted for; but the non specific alkaline phosphatase will cleave any phosphate group regardless of its position on the sorbitol molecule.

The main problem with enzyme assays when used to determine the concentration of a substrate is one of specificity. Generally there is no such problem with samples in pure solution. Biological matrices, such as tissue homogenate, whole blood or plasma contain many other compounds, for example amino acids, carbohydrates, lipids and proteins, all of which can potentially interfere by acting as substrates or inhibitors of the enzyme. Some of these potential sources of interference can be overcome by their removal during sample preparation. For example, proteins can be precipitated by acid
or base and lipids and fats removed by their extraction with a non-polar solvent. In addition, assay conditions can be chosen to favour the maximum efficiency of a particular enzyme which has its own optimum for pH, temperature and ionic environment.

The purity of the enzyme preparation, however, is singly the most important factor in considering the specificity of enzyme assays. Crude enzyme preparations are more likely to contain substantial amounts of other enzymes. So for polyol analysis any particular dehydrogenase enzyme preparation should be substantially free of other oxidoreductase activity. Other polyols have been reported to be converted by SDH and include xylitol and L-threitol (Beutler 1984b, Goedde and Langenbeck 1984). In this case, no matter how pure the enzyme preparation of SDH, these polyols are still converted which has led some workers to rename SDH as Polyol Dehydrogenase.

Overall the advantages of enzymatic methods for the determination of substrates out weigh the disadvantages. The advantages include, cost effective, fast analysis times with automation for large sample number turnover, good precision and high sensitivity. The disadvantages include lack of specificity and only single component analysis. Enzyme assays are the method of choice for routine screening of large numbers of samples in hospital laboratories. Enzyme assays would be the method of choice if pre-purification of the biological sample rendered only the single component of interest. It is this latter consideration that in part has led to the use of chromatographic separation techniques for the analysis of complex mixtures in general and polyols in particular.
1.6.2 Chromatographic Separation of Polyols

Chromatography is a term used to describe systems by which components of a liquid mixture may be separated. Separations can be achieved by dissolving the mixture in a mobile phase, classically a liquid or a gas, and allowing this mobile phase to flow through a stationary immobile phase which is either a liquid or a solid. Separations are achieved by the relative affinity of the different solute molecules for the stationary phase which retains them to different extents. Thus, molecules which are retained by the stationary phase are separated from those which have more affinity for the mobile phase.

In liquid chromatography (LC) the mobile phase is an aqueous solution, organic solvent or a mixture of these which carries the solutes over a bed of stationary phase which can be in the form of a thin layer or packed in a column. The latter is nowadays the most popular form of LC because modern instrumentation, with continuous flow, automatic injectors and sensitive detectors, facilitates the on line quantitation of solutes as they elute from the column. Such systems together with highly efficient columns (available commercially from many sources) have now become known variously as high performance, high speed or high pressure liquid chromatography (HPLC).

With paper and thin layer chromatography (TLC) the major drawback has been the accurate quantitation of separated components. The situation for TLC is, however, changing with the progressive use of this technique coupled to the flame ionisation detector (FID) for enhanced quantitation. In addition, when used in combination with nuclear magnetic resonance (NMR), TLC can serve as a powerful qualitative method
for the initial identification of drug metabolites and for screening purposes in forensic toxicology.

There are four or five modes of operation of HPLC based upon the principles by which components are selectively retained. These modes are adsorption, partition, ion pairing, ion exchange and size exclusion. In adsorption chromatography (solid liquid chromatography) separation occurs due to interaction between the solutes and the surface of the solid porous stationary phase.

The most widely used solid phase in adsorption chromatography is silica which has polar silanol groups on its surface. Generally, eluents used for adsorption chromatography are non-polar organic solvents and non-polar solutes soluble in the eluent are separated by this means; which is often referred to as normal phase chromatography. Polar solutes can interact with the free silanol groups of silica which are slightly acidic and hence basic groups are very strongly adsorbed often resulting in their complete retention. Water present in such a system is adsorbed by the free silanols. As a result of this the irradiation of water in the system is essential for the effective elution of non-polar compounds. For some applications there is the possibility of adding small amounts of water or other polar solvents to the eluent in an attempt to partially control their presence. However, in so doing the nature of the silica surface is altered dramatically and the separations obtained can be irreproducible because of competition between the partially solvated silanol groups and the solute. It is partly due to this problem that much attention has been devoted to partition (liquid:liquid) chromatography as an alternative means of separating polar components.
In partition chromatography, separation is achieved by a liquid stationary phase which is immiscible with the eluent. The most common form that this takes is with a non-polar stationary liquid phase, such as the octadecyl (ODS, C18) phase and a polar mobile phase, such as a methanol/water mix. When used in this form partition chromatography is classically used for the separation of polar components which include many drugs and their metabolites. This is particularly useful because many modern drugs are low molecular weight, non volatile, polar compounds which when metabolised in-vivo become more polar to aid their excretion.

Originally in partition chromatography the non-polar phase was coated on either silica or some other inert porous particle. More usual now is the attachment of non-polar alkyl groups to the free silanols of silica to produce bonded non-polar phases containing alkyl chains of various lengths; typically from C5 up to C18. In normal phase (adsorption) chromatography the polar components are the last to elute if at all whereas in partition chromatography with a polar eluent the polar solutes elute first and this has led to the use of the term reversed phase chromatography. However, the advent of bonded phases for HPLC is not confined to non-polar alkyl derivatives. There is a vast range of intermediate polarities arising from the bonding to silica of other compounds which contain groups such as phenyl, cyano, nitro, amino and hydroxyl which are often situated on alkyl moities acting as spacers.

As a consequence of the various stationary phases now available partition chromatography can be used in a variety of modes where selectivity is further enhanced by modification of the eluent. In drug analysis, for example, the pH of the eluent and
the pKₐ of the drug are important since in reversed phase HPLC the non-ionised species of the drug exhibits greater retention. Thus, acidic drugs show an increase in retention as the pH of the eluent is lowered whereas under these conditions bases would show a decreased retention.

An alternative eluent modification is the addition of hydrophobic cations or anions to the polar eluent in reversed phase LC. For drugs that have positive or negative charges the addition to the eluent of an oppositely charged hydrophobic counter ion results in the formation of a neutral ion pair with the drug and an increase in retention. For example, the use of sodium salts of alkylsulphonic acids, such as sodium octylsulphonate, can be used as an ion-pair for basic drugs. Quaternary ammonium compounds, such as tetrabutylammonium salts are used as ion-pairs for acidic drugs. This form of LC is known as ion-pairing and generally the concentration of the counter ion added to the eluent is between 1 to 5 mM. With prolonged use of eluents containing pairing ions or when the ion-pair is present in high concentrations the surface of the packing material can become saturated producing a stationary phase which can act like an ion exchanger.

Ion exchange is another mode of LC separation which consists of a stationary phase containing anionic or cationic groups on the surface to which solutes of opposite charge are attracted. There are two types of widely used ion exchange column packings based on either ion exchange resins or bonded phase silicas. A third kind of ion exchange column consists of ion exchange micro beads of up to 0.1 μm diameter bonded to nonporous resin particles with a uniform radius of approximately 5 μm. The latter type of columns (patented by Dionex) have the advantages of rapid diffusion, high efficiency
and mechanical strength. These columns have been used for a wide variety of separations but so far their high cost can be prohibitive.

Size exclusion LC makes use of a stationary phase that has a controlled pore size such as rigid minute glass beads or silicas. Alternatively the stationary phase can be semi-rigid; made from copolymers such as polystyrene-divinyl benzene. When silica of controlled pore size is used adsorption effects can occur so these exclusion phases are best used as normal phase systems with organic solvents as mobile phase. In size exclusion LC solutes are separated according to their molecular size and ability to enter the pores. Larger molecules unable to enter the pores elute first and the smaller molecules are retained by specific interactions occurring within the pore. The introduction of microparticulate packings in size exclusion allows large molecules or polymers of molecular weights over 1000 to be separated. As a consequence size exclusion has been used as a technique to determine molecular size and weight and is especially useful for the characterisation of large biomolecules such as globulins, proteins and oligosaccharides.

Separation of solutes in adsorption chromatography is primarily due to hydrogen bonding between free silanol groups and the solute. Separation in partition chromatography is due to intermolecular forces, which include hydrogen bonding, induced dipole interaction, dispersion forces and specific interaction forces. In addition, in many separations irrespective of the mode of operation, there are elements of ion exchange and size exclusion which also contribute to the separation mechanism. In LC a prime requirement is that the solutes are soluble in the mobile phase and a first
indication of the type of mode to be selected can be summarised in the rule that like polarity dissolves like.

The advantage of HPLC is the wide selectivity available by choosing various combinations of stationary and simple or modified mobile phases. This is enhanced by the use of gradient elution which is particularly appropriate to bonded phase columns which are able to equilibrate quickly between the original and final amounts of binary or ternary solvent combinations. Furthermore, sensitive on line detectors can be used in sequence to add to the selectivity of the technique. The most common detectors used in HPLC are the ultraviolet-visible detector, the fluorescence detector, the refractive index detector and the electrochemical detector.

The disadvantages of HPLC include the relatively narrow pH range for bonded phases typically between 2 to 8. Non-polar bonded phases can be stripped from the support at high pH. A further disadvantage of HPLC is the lack of a sensitive detector such as the highly sensitive and specific electron capture detector (ECD) used in gas liquid chromatography. To a certain extent this problem is being overcome by the apparent technical feasibility of coupling mass-spectrometry as a detector to HPLC. Once the final problem of removing the liquid eluent before it enters the detector is solved then HPLC-MS, in terms of selectivity and detection, will be one of the ultimate chromatographic techniques limited only by its high cost.

By contrast to HPLC, gas-liquid chromatography (GLC), in more recent times less popular, is gaining favour because of the introduction of highly efficient capillary columns containing cross-linked and immobilized liquid stationary phases. These phases
cover a range of polarities from the non-polar methyl silicones to the polar carbowax types. However, the most common commercially available phases, obtained already immobilised in capillary columns, are of the non-polar to mid-polar variety. This is partly due to technical difficulties in bonding polar phases to the internal silica wall. The dimensions of capillary columns are typically 10, 25 or 50 metres in length with internal diameters of from 0.25 mm (narrow bore columns) to 0.5 mm (mega bore columns). The phase thickness, either coated or bonded onto the inside of the capillary, are usually thin 0.25 μm in narrow bore columns and anything up to 2 or 3 μm in mega bore columns. Extra strength is given to fused silica columns by coating the outside of the column with a heat resistant polymer such as polyimide.

In capillary GLC the mobile phase is an inert gas, such as helium or nitrogen, which carries the gaseous mixture through the column. Separations are carried out by injecting the sample into the inlet of the column which is held at a constant temperature in an oven. Each component of the mixture is swept towards the detector by the carrier gas and the solutes partition between the stationary and mobile phase. Molecules with the greatest affinity for the stationary phase spend a longer period in it and consequently take longer to reach the detector. The column temperature can be increased by temperature programming, to facilitate the elution of components retained in the stationary phase, which is the GLC equivalent to gradient elution in HPLC. The commonly used detectors in GLC are the universal flame ionisation detector (FID), ECD and mass-spectrometer.

The important difference between HPLC and GLC is the degree of selectivity available. In HPLC eluent modification can dramatically alter the selectivity and
therefore the separation obtained. Whereas in GLC only the stationary phase and the temperature have any significant effect on the separation. Although with capillary GLC the injection technique is an important factor in obtaining efficient separations. This is because it is technically difficult to inject directly onto the top of a capillary column particularly those that have narrow internal diameters. This problem does not occur with packed GLC columns. With HPLC there is no one universal elution system, universal detector or unified retention scale. Whereas GLC has this in the form of low polarity phases, the universal FID detector and the retention index scale.

GLC complements HPLC and is used for compounds that are readily volatilised such as organic solvents and alcohols. GLC is less suitable for those compounds that are thermally labile or of low volatility or compounds that have polar functional groups such as many drugs. However, in between these extremes there are drugs that can be separated by both chromatographic techniques and the choice depends upon the availability of detection and the ability to separate the compounds of interest.

Sample preparation of biological matrices is important in HPLC and GLC chromatographic separations. Techniques are used in order to remove sources of potential interference, to render the solute in a suitable form for a given application, to enhance detection limits, to reduce the time required for an analysis and to reduce contamination preserving the working life of the column. Sample preparation procedures include protein precipitation with or without pH adjustment, solvent extraction with or without back extraction, derivatisation and sample concentration. These can all be used in various combinations depending upon the nature of the sample, the chromatographic conditions and the type of detector used to monitor the
eluting components. Sample preparation procedures in combination with HPLC or GLC are extensively used in the determination of drugs in biological fluids as has been reviewed by Chamberlain (1985). The use of these techniques for the separation of sugars and polyols are discussed below in sections 1.6.2.1 and 1.6.2.2.

1.6.2.1 Trace Analysis of Polyols by HPLC

The analysis of polyols by HPLC is complicated by the fact that low concentrations of carbohydrates and polyols are difficult to detect by conventional techniques. Many drugs contain chromophores or fluorophores which enable them to be detected by spectrophotometry or fluorimetry. Simple carbohydrates and polyols, such as those already described, do not contain chromophores or fluorophores in the molecule and cannot be detected by spectro-fluorometric detectors. In order to enhance polyol detection one or more of the hydroxyl groups can be derivitized with a chromophore or fluorophore. One approach for the HPLC analysis of trace levels of polyols might employ either derivatisation of the sample prior to chromatography or post-column.

The derivatisation of carbohydrates and polyols, including sorbitol, has been described by Nachtman and Budna (1977) who prepared the nitrobenzoate derivatives. The derivatives of glucose, fructose and sorbitol were prepared in pyridine using nitrobenzoyl chloride and separated on a normal phase partition system using a silica column with n-hexane-chloroform-acetonitrile (10:3:2.5 v/v) as eluent. Detection of the derivatives was by UV at 260 nm with a reported increase in sensitivity of four orders of magnitude greater than refractive index. A diabetic confectionary containing sorbitol was analysed by this method which had a relative standard deviation of 2.3%.
The separation of some polyols as their nitrobenzoate derivatives has also been described by Schwarzenbach (1977) who used a modified partition system; with a polar amino-alkyl modified silica stationary phase and water-acetonitrile (1:4 v/v) as a polar eluent. The system was used to separate polyols in apple juice by direct injection of the derivatized sample onto the column. The same chromatographic conditions were used to separate and measure xylitol from glycerol in a tooth-paste. Petchey and Crabbe (1984) have shown that tissue extracts of polyols and glucose, as the nitrobenzoate derivatives, can be separated and analysed using normal phase HPLC. They found that human cataractous lens contained glucose, sorbitol and inositol at 1.01, 0.35 and 11.74 mg/g dry weight respectively. They found that ultra filtration of the homogenised tissue sample, lens, plasma or erythrocyte, provided a cleaner extract which was more easily and fully derivatized. The method was linear over the range 3-60 µg/ml with a limit of detection of 2 ng.

An alternative derivatisation of polyols was used by Dethy et al., (1984) to determine tissue concentrations of sorbitol and galactitol. Protein precipitates of rat lens, sciatic nerve and erythrocytes and human skin fibroblasts were lyophilised to dryness and derivitised with phenylisocyanate. The derivitized samples were diluted with methanol and pyridine and injected onto a reversed phase C18 column with acetonitrile phosphate buffer at pH 7.0 (60/40%). Detection was with UV at 240 nm with a limit of detection of 3 ng. The method was used to determine the concentrations in human skin fibroblasts of sorbitol and galactitol, which were found to be raised, when incubated with high levels of glucose or galactitol respectively.
Recently the polyol myo-inositol has been separated as the anthraniloyl derivative using a reversed phase system with a C8 column as stationary phase and acetonitrile-phosphate buffer as eluent, Karagacin et al., (1987). The anthraniloyl derivative was detected using fluorimetry with a detection limit of 0.1 nmoles (approximately 18 ng).

An example of the post-column derivatisation of carbohydrates has been reported by Honda et al., (1984). Carbohydrates were initially separated by gel permeation, ion exchange or partition and derivitized post column by 2-cyano-acetamide. Electrochemical detection of the carbohydrates was obtained by the ready oxidation of the derivatives at a glassy carbon electrode. The limit of detection was given as 20 pmol for glucose. The cyano-acetamide derivatisation is only applicable to reducing sugars and polyols do not react to form derivatives. Therefore, even though polyols may be present in the sample, the method is selective for reducing carbohydrates only. Other methods for the post column detection of polyols include colorimetric detection using a cuprammonium reagent, McKay et al., (1987) and conductivity detection of the ionized polyol-borate complex, Okada and Kuwamoto (1986).

By contrast to partition chromatography Ohsawa et al., (1986) used ion chromatography to measure xylitol in human saliva and serum. The method utilizes the ionization of polyols and sugars, in strong alkali which is used as the eluent, with pulsed amperometric detection. The method was linear between 0.025 and 1.0 μg/ml with a limit of detection of 0.01 μg/ml. Using the method peak levels of xylitol were found at 30 and 150 minutes in serum and saliva after oral administration of xylitol. As yet no further information is available on the specificity of polyol separation in other biological matrices with this method.
With large sample volumes containing relatively high amounts of polyols their concentrations can be rapidly determined, without derivatisation, as in the method of Dokladalova et al., (1980). The separation of sorbitol from many other polyols and carbohydrates was achieved using an Aminex column (amine modified silica) and water as the eluent with refractive index detection. Furthermore, the system can be used as an initial preparatory separation of monosaccharides from polyols. The disadvantage of this system is the inability to separate sorbitol and xylitol which have the same retention. A similar separation has been described by Brandao et al., (1980) in which sorbitol was poorly resolved from glucose using water-acetonitrile as eluent. However, the advantage of a ternary mobile phase was demonstrated, with the addition of ethanol to the eluent, which resolved sorbitol from mono and disaccharides. No other polyols were investigated for possible co-elution with sorbitol.

The difficulty of HPLC separations of polyols from carbohydrates has been investigated by Shaw and Wilson (1982) who used several different partition systems. The best separation was achieved using a carbohydrate column, which resolved glucose, fructose, sorbitol, manno-heptulose and sucrose. In addition, the authors demonstrated the use of UV detection at 197 nm for the determination of the above components in apple juice. Sorbitol was present in freshly pressed apple juice at a concentration of 0.25% (w/w) and in pear juice, depending on the type of pear, between 2 and 3%.

In summary, the liquid chromatographic separations of polyols is mostly confined to their separation from carbohydrates. Especially noticeable is the greater difficulty of separating similar polyols from each other; for example, the resolution of hexitols. Methods exist where at least two polyols can be separated but with a lack of specificity.
when other polyols of unknown identity are present in the sample. In general, polyols and carbohydrates can be modified, by introducing a chromophore into the molecule, to enhance their detection and to overcome the lack of sensitivity associated with RI detection or the lack of selective detection with low wavelength UV. The HPLC of carbohydrates and polyols has been reviewed by Robards and Whitelaw (1986).

In order to overcome problems associated with HPLC separation or detection of polyols an alternative approach is the use of gas-liquid chromatography (GLC). GLC complements HPLC for a wide variety of applications; in particular in cases where volatile compounds are not easily separated, or detected at the desired levels, by HPLC.

1.6.2.2 Trace Analysis of Polyols by Gas-Liquid Chromatography

Almost all volatile organic compounds respond to the flame ionization detector (FID). The FID has been used as a general detector in GLC, for a wide variety of compounds including alkanes, fatty acids, steroids, pesticides and many types of drugs. Consequently the FID can be used to detect most types of organic compound and as such is a universal non-selective detector. The major limitation of the FID with packed columns is a detection limit, on column, of the order of 50 ng amounts. By contrast, the electron capture detector (ECD) is a selective detector responding to electron capturing species present in the molecule presented to the detector. The most strongly electron capturing compounds are those containing halogens, nitro groups or highly conjugated systems. Certain compounds such as chlorinated pesticides and polychlorinated biphenyls are highly electron capturing and when extracted from biological matrices have on column detection limits at the low picogram level.
Alternatively compounds can be derivatised with reagents containing electron capturing species to enhance sensitivity and their selective detection in the presence of other non responding compounds.

The use of MS detection in GLC has been extensive because of the highly specific nature of the detector. The detector can be set to monitor a particular ion fragment, characteristic of the compound, for absolute specificity. Furthermore, the detector can be set to monitor the total ion fragmentation pattern, of components eluting in sequence from the GLC, to identify the nature of unknown components in the sample. The cost of these detectors does not make them readily available for routine use. However, MS detection is used in forensic science, drug metabolism, toxicology and medical research with enormous benefit to man.

Polyols require derivatisation in order to make them volatile and amenable to gas-liquid chromatographic separation processes. The choice of derivatisation reagent in GLC is important and is related to the type of detector at the outlet of the column. Derivatisation is achieved by introducing a masking moiety at each hydroxyl group producing a volatile hydrocarbon of increased molecular weight. Once carbohydrates and polyols have been derivatised the products can often be injected onto the GLC column directly, or they can be extracted from the derivatising reagents and further concentrated to obtain cleaner samples with a gain in detector sensitivity.

The difficulty of preparing volatile derivatives of polyhydroxy compounds has been investigated by Sweeley et al., (1963) who described the rapid derivatisation of this class of compounds with silylating reagents. Sugars were treated with hexamethyl-
dichlorosilazane and trimethylchlorosilane to produce the trimethylsilyl (TMS) derivatives. The TMS derivatives were separated on a variety of packed column stationary phases and in general where a single anomer was present the elution of a single peak was observed. In the case of glucose more than one peak of the TMS derivative was observed. The authors also reported that the TMS derivatives of pentitols and hexitols were less well resolved. Moreover, the corresponding acetyl derivatives could not be separated on any of the stationary phases studied: which included a range of polarity from the non-polar SE 52 to the polar Carbowax 1540 and nitrile silicone, XE 60. An example of TMS derivatisation applied to a biological matrix was reported by Clements and Starne (1975) who analysed myo-inositol from human urine as the TMS derivative on a non-polar packed column using methylmannoside as an internal standard. The method was in good agreement with a yeast bio-assay for myo-inositol.

Because of the multiplicity of derivative peaks produced from aldose anomers Sawardeker et al., (1965) reduced the aldose sugars to their corresponding alditols by sodium borohydride reduction. The alditols were treated with acetic anhydride and pyridine to produce the acetates which were found to be more polar than the corresponding TMS derivatives. In addition, the alditol acetates could not be eluted without their degradation on the polar stationary phase Carbowax 20M. The ideal polarity phase was found to be ECNSS-M (a phase consisting of ethylene glycol succinate chemically combined with a cyanoethyl silicone) and the method was reported to have an overall recovery of 98%. The use of silylation to derivatise polyols has been reviewed by Poole (1977).
Szafranek et al., (1973) separated polyols as the acetates using a glass capillary column coated with a non-polar phase. Dry sodium acetate was found to be a quicker catalyst than pyridine but the analysis time required to separate hexitols was 55 minutes. Other catalysts have been used in the formation of acetate derivatives and include; methoxylamine hydrochloride in pyridine Mount and Laker (1981) and methylimidazole Henry et al., (1983). The advantage of some of these catalysts is the apparent increased rate of acetylation, above pyridine alone, and their ability to catalyse acetylation when traces of water are present in the sample. Acetylation procedures for sugars and polyols have been reviewed by Blau and King (1977).

An example of the separation of polyols from a biological matrix is that of Popp-Snijders et al., (1983) who separated hexitols extracted from human erythrocytes as the acetates on a non-polar capillary column using galactitol as the internal standard. The polyol acetate derivatives were reported to be stable for at least three months. Furthermore, the identity of the eluted sorbitol hexa-acetate was confirmed by mass spectrometry.

In addition, to the use of TMS and acetate derivatisation are the use of electron capturing reagents for the preparation of highly volatile derivatives resulting in increased selectivity and sensitivity with ECD detection. When applied to polyols the added advantage of enantiomeric separation, using modified enantiomeric selective phases, has become apparent. Leavitt and Sherman (1982) prepared the heptafluorobutanoyl derivative of chiro-inositol and separated the D- and L-enantiomers on a capillary chiral column (Chirosil-Val) at an isothermal temperature of 115°C. In
addition, König and Benecke (1983) prepared the trifluoroacetyl derivatives of polyols and were the first to separate the D- and L-enantiomers of mannitol, arabitol and fucitol on a modified polysiloxane phase (XE 60-L-Valine-phenylethylamide) where the L-enantiomer eluted first, although unfortunately no biological examples were given. Previous reports of the non-existence of certain D- or L-enantiomers of polyols in plants (section 1.2) may well be due to the lack of suitable separations available at the time. In addition, the ability to perform such separations, due to modern column technology, demonstrates a further advantage of capillary over packed column GLC.

One of the disadvantages of packed column GLC is inefficiency in separating solutes that have increased and similar retentions. With long elution times large carrier gas flow rates are required resulting in a high pressure drop along the column. With a raised pressure drop the carrier gas flow becomes turbulent which disrupts the partition process. These factors in conjunction with finite maximum operating temperatures of the phase make some separations difficult to achieve.

Where analyses have long elution times and inefficient separation the problem can sometimes be overcome by the use of glass or fused silica capillary columns. Such columns are typically 25 metres long with internal diameters of the order of 0.1-0.3 mm. Glass capillary columns have been superceded by fused silica capillary columns; which are drawn from silica at high temperatures using fibre-optic technology, reviewed by Tarbet et al., (1988). The advantage of silica capillary columns is in the ability to coat the internal wall with intra-molecular crossed linked phases resulting in higher maximum working temperatures. Furthermore, Grob et al., (1981) were the first to demonstrate that some phases can be immobilized, by chemical linking to residual
active sites on the fused silica internal wall, resulting in greater thermal and chemical stability.

The rationale of using capillary columns is that the efficiency of a column, the height equivalent to a theoretical plate, is directly related to the diameter of the column. For a given distribution coefficient of a solute between the gas and liquid phase, which is a constant, the smaller the diameter the less the gas volume and hence an increase in the partition ratio of the solute between the stationary and mobile phase, Jennings (1978). The partition ratio can further be increased by increasing the overall length of the column and film thickness of the phase; although any gain in efficiency is cancelled by resistance to mass transfer effects for columns with film thicknesses above 1 μm, Davies (1988). Thus highly efficient columns can be obtained by decreasing the diameter, increasing the length of the column, and or increasing the film thickness of the phase. In addition, wall coated capillary columns have negligible carrier gas pressure drops along the column because the carrier gas does not flow through a packing and the height equivalent to a theoretical plate is unaffected by eddy diffusion effects.

The major problem with capillary GLC is one of sample injection. There is greater difficulty in introducing the sample onto the top of the capillary column than in the wider bore packed columns. As a consequence several different injection techniques have been devised to overcome the problem. The major injection techniques currently available are; split, splitless (with or without solvent effect), cool-on-column and programmable temperature vaporization (PTV).
Split injection employs an inert glass liner, which can be heated and through which the carrier gas flow can be directed towards the column inlet. A GLC syringe can be easily introduced through a septum to deliver the sample into the vaporizing liner. The design of the injector allows the carrier gas to be split so that the majority of the incoming carrier gas can be vented through an exhaust without continuing down the column. The carrier gas flow rate through the split vent can be adjusted to give preset split ratio's, typically 1 in 100, of flow down the column to flow through the vent. The use of split injection technique is its application to relatively concentrated samples for semi quantitative analysis; such as volatiles in petroleum. Because the sample is split the resultant initial band on the column is very narrow often resulting in very efficient separations not affected by carrier solvent. The danger is in attempting to perform quantitative analysis for sample components covering a wide boiling point range where different components are split to differing extents (Grob K. Jnr 1988). Therefore, for quantitation, the use of internal standards is mandatory when using split injection for samples containing solutes of widely differing volatility.

Splitless injection is an adaptation of the split technique where the split vent is closed during injection and opened at a given time after injection. The exact conditions depend upon the application but the technique is often used for samples where split injection does not give the desired sensitivity and where solvent effects are minimal. Injection using splitless with solvent effect is one of the definitive techniques for quantitative capillary GLC analysis. The same injection technique is used as in splitless but with the initial column temperature lower than that of the boiling point of the carrier solvent used to introduce the sample. Upon injection the sample vaporizes in the injector liner and the solvent condenses on the first one or two metres of the
column, thereby concentrating the sample onto the column. After a given period the split vent is opened to purge any excess solvent vapour and in doing so cleans the vaporizing liner. The choice of solute carrier solvent is important in this injection mode because some solvents flood the column, which spreads the initial band and leads to decreased efficiency. The advantage of the above injection techniques is that automatic samplers are commercially available.

Cool-on-column is an injection technique where the sample is introduced directly into the column via a capillary needle of smaller diameter than the column. In most cases the best results are obtained using the splitless with solvent effect column conditions where similar results are observed. However, this type of injection is difficult to automate. Parameters affecting the quantitative performance of cool-on-column and splitless injection systems have been investigated by Snell et al., (1987). Programmable temperature vaporization is a recent extension to the split and splitless technique and when used in those modes offers no advantage. However, the PTV has the distinct advantage that low boiling solute carrier solvent, such as chloroform, can be dumped, via the split vent, at a vaporizing temperature well below that of the boiling points of the solutes. The split is then closed and the temperature of the injector raised to volatilize the solutes. There is the possibility that other low boiling components, which could be of interest, will be lost in the initial solvent dumping. However, delirious solvent effects can be reduced to a minimum. The parameters affecting PTV injection of n-alkanes have been investigated by Poy (1982).

The problems associated with capillary injection systems has been extensively reviewed by Grob K. Jnr (1988) who advocates the use of retention gaps to overcome some of
the solvent flooding effects observed with various injection conditions, Grob (1982), Grob and Schilling (1987). Modern inlets for capillary GLC have been reviewed by Hinshaw (1987). Recent fundamental developments in gas chromatography have been reviewed by Clement et al., (1986). The use of GLC for the estimation of sugar alcohols in biological fluids has been reviewed by Laker (1980). The chromatography of monosaccharides and disaccharides has been reviewed by Robards and Whitelaw (1986). Table 2 summarises some of the representative uses of GLC for polyol analysis.

The use of GLC and HPLC methods for the separation and determination of almost all classes of compounds has had an enormous impact on man. This impact can be assessed by the fact that in 1988 there were 1272 GLC and 2487 HPLC abstracted publications covering all aspects of the techniques. In terms of health care modern chromatographic advances now make it possible to separate and determine, for example; individual enantiomers of chiral compounds. This enables the study of racemic mixtures, or the individual enantiomers, to be fully investigated before their use as drugs so that disasters such as thalidomide may in the future be avoided.
Table 2 Summary of Some Gas-Liquid Chromatographic Separations of Polyols

<table>
<thead>
<tr>
<th>YEAR</th>
<th>AUTHOR</th>
<th>DERIVATIVE</th>
<th>COLUMN</th>
<th>MATRIX</th>
<th>COMMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1963</td>
<td>Sweeley et al.</td>
<td>TMS more anomerisation when sugars refluxed with pyridine</td>
<td>Various packed in lab</td>
<td>Pure</td>
<td>Multiple peaks sugar anomers polyol acetates not resolved</td>
</tr>
<tr>
<td>1965</td>
<td>Sawardeker et al.</td>
<td>ACETATES refluxed</td>
<td>Various packed 10', 3% ECNSS-M</td>
<td>Pure</td>
<td>Degradation on Carbowax 20M 75 min analysis time</td>
</tr>
<tr>
<td>1971</td>
<td>Travis et al.</td>
<td>TMS</td>
<td>12', 15% glycol succinate</td>
<td>Lysed RBC</td>
<td>Hexitols not resolved</td>
</tr>
<tr>
<td>1972</td>
<td>Blau</td>
<td>TMS and ACETATES</td>
<td>Various packed OVI, OV17 ECNSS-M</td>
<td>Urine</td>
<td>GC-MS</td>
</tr>
<tr>
<td>1973</td>
<td>Szafranek et al.</td>
<td>ACETATES as catalyst</td>
<td>Glass capillary 60 m SE30</td>
<td>Pure</td>
<td>55 min for hexitols Split 5:1</td>
</tr>
<tr>
<td>1973</td>
<td>Aloia</td>
<td>TMS and ACETATES</td>
<td>Packed 3% SE30 3% poly101A</td>
<td>Serum Urine</td>
<td>Poor hexitol resolution removal of glucose by enz</td>
</tr>
<tr>
<td>1974</td>
<td>Varma and Kinoshita</td>
<td>TMS</td>
<td>Packed 3% SE30</td>
<td>Rat Lens</td>
<td>Sorbitol raised</td>
</tr>
<tr>
<td>1975</td>
<td>Heaf and Galton</td>
<td>TMS</td>
<td>Packed 7' 3% SE 30</td>
<td>Human urine lens</td>
<td>Hexitols raised Urea removed by urease enzyme</td>
</tr>
<tr>
<td>1978</td>
<td>Smith et al.</td>
<td>TMS</td>
<td>Glass capillary 30 m x 0.2 mm SE52</td>
<td>Human spinal fluid</td>
<td>GC-MS, 65 min analysis time sorbitol elutes between glucose anomers</td>
</tr>
<tr>
<td>1981</td>
<td>Allen et al.</td>
<td>ACETATE</td>
<td>Packed 7', 3% SP2340</td>
<td>Amniotic fluid</td>
<td>Ion exchange removal of glucose</td>
</tr>
<tr>
<td>1981</td>
<td>Mount and Laker</td>
<td>ACETATE</td>
<td>Packed 2 m, 3% XE60</td>
<td>Human urine</td>
<td>Hexitols not resolved</td>
</tr>
<tr>
<td>YEAR</td>
<td>AUTHOR</td>
<td>DERIVATIVE</td>
<td>COLUMN</td>
<td>MARTIX</td>
<td>COMMENT</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
<td>------------</td>
<td>--------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>1983</td>
<td>Mayhew et al.</td>
<td>TMS</td>
<td>Packed</td>
<td>Human sciatic nerve</td>
<td></td>
</tr>
<tr>
<td>1983</td>
<td>Popp-Snijders et al.</td>
<td>ACETATES</td>
<td>Capillary 25 m X 0.22 mm CpdSi15</td>
<td>Human RBC</td>
<td>GC-MS, 11 min analysis time lack of assay data</td>
</tr>
<tr>
<td>1984</td>
<td>Roboz et al.</td>
<td>ACETATES</td>
<td>Packed and capillary serum 50 m OV1701</td>
<td>Human plasma</td>
<td>GC-MS extensive validation methanol ppt.</td>
</tr>
<tr>
<td>1984</td>
<td>Johnson &amp; Mayersohn</td>
<td>TMS</td>
<td>Capillary 25 m OV1</td>
<td>Human plasma</td>
<td>Precision and recovery splitless</td>
</tr>
<tr>
<td>1986</td>
<td>Jansen et al.</td>
<td>TMS</td>
<td>Capillary 25 m X 0.22 mm OV1, 1 μ phase split 1:15</td>
<td>Human urine Washed RBC</td>
<td>25 min analysis time per sample polyols not lost during washing methanol ppt. Hexane extract to remove lipids</td>
</tr>
</tbody>
</table>

Note: TMS = trimethyl silyl derivative, RBC = red blood cell enz = enzyme, ppt = protein precipitation
1.7 The aims of the present study

The aims of this research are to establish and validate methods for the determination of sorbitol and other polyols in biological matrices. To date, no method comparison for sorbitol determination has been carried out. A particular aim, therefore, is to set up and compare an enzyme and GLC assay for the determination of erythrocyte sorbitol concentrations in samples from non-diabetic and diabetic human subjects.

Further aims of this study are to use the analytical methods to determine the erythrocyte sorbitol concentrations in diabetic patients taking part in clinical trials of the novel aldose reducase inhibitor Sorbinil. Additionally, there is a need to establish the rapidity and daily consistancy of the erythrocyte sorbitol and myo-inositol response to Sorbinil therapy.

It is anticipated that there will be considerable variation in erythrocyte sorbitol levels both in individuals and in response to therapy. Some of the sorbitol levels may be low, possibly as a consequence of Sorbinil therapy, and the analytical methods may have to be modified to cope with low concentrations. Therefore, a continuing aim of this research is to develop improved methods capable of the desired sensitivity. Furthermore, it is important that they be fully assessed in terms of performance because there is a general lack of information regarding the quality of these methods at low sorbitol concentrations.

In humans the daily variation of erythrocyte sorbitol and myo-inositol has not been investigated. It is especially important to establish a normal human range for any blood
parameter before assumptions are made concerning abnormal levels. Therefore, a further aim of this research is to determine the daily variation of erythrocyte sorbitol and myo-inositol in normal, healthy non-diabetic volunteers and to compare these levels with those in diabetic subjects.

A final aim of this research is to attempt to establish an in-vitro incubation system to examine the activity of aldose reductase in erythrocytes, from normal and diabetic subjects, with a view to its possible use as a diagnostic test. Moreover, to try to determine whether there are differences in AR activity between normal and diabetic subjects which may support the hypothesis that the underlying cause of diabetic complications is biochemical in origin.
CHAPTER 2

MATERIALS AND METHODS

2.1 General Reagents and Compounds

2.1.1 General Reagents

Acetonitrile (J T Baker, Phillipsburg), chloroform, ethyl acetate and n-butyl acetate (BDH, Poole, Dorset), methanol (Fisons, Loughborough) and diethyl ether (May and Baker, local supplier) were all of AR grade. Perchloric acid 6% was obtained by diluting 60% general reagent grade perchloric acid (BDH, Poole) with water. Distilled water was prepared in the laboratory from a Fi-streem water still (Fisons Scientific Equipment, Loughborough). Reverse osmosis purified water was obtained using an Elgastat Spectrum, (Elga products, Buckinghamshire). All compounds for making buffers were obtained in AR grade and used according to the methods in the text. Adjustment of buffer pH was followed using a model PTI pH meter (EDT Instruments Ltd, Kent). Saline solution (0.85% w/v NaCl) was obtained from Sigma or generated in the laboratory using AR grade NaCl dissolved in distilled water to the required concentration.

Krebs-Henseleit buffer consisting of 118 mM NaCl, 4.8 mM KCl, 3.2 mM CaCl₂, 1.2 mM MgSO₄, 1.0 mM KH₂PO₄, 24 mM NaHCO₃, and 1.2 mM HEPES (N-[2-hydroxyethyl]piperazine-N-[2-ethanesulphonic acid] sodium salt), pH 7.4 was made from AR grade reagents supplied by BDH.
2.1.2 Compounds

The following polyols (with their % purities in parentheses) sorbitol (99.0), galactitol (99.0), myo-inositol (99.9), d-mannitol (99.6), xylitol (99.0), ribitol, and the sugars α-(+)-glucose (94.5 with approximately 5% as the β anomer), galactose (98.2, 0.01% as glucose), d-(+)-mannose (99.0), d(-)-fructose (99.8) and d-(+)-xylose (99.0) were obtained from Sigma Chemical Co., Poole, Dorset. According to the manufacturers information the purity was obtained by packed column gas-liquid chromatography, except for ribitol which was found to be one spot by thin-layer chromatography, but no further details were available.

D-sorbitol hexaacetate (99.8%), α-d-(+)-glucose pentaacetate (99.0%), α-methylmannoside (99.9%, GLC), 1-O-methyl-α-d-glucopyranoside (99.0%, GLC) the enzyme Sorbitol dehydrogenase (activity units as specified) and ethylenediaminetetraacetic acid (EDTA, AR grade) were obtained from Sigma Chemical Co., Poole. Sorbinil, CP-45,634 (analytical standard) and CP-43,659; Sorbinil analogue for use as an internal standard (analytical standard) were gifts from Pfizer Central Research, Sandwich, Kent. Compounds used for calibration standards were accurately weighed using a model 2024MP five place balance (Sartorius GmbH, Gottingden) and made up to volume with appropriate solvent, as specified in the text.

2.1.3 Derivatising Reagents

Acetic anhydride (AR Grade) was obtained from BDH, Poole, Dorset, and stored in stoppered dark containers in a desiccator. Pyridine (AR grade) and 4-dimethyl amino pyridine (99%) were obtained from Aldrich Chemical Co., Gillingham. Pyridine was
redistilled before use and stored over KOH (AR grade, BDH) in a dessicator. 1-methyl imidazole was obtained from Sigma. Trimethyl silane and hexamethyl disilazane were obtained from Pierce Chemical Co., Ond-Beijerland, Holland. Trifluoro-acetic anhydride (AR grade) was obtained from Sigma.

Derivatisations requiring heating were carried out in a model BT3 test tube heater (Grant Instruments, Cambridge). Where specified, a 2% solution of dimethyl-dichlorosilane in 1,1,1-trichloroethane (GPR grade, BDH, Poole) was used to silylate glassware.

2.2 Instrumentation

A Perkin-Elmer LS5 fluorimeter (Perkin-Elmer Corp., Beaconsfield) with sipper cell attachment was used for enzyme assays. A thermostated water bath (Grant Instruments, Cambridge) was used for incubating the enzyme reactions. For in-vitro incubation studies of aldose reductase activity a model UE3 metabolic shaking water bath (Grant Instruments, Cambridge) was used.

Packed column gas chromatography was performed on a Pye 104 series GC with FID detection (Pye Unicam, Cambridge). Capillary GLC was carried out using either, a Sigma 3B capillary GC with FID and ECD detectors, equipped with Grob type and cool-on-column injectors, or a model 8320 capillary GC with FID detection and Programmable Temperature Vaporiser (both capillary GC instruments supplied by Perkin-Elmer Corp., Beaconsfield). The capillary GLC systems used helium (Air Products) as carrier gas. Oxygen free nitrogen (OFN) for detector make-up, hydrogen and air for FID were supplied by BOC, London. Sample injection was carried out using
1 or 5 μl plunger in needle syringes (Scientific Glass Engineering, Milton Keynes). The syringe needles were cleaned between injections using a syringe cleaner (Scientific Glass Engineering, Milton Keynes). A model CR650 chart recorder (JJ Lloyd, Southampton) or an SP4270 computing integrator (Spectra Physics, California, USA) was used to record the detector output from the packed column and Sigma 3B instruments. Capillary GLC columns of various lengths, diameters and phases were obtained from SGE, Milton Keynes, or SAC Chromatography, Hertfordshire, and the particular type of column used, along with the analytical conditions, are described in the text.

2.2.1 Capillary GLC Column Assessment

Capillary columns were installed into the capillary gas chromatographs according to the instrument manufacturers specifications. The helium carrier gas inlet pressure was adjusted to give a linear carrier gas velocity of between 30-35 cm/sec. This was approximated by measuring the time taken from the injection point to the emergence of the solvent front of a volatile solvent such as chloroform, at an isothermal column oven temperature of 130°C.

The efficiency of the columns was measured by making a 1 μl split injection of an activity mix; for example, Activity mix H (SGE) or a mix consisting of C7,8,9,10,12,14 and 16 n-Alkanes made in the laboratory. The theoretical efficiency was determined by measuring the peak width, in mm, at half height for the last eluted component of the mix, and the retention, in mm, from the injection point. The number of theoretical plates, N, was calculated according to the method of Jennings (1978) as follows:
\[
\left( \frac{\text{Retention time (mm)}}{\text{Peak width at half height (mm)}} \right)^2 \times 5.54 = N
\]

The adjusted retention time for a component was approximated by measuring the time, in mm, from the injection point to the emergence of the solvent front and subtracting this from the component retention. The number of effective plates, \( n \), was then calculated as:

\[
\left( \frac{\text{Adjusted retention time (mm)}}{\text{Peak width at half height (mm)}} \right)^2 \times 5.54 = n
\]

The resultant column efficiencies were used to compare the column manufacturers efficiency specifications obtained under similar conditions. Capillary columns of 25 metre lengths with 0.32 mm internal diameters with a phase thickness of, typically, 1.0 \( \mu m \) were used. When tested as described these columns had average \( N \) values of between 50 to 60 thousand theoretical plates resulting in a height equivalent to a theoretical plate of 0.5 mm or less.

\section*{2.3 General Laboratory Equipment}

For mixing individual samples a vortex mixer (Fisons, Loughborough) was used and where large numbers of tubes required simultaneous mixing a model 2601 multitube vortexer (SMI, Emeryville, USA) was used. Solvent extraction was performed using a home made horizontal shaker, whirlimixing or a rotary mixer (Luckham Ltd, Burgess Hill, Sussex). Separation of non miscible layers was carried out in a Mistral 6L refrigerated centrifuge (MSE, England). Solvent evaporation was carried out using a multi tube solvent evaporator with heated air bath (Robens Institute workshop) with OFN to aid solvent evaporation. Occasionally a Buchler vortex evaporator (Buchler
Instruments, USA) was used instead. A Modulyo model 4, with high vacuum pump, freeze drier (Edwards High Vacuum Co. Ltd., Crawley, Sussex) was used to obtain freeze dried samples.

Glass screwneck test tubes 100 X 16 mm with teflon lined screw caps (Corning, New York) were used for sample preparation and storage, derivatisation reactions and solvent extractions. Glass culture tubes 100 X 16 mm (Corning, New York) were used for enzyme assays. Specific precautions taken with the glass ware, such as silylation prior to derivatisation, are indicated in the text. Volumetric flasks, volumetric pipettes and graduated pipettes (FSA, Fisons Scientific Equipment, Loughborough) of various volumes were used for generating aqueous and organic standard solutions and for dispensing organic solvents. Aqueous volumes were dispensed using either 5 ml, 1 ml or 200 μl adjustable volume hand dispensers (Gilson Medical Electronics, Villiers-Le-Bel) which were calibrated on water before use. Repeat dispensing of blood or aqueous solutions was done using a repeating dispenser (Eppendorf, Hamburg) or a Falcon Pipet-Aid automatic dispenser (Beckton Dickinson, Oxnard, USA).

2.4 Blood Collection

For the generation of plasma or erythrocyte assay standards whole blood from healthy volunteers; approximately 500 ml from each donor, was obtained by venepuncture. The blood was collected into vessels containing anticoagulant; 0.5 g EDTA dissolved in approximately 10 ml of distilled water. The whole blood was separated by centrifugation in a Mistral 6L refrigerated centrifuge (MSE Scientific Instruments, Crawley) at 2000 r.p.m. and 5°C. Plasma was pooled and stored at -20°C until required and the erythrocytes treated according to the following protocol: Erythrocytes
from healthy volunteers were washed twice with ice cold isotonic saline, centrifuging between washes, and the buffy coat removed. The washed erythrocytes were then pooled and used to generate standards or for incubation studies. Methods for the generation of erythrocyte calibration standards are given in sections 3.1.2, 3.2.2.1 and 3.2.2.2. The details of the incubation studies are given in section 2.7. Packed cell erythrocyte haemoglobin concentration was determined spectrophotometrically by the method of van Kampen and Zijlstra, (1961).

Venous blood samples from diabetics, for polyol and Sorbinil determinations, was obtained by venepuncture and collected into 10 ml vacutainer tubes containing lithium heparin as anticoagulant (Beckton Dickinson, Meyland, France). The tubes were immediately placed on ice and processed, as above, as quickly as possible. Plasma was stored at -20°C until taken for analysis. Aliquots of washed erythrocytes, 1.0 ml, were dispensed into tubes containing ice cold 6% perchloric acid, vortex mixed, capped and stored at -20°C until taken for analysis. As a precaution, the erythrocyte sample preparation method for all polyol determinations was carried out as quickly as possible to minimise any possibility of alterations in the polyol content.

2.5 Determination of Plasma Sorbinil Concentrations by High Performance Liquid Chromatography

Plasma Sorbinil concentrations were determined using an adaptation of the method of Foulds et al., (1981) and was as follows: 1.0 ml of plasma sample or standard was dispensed into a 10 ml glass screw neck test tube. Using a 1.0 ml Gilson hand dispenser 1.0 ml of internal standard (5 μg CP-43,659 per ml phosphate buffer 0.25M, pH 7) was added and vortex mixed for 10 seconds. 5 ml of diethyl ether was then
added and the samples mixed on a rotary mixer for 10 minutes. The samples were
centrifuged for 5 minutes at 2000 r.p.m. at 15°C and the upper ether layer transferred
to clean 10 ml culture tubes. The ether was evaporated to dryness in a multtube
solvent evaporator under a gentle stream of OFN at 37°C. The residue was
resuspended in 100 µl of eluent and vortex mixed on a whirlimixer for 10 seconds. The
solution was transferred to a limited volume insert (Chromacol, London) and 50 µl
injected by a WISP auto sampler (Waters Associates, Milford, Massachusetts) onto the
HPLC with the following conditions: Column; Spherisorb ODS 5 µm, 12.5 cm by 5mm
(Hichrom, Reading) with a mobile phase consisting of methanol-water-acetic acid,
35:65:1 % v/v delivered at 1.0 ml/minute by an Altex 110A pump (Beckman
Instruments Inc., San Ramon, California). Detection was by a Pye LC-UV variable
wavelength detector (Pye Unicam, Cambridge) at 284 nm with the output recorded on
a JJ Lloyd chart recorder.

Stock solutions of Sorbinil (whether for calibration standards or blind spikes) were
made by accurately weighing 10 mg into 10 ml volumetric flasks and making up the
volume with methanol to give 1 mg/ml stock solutions. The working solutions used for
spiking Sorbinil into plasma were serially diluted from the stock solutions with
methanol to the required concentrations. Stock solutions of internal standard in
methanol (1 mg/ml concentrations) were generated in a similar manner and stored at
4°C. The internal standard stock solution was diluted with phosphate buffer to the
required concentration on the day.

Plasma calibration standards were prepared in the following way. Pooled, control
plasma from healthy volunteers was collected as described above. 10.0 ml plasma
aliquots were accurately dispensed into screw neck test tubes and to each was added
100 μl of 100, 200, 300, 400 and 500 μg/ml Sorbinil in methanol, via a 100 μl spiking syringe (Hamilton Co., Reno, Nevada) to give concentrations of 1, 2, 3, 4 and 5 μg Sorbinil per ml plasma. The tubes were capped, mixed well by vortexing and approximately 1.2 ml aliquots dispensed into small containers. In this way, eight sets of calibration standards were obtained and stored at -20°C until required for analysis. Plasma blind spikes, the concentrations of which were unknown to the analyst, were generated in the same way as plasma Sorbinil calibration standards. On each day of analysis, authentic samples were analysed with a set of calibration standards and blind spikes. In addition, approximately 10% of the patient samples were analysed on a second day.

2.6 Screening of Diabetic Subjects for Sorbinil Trials

Diabetic subjects for inclusion in the Sorbinil trials were recruited from the diabetic out patients clinic at St. Luke’s Hospital, Guildford, by Dr. J. Wright (consultant chemical pathologist). Patients were fully informed as to the purpose of the trial and a full medical and clinical screening performed.

Routine haematological and clinical biochemistry tests, including glycosylated haemoglobin (HbA1c) were performed by the district pathology laboratories of St. Luke’s Hospital. Patients with normal results were included in the trial. However, patients with histories of heart complaints, high blood pressure, liver disease, alcohol abuse or severely poor diabetic control were excluded from participation. Written consent was obtained from each subject willing to take part in the trial and they were free to withdraw at any time. Ethical approval for the trials was obtained from the ethical committees of the University of Surrey and St. Luke’s Hospital.
Diabetic patients taking part in the Sorbinil trials were screened for haematological and clinical chemistry parameters regularly throughout the trials: a full blood count and heart and liver function tests and a medical examination were performed weekly. Any patient with abnormal results or signs of intolerance to the drug were immediately withdrawn from the trial. Normal healthy non-diabetic subjects were recruited from the Robens Institute volunteer panel. For studies conducted on healthy volunteers they were fully informed of the purpose of the study and free to withdraw at any time.

2.7 **In-vitro erythrocyte incubation studies**

For incubation studies whole blood was collected from volunteers (both diabetic and non-diabetic subjects) and the erythrocytes prepared and washed in saline as described above (section 2.4). Pooled washed erythrocytes (20 ml) were added to an equal volume of Krebs-Henseleit buffer using a Gilson 5 ml hand dispenser. Alternatively, small volumes of washed erythrocyte samples (2 ml), obtained from individual volunteers, were added to equal volumes of Krebs-Henseleit buffer using a 1 ml Gilson hand dispenser. D(+)glucose (AR grade obtained from BDH) was added to Krebs-Henseleit buffer in 100 ml volumetric flasks. The volume was made up to the mark with reverse osmosis water to give glucose concentrations in the buffer ranging from 20-100 mM. A volume of the Krebs-Henseleit buffer containing glucose was added to an equal volume of erythrocytes to give final glucose concentrations in the incubation mixture of between 10-50 mM. The particular glucose concentrations and the reason for using them in different studies are given in the text.

To one flask containing glucose (100 mM) in buffer was also added Sorbinil to give a concentration of 20 μg/ml. Krebs-Henseleit buffer without glucose or Sorbinil acted
as the control buffer. For each time point at least two separate erythrocyte samples were prepared. For incubation studies a 30 minute equilibration time was allowed, at room temperature, followed by incubation of the samples for various times. All samples were incubated in 10 ml clean glass culture tubes at 37°C in a shaking water bath. After the appropriate incubation time each individual sample was collected, using a Gilson hand dispenser, and centrifuged at 4°C, the supernatant was removed and checked by eye for any sign of haemolysis. The erythrocytes were then washed twice with ice cold saline, centrifuging between washes, and prepared for sorbitol assay by adding 1.0 ml of packed erythrocytes to 2.0 ml 6% ice cold perchloric acid. The samples were mixed by vortexing to ensure protein precipitation which was taken to be complete when a smooth brown suspension was obtained. The precipitated erythrocyte samples from incubation studies were then stored at -20°C until taken for analysis by either the enzyme or capillary GLC assays. Details of these methods are given in the results (chapter 3).

2.8 Statistical Methods

The data obtained from these studies was treated using the Minitab (Copyright, Pennsylvania University) statistical data base on the Prime mainframe computer of the University of Surrey. The data base was used to compute means, standard deviations (SD), standard errors of the mean (SEM), paired and un-paired student t’tests and for obtaining correlation coefficients. Least squares linear regression analysis was performed using a Casio scientific calculator (for example, Casio fx-85N) and the equation of the line and correlation coefficient (r), were obtained. Other statistical tests, such as % difference from the mean, are described in the text. Plots of results were obtained using the GrafP programme on the mainframe computer, University of Surrey.
CHAPTER 3

RESULTS AND DISCUSSION

The results presented in this chapter have been arranged to approximate the order in which the work was carried out. However, there was considerable overlap between certain studies. Firstly, the enzyme method development and validation are presented (section 3.1) followed by the development and validation of a capillary GLC method for erythrocyte sorbitol (section 3.2). Attempts to improve the capillary GLC assay (which was an ongoing aim throughout the project) are then presented (section 3.2.3). This is followed by a method comparison between the enzyme and capillary GLC assays for erythrocyte sorbitol determination (section 3.3).

For continuity the Sorbinil clinical trials and the polyol normal range trial are presented together (section 3.4); although these trials were conducted after the respective method validations. Thus, Sorbinil trial No. 1 (using the enzyme method for erythrocyte sorbitol determination) was carried out at the end of the enzyme assay validation and Sorbinil trial No. 2 at the end of the capillary GLC validation. The polyol normal range trial was carried out at a later stage using the capillary GLC method with various modifications. Finally, the results from pilot studies to examine the feasibility of an erythrocyte aldose reductase assay as a possible diagnostic test for diabetic neuropathy are presented (section 3.5).
3.1 Development of an Enzyme Assay for Erythrocyte Sorbitol Determination

In order to determine suitable conditions for an enzyme assay for sorbitol the following were examined:

i. the specificity of the enzyme sorbitol dehydrogenase (SDH) and

ii. validation by assessing the linearity, accuracy and precision of erythrocyte sorbitol standards and blind spikes both within and between days.

3.1.1 Specificity of Sorbitol Dehydrogenase

The specificity of SDH was determined in the following way: Sorbitol, 282.4 mg, was accurately weighed into a boat and the contents transferred to a 250 ml volumetric flask. The volume was made up to the mark with distilled water, to give a 6.2 mM solution. Aliquots of 100, 200, 400, 600 and 1000 μl of the stock sorbitol solution were spiked into separate 10 ml volumetric flasks and the volume made up to 10 ml with distilled water. Sorbitol calibration standards were obtained by spiking 50 μl of each of the above dilutions into 0.95 ml of 6% perchloric acid (PCA) to obtain the following concentrations 3.1, 6.2, 12.4, 18.6 and 31.0 nmol/ml with 1.0 ml 6% PCA as the zero standard.

A series of xylitol calibration standards (covering the same molar concentrations as for sorbitol) were prepared by transferring 235.9 mg of xylitol into a 250 ml volumetric flask and processing the standards as described for sorbitol. Similarly, the sugars xylose, fructose, galactose and the polyols ribitol, galactitol, mannitol and myo-inositol were prepared as solutions in distilled water so that 50 μl spikes into 0.95 ml 6% PCA gave
12.4 nmol/ml solutions. Each sugar and polyol was analysed in duplicate against a sorbitol calibration curve.

The method of analysis was as follows: to each 1.0 ml standard or test sample was added 2.0 ml 6% PCA and the samples vortex mixed for 5 seconds. An aliquot of the sample was transferred to a fresh culture tube and neutralized, by the addition of 3M K$_2$CO$_3$, till the pH was between 7 and 9. Typically, for 2.0 ml of 6% PCA, 300 µl of 3M potassium carbonate was required to bring the pH to about 8. The samples were vortex mixed and centrifuged at 2000 r.p.m. for 10 minutes. Three 0.5 ml aliquots of the precipitated supernatant were quantitatively transferred to three separate culture tubes; each containing 1.0 ml glycine (50 mM)/ NAD (1.2 mM) buffer, pH 9.4. The enzyme SDH was added to two of the tubes, to provide duplicate determinations for each sample, and the third tube acted as a blank without the addition of enzyme. The samples were pre-incubated for 10 minutes, at 25°C, before the addition of 10 µl of enzyme solution; containing 2.5 units of enzyme activity.

[Note: The enzyme sample was diluted with distilled water so that 10 µl contained 2.5 units of enzyme activity. The actual dilution depended upon the specific activity of the enzyme as supplied, but was typically 4.7 units/mg solid, so that 60 mg solid was diluted in 1.128 ml of distilled water to provide the required concentration. One unit of enzyme activity was described, by the supplier, as the amount of protein required to convert 1 µmole of substrate to product per minute at 25°C and pH 9.]

The samples containing enzyme and their blanks were vortex mixed for 5 seconds, stood in a water bath, and incubated at 25°C for 30 minutes. After incubation the two samples and relevant blank were read in a fluorimeter (Perkin-Elmer, LS5) using a
sipper cell for sampling in the following manner. Glycine/NAD buffer was sampled into the cuvette using the sipper attachment (a peristaltic pump that can sample approximately 0.5 ml when activated by a switch). The fluorescence reading obtained from the glycine/NAD buffer was recorded on the digital printer and the value used to zero the instrument readout using the autozero facility. Each tube, containing enzyme, was then sampled in turn through the sipper into the cuvette and the reading recorded on the digital printer. The excitation and emission wavelengths of the fluorimeter were set at 366 and 452 nm respectively with 10 nm slit widths. The fixed scale sensitivity of the instrument was usually set to give 60-70% of the maximum output (999.9) on the highest concentration standard.

The sample blank, without enzyme, was then read in the same way and the value recorded. Thus, the two samples containing enzyme were read followed by their relevant blank. The zero was then checked by resampling spare glycine/NAD buffer and if necessary re-zeroed. In practice it was rarely found that re-zeroing on the buffer was required. The results were calculated by averaging the duplicate fluorimeter readings, of samples containing enzyme, and subtracting the relevant blank value. The adjusted fluorescence values obtained for calibration standards were subjected to least squares linear regression analysis, using a suitable calculator, and the y intercept, slope and correlation coefficient, r obtained. The results for the specificity of SDH, for the compounds tested, are shown in table 3. Plots of the sorbitol and xylitol calibration standards, in perchloric acid, are shown in figure 3.
Table 3. Substrate Specificity of Sorbitol Dehydrogenase (SDH)

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>AMOUNT ADDED (nmol/ml)</th>
<th>AMOUNT FOUND (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i</td>
<td>ii</td>
</tr>
<tr>
<td>XYLITOL</td>
<td>12.4</td>
<td>12.4</td>
</tr>
<tr>
<td>RIBITOL</td>
<td>8.7</td>
<td>10.4</td>
</tr>
<tr>
<td>GALACTITOL</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>MANNITOL</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>MYO-INOSIL</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>XYLOSE</td>
<td>0.8</td>
<td>3.6</td>
</tr>
<tr>
<td>GALACTOSE</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MANNOSE</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GLUCOSE</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FRUCTOSE</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

50 µl of substrate spiked into 0.95 ml of 6% PCA
i and ii are two separate samples analysed against a sorbitol standard curve, in 6% PCA, covering the range 0-31.0 nmol/ml. ND = none detected.
Figure 3. Calibration Curves for Sorbitol and Xylitol in 6% Perchloric Acid: Analysis by Sorbitol Dehydrogenase Enzyme Assay
3.1.2 Validation of an Enzyme Assay for Erythrocyte Sorbitol Determination

The performance of the enzyme assay for erythrocyte sorbitol concentrations was determined in the following way. Venous blood was taken from healthy volunteers and processed as described in chapter 2 to obtain pooled washed erythrocytes. As a precaution the red blood cells were maintained on ice throughout the following procedure. Erythrocytes were aliquoted, in 0.95 ml amounts, into separate tubes and 50 µl of sorbitol standards in distilled water spiked to obtain standards covering 0-31.0 nmol/ml in exactly the same way as described for the specificity study. In addition, erythrocyte blind spikes (the concentrations unknown to the analyst) were generated by another operator using the same pool of washed erythrocytes. Four sets of sorbitol calibration standards and 36 blind spikes were generated. The erythrocyte protein was then precipitated by the addition of 2.0 ml of 6% PCA to each sample, which was vortex mixed to obtain a smooth suspension. The erythrocyte calibration standards and all the blind spikes were stored at -20°C. On the day of analysis stored samples were thawed and maintained on ice, as a precaution, throughout the following procedure. Samples were vortex mixed thoroughly and centrifuged at 2000 r.p.m. for 10 minutes. Difficulty was found in removing 2.0 ml of the perchloric acid supernatant and 1.8 ml aliquots were removed and neutralised by the addition of 3M K₂CO₃; typically 150 µl of potassium carbonate being required. From this stage on the enzyme assay adopted was exactly as described for the specificity study.

One of the erythrocyte sorbitol standard curves, with a set of similar standards in isotonic saline, was analysed on the day of generation. Two of the sets of erythrocyte sorbitol calibration standards were analysed, after 1 and 4 days storage at -20°C (called
day 1 and day 4 respectively), along with half of the blind spikes on each of those days. The remaining calibration set was saved for future use.

Fluorimeter readings for each standard and sample were obtained and treated as already described. The equation of the curve for calibration standards was used to obtain the concentration of sorbitol in unknown samples. Erythrocytes contain a small amount of sorbitol already present in the matrix before the addition of known amounts of sorbitol to generate calibration standards. The concentration of this amount of sorbitol is called the endogenous level and has to be taken into account in the calculation of amounts of sorbitol in unknown samples. Therefore the results of sorbitol concentrations in unknown samples was adjusted by adding the value obtained for the endogenous sorbitol level present in the calibration standards. This latter step was unnecessary, when calibration standards were generated in pure aqueous solutions; where endogenous sorbitol is not present.

The results of least square linear regression analysis for the calibration standards in isotonic saline and erythrocytes gave r values of 0.999 and 0.990 respectively. The correlation coefficients for erythrocyte calibration standards, used to analyse blind spikes after 1 and 4 days storage (day 1 and day 4 respectively) were 0.994 and 0.998 respectively. The results for the analysis of calibration standards and a set of blind spikes, after 1 days storage (called day 1), are shown in detail in table 4. The calibration plot for these results is shown in figure 4. The results obtained for all the blind spikes, stored for 1 and 4 days (called day 1 and day 4 respectively), are shown in table 5.
Table 4. Fluorimeter Readings Obtained for Erythrocyte Standards and Blind Spikes by Enzyme Assay, after 1 Days Storage (day 1).

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>READING 1</th>
<th>READING 2</th>
<th>BLANK</th>
<th>MEAN - BLANK (nmol/ml)</th>
<th>VALUE (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard amount added</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-E</td>
</tr>
<tr>
<td>A</td>
<td>0.0</td>
<td>147.1</td>
<td>167.3</td>
<td>30.2</td>
<td>127.0</td>
</tr>
<tr>
<td>B</td>
<td>3.1</td>
<td>276.1</td>
<td>286.8</td>
<td>88.8</td>
<td>192.7</td>
</tr>
<tr>
<td>C</td>
<td>6.2</td>
<td>346.1</td>
<td>360.9</td>
<td>98.5</td>
<td>255.0</td>
</tr>
<tr>
<td>D</td>
<td>12.4</td>
<td>396.8</td>
<td>441.1</td>
<td>53.7</td>
<td>365.3</td>
</tr>
<tr>
<td>E</td>
<td>18.6</td>
<td>564.4</td>
<td>597.1</td>
<td>87.2</td>
<td>493.6</td>
</tr>
<tr>
<td>F</td>
<td>31.0</td>
<td>816.2</td>
<td>808.5</td>
<td>169.3</td>
<td>643.1</td>
</tr>
</tbody>
</table>

Blind spike sample

<table>
<thead>
<tr>
<th>No.</th>
<th>READING 1</th>
<th>READING 2</th>
<th>BLANK</th>
<th>MEAN - BLANK (nmol/ml)</th>
<th>VALUE (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>761.2</td>
<td>835.8</td>
<td>121.6</td>
<td>676.9</td>
<td>31.5</td>
</tr>
<tr>
<td>2</td>
<td>522.0</td>
<td>561.8</td>
<td>102.6</td>
<td>439.3</td>
<td>17.4</td>
</tr>
<tr>
<td>3</td>
<td>523.2</td>
<td>553.3</td>
<td>79.3</td>
<td>459.0</td>
<td>18.6</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>689.9</td>
<td>56.8</td>
<td>633.1</td>
<td>28.9</td>
</tr>
<tr>
<td>5</td>
<td>690.5</td>
<td>775.1</td>
<td>52.9</td>
<td>679.9</td>
<td>31.7</td>
</tr>
<tr>
<td>6</td>
<td>291.1</td>
<td>314.3</td>
<td>96.1</td>
<td>206.6</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>487.0</td>
<td>482.4</td>
<td>69.9</td>
<td>414.8</td>
<td>16.0</td>
</tr>
<tr>
<td>8</td>
<td>682.1</td>
<td>741.2</td>
<td>50.2</td>
<td>661.5</td>
<td>30.6</td>
</tr>
<tr>
<td>9</td>
<td>312.1</td>
<td>321.8</td>
<td>116.2</td>
<td>200.8</td>
<td>3.3</td>
</tr>
<tr>
<td>10</td>
<td>284.2</td>
<td>309.7</td>
<td>112.8</td>
<td>184.2</td>
<td>2.3</td>
</tr>
<tr>
<td>11</td>
<td>280.5</td>
<td>308.9</td>
<td>103.0</td>
<td>191.7</td>
<td>2.7</td>
</tr>
<tr>
<td>12</td>
<td>663.6</td>
<td>732.9</td>
<td>105.8</td>
<td>592.5</td>
<td>26.5</td>
</tr>
<tr>
<td>13</td>
<td>491.2</td>
<td>530.9</td>
<td>89.2</td>
<td>421.9</td>
<td>16.4</td>
</tr>
<tr>
<td>14</td>
<td>462.9</td>
<td>504.8</td>
<td>66.0</td>
<td>417.9</td>
<td>16.1</td>
</tr>
<tr>
<td>15</td>
<td>259.3</td>
<td>272.0</td>
<td>70.2</td>
<td>195.5</td>
<td>2.9</td>
</tr>
<tr>
<td>16</td>
<td>653.7</td>
<td>664.7</td>
<td>124.4</td>
<td>534.8</td>
<td>23.1</td>
</tr>
<tr>
<td>17</td>
<td>501.1</td>
<td>537.3</td>
<td>100.9</td>
<td>418.3</td>
<td>16.2</td>
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<tr>
<td>18</td>
<td>269.9</td>
<td>297.3</td>
<td>98.3</td>
<td>185.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Standard B</td>
<td>297.1</td>
<td>312.7</td>
<td>105.9</td>
<td>199.0</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Mean = mean of reading 1+2

$r = 0.994$ from least square linear regression of the standards

$E = $ Endogenous level obtained from 0 standard = 8.7 nmol/ml

Standards and blind spikes were stored for 1 day at -20°C before analysis.
Figure 4. Calibration Plot of Erythrocyte Sorbitol Concentration against Fluorescence Value after 1 Days Storage (day 1).
Table 5. Precision Data for Erythrocyte Blind Spike Analysis

DAY 1 The endogenous erythrocyte sorbitol level obtained from the calibration standards on this day was 8.7 nmol/ml.

<table>
<thead>
<tr>
<th>Added + endogenous</th>
<th>11.1</th>
<th>22.7</th>
<th>34.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>12.3</td>
<td>26.1</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>27.3</td>
<td>37.6</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>24.7</td>
<td>40.4</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>25.1</td>
<td>39.3</td>
</tr>
<tr>
<td></td>
<td>11.6</td>
<td>24.8</td>
<td>35.2</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>24.9</td>
<td>31.8</td>
</tr>
<tr>
<td>Mean</td>
<td>11.6</td>
<td>25.5</td>
<td>37.4</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.53</td>
<td>1.02</td>
<td>3.36</td>
</tr>
<tr>
<td>C.V. %</td>
<td>4.6</td>
<td>4.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Mean recovery %</td>
<td>103</td>
<td>111</td>
<td>108</td>
</tr>
</tbody>
</table>

DAY 4 The endogenous erythrocyte sorbitol level obtained from the calibration standards on this day was 6.4 nmol/ml.

<table>
<thead>
<tr>
<th>Added + endogenous</th>
<th>8.9</th>
<th>20.5</th>
<th>32.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>9.7</td>
<td>20.6</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>9.2</td>
<td>20.2</td>
<td>31.5</td>
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<td></td>
<td>8.9</td>
<td>20.5</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>20.0</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>21.1</td>
<td>30.6</td>
</tr>
<tr>
<td></td>
<td>9.6</td>
<td>19.9</td>
<td>30.6</td>
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<tr>
<td>Mean</td>
<td>9.3</td>
<td>20.4</td>
<td>30.3</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.33</td>
<td>0.44</td>
<td>0.80</td>
</tr>
<tr>
<td>C.V. %</td>
<td>3.6</td>
<td>2.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Mean recovery %</td>
<td>104</td>
<td>100</td>
<td>94</td>
</tr>
</tbody>
</table>

All results are expressed as nmol/ml erythrocyte
Results for blind spikes obtained from different calibration standards were determined in the following way. Fresh blind spikes in perchloric acid and erythrocytes were generated along with a new set of erythrocyte calibration standards from freshly collected blood. The standards and blind spikes were generated in exactly the same way as described above. These standards and samples were analysed with the remaining set of erythrocyte calibration standards; where the latter had been stored (at -20°C) for 4 weeks. The correlation coefficients for the "old" and "new" standards were 0.996 and 0.992 respectively. The results for the blind spike levels, obtained using each set of calibration standards, are shown in table 6.

Table 6. Comparison of Blind Spike Results Obtained by two Different Sets of Erythrocyte Calibration Standards

<table>
<thead>
<tr>
<th></th>
<th>&quot;OLD&quot; STANDARDS</th>
<th>&quot;NEW&quot; STANDARDS</th>
<th>% DIFFERENCE FROM MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perchloric</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blind spikes</td>
<td>9.9</td>
<td>9.2</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>2.6</td>
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<td>17.9</td>
<td>16.6</td>
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<tr>
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<td>10.0</td>
<td>9.3</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>25.7</td>
<td>23.8</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>9.9</td>
<td>8.6</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>30.3</td>
<td>29.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blind spikes</td>
<td>14.4</td>
<td>13.4</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>9.8</td>
<td>9.1</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
<td>22.3</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>19.4</td>
<td>18.0</td>
<td>1.6</td>
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<td></td>
<td>12.7</td>
<td>11.8</td>
<td>3.7</td>
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<td></td>
<td>31.5</td>
<td>29.2</td>
<td>3.8</td>
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<td></td>
<td>14.7</td>
<td>13.7</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>35.1</td>
<td>32.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Results are nmol/ml and include the endogenous level obtained from the calibration standards

% difference from the mean is calculated as

\[
\frac{\text{higher} - \text{lower result}}{\text{higher} + \text{lower result}} \times 100
\]
3.1.3 Discussion

The SDH used in these studies is a relatively impure, non-specific preparation obtained from sheep liver. The non-specific nature of this enzyme has resulted in its use to determine either sorbitol or xylitol using the relevant substrate calibration standards (Beutler, 1984a and b). The slopes of the calibration standards, for the same molar concentrations of sorbitol and xylitol, were 10.39 and 8.34 respectively (figure 3). The optimum pH for xylitol assay is 8.6 rather than 9.4, using SDH purified from sheep liver, (Beutler, 1984b) and this may account for the difference in slopes obtained. The recovery of ribitol, at 12.4 nmol/ml, was 77%. Galactitol, mannitol and xylose were not expected to act as substrates for this assay and the finding that they do so may be due to these samples containing small amounts of impurities, which are polyols, that do act as substrates. Myo-inositol and the other sugars tested did not act as substrates for this enzyme assay.

In studies where sorbitol, in biological matrices, is to be analysed by this particular enzyme preparation the possible existence of co-substrates, such as xylitol, in the matrix will have to be taken into account. In this respect, Travis et al., (1971) found that the existence of xylitol in human erythrocytes was limited to less than 2.5 nmol/ml if at all. Alternative analytical techniques, such as chromatographic separations, may have to be adopted to overcome the lack of specificity shown by this enzyme.

The use of SDH to determine sorbitol concentrations in biological matrices was first reported by Clements et al., (1969) who examined sorbitol levels in rabbit aorta and plasma. The method was adapted by Malone et al., (1980) to determine sorbitol
concentrations in erythrocyte hemolysates, and forms the basis of the method assessed here, but no precision data was reported by them.

In the current study the precision and accuracy of sorbitol blind spikes fortified into erythrocytes gave the following results. The overall C.V. for blind spikes, after 1 and 4 days storage, was found to be 4.3% with a mean recovery of 103% (table 5). The slopes and correlation coefficients of the calibration curves, on these two days, were in close agreement; 16.85 and 17.3, and 0.994 and 0.998 respectively, indicating near identical sensitivities. The blind spikes assessed three levels corresponding approximately to low, middle and high calibration points. The only other validation data for an enzyme method for erythrocyte sorbitol gave a within day reproducibility of 6.7% and a between day reproducibility of 13%, but the actual levels determined were not specified, Lapolla et al., (1985).

A limit of detection of 0.3 nmol/ml for erythrocyte sorbitol was reported by Malone et al., (1980) using 1.0-50 nmol/ml calibration standards. The limit of detection for the current method is approximately the endogenous level found on the day using the twice y intercept method described by McAinsh et al., (1981). However, using half the adjusted fluorescence value (mean reading - blank reading) of the bottom standard, approximately 65, (see table 4) gives a limit of detection of half the endogenous level; approximately 3.0 nmol/ml.

It may be possible to improve the limit of detection for the enzyme assay described in this work. The relatively high and variable blank values so far found limits the minimum detectable amounts to a value which is clearly above this background noise. Taking the data from table 4, for all 25 samples, the mean blank value was found to be 90 with a
standard deviation of 30 (CV 33%). A value of 3X the standard deviation of the background noise added to the mean noise level would give 180 as the cutoff value above which a response may be due to analyte. Computing this value into the linear regression equation obtained for the standards (table 4) gives a value of 2.0 nmol/ml as the limit of detection. This concentration is slightly lower than the value obtained by the empirical method of extrapolation from the calibration curve and represents approximately one quarter of the endogenous sorbitol level.

The accuracy between different, fresh and stored, standards was assessed by comparing the results obtained for them from a series of blind spikes. The following values for the slopes, 11.2 and 12.11, correlation coefficients, 0.996 and 0.992, and endogenous sorbitol levels, 6.9 and 5.6 (nmol/ml) were found for the old and new calibration standards respectively. The mean percent difference from the mean for perchlorate and erythrocyte blind spikes was 4.1 and 3.4 % respectively (table 6). When calculated using the stored standards, compared to calculation using fresh standards, all the blind spikes values were found to be higher with an overall recovery of 108%. Slope differences between the two calibration sets were ± 8% and can reflect differences between original calibration solutions, pools of erythrocytes, storage effects or any combination of these. However, the effect of sample storage has not been fully investigated and will require careful consideration. Sample storage effects were investigated by performing a method comparison study and the results are discussed below (see section 3.3).

Large sample numbers analyzed over several days requires several sets of calibration standards which should be prepared simultaneously using the same pool of erythrocytes. Furthermore, the standards should be stored under identical conditions to the real
samples. The standard curve slopes and endogenous levels can then be monitored to give an indication of daily changes in sensitivity and precision at or near to the limit of detection. In addition, it is important to carry out checks of standard accuracy which may help to determine any sample storage effects.

One of the possible sources of error for the erythrocyte sorbitol enzyme assay was considered to be the accuracy of pipetting washed packed erythrocytes. This was examined by accurately weighing 1.0 ml aliquots of erythrocytes repeatedly dispensed by a p1000 Gilson hand dispenser. The haemoglobin content for each dispensed sample was also determined. The mean weight of erythrocytes, for 8 samples, was 1.0516 g with a C.V. of 1.9% and the mean haemoglobin content 26.2 g/100ml with a C.V. of 1.8%. The respective results in whole blood, for 8 samples, were 1.0325 g, C.V. 1.92% and 16.2 g/100ml, C.V. 2.25%. From these results it would appear that the errors in pipetting a viscous matrix account for only a small proportion of the daily variation in endogenous sorbitol levels.

The range of standards adopted was based on literature values for human normal and diabetic erythrocyte sorbitol levels (Malone et al., 1980, Morrison et al., 1970 and Popp-Snijders et al., 1983). In particular, calibration standards are required to cover a range that would include high levels, encountered in some diabetic samples, down to low levels in normal blood. These levels are anticipated to be of the order of 25.0 down to 5.0 nmol/ml respectively. Erythrocyte sorbitol results are often expressed in terms of the haemoglobin content of washed packed red blood cells where 1 nmol/ml equals approximately 3 nmol/g haemoglobin. Thus the expected range is 15-75 nmol/g haemoglobin.
In general, each batch of enzyme allows for 100 sample analysis, including standards and duplicate determinations, with the feasibility of completing that number in a day. The enzyme assay was applied to the analysis of erythrocyte samples from diabetic patients participating in a clinical trial of the AR inhibitor Sorbinil and the results are discussed below, (see section 3.4.1).

3.2 Development of a Capillary Gas-Liquid Chromatographic Assay for Erythrocyte Sorbitol Determinations

3.2.1 Initial GLC Studies

3.2.1.1 Packed Column GLC

Preliminary studies to investigate the GLC separation of polyols were performed using packed column systems. Packed column GLC was carried out on a Pye 104 gas chromatograph with flame ionisation detection. The retention of sorbitol hexaacetate (SHA) was determined for various columns and oven temperature conditions, with oxygen free nitrogen (OFN) as carrier gas. Initially, this was done by dissolving SHA in various solvents, eg; chloroform, to give an accurate solution consisting of 1.0 mg/ml. A 1 μl injection of this solution onto, for example; a 2 m x 4 mm ID column packed with 3% SP2250 on 80-100 Supelcoport, with carrier gas at 14 ml/min and an isothermal oven temperature of 205°C, resulted in a retention time of 22 minutes for SHA. Measurement of the retention and peak width at half height for sorbitol, under these conditions, resulted in estimates for the number of theoretical and effective plates to be 3,350 and 3,000 respectively.
Several polyols were derivatised and a retention study, using the above GLC system, carried out as follows. Sorbitol, galactitol, mannitol, xylitol, ribitol and glucose were acetylated individually, and as a mixture, in the following way. Each compound was accurately weighed and prepared as a 1 mg/ml solution in water. Using a Hamilton syringe 10 μl spikes of each solution was added to separate tubes; one containing them all as a mixture, and the volume made up to 1.0 ml with distilled water. 2.0 ml 6% perchloric acid was added and an aliquot (2.0 ml) of the solution neutralised by the addition of potassium carbonate as previously described. A 1.8 ml aliquot of the neutralised perchlorate was located in fresh tubes and the contents taken to dryness, under vaccum at 70°C for 1.5 hours, in a Buchler vortex evaporater. Acetate derivatives were prepared according to the method of Popp-Snijders et al., (1983). Samples were derivatised, at 70°C for 1 hour, by the addition of 0.25 ml pyridine and 0.25 ml acetic anhydride. The reaction was stopped by the addition of 0.5 ml distilled water and 1.0 ml chloroform was added. The polyol derivatives were extracted into the chloroform by vortex mixing for 30 seconds. The samples were centrifuged, for 10 minutes at 2000 r.p.m., and the chloroform layer taken to dryness, in fresh tubes, under a gentle stream of OFN. The samples were resuspended in 100 μl chloroform and 1 μl injected onto the GC. Results of the packed column separation of acetate derivatives are shown in figure 5 and demonstrates incomplete separation of the alditols.
Figure 5. Packed Column GLC Separation of Alditol Acetates

Samples were 10 μg of each sugar derivatised and resuspended in 100 μl of chloroform with 1 μl injection onto the column
A set of erythrocyte standards, covering the range 0-10 μg/ml, containing 5 μg α-methyl mannoside as internal standard, was taken through the whole procedure; from perchlorate precipitation to acetate derivatisation. The resulting correlation coefficient for the standards was 0.994. However, the retention study showed that sorbitol, galactitol and mannitol could not be completely resolved using various column temperature programming conditions. For this method to be applied to pathological samples, where changes in hexitols other than sorbitol may occur, a much better degree of separation is required.

Packed column GLC of tri-methyl silyl derivatives of polyols was investigated, both in perchlorate and erythrocyte suspensions. Extracts containing sorbitol and internal standard were prepared and taken to dryness as described above. The method of Sweeley et al., (1963) was used to form the TMS-polyol derivatives. The correlation coefficients of calibration standards in these extracts were found to be less than 0.94 and not reproducible.

3.2.1.2 Capillary GLC of Polyols

Packed column GLC of polyols resulted in the problem of inadequate resolution of closely related hexitol acetates. Therefore, capillary GLC columns were investigated to see if the problem of hexitol resolution could be overcome. Sorbitol hexaacetate (SHA) was accurately weighed and diluted to obtain 100 and 50 μg/ml solutions in n-butyl acetate (NBA). Elution conditions for SHA were examined by injecting 1 μl onto the GLC.
An initial retention study was carried out as follows: Polyols and sugars were accurately weighed and made up in volumetric flasks to give 1.0 mg/ml solutions in distilled water. Aliquots, consisting of 5 µl of each polyol and sugar, were dispensed into separate tubes and into a tube containing them all as a mixture. The tubes were taken to dryness by vortex evaporation, acetylated, extracted into chloroform and dried down under OFN as already described. A tube taken through the whole procedure served as a reagent blank. The dried derivatives were resuspended in 100 µl of NBA and 1 µl injected onto a Perkin-Elmer Sigma 3B capillary GLC with FID detection. Retention data is shown in table 7. It was found that repeat injections of the individual sugar and polyol samples, as well as injections of the mixtures, gave consistent retention times. Separation of the hexitol acetates were almost completely baseline resolved and an example, along with the analytical conditions, is shown in figure 6.

### Table 7. Retention Data for Some Sugars and Polyols Separated by Capillary GLC

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>RETENTION (min)</th>
<th>PEAK HEIGHT (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XYLOSE</td>
<td>8.0 &amp; 8.3</td>
<td>36 &amp; 56</td>
</tr>
<tr>
<td>RIBITOL</td>
<td>9.3 &amp; 10.0</td>
<td>186 &amp; 16</td>
</tr>
<tr>
<td>XYLITOL</td>
<td>9.7 &amp; 10.0</td>
<td>141 &amp; 26</td>
</tr>
<tr>
<td>METHYL-α-D-GP</td>
<td>11.0</td>
<td>&gt;200</td>
</tr>
<tr>
<td>D(+)MANNOSE</td>
<td>12.2 &amp; 12.6</td>
<td>62 &amp; 34</td>
</tr>
<tr>
<td>D(+)GALACTOSE</td>
<td>12.6</td>
<td>59 &amp; 63</td>
</tr>
<tr>
<td>D(+)GLUCOSE</td>
<td>12.6 &amp; 12.8</td>
<td>70 (peaks fused)</td>
</tr>
<tr>
<td>MYO-INOSETOSE</td>
<td>14.3</td>
<td>81</td>
</tr>
<tr>
<td>D-MANNITOL</td>
<td>14.6</td>
<td>125</td>
</tr>
<tr>
<td>SORBITOL</td>
<td>15.0</td>
<td>102</td>
</tr>
<tr>
<td>GALACTITOL</td>
<td>15.3</td>
<td>67</td>
</tr>
<tr>
<td>SHA (50 µg/ml)</td>
<td>15.0</td>
<td>43</td>
</tr>
</tbody>
</table>

Methyl-α-D-GP = 1-O-methyl-α-D-glucopyranoside (2 mg/ml solution)  
SHA = sorbitol hexaacetate  
Analytical conditions are given in figure 6.
Figure 6. Capillary GLC Separation of Polyol and Sugar Acetates

Samples were 5 μg each of compounds derivatised and resuspended in 100 μl NBA with 1 μl injected onto the column.

Key:
1 and 2 Xylose
3 Ribitol
4 Xylitol
5 α-mGp
6 D+ Mannose (2 peaks)
7 D+ Galactose
8 and 9 D+ Glucose
10 Myo-inositol
11 D-Mannitol
12 Sorbitol
13 Galactitol

α-mGp = 1-O-Methyl-α-D(+) Glucopyranoside

Conditions:
Column 25 m X 0.32 mm (ID) BP1, 1 μ phase (S.G.E.) programmed from 110°C for 1 min at 39.9°C/min to 220°C held for 12 min with Helium carrier gas at 5.0 ml/min. Injector 250°C with the split vent opened 0.7 min after injection. FID detection range X1, amplification X16 with JJ Lloyd chart recorder (10mv) at 5 mm/min. GC, Perkin Elmer Sigma 3B with OFN makeup gas. (Retention Data is given in table 7.)
Calibration standards were generated in perchloric acid and erythrocytes, covering 0-31.0 nmol/ml, and processed to obtain the acetate derivatives as previously described. Internal standard (5 μl of 0.2 mg/ml Methyl-α-D-(+)-glucopyranoside in water) was added at the beginning of the sample workup procedure. The capillary GLC analytical conditions were as for the retention study. The results were calculated by obtaining the peak height ratio of sorbitol to internal standard and regressing the values obtained for calibration standards against their concentrations. The correlation coefficients were 0.987 and 0.959 for PCA and erythrocte standards respectively. No interference at hexitol retentions was observed in perchlorate or erythrocyte samples. The results showed some interference at the internal standard retention; which could explain the poor linearity of the standards. In addition, no peak was observed in erythrocytes at the galactitol retention which suggested the use of this hexitol as an alternative internal standard. From these initial findings a more extensive capillary GLC method validation was carried out (see section 3.2.2).

3.2.1.3 Discussion

The packed column separation of alditol acetates was first reported by Sawardeker et al., (1965) who reported complete degradation of these derivatives on the polar Carbowax 20M column. The best separations were achieved using a 10 foot column packed with ECNSS-M (Ethylene glycol succinate chemically combined with cyanoethyl silicone) with the hexitols resolving after approximately 75 minutes. Several similar packed column separations in biological matrices, such as human urine, serum and cerebro-spinal fluid, have been attempted (Blau, 1972, Mount and Laker, 1981, and Roboz et al., 1984). Some include the prior removal of glucose by the enzyme glucose
oxidase (Aloia, 1973) or by ion exchange (Allen et al., 1981). In most cases the resolution of hexitols was poor and incomplete after long retention times (40 minutes or more) which makes for lengthy, non cost effective analyses.

In the case of packed column separations of TMS-polyol derivatives difficulty has arisen in separating hexitols (Sweeley et al., 1963 and Travis et al., 1971). However, Varma and Kinoshita (1974) and Mayhew et al., (1983) have used such separations to examine polyol levels in rat lens and human sciatic nerve. As with acetylation some of these methods remove potentially interfering compounds present in the matrix, particularly glucose in blood, during sample preparation. Another example of matrix interference is the presence of urea in urine. Its removal by the enzyme urease, prior to TMS ether formation, has enabled Heaf and Galton (1975) to estimate changes in hexitol excretion in human urine.

The advent of glass capillary GLC as introduced by Golay in 1958 (Jennings, 1978) has led to some previously difficult separations being achieved. Glass capillary separations of pure polyol acetates was first described by Szafranek et al., (1973) who achieved the complete resolution of hexitols in approximately 55 minutes using a 60 metre column coated with SE 30 phase. They reported that fructose forms non-volatile acetylation products. The glass capillary separation of TMS derivatives of polyols was reported by Smith et al., (1978) who found that sorbitol from human cerebro-spinal fluid eluted between the α and β-glucose anomers without interference from these or other constituents.

Glass capillary separations, lengthy in terms of polyol elution times, have generally been superseded by the use of fused silica capillary columns. The latter offer the
additional advantages of greater chemical and thermal stability through immobilised and cross-linked stationary phases (Grob et al., 1981).

Various commercial fused silica capillary columns, available in our laboratory, were investigated and the initial hexitol separations obtained on a methyl silicone phase were encouraging (table 7 and figure 6). Due to the unsuitability of methyl-glucopyranoside as an internal standard, galactitol was substituted instead in further studies to determine assay performance. Galactitol has the added advantage that on the systems so far tested its elution was close to other hexitols and its endogenous presence in the matrix was not evident. In addition, factors such as sample preparation, column injection technique and derivatisation procedures were investigated to determine their influence on the analysis.

3.2.2 Capillary GLC Method Validation for Erythrocyte Sorbitol Determination

3.2.2.1 Sample Preparation using Vortex Evaporation

The aim of this study was to determine the within day and between day linearity, accuracy and precision of calibration standards and blind spikes generated in erythrocytes. The experimental protocol adopted was similar to that used for the enzyme assay validation. Pooled washed erythrocytes were obtained from healthy non-diabetic volunteers and prepared as described above, (see section 3.1.). Enough erythrocyte calibration standards, covering the range 0-31.0 nmol/ml, were generated to provide for 6 days analysis. Blind spikes were generated, in the same pooled erythrocytes, by another operator using a separate accurately prepared sorbitol solution. Standards and blind spikes were stored at -20ºC.
The capillary GLC sample work up procedure was as already described with the exception that on the day of analysis 100 µl of 20 µg/ml galactitol in water was added, as internal standard, to each thawed standard and sample. Neutralised perchloric supernatants were taken to dryness by vortex evaporation, then derivatised to form the peracetates, extracted into chloroform and taken to dryness. Samples were resuspended in 100 µl of NBA and vortex mixed prior to 1 µl injections onto a Perkin-Elmer Sigma 3B capillary GLC with FID. Peak heights for sorbitol and galactitol were obtained for calibration standards and subjected to least squares linear regression analysis. The equation of the curve for calibration standards was used to obtain the sorbitol concentrations in unknown samples. In total, five standard curves were analysed on separate days. Eighteen blind spikes were analysed on each of two days (36 in total). In order to obtain an indication of the sorbitol levels in diabetic blood 24 diabetic patient samples were randomised, by another operator, and 8 analysed on each of three consecutive days. These samples were also analysed by the enzyme assay and the results are discussed in detail below (see section 3.3).

The recovery and precision data obtained for blind spikes is shown in table 8, and the performance of the standard curves is summarised in table 9. The results are discussed below (see section 3.2.2.3). An example erythrocyte sorbitol calibration curve is shown in figure 7. The capillary GLC analytical conditions with example chromatograms are shown in figure 8.
Table 8. Recovery and Precision Data for Blind Spikes and Endogenous Erythrocyte Sorbitol Level

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>Endogenous level found</th>
<th>12.4 nmol/ml standard spiked</th>
<th>18.6 nmol/ml standard spiked</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% recovery</td>
<td>% recovery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6</td>
<td>103.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>104.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.8</td>
<td>102.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5</td>
<td>86.5</td>
</tr>
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<td></td>
<td></td>
<td>4.2</td>
<td>106.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.7</td>
<td>103.5</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td>3.6</td>
<td>101.2</td>
</tr>
<tr>
<td>S.D.</td>
<td></td>
<td>0.57</td>
<td>7.3</td>
</tr>
<tr>
<td>C.V. %</td>
<td></td>
<td>15.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DAY 2</th>
<th>3.1 nmol/ml standard spiked</th>
<th>6.2 nmol/ml standard spiked</th>
<th>18.6 nmol/ml standard spiked</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% recovery</td>
<td>% recovery</td>
<td>% recovery</td>
</tr>
<tr>
<td></td>
<td>85.4</td>
<td>97.7</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>110.1</td>
<td>115.8</td>
</tr>
<tr>
<td></td>
<td>91.0</td>
<td>88.4</td>
<td>110.5</td>
</tr>
<tr>
<td></td>
<td>98.9</td>
<td>124.8</td>
<td>101.0</td>
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<td></td>
<td>93.3</td>
<td>93.0</td>
<td>110.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93.0</td>
<td>107.7</td>
</tr>
<tr>
<td>MEAN</td>
<td>93.7</td>
<td>101.2</td>
<td>107.4</td>
</tr>
<tr>
<td>S.D.</td>
<td>6.0</td>
<td>13.8</td>
<td>6.3</td>
</tr>
<tr>
<td>C.V. %</td>
<td>6.4</td>
<td>13.6</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Day 1 = samples stored for two days at -20°C
Day 2 = samples stored for one week at -20°C

Table 9. Performance of Erythrocyte Sorbitol Standard Curves

<table>
<thead>
<tr>
<th>NO</th>
<th>SLOPE</th>
<th>CORRELATION COEFFICIENT</th>
<th>ENDGENOUS SORBITOL FOUND nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0419</td>
<td>0.995</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>0.0413</td>
<td>0.999</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>0.0478</td>
<td>0.993</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>0.0415</td>
<td>0.996</td>
<td>3.7</td>
</tr>
<tr>
<td>5</td>
<td>0.0413</td>
<td>0.993</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Mean endogenous erythrocyte sorbitol level = 3.5 nmol/ml, C.V. % = 19.2
Mean slope = 0.0427, C.V. % = 6.6
Figure 7. Example Calibration Curve for Erythrocyte Sorbitol Standards Analyzed by Capillary GLC

\[
y = 0.153 \\
\text{slope} = 0.041 \\
r = 0.999 \\
E = -3.7 \text{ nmol/ml}
\]
Figure 8. Capillary GLC Separations of Erythrocyte Polyols and Sugars

Key:
1 d(+)Glucose (2 peaks)
2 Myo-inositol
3 d-Mannitol
4 Sorbitol
5 Galactitol
(internal standard)

Conditions:
All conditions as for figure 6 but with initial column temperature at 110°C for 1 min and programmed at 39.9°C/min to 230°C and held for 12 min. Samples were resuspended in 100 µl NBA and 1 µl injected.

a) Zero, Endogenous Erythrocyte Calibration Standard
As figure 8a).

Conditions:
As figure 8a).

Figure 8 b). Erythrocyte Sorbitol Standard 6.2 nmol/ml
Key:
As figure 8a).

Conditions:
As figure 8a).

c). Erythrocyte Sample from a Diabetic Subject
3.2.2.2 Sample Preparation using Freeze Drying

The aim of this study was to carry out a method validation, for erythrocyte sorbitol determination, using freeze drying to obtain dry samples prior to derivatisation. Enough erythrocyte calibration standards were generated so that the between day precision of blind spikes, at three different erythrocyte sorbitol concentrations, could be determined on 6 different days. In order to facilitate rapid dispensing of calibration standards they were prepared in bulk as described below. In addition, freeze drying of the neutralised perchlorate supernatants was investigated, as an alternative to drying by vortex evaporation, to examine whether this alternative improved either the qualitative or quantitative performance of the assay.

Bulk sets of calibration standards were generated in washed erythrocytes from healthy volunteers. This was achieved by accurately weighing sorbitol to obtain a 1.0 mg/ml solution in distilled water. A volume of 0.625 ml of this solution was accurately transferred to a 250 ml volumetric flask and the volume made up to the mark with freshly prepared 6% perchloric acid to give a 2.5 μg/ml solution. Aliquots of this solution were accurately diluted with 6% PCA to obtain 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 μg/ml solutions in 100 ml volumetric flasks. Aliquots, 2.0 ml each, of these PCA standards were accurately dispensed into separate tubes. To each tube was accurately dispensed 1.0 ml of pooled, washed erythrocytes; using an Eppendorf repeating dispenser. The zero sorbitol calibration standard consisted of 2.0 ml PCA only; to which 1.0 ml erythrocytes were added. In this way erythrocytes were fortified, with PCA containing sorbitol, to produce calibration standards equivalent to 0 - 5.0 μg/ml. There were enough PCA sorbitol standards to generate up to 40 complete calibration sets. Blind spikes were generated using the same pool of erythrocytes, by another operator,
in an analogous manner. Separate sorbitol solutions were used to fortify the blind spikes. All the tubes were capped, vortex mixed to obtain a smooth suspension and stored at -20°C. Individual sets of calibration standards and blind spikes were analysed on each day; 6 in total over a 2 week period.

On the day of analysis samples were thawed and 100 μl of 25 μg/ml galactitol in water added (to each tube) as internal standard. As on previous occasions the samples were maintained on ice as a precaution. Samples were mixed, centrifuged and the perchlorate supernatant neutralised as described earlier. Aliquots, consisting of 1.8 ml neutralised extracts, were placed in screw neck tubes and the tops covered with gauze bolting cloth secured by elastic bands. The samples were frozen at -20°C for at least three hours prior to placing them in a freeze drier. The samples were freeze dried overnight with the cold trap operating at -55°C. Freeze dried samples were acetylated, extracted, dried and resuspended in 100 μl NBA as already described. Samples were injected onto a Perkin-Elmer 8320 GLC equipped with PTV injector, FID detector and automatic data handling using a GP2000 plotter. Peak height ratios of sorbitol/galactitol for calibration standards were taken from the integrator and subjected to least squares linear regression analysis. The equation of the curve, for the calibration standards, was used to obtain the sorbitol concentrations in unknown samples. The results for blind spike analysis and standard curve performance are shown in table 10, and discussed in section 3.2.2.3. The method was applied to the analysis of erythrocyte samples taken from healthy, non-diabetic subjects and these results are shown and discussed in section 3.4.3.
Table 10. Performance of Blind Spikes and Standards for Freeze Dried Erythrocyte Samples Analysed by Capillary GLC.

**BLIND SPIKE PRECISION**

<table>
<thead>
<tr>
<th>DAY</th>
<th>1.2 (+E)</th>
<th>4.0 (+E)</th>
<th>Endog (+E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>2.6</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>1.1</td>
<td>2.3</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>1.1</td>
<td>2.4</td>
<td>3.9</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>2.3</td>
<td>4.0</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>2.3</td>
<td>4.2</td>
</tr>
<tr>
<td>6</td>
<td>1.3</td>
<td>2.6</td>
<td>4.5</td>
</tr>
</tbody>
</table>

**MEAN** | 2.4 | 5.4 | 1.2
**S.D.** | 0.147 | 0.307 | 0.08
**C.V. %** | 6.1 | 5.7 | 6.7

**STANDARD CURVES**

<table>
<thead>
<tr>
<th>DAY</th>
<th>SLOPE</th>
<th>r</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.309</td>
<td>0.997</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>0.352</td>
<td>0.997</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>0.346</td>
<td>0.997</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>0.371</td>
<td>0.998</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>0.359</td>
<td>0.994</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>0.349</td>
<td>0.997</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**MEAN** | 0.347 | 1.2
**S.D.** | 0.020 | 0.075
**C.V. %** | 6.0 | 6.3

E = Endogenous erythrocyte sorbitol level obtained on the day.
The mean endogenous sorbitol level of 1.2 µg/ml is equivalent to 6.6 nmol/ml.
3.2.2.3 Discussion

The performance of the capillary GLC assay for erythrocyte sorbitol, with sample preparation using vortex evaporation or freeze drying prior to acetylation, has been assessed. The overall recovery and relative standard deviations for blind spikes were 101.5%, ± 8.7% and 101.9% ± 6.2% for the two drying methods respectively (tables 8 and 10). Both sample preparation methods gave very good linearity with correlation coefficients above 0.993 (tables 9 and 10). The relative standard deviation of the slopes of the standard curves, for both methods, was approximately ± 6% indicating consistent between day sensitivities for the two procedures.

The limit of quantitation was estimated as half the endogenous level between 2 and 3 nmol/ml erythrocytes. There is the possibility of improving the limit of detection; for example, if more sample was analysed, resuspension in a smaller volume or greater detector amplification were employed. However, the limit of detection is dependent upon the background noise of the detector. This can be estimated by performing a blank run, temperature programming in this particular application, without injecting a sample as described by Knoll (1985). Using this method the largest noise fluctuation is measured in a chart interval that is a multiple of the analyte’s chromatographic peak width at half height. The concentration for the analyte at the limit of detection ($C_{LOD}$) can then be estimated by the following equation:

$$C_{LOD} = K_{LOD} \cdot \frac{h_n \cdot C_s}{h_s}$$

where $K_{LOD}$ is a constant, $h_n$ is the largest noise fluctuation observed in the measurement interval and $C_s$ is a calibration sample of known concentration resulting
in a peak height of \( h_p \). Knoll has derived values for the constant \( K_{LOD} \) which equals 1.9718 when the peak width multiple is 10. Using this equation the limit of detection for the capillary GLC method is estimated to be 0.1 \( \mu g/ml \). In addition, Knoll has derived constants for the estimation of the limit of quantitation which is equal to 3 times the limit of detection. Thus the limit of quantitation for the current method can be estimated to be 0.3 \( \mu g/ml \) which is approximately one tenth the value when determined by extrapolation from the calibration curve.

The endogenous erythrocyte sorbitol concentrations, from different pools of washed red blood cells, averaged 3.6 and 6.6 nmol/ml by vortex and freeze drying respectively (tables 8 and 10). Vortex evaporation gave the greatest imprecision, observed at the endogenous level; C.V. 19%, compared to 6.3% by freeze drying. However, the method of bulk standard generation may also contribute to the improved precision observed for the freeze drying validation study. The importance of using the matrix endogenous level as a daily quality control parameter has been stressed by Roboz et al., (1984) who found the daily variation in endogenous serum polyols to vary by an average of 9% when samples were dried down under OFN after solvent precipitation of the proteins.

The overall recovery of sorbitol at the highest spiked calibration level compared to unextracted sorbitol hexaacetate was better than 90% for both sample preparation methods. The recovery C.V., assessed by the variation in peak height of galactitol as internal standard, was typically 30% for both drying methods and this variation reflects all stages of the assay.

Other examples of sample drying of biological matrices include protein precipitation with methanol or acetonitrile followed by drying down under OFN (Roboz et al., 1984,
Johnson and Mayersohn, 1984 and Jansen et al., 1986). Unextracted aqueous polyol solutions, at 100 \( \mu g/ml \) concentrations, taken to dryness directly by vortex evaporation were recovered quantitively. And in conjunction with the speed of this drying method was felt adequate to determine sorbitol levels in samples from a Sorbinil clinical trial (see section 3.4.2). It was noticed that un-neutralised perchlorate supernatants could not be taken to dryness within 12 hours of continual vortex evaporation. Whereas neutralised perchlorate supernatants could generally be dried within 2 hours. Therefore the neutralisation step following perchlorate precipitation is essential in this sample workup procedure.

No interference was seen at any of the hexitol retentions and the analysis does not suffer from the lack of specificity shown by the enzyme method with xylitol. However, the possibility of coelution between naturally occurring chiral polyol enantiomers, for example; chiro-inositol with myo-inositol, has still to be examined.

Capillary GLC separations of erythrocyte polyols were first reported by Popp-Snijders et al., (1983) using acetate derivatisation with mass-spectrometry detection to confirm the identity of eluting hexitols. The current method is partially based on the above although there are differences in sample preparation and calibration. Firstly, the method of Popp-Snijders splits the original precipitated erythrocyte sample (2.5 ml) into three with the third aliquot acting as a blank without the addition of galactitol as internal standard. This was particularly useful because very little endogenous galactitol (galactitol already present in the matrix) was found by them confirming the choice of this polyol for use as an internal standard.
Their method uses drying down under OFN of the neutralised perchlorate supernatant but no further information was given by them. As a consequence two alternative drying methods were investigated in the current study in order to determine the effect of this stage of sample workup on the assay performance. The evidence would support either of the three methods for obtaining dry samples prior to derivatisation. Although it would appear that of the two methods investigated in the current study freeze drying gave better results.

The division of the original sample into three (as described by Popp-Snijders) in conjunction with resuspension of the derivatised extract in 0.5 ml chloroform results in a final concentration which is more dilute than that used in the current study. In addition, the final resuspended polyol acetate derivatives (in the Popp-Snijders method) were injected at an isothermal column temperature held at 180°C. It had been noticed, during the initial set up of the current method, that polyol acetates resuspended in chloroform gave reduced peak heights with high initial column temperatures and that this could effect the ultimate sensitivity of the method. Somewhat surprisingly limits of both quantitation and detection were not reported by them. In addition, there appeared to be a lack of assay performance data particularly at low levels close to the endogenous sorbitol level. As this level is close to the limit of quantitation an indication of the assay variation at such low levels is required. This is especially needed if low sorbitol levels are expected to be produced as a result of therapy designed to inhibit aldose reductase activity.

A further difference between these two capillary GLC methods is the choice of column for the separation. The method of Popp-Snijders used a 25 m narrow bore (0.25 mm ID) column with the phase (Cp sil 5) coated on the inside. The actual phase thickness
was not quoted by them but as they encountered large predominating glucose peaks there is the danger of overloading the phase. Such overloading may affect the separation and long term performance of capillary GLC columns. As an attempt to safeguard against deleterious effects occurring due to column overload a wider bore column, generally 0.32 mm ID, with a thicker 1 μm immobilised phase was used in the current studies.

Although there are differences in methodological approach, and considering the data available thus far, the two capillary GLC methods agree well with very similar results obtained for assay performance in erythrocyte samples. Further experiments to determine assay factors, such as capillary GLC injection technique (see section 3.2.3), were designed to add data to the capillary GLC method for polyol determination already obtained by Popp-Snijders and these validation studies.

The only other example of capillary GLC separations of erythrocyte polyols, that of Jansen et al., (1986), used TMS derivatisation with FID detection on a limited number of blood samples. They did not examine any performance criteria and no precision data was reported. However, these authors found that mannose and glucose but not the hexitols, were lost by extensive sample pre-washing with saline.

The range of standards adopted was based on literature values already cited; as for the enzyme assay. The overall length of analysis is more time consuming than the enzyme method especially with freeze drying overnight. The complete analysis of 30 samples would take approximately 2 days from start to finish and is fairly labour intensive. The time can be cut down using an automatic injector allowing samples to be run unattended. Although taking considerably longer than the enzyme method the capillary
offers the advantage that other polyols can be determined from each chromatographic trace. This was investigated further when the capillary GLC method was used to determine erythrocyte sorbitol and myo-inositol concentrations in diabetics participating in a clinical trial of the aldose reductase inhibitor, Sorbinil (section 3.4.2).

3.2.3 Further Studies to Optimise the Capillary GLC Method

3.2.3.1 Capillary Column Injection Techniques

Different injection techniques can be used to introduce the sample into the capillary column. Several techniques, available in our laboratory, were investigated to determine which would be most suitable for the routine analysis of large numbers of polyol samples. Furthermore, whichever technique was found to be most appropriate would require optimisation.

The techniques available were split or splitless, cool-on-column and programmable temperature vaporisation (PTV). All of these techniques were investigated with different combinations of column temperature programming and carrier solvents. In general, acetate derivatives of blood standards were used to assess the techniques. Initially, a 10 m x 0.32 mm ID, BP1, 1 µ phase column was connected to each injector in turn and a sorbitol hexaacetate solution (50 µg/ml NBA) chromatographed under isothermal conditions with FID detection. The results are shown in table 11. Capillary injection techniques were compared using the same erythrocyte calibration standards, firstly resuspended in 100 µl chloroform and subsequently dried down under OFN and resuspended in 100 µl NBA. The results are shown in table 12.
Table 11. Comparison of Capillary Column Injection Techniques

<table>
<thead>
<tr>
<th>TECHNIQUE</th>
<th>EFFICIENCY (Effective plates/m)</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPLIT</td>
<td>2,334</td>
<td>Ratio 1:50, automatable, Used for concentrated samples, 0.1 μl injection</td>
</tr>
<tr>
<td>SPLITLESS</td>
<td>2,262</td>
<td>Used for dilute samples, automatable, 1.0 μl injection volumes</td>
</tr>
<tr>
<td>COOL-ON-COLUMN</td>
<td>1,921</td>
<td>Definitive technique, difficult to automate, 1-2 μl injection volumes</td>
</tr>
<tr>
<td>PTV</td>
<td>2,423</td>
<td>Solvent dumping possible for large injection volume probably automatable</td>
</tr>
</tbody>
</table>

Column 10 m x 0.32 mm ID, BP1, 1 μ phase operated at 200°C with helium carrier gas at 35 cm/sec.

Table 12. The Influence of Solvent Effects on the Results of Erythrocyte Calibration Standards

<table>
<thead>
<tr>
<th>STANDARD μg/ml</th>
<th>SORBITOL Peak height mm</th>
<th>GALACTITOL (IS) Peak height mm</th>
<th>PEAK HEIGHT RATIO Sorbitol/Galactitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₂ NBA</td>
<td>CHCl₂ NBA</td>
<td>CHCl₂ NBA</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.5 6</td>
<td>9.5 47</td>
<td>0.157 0.127</td>
</tr>
<tr>
<td>1</td>
<td>6 15</td>
<td>8 45</td>
<td>0.750 0.333</td>
</tr>
<tr>
<td>2</td>
<td>7.5 20</td>
<td>5.5 49</td>
<td>1.363 0.408</td>
</tr>
<tr>
<td>3</td>
<td>10.5 28</td>
<td>8.5 47</td>
<td>1.235 0.595</td>
</tr>
<tr>
<td>4</td>
<td>8 32</td>
<td>6 38</td>
<td>1.333 0.842</td>
</tr>
<tr>
<td>5</td>
<td>6 56</td>
<td>3 55</td>
<td>2.000 1.018</td>
</tr>
</tbody>
</table>

\[ y = 0.366 0.113 \]
\[ \text{Slope} = 0.309 0.176 \]
\[ r = 0.926 0.992 \]

Column 25 m x 0.32 mm ID BP1, 1 μ Phase, Injection volume 1 μl
The acetate derivative of a 5 μg/ml erythrocyte standard, resuspended in NBA, was injected in the split mode (with a split ratio of approximately 1:25) and eluted with various initial column temperatures, held for 1 minute, then programmed at 30°C/min to a final temperature of 210°C. The column used was a 25 m x 0.2 mm (ID) BP5 (phenyl methyl silicone) with a 0.25 μ phase. This column was chosen because it was thought to be a similar column to that used by Popp-Snijders et al., (1983) as already discussed (see section 3.2.2.3). The peak height of sorbitol was found to decrease with increasing initial column temperature and this effect is shown in figure 9, galactitol and myo-inositol were similarly affected.

Cool-on-column injection was performed using a 25 m OV101 column to determine the accuracy of this injection technique. A set of erythrocyte standards, without internal standard added, was injected and it was found that linear regression analysis of the standards gave a correlation coefficient of 0.95. In addition, because galactitol was not added any peak elution at this retention could be determined. It was necessary to do this because if galactitol was present to any extent the choice of this polyol as internal standard would have to be re-examined. No peak was observed to elute at the galactitol retention giving further evidence that galactitol may be a suitable choice as internal standard. Therefore, the finding by Popp-Snijders of negligible levels of endogenous galactitol present in the matrix has been confirmed in this study by the use of an alternative phase and injection technique. Example chromatograms are shown in figure 10 and it would appear that an aqueous solution of sorbitol is chromatographically pure eluting as a single peak.
Figure 9. The Relationship Between Sensitivity and Cold Trapping in the Split Mode

Chromatographic conditions are given in the text and were obtained using a Perkin Elmer Sigma 3B capillary GLC with helium carrier gas at 2 ml/min.
Conditions:

Column 25 m X 0.2 mm (ID) OV 101, 0.25 µ Phase (S.G.E. Ltd.) with helium carrier gas at 0.6 ml/min. Programmed from 120°C for 1 min at 39.9°C/min to 230°C hold for 10 min. FID detection (300°C) X1, X16 with JJ LLoyd chart recorder (10mV) at 10 mm/min. GC, Perkin Elmer Sigma 3B with Cool-on-Column injecter.

Figure 10. Chromatograms of Standards using Cool-on-Column Injection

a) Sorbitol (2 µg/100 µl water) taken to dryness by vortex evaporation, acetylated, extracted and resuspended in 100 µl NBA and 1 µl injected onto the column
1 d(+)Glucose
2 Myo-inositol
3 d-Mannitol
4 Sorbitol
5 Galactitol

Key:

Conditions:
As Figure 10a).

Figure 10 b). 5 µg/ml Erythrocyte Sorbitol Standard Without Galactitol as Internal Standard
Programmable temperature vaporisation (PTV) was assessed using a standard activity mix (in n-pentane), with the injector preheated to 250°C in the split mode. The results were similar to those obtained for conventional split type injectors with the same column. However, when the activity mix was injected with the PTV conditions set to dump solvent (initial PTV temperature 100°C) the lower boiling point volatiles, including n-tridecane, were virtually completely lost from the chromatogram (sample discrimination). This effect was observed in erythrocyte samples but the hexitols were not affected. This injection technique was used for the analysis of a series of polyol samples obtained from normal non-diabetic subjects and an example chromatogram is given in figure 15, section 3.4.3.

3.2.3.2 Discussion of Injection Techniques

The choice of injection technique used in capillary GLC depends on the type of analysis to be performed. Concentrated samples; for example, petroleum, can be analysed by making 0.1 μl injections with high split ratio's. For dilute samples containing low concentrations of solutes split injections are not recommended. The samples are better transferred completely onto the column using cool-on-column injection. However, this technique is not easily automated and requires delicate expertise in handling which is considerably labour intensive. Furthermore, applications requiring a rapid turnover of large numbers of samples are better injected automatically using split or splitless; provided the solutes are not heat labile at high volatilisation temperatures. In which case they would have to be injected by cool-on-column or possibly solvent dumping using PTV.
The choice of carrier solvent is important in splitless injection especially when using solvent effect. The disadvantage of using chloroform with solvent effect is its low boiling point, 70°C. The initial column temperature would have to be about 40°C, to obtain the concentration effect onto the column, and this increases the analysis time. Also, the recycling time for some gas chromatographs, to return to low initial temperatures, prolongs the equilibration time between injections. These factors do not produce a cost effective method. Furthermore, chloroform when used in place of n-butyl acetate as carrier solvent was seen to affect both the performance of erythrocyte standards, producing non-linear calibrations (table 12), and the sensitivity (measurable peak height). These effects have not been reported by others.

Butyl acetate is not an ideal solvent for splitless injection (Grob, K. Jr., 1983) because it has the disadvantage of spreading through the first metre or so of non polar columns, (Saravalle, et al., 1983). This effect can be overcome by using retention gaps at the capillary inlet (Grob, K. Jr., 1982 and Grob, K. and Schilling, B., 1987) but when used in this laboratory no advantage was observed for columns of 25 m or more in length. Moreover, the large n-butylacetate (NBA) solvent band produced by splitless (with solvent effect) did not affect the separation of hexitols; where retentions were dependent on the length of column, type and thickness of phase and column temperature programming. The advantages of using NBA are the higher initial oven temperature, typically 110°C to obtain the concentration effect, and much reduced recycling and equilibration times; which all combine to produce a shorter more cost effective analysis. In addition, the performance of the standard curves does not appear to be affected by this solvent (table 12).
The large NBA solvent band obtained by splitless injection is reduced when a split injection is made using cold-trapping column temperature programming (column programming as for splitless with solvent effect). Split injection, with samples resuspended in NBA, resulted in reduced hexitol peak heights, as expected, when compared to concentrating the sample with splitless. This effect was even more apparent with high initial column temperatures where less cold trapping occurs (figure 9) even when low split ratios were used. Therefore, split injections with high initial oven temperatures should be avoided for analyses requiring trace determinations. The results of investigations into capillary GLC injection techniques are summarised below in table 13.

Table 13. Summary of Findings for Capillary GLC Injection Techniques

<table>
<thead>
<tr>
<th>INJECTION TECHNIQUE</th>
<th>DECREASED SENSITIVITY WITH</th>
<th>INCREASED SENSITIVITY WITH</th>
<th>COMMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPLIT</td>
<td>High initial column</td>
<td>Low initial column</td>
<td>? Low split ratio’s with solvent</td>
</tr>
<tr>
<td></td>
<td>temperatures using</td>
<td>temperatures if using</td>
<td></td>
</tr>
<tr>
<td></td>
<td>chloroform and NBA</td>
<td>chloroform</td>
<td></td>
</tr>
<tr>
<td>SPLITLESS</td>
<td>Chloroform not a good</td>
<td>NBA a reasonable</td>
<td>Beware of column overload solvent</td>
</tr>
<tr>
<td></td>
<td>choice unless very</td>
<td>choice but floods</td>
<td></td>
</tr>
<tr>
<td></td>
<td>low initial column</td>
<td>non-polar phases</td>
<td></td>
</tr>
<tr>
<td></td>
<td>temperature</td>
<td>higher initial column temp</td>
<td></td>
</tr>
<tr>
<td>COOL-On-COLUMN</td>
<td>As for splitless</td>
<td>As for splitless</td>
<td>Labour intensive</td>
</tr>
<tr>
<td>PTV</td>
<td>Loss of solutes with NBA</td>
<td>Chloroform or even lower</td>
<td>Inaccurate temperature control when</td>
</tr>
<tr>
<td></td>
<td>and solvent dumping</td>
<td>boiling point solvents but</td>
<td>programming can be used for normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>solutes must be soluble in solvent</td>
<td>split/less</td>
</tr>
</tbody>
</table>
The particular studies envisaged in this work will require as much detector sensitivity as possible: because it is anticipated that diabetic erythrocyte sorbitol concentrations could be reduced by treatment with aldose reductase inhibitors. There is the additional requirement of a high turnover of large numbers of samples with the eventual aim of providing automated sample injection. Therefore, sample injection using splitless with solvent effect is probably a reasonable compromise; resulting in most of the sample transferring to the column without undue loss of detector sensitivity.

3.2.3.3 Investigations of Polyol Derivatisation

A study of the relationship between acetylation and time for the derivatisation of sorbitol and galactitol was performed, (acetic anhydride and pyridine, 0.25 ml each was used). Individual tubes containing 20 and 10 µg sorbitol and galactitol respectively were incubated at 100°C in an oven for various times; from 0 to 60 minutes up to 6 hours. Samples, at each time point, were extracted with chloroform containing myo-inositol hexaacetate as unextracted standard. No difference in sorbitol or galactitol recovery was found from between 15 minutes up to 6 hours.

Different amounts of pyridine were used to catalyse acetylation of dried PCA extracts containing 5µg sorbitol and 2.5 µg galactitol. No difference was found, in the peak height ratio's for sorbitol to galactitol, when the reaction was catalysed with either 50, 100, 150 or 200 µl pyridine. When biological sample extracts were acetylated as already described discoloration was observed. The degree of discoloration, which was less when smaller amounts of pyridine was used, had no effect on polyol recovery from spiked samples. No difference in recovery was found between new pyridine used
straight from the bottle and the same pyridine distilled before use. As a precaution, however, freshly distilled pyridine, stored over KOH, was used in all studies.

Two peaks were observed, both on packed and capillary GLC, for the acetylated single α-D-glucose anomer. Commercially obtained α-D-glucose pentaacetate gave only one peak indicating that mutarotation of α-D-glucose was occurring under the acetylation conditions used. Acetylated fructose was observed to break down producing several small peaks on the capillary system making quantitation of this sugar impossible, also observed by Szafranek et al., (1973).

Single sample extractions with chloroform were found to extract polyols completely. Silylating of glassware, prior to their use for reactions, was found to be unnecessary there being no qualitative or quantitative differences to clean tubes.

Alternative catalysts for acetylation were examined. These included N-methyl imidazole and 4-dimethyl aminopyridine. Acetylation with N-methyl imidazole, using the method of Wachowiak and Conners (1979) was not successful. Polyols could not be extracted from the dark brown oily residue that resulted. No such problem was reported by Henry et al., (1983) who used this alternative catalyst. Acetylation with 4-dimethyl aminopyridine (DMAP) was carried out on erythrocyte and plasma samples. Using the method of Höfle et al., (1978) it was found that the reaction was complete in 15 minutes at 40°C with very good recovery. A set of erythrocyte standards acetylated with DMAP as catalyst gave 95% recovery with a correlation coefficient of 0.992. In blank plasma samples a small peak at the galactitol retention can be seen but sorbitol is hardly discernable and example chromatograms are shown in figure 11.
Figure 11. Capillary Separations of Plasma Polyols and Sugars - Acetylation using DMAP Catalyst

Key:
1 Unknown from DMAP
2 d(+)-Glucose
3 Unknown
4 Unknown
5 Myo-inositol
6 d-Mannitol
7 Sorbitol
8 Galactitol

Conditions:
Column, 25 m X 0.32 mm (ID) BP1, 2 μ phase (S.A.C. Ltd.) programmed from 110°C for 1 min at 39.9°C/min to 240°C hold for 20 min with helium carrier gas at 1.4 ml/min. Injector slitless (280°C) with vent opened 1 min after injection. FID detection X1, X8 with J Lloyd chart recorder (10mV) at 5 mm/min. GC: Sigma 3B.

Samples extracted in 0.5 ml NBA and 1 μl injected onto the column. Sample recovery is greater than for the same standard injected with an initial temperature of the column at 160°C (fig 11b).

a) 1.0 ml plasma standard with 5μg each sorbitol and galactitol
Key:

As Figure 11 a).

Conditions:

As Figure 11 a) except initial temperature of the column was 160°C for 1 min.

**Figure 11 b).** The same 5 μg/ml plasma standard as fig 11 a) but injected with initial column temperature at 160°C
Key:
As Figure 11 a).

Conditions:
As Figure 11 b).

Figure 11 c). Blank plasma standard
Acetylation using trifluoroacetic anhydride (TFA) to produce the trifluoroacetyl derivatives of polyols was attempted. TFA acetylation using the method of Shapira (1969) on small amounts of pure polyol was not successful. The method of Ando and Yamakawa (1971) was used to produce the hexakis derivative of sorbitol which eluted after 5.4 minutes on a non polar capillary column programmed from 110°C for 2 minutes at 10°C/min to 150°C with FID detection. Some components were seen to elute after the sorbitol and when hexitol mixtures were derivatised a considerable number of extraneous peaks was observed.

3.2.3.4 Discussion of Derivatisation Procedures

Acetylation of sugars and polyols using acetic anhydride and pyridine, in various proportions and under various reaction conditions, has been widely used (Blau and King 1977). Pyridine is used because it is an excellent solvent for sugars, polyols and their acetate derivatives, and it acts as a base for protons produced by the reaction. The production of two peaks for a single anomer of glucose has been reported (Blau and King 1977). To avoid this problem sugars are often chemically reduced to their corresponding alditols which when acetylated elute as single peaks. This latter approach cannot be adopted in the present studies because glucose present in the blood would be reduced to sorbitol. An alternative is to carry out the reaction overnight at room temperature or for 24 hours on ice - both very time consuming - which results in the formation of a single peak. This was unnecessary in this study, because no interference between the two glucose peaks and the hexitols was observed for any capillary separation so far investigated. This result can be compared to TMS derivatisation where sugars are known to produce multiple peaks which can have considerably different retentions on the same column, (Smith et al., 1978). There is the
possibility of coelution of peaks derived from both polyol and sugars which could affect quantitation of the compound under investigation. Furthermore, polyol acetate derivatives are more stable than the TMS ethers (Popp-Snijders et al., 1983) and this was a further factor in choosing to pursue capillary separations of acetates.

Pyridine has male specific toxic effects and its use should be limited and carefully carried out. DMAP has been found to be a viable alternative; catalysing acetylation by $10^4$ times pyridine (Höfle et al., 1978, Wachowiak and Conners, 1979). DMAP was investigated for its usefulness as a polyol acetylation catalyst and the initial results were encouraging. Another distinct advantage of DMAP is that the reaction time is rapid at room temperature and upon completion the reactants can be evaporated off without having to resort to solvent extraction. However, before DMAP can be adopted in place of pyridine an extensive method validation in biological matrices would have to be performed. Dry sodium acetate was used as an alternative to pyridine by Szafranek et al., (1973) who reported its faster catalytic action. However, the application was performed on pure compounds and its catalytic effect on biological extracts remains to be determined.

The trifluoroacetyl derivatives of polyols and sugars are much more volatile than the acetates. The reaction was carried out using 0.25 ml each of ethyl acetate and trifluoroacetic anhydride. The presence of extraneous peaks, which may interfere with hexitol retentions, is probably due to impurities in the solvent and emphasises the need to use very pure reagents. Separations of TFA derivatives of hexitols have recently been reported for cyanopropyl bonded capillary columns by Haga and Nakajima (1988). These authors achieved excellent separations of the pure hexitols with elution times of up to 16 minutes. Possible interference from glucose and other components in
biological matrices was not investigated. Nevertheless, the principle has been established that TFA derivatisation could be an alternative approach. This is significant if extra sensitivity is required which can be gained using electron capture detection instead of FID.

3.3 Method Comparison Between the Enzyme and Capillary GLC Assays for Erythrocyte Sorbitol Determinations

Some samples were analysed by both the enzyme and capillary GLC methods and the results compared. Samples were collected from diabetic patients attending the out-patients clinic at St. Lukes Hospital, Guildford. The samples were stored on ice and treated immediately on arrival back at the laboratory. The red blood cells were washed and processed as described previously with the exception that 2.0 ml erythrocyte aliquots from each sample were added to 4.0 ml 6% ice cold PCA. Pooled normal non-diabetic blood was processed to obtain calibration standards using the same volumes of blood and PCA. The samples and standards were stored at -20°C until taken for analysis. For the enzyme analysis 1.8 ml aliquots of thawed sample were taken and analysed by the method already described for the enzyme validation study (see 3.1.1). The remainder of the sample was analysed by the capillary GLC method with drying by vortex evaporation as detailed above (see 3.2.2.1). The results are shown in table 14 and the correlation between them (r value) is not particularly high at 0.85. Furthermore, all the capillary results gave lower concentrations than the enzyme results. One explanation for this is that the enzyme result gives a falsely high value possibly because another component in the matrix also acts as a substrate for the enzyme.
<table>
<thead>
<tr>
<th>ENZYME</th>
<th>CAPILLARY GLC</th>
<th>% DIFFERENCE FROM MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.4</td>
<td>8.3</td>
<td>6.2</td>
</tr>
<tr>
<td>14.6</td>
<td>14.4</td>
<td>0.7</td>
</tr>
<tr>
<td>14.6</td>
<td>10.3</td>
<td>17.3</td>
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<tr>
<td>10.1</td>
<td>10.0</td>
<td>0.5</td>
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<td>12.4</td>
</tr>
<tr>
<td>19.6</td>
<td>14.4</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Results are nmol/ml erythrocyte
% DIFFERENCE FROM MEAN calculated as
\[
\frac{\text{Enzyme result} - \text{Capillary result}}{\text{Enzyme result} + \text{Capillary result}} \times 100
\]

A further comparison was made on a random selection of diabetic samples left over from patients who had participated in an aldose reductase clinical trial. The original analysis, by capillary GLC, was performed near to the date of collection. The subsequent enzyme assay was performed on duplicate samples which had been stored at -20°C for several months. The results are shown in table 15.

The mean % difference from the mean for the method comparison on the same sample was +8.8%, from the enzyme to capillary assay, with the enzyme giving consistently higher results than the capillary assay (table 14). This result is not unexpected considering the non-specific nature of the SDH enzyme. The two methods are significantly different when tested using student t test, p < 0.07, and are presumed to reflect true systematic differences in the assays. The levels found were all between
approximately 9 and 20 nmol/ml. Furthermore, from the validation studies the precision at these levels is better than 5% for both methods. These values are all above levels found in normal non-diabetic pooled erythrocytes and are in agreement with the diabetic range found by Malone et al., (1980) and Popp-Snidjers et al., (1983).

Table 15. Method Comparison on Duplicate Samples Originally Assayed by Capillary GLC and Stored Before Enzyme Assay

<table>
<thead>
<tr>
<th>1st ASSAY CAPILLARY GLC</th>
<th>2nd ASSAY ENZYME</th>
<th>% DIFFERENCE FROM MEAN Capillary - Enzyme</th>
</tr>
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<tbody>
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<td>12.1</td>
<td>10.6</td>
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<td>15.6</td>
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<tr>
<td>2.7</td>
<td>2.4</td>
<td>5.9</td>
</tr>
<tr>
<td>6.0</td>
<td>7.3</td>
<td>-9.8</td>
</tr>
<tr>
<td>6.8</td>
<td>7.2</td>
<td>-2.9</td>
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<td>9.9</td>
<td>3.9</td>
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<tr>
<td>6.7</td>
<td>6.8</td>
<td>0.7</td>
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<tr>
<td>8.3</td>
<td>7.1</td>
<td>7.8</td>
</tr>
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<td>13.8</td>
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<tr>
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<td>-4.0</td>
</tr>
<tr>
<td>6.8</td>
<td>8.6</td>
<td>-11.7</td>
</tr>
</tbody>
</table>

Results are nmol/ml erythrocyte
1st Assay by Capillary GLC
2nd Assay by Enzyme after storage at -20°C

% DIFFERENCE FROM MEAN calculated as

\[
\text{\% Difference from mean} = \frac{1\text{st Assay} - 2\text{nd Assay}}{1\text{st Assay} + 2\text{nd Assay}} \times 100
\]

Method comparison on duplicate clinical trial samples, where the enzyme assay was performed after several months sample storage, gave a mean % difference from the mean of -0.5% from the capillary to enzyme assay (table 15). The results obtained by both methods were not statistically significant when subjected to student t test (p, > 0.9) neither does there appear to be any particular bias towards one method.
The method comparison studies showed that there is a small, systematic difference between the values obtained from the same sample by the capillary and enzyme assays. However, there appears to be no difference between the results obtained by both methods on replicate clinical trial samples. The enzyme analysis of the replicate samples was carried out after prolonged storage. In an earlier experiment using the enzyme assay the results for freshly generated blind spikes were greater when calculated using values obtained from standards stored for a month (see 3.2.1). One possible explanation for these results is that there is a component in the matrix which scores to about 10% on the enzyme assay. Therefore what is observed is the concentration of this component decreasing slightly upon storage. The exact nature of this component is unknown but could be a polyol other than sorbitol. A storage trial, with analysis by the capillary method, may help to elucidate whether this phenomena is repeated and solely attributed to sorbitol.

To date this is the only method comparison to be carried out on sorbitol analysis and it may help to provide useful data, in the future, for determining the choice of assay to be adopted in particular studies. The only other literature data on polyol method comparisons is that of Soyama and Ono (1988) who investigated plasma D-arabinitol by an enzyme and a GLC method. Correlation between the two methods was good (0.99) but the enzyme method gave higher results due to mannitol acting as a substrate as well as arabinitol.

In summary, it is concluded that the enzyme and capillary GLC methods developed for the analysis of erythrocyte sorbitol are suitable to determine this component with a reasonable degree of accuracy. Both methods have been found to give consistent linear calibrations over the expected range of sorbitol concentrations in erythrocytes.
However, there is a need to determine the reproducibility of the methods when using them for the analysis of authentic samples. This was done when both these methods were applied to the analysis of erythrocyte samples from diabetic patients taking part in clinical trials of the AR inhibitor Sorbinil. The results of these clinical trials and the performance of the assays for sorbitol determination are presented in the following section.

3.4 Clinical Trials

3.4.1 The Effect of Oral Sorbinil on Erythrocyte Sorbitol Levels in Diabetic Subjects: Trial No.1. Sorbitol Determination by Enzyme Assay

3.4.1.1 Trial Design and Methods

The aim of the study was to determine the effect of oral Sorbinil, 250 mg daily, on erythrocyte sorbitol levels in diabetic subjects. The sorbitol levels were determined using the enzyme assay and an additional aim was to assess the precision of that method.

The trial was a double-blind placebo controlled study over 8 weeks with a two week run-in and two week run-out period when patients received placebo. Between these periods patients received either Sorbinil 250 mg daily or placebo daily for 4 weeks. Thus the study lasted 8 weeks in total. Diabetic patients were recruited from the out-patient clinic of St. Lukes Hospital, Guildford.
Blood samples were taken at the end of each week by venepuncture and separated to obtain plasma and washed erythrocytes. Plasma was saved for Sorbinil analysis to confirm compliance with medication. Erythrocyte sorbitol concentrations are often reported in nmol/g haemoglobin. Therefore packed cell haemoglobin determinations were performed on washed erythrocytes so that their sorbitol concentrations could be referenced to the packed cell haemoglobin content. Perchlorate precipitates of erythrocytes and plasma samples were stored at -20°C until taken for assay. At each time point enough blood was taken and processed so that at least two replicate erythrocyte sorbitol samples were generated. In addition, blood was taken each week for the determination of haematological and clinical chemistry parameters. The percentage of glycosylated haemoglobin (HbA1c) was also determined for each patient at each time point. The SDH enzyme assay, as described above (section 3.1.2) for the validation study, was used without modification to determine erythrocyte sorbitol concentrations. Blind spikes were assayed with every set of samples and standards to determine day to day precision.

3.4.1.2 Trial Results

A total of 19 patients were recruited for the trial of which 16 successfully completed the protocol and 3 were withdrawn. Of the withdrawn patients one sustained a broken leg, another had suspect liver function tests and the third developed signs of an adverse reaction to the drug. In all other patients the drug or placebo was well tolerated.

The trial results are shown in figure 12, and show a drop in erythrocyte sorbitol levels during weeks 3-6 for the Sorbinil treated group compared to the group receiving placebo. This drop is significant, p < 0.001, and even more so if two high results from
a Sorbinil treated patient (No. 7, week 3 and 5) are omitted. However, re assay confirmed the two high values found in this individual so they remain included in calculations. Therefore, the overall fall in erythrocyte sorbitol, observed in the treated group during weeks 3-6, is of the order of 40% from the corresponding placebo level.

Figure 12. Placebo and Sorbinil Treated Erythrocyte Sorbitol Levels - Clinical Trial No.1

Sorbinil was given orally at a daily dose of 250 mg.

*p, < 0.001 between Sorbinil and placebo groups for weeks 3-6 and week 7

All results are expressed as nmol/g Haemoglobin
In all 10 patients received placebo and 9 Sorbinil. The three patients withdrawn from the study were found to belong to the Sorbinil treated group and none of their parameters are included in any calculations. There was no clinical evidence of changes in diabetic control during the course of the study. Glycosylated haemoglobin (HbAlc) levels were monitored weekly and did not change on Sorbinil treatment. The values for all patients (± SD) were respectively, 12.0% ± 4% and 11.9% ± 3% at the start and end of the study. However, very poor diabetic control was observed in some patients as indicated by high blood glucose and HbAlc values. This was particularly evident in the individual with spuriously high sorbitol levels, referred to above, whose HbAlc over the 8 week period was 17.5% ± 1.5%.

Sorbitol assays were performed on 9 separate days and the erythrocyte standard curves monitored on each of those occasions. The correlation coefficients were all better than 0.990, the mean slope was 11.6 with a C.V. of 15.8%, and the mean endogenous sorbitol level was found to be 6.3 nmol/ml with a C.V. of 8.5%.

Blind spikes were generated to determine precision at 4 different levels equal to 3.7, 5.0, 20.5 and 24.8 nmol added /ml erythrocyte and the total number of determinations made at each level were 4, 6, 4 and 6 respectively. The precision at these levels gave C.V.% values of 17.2, 9.0, 8.8 and 5.9 respectively, and corresponding mean recoveries of 118, 100, 107 and 103%. The blind spikes overall gave a mean recovery of 107% with a C.V. of 10.2%. However, when daily differences in endogenous sorbitol levels are taken into account the overall blind spike recovery was 105% with a C.V. of 12.8%. The overall assay performance is in good agreement with the values obtained in the validation study.
In addition, sorbitol determinations were carried out on two different days on approximately 14% of the samples. The overall % difference from the mean between the two assays was found to be 13%. This value includes one sample near to the limit of detection (values below the endogenous level) and one sample where the two determinations were above the upper range of the standard curve. If the duplicate results for these two samples are excluded then the overall % difference from the mean was found to be 9.7%. However, these two results are not excluded from any calculations.

Compliance with medication was confirmed by analysis of the plasma samples from all Sorbinil treated patients. The average value for the treatment period was found to be 14.2 ± 0.8 µg/ml (result ± SEM). The Sorbinil levels in the treated group fell to a mean value of 1.9 ± 0.6 µg/ml at week 7, when treatment had ceased, with trace levels detectable in some patients at the end of the study. The Sorbinil method gave good linear correlation coefficients for each day of analysis and the precision was found to be ± 9.4% by repeat sample analysis with a limit of detection of 0.2 µg/ml.

3.4.1.3 Discussion of Results

The compound Sorbinil, CP-45,634, has been found to be a potent inhibitor of aldose reductase in human placental tissue and various diabetic animal models (Peterson et al., 1979, Finegold et al., 1983, Gillon et al., 1983 and Yue et al., 1984). The pharmacokinetics of Sorbinil in non-diabetic humans was first investigated by Foulds et al., (1981) who found it to be rapidly absorbed, with peak serum levels strongly correlated with dose, and a long biphasic half life of elimination of 38-51 hours. After oral administration of 250 mg daily for 10 days to healthy subjects the mean peak
serum concentration was found to plateau at 10.6 μg/ml. Similar results were found in the present study where the level of Sorbinil was found to average 12.6 μg/ml after 7 days oral administration to diabetics at the same dose level. In addition, detectable levels of Sorbinil were found in some diabetics, after the two week washout period, confirming a long half life for the drug. However, it still remains to be determined if there is any significant difference in Sorbinil pharmacokinetic profiles between normal non-diabetic and diabetic subjects.

Previous clinical trials of Sorbinil in diabetics has concentrated on its effect upon motor nerve conduction velocity. Sorbinil was found to improve motor nerve conduction velocity to a small extent in 39 stable diabetics where the beneficial effect was seen to be diminished upon cessation of treatment (Judzewitsch et al., 1983). This effect has been observed, at the same dose (250 mg daily), by Fagius et al., (1985) and Guy et al., (1988) who both conclude that the benefit of Sorbinil is less in diabetic patients with established neuropathy.

The effect of Sorbinil on human erythrocyte sorbitol levels in a clinical trial of diabetics was first reported by Malone et al., (1984) who used a modification of the enzyme assay of Clements et al., (1969) to monitor sorbitol levels. They demonstrated a suppression of erythrocyte sorbitol of approximately 50% from placebo levels on treatment with 250 mg oral Sorbinil daily. The results obtained by them are in very good agreement with our findings. However, in the current study, diabetics were recruited irrespective of their degree of glycaemic control; which in some cases was very poor. Whereas, in the Malone study all the diabetic volunteers were males with very good glycaemic control; with a mean HbA1c of 10%. Thus the 40% suppression of sorbitol levels, observed in our study, may be a reflection of an overall poorer level
of glycaemic control which in turn increases the chances of excess glucose conversion to sorbitol.

One criticism of the Malone Study is the complete absence of any assay data for the erythrocyte sorbitol determinations. Assay performance is crucial to the interpretation of experimental results obtained from the analytical method used. The performance of the enzyme assay was examined in the current study to give an indication of its suitability to this type of clinical trial. In general the performance of the enzyme assay was found to be the same as obtained in the validation study (see section 3.1.2). The day to day precision of the endogenous level was found to be 8.5% which is acceptable for these low levels which tend towards the limit of quantitation. Accuracy standards, fortified by another operator (blind spikes), gave an overall accuracy to within 5% of the expected levels. The greatest imprecision of these standards was found at low concentrations close to the endogenous level. The mean lowest sorbitol levels in the Sorbinil treated group corresponded to about 7.4 nmol/ml and the precision at this level was found to be 9%. The precision above this level was found to be the same or better. In addition, the day to day reproducibility was found to average 13%. Consequently, it is believed that the performance of the enzyme assay was reliable enough to indicate that the fall in erythrocyte sorbitol (observed in the Sorbinil treated group and not seen in placebos) was due to inhibition of the AR enzyme and not attributable solely to variations in the analytical method.

The fact that two independent laboratories obtained similar clinical trial results (our study and that of Malone et al.) could be interpreted as further evidence for the sorbitol hypothesis. However, whether the erythrocyte sorbitol model truly reflects biochemical events in less accessible tissues remains unclear. To elucidate the
erythrocyte model further requires the analysis of other intracellular components; in particular myo-inositol. This could be achieved by using capillary GLC which has the potential for measuring several components in the sample. This technique was used in a second clinical trial where diabetic patients were given a reduced daily dose of oral Sorbinil. The reason for using a lower Sorbinil dose was the finding, in some studies (for example Jaspan et al., 1985) including our own, of poor drug tolerance by approximately 11% of treated diabetic patients. Therefore the polyol hypothesis, and the erythrocyte sorbitol model in particular, could be further tested by both a lower dose of Sorbinil and by the use of an alternative analytical technique to measure the response of polyols to the decreased dosing regimen.

3.4.2 The Effect of Oral Sorbinil on Erythrocyte Sorbitol and Myo-inositol Levels in Diabetic Subjects: Trial No.2. Analysis by Capillary GLC

3.4.2.1 Trial Design and Methods

The aim of the study was to determine the effect of oral Sorbinil given at two doses on erythrocyte sorbitol and myo-inositol concentrations in diabetic subjects. The sorbitol and myo-inositol levels were determined by capillary gas-liquid chromatography and a further aim was to determine the accuracy and precision of that method. An additional aim was to establish a range for erythrocyte sorbitol and myo-inositol concentrations in healthy, normal non-diabetic subjects.

The trial design was a double blind placebo controlled study consisting of a one week run-in, followed by a two week treatment, and a final one week run-out period. During the treatment period patients received either, oral Sorbinil 125 mg daily for the first
week stepped up to 250 mg daily for the second week, or placebo throughout. All patients received placebo during the run-in and run-out periods. The trial lasted 28 days with a further sample taken on day 35 for the determination of residual drug levels. Diabetic patients were recruited from the outpatient clinic at St. Lukes Hospital, Guildford.

Blood samples were taken at the beginning of the study (day 0) and every week day during the treatment period (days 7-11, 14-18) and at the start (day 21) and end of the run-out period (day 28). At each time point enough blood was collected by venepuncture to generate duplicate samples for the capillary GLC assay. Additional blood was taken each week for the determination of haematological and clinical chemistry parameters. In addition, HbA1c was determined in each patient at the start and end of the study. On sample days patients attended the Robens Institute, University of Surrey, where the blood was collected and immediately processed to obtain plasma and erythrocyte perchlorate precipitates. Plasma samples were saved for Sorbinil determinations to confirm compliance with medication. In addition, blood samples were collected from healthy, normal, non-diabetic volunteers to establish a normal range for erythrocyte sorbitol and myo-inositol. These samples were processed in exactly the same way as for diabetic samples. All samples were stored at -20°C until taken for assay.

Sorbitol and myo-inositol acetate derivatives were analysed by the capillary GLC method following vortex evaporation to obtain dry samples. The method, as described for vortex drying in the validation study, was used without modification with the exception that myo-inositol was added to the sorbitol calibration standards in the following way. Accurate solutions of myo-inositol in water covering the concentrations
0-100 μg/ml were prepared and 100 μl spikes of each concentration added to the erythrocyte sorbitol standards, in ascending order, to give 2-10 μg/ml levels. In this way the highest sorbitol calibration standard was also the highest myo-inositol standard. Zero standards contained 1.0 ml erythrocytes and 2.0 ml 6% PCA only. Erythrocyte blind spikes were generated containing sorbitol and myo-inositol; the concentrations of which were unknown to the analyst. Erythrocyte standards and blind spikes were stored at -20°C until taken for analysis. Packed cell haemoglobin determinations were made on all washed erythrocyte samples. A random selection of these samples were determined in duplicate and the overall within day precision found to be ± 2%.

3.4.2.2 Trial Results

A total of 13 patients were recruited to the trial and 11 completed the protocol successfully. Two patients were excluded from the trial because of significant deviations from the protocol. A total of 8 patients received Sorbinil and 3 placebo. No evidence was found of any adverse reaction to Sorbinil and both drug and placebo were well tolerated. The results for erythrocyte sorbitol and myo-inositol levels in the treated group are shown in figures 13a and b and are given in detail in table 16.

The results show a drop in erythrocyte sorbitol levels, from 26.6 to 10.2 (nmol/g Hb) between day 7 and 8, for patients who received a single 125mg oral dose of Sorbinil (table 16). This drop is highly significant, p < 0.0001, representing a fall of approximately 62% and has not been demonstrated in previous Sorbinil trials. There was a further fall in sorbitol levels to the lowest mean value of 5.7 found on day 17 after three consecutive doses of 250 mg Sorbinil. There was no significant difference
in the overall mean sorbitol levels for the Sorbinil treated group between the first and second week of active therapy, p > 0.35.

Figure 13 a. Daily Erythrocyte Sorbitol Levels in Sorbinil Treated Diabetics

Figure 13 b. Daily Erythrocyte Myo-inositol Levels in Sorbinil Treated Diabetics
Table 16. Sorbinil Trial No.2 Erythrocyte Sorbitol and Myo-inositol Levels in Sorbinil Treated Diabetics

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<thead>
<tr>
<th>Day</th>
<th>Placebo run-in</th>
<th>Sorbinil 125 mg daily</th>
<th>Sorbinil 250 mg daily</th>
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</thead>
<tbody>
<tr>
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<tr>
<td>Day 7 - 13</td>
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<td>Day 14 - 20</td>
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<td>Day 21 - 35</td>
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**SORBITOL (nMOL/g HAEMOGLOBIN)**

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<th>9</th>
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<td>8</td>
<td>7</td>
<td>11</td>
<td>11</td>
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</tr>
</tbody>
</table>

--- Sorbinil 125 mg daily --- Sorbinil 250 mg daily ---

**MYO-INOSITOL (nMOL/g HAEMOGLOBIN)**

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<th>16</th>
<th>17</th>
<th>18</th>
<th>21</th>
<th>28</th>
<th>35</th>
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<tbody>
<tr>
<td>Mean</td>
<td>141</td>
<td>140</td>
<td>130</td>
<td>119</td>
<td>124</td>
<td>125</td>
<td>130</td>
<td>132</td>
<td>119</td>
<td>125</td>
<td>136</td>
<td>130</td>
<td>146</td>
<td>141</td>
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<tr>
<td>SEM</td>
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<td>12</td>
<td>8</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>11</td>
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<td>9</td>
<td>8</td>
<td>11</td>
<td>7</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>11</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
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<td>7</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

Days 0, 7, 28 and 35 include placebo values and are compared to days 8-21 where * p < 0.0001
No significant drop in erythrocyte sorbitol levels was observed in any of the three patients receiving placebo. The lowest individual placebo level was 15 with an overall mean value (± SD) of 38.1 ± 17.6 nmol/g Hb. The two patients that were excluded from the trial, because of deviations from the protocol, were found to belong to the placebo group. Consequently, the actual number of placebo treated patients is small and it is unfortunate that not enough patients were recruited to the trial to protect against such exclusions occurring.

The erythrocyte myo-inositol concentrations were not altered by Sorbinil treatment at either dose and there was no significant difference between treated and placebo groups. However, a true comparison can only be made if there were more subjects belonging to the placebo group. Consequently only the average myo-inositol levels can be examined. The overall mean myo-inositol level (± SEM), for all diabetics, was found to be 138 ± 3.0 nmol/g Hb. The % haemoglobin in the glycosylated form was found to be unchanged from the start to finish of the study. The values (± SEM) were 10.6% ± 1.1% and 10.0% ± 0.7% and not significantly different p > 0.6.

In order to obtain an indication of the erythrocyte sorbitol and myo-inositol concentrations in healthy, non-diabetic subjects whole blood was collected by venepuncture from 11 volunteers. A small portion of the blood was separated for glucose determinations and all the subjects were found to have normal glucose levels except for one individual who was found to have an abnormally low level of blood glucose. (Subsequent clinical investigation of this subject confirmed the low blood glucose level for which no cause has so far been found). The blood was treated to obtain the perchlorate precipitates as already described and analysed with trial samples. Results for the erythrocyte sorbitol and myo-inositol levels in normal, healthy
non-diabetic volunteers is summarised in table 17. However, not enough samples were taken to establish a full normal range for non-diabetics and this was examined in more detail in a separate study (see section 3.4.3).

Table 17. Normal Range for Erythrocyte Sorbitol and Myo-inositol in Healthy Non-diabetic Subjects

<table>
<thead>
<tr>
<th>SORBITOL</th>
<th>MYO-INOSITOL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>nmol/g Hb</strong></td>
<td><strong>nmol/g Hb</strong></td>
</tr>
<tr>
<td>MEAN</td>
<td>20.0</td>
</tr>
<tr>
<td>SEM</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>11</td>
</tr>
</tbody>
</table>

Analysis of plasma for Sorbinil showed compliance in the treated group where levels were found to rise steadily from the onset of therapy. The mean Sorbinil levels are compared in figure 14, to the corresponding mean erythrocyte sorbitol level and there was a negative correlation of -0.86 between the two. No residual Sorbinil was detected in any sample at day 35. The capillary GLC assay was assessed by the daily performance of the standard curves, blind spikes and repeat analysis and these results are summarised in table 18. Example chromatograms from a Sorbinil treated diabetic subject are shown in figure 15 and compared to a chromatogram from a healthy, normal non-diabetic subject.
Figure 14. Comparison of Mean Erythrocyte Sorbitol and Mean Plasma Sorbinil in Diabetic Subjects
Table 18. Performance of Capillary GLC Assay for Erythrocyte Sorbitol and Myo-inositol Determination.

<table>
<thead>
<tr>
<th></th>
<th>SORBITOL</th>
<th></th>
<th>MYO-INOSITOL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLOPE</td>
<td>r</td>
<td>E</td>
<td>SLOPE</td>
</tr>
<tr>
<td>MEAN</td>
<td>0.042</td>
<td>0.996</td>
<td>5.26</td>
<td>0.257</td>
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<tr>
<td>S.D.</td>
<td>0.003</td>
<td>0.002</td>
<td>0.71</td>
<td>0.020</td>
</tr>
<tr>
<td>C.V.%</td>
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</table>

**BLIND SPIKES**

<table>
<thead>
<tr>
<th>MEAN RECOVERY %</th>
<th>100.6</th>
<th>98.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.V. %</td>
<td>11.8</td>
<td>10.7</td>
</tr>
<tr>
<td>n=11</td>
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</table>

**REPEAT ASSAY**

<table>
<thead>
<tr>
<th>OVERALL % DIFFERENCE FROM MEAN</th>
<th>10.9</th>
<th>9.0</th>
</tr>
</thead>
</table>

*r* = Correlation coefficient from least squares linear regression  
*E* = Endogenous level in nmol/ml erythrocyte
Key:
1 d(+)Glucose
2 Myo-inositol
3 d-Mannitol
4 Sorbitol
5 Galactitol
(internal standard)

Conditions:
Column 25 m X 0.32 mm (ID) BP1, 1 μ (S.G.E. Ltd.) programmed from 110°C for 1 min at 39.9°C/min to 230°C hold for 11 min with helium as carrier gas at ≈4 ml/min. Injector (275°C) with the split vent opened 0.7 min after injection. FID detection (300°C) X1, X8 with data recording using a Spectra Physics SP4270 computing integrator at 5 mm/min. GC: Sigma 3B.

Figure 15. Example Chromatograms of Erythrocyte Polyols from a Diabetic Patient Treated with Sorbinil and a Healthy, Normal Non-diabetic Subject

a) Normal non-diabetic subject. Notice the shape of the myo-inositol peak (peak number 2) in this normal blood compared to the diabetic Samples resuspended in 100 μl NBA and 1 μl injection volume
Key:
As Figure 15 a).

Conditions:
As Figure 15 a).

Figure 15 b). Diabetic patient day 21 after the last day of Sorbinil treatment
As Figure 15 a).

Conditions:
As Figure 15 a).

Key:
As Figure 15 a).

Figure 15 c). Same diabetic patient (as in b above) at day 35 two weeks after the end of Sorbinil treatment
3.4.2.3 Discussion of Trial Results

Due to the adverse reactions to Sorbinil observed in about 10% of diabetic patients Martyn et al., (1987) investigated the extended use of 125 mg oral Sorbinil daily over a 6 month period. Their study was primarily designed to observe changes in peripheral nerve function but no improvement was seen during treatment with Sorbinil. Furthermore, measurement of erythrocyte sorbitol levels, after 3 and 6 months of Sorbinil therapy, had fallen to within and below the normal non-diabetic range; with average values of 3 nmol/ml. Erythrocyte sorbitol was only determined at these two time points and no information was available on the speed of erythrocyte sorbitol response or its daily variation with respect to either Sorbinil or placebo therapy.

The second Sorbinil trial conducted at the Robens Institute was designed to address the latter questions of the initial and subsequent daily response of erythrocyte sorbitol and myo-inositol to Sorbinil therapy at lower doses. Myo-inositol was specifically included to see whether its response changed upon therapy in any way which might reflect the pattern of events in less accessible tissues.

The results of this second trial showed that erythrocyte sorbitol levels in Sorbinil treated diabetics fell after the first day of drug administration (table 16). Exactly how quickly within the first 24 hours this occurs has not yet been established. Myo-inositol, however, was unchanged upon Sorbinil treatment but significantly different to the levels observed in normal non-diabetic subjects. The difference was significant, p<0.001, and approximately 20% greater for diabetics (mean 138 nmol/gHb) than normal non-diabetics (mean 114 nmol/gHb, table 17). The myo-inositol levels in diabetic
erythrocytes might reasonably have been expected to be lower before the onset of Sorbinil therapy (first week run-in period with placebo treatment) and rising to a higher level during Sorbinil therapy. If this had occurred then the erythrocyte polyol changes would model the polyol changes observed in animal models, particularly the streptozotocin induced diabetic rat. However, the evidence from this trial does not appear to support that hypothesis. A similar conclusion was reached by Popp-Snijders et al., (1984) who measured the erythrocyte sorbitol and myo-inositol changes in diabetics receiving 200 mg oral Sorbinil daily.

The capillary GLC analysis was carried out on 11 different days and found to perform very well. Standard curves for calibration standards for sorbitol and myo-inositol gave excellent linearity with r values for both polyols greater than 0.993. The precision of the endogenous level was found to have a C.V. of 7 and 17% for sorbitol and myo-inositol respectively. The endogenous levels of myo-inositol are quite high approximately five times the endogenous sorbitol level. Detection at the high endogenous myo-inositol levels is not a problem and does not account for the imprecision seen. The accuracy was assessed by blind spikes generated at several concentrations and the recoveries for sorbitol and myo-inositol were found to be 100 and 102% respectively. Reproducibility was found to give mean % differences from the mean of 11 and 9% for sorbitol and myo-inositol respectively. All the repeat diabetic samples were found to have values for sorbitol and myo-inositol close to the respective endogenous levels found in healthy, non-diabetic erythrocytes.

For sorbitol the accuracy is slightly better than the SDH enzyme assay with comparable linearity, precision and reproducibility. For myo-inositol the capillary GLC assay is accurate and reproducible but imprecise at the endogenous level. Further validation
of the myo-inositol assay is required particularly to determine factors that may affect precision.

During this trial attempts were made to establish a normal range for erythrocyte sorbitol and myo-inositol. However, the number of determinations made on normal blood was small. Furthermore, only single samples were taken; so that no information could be gained on the daily variation of these levels in normal non-diabetic subjects. In order to be more specific about the daily variation of erythrocyte sorbitol and myo-inositol in normal non-diabetic subjects a trial was carried out on blood taken from healthy volunteers.

3.4.3 The Daily Variation of Erythrocyte Sorbitol and Myo-inositol in Healthy, Normal Non-diabetic Subjects

3.4.3.1 Trial Design and Methods

The trial was designed to collect approximately six samples each, on different days spread over two weeks, from healthy, normal non-diabetic volunteers. Participants in the trial were instructed to continue with their normal daily activities. These activities were recorded by completing a proforma on each day that a sample was taken indicating, diet (including alcohol consumption), exercise and feelings of well being. On each day that samples were taken volunteers attended the Robens Institute in the morning and whole blood was drawn by venepuncture. Samples for erythrocyte polyol determination were maintained on ice and prepared in duplicate as already described. The plasma glucose level was determined for each volunteer on the first day and in
all cases the values were found to be normal. Therefore, no individual was excluded because of a possible tendency towards hyperglycaemia.

Erythrocyte sorbitol and myo-inositol were measured using capillary GLC, after freeze drying and acetate derivatisation, as described above in section 3.2.2.2. Myo-inositol concentrations were determined by response factors for a 10 μg/ml myo-inositol standard (with galactitol as internal standard) taken through the whole procedure. Derivatised samples were injected onto the capillary column using a PTV injector. The PTV was programmed at an initial temperature of 100°C with the split vent opened for 30 seconds. The split vent was then closed and the injector temperature increased to and held at 300°C. The split vent was then re-opened at 1.5 minutes after injection to clear any residual solvent from the injector lining. The aim with this particular PTV injector programme was to dump the solvent, through the split valve, immediately when the sample was injected. The method performance was assessed by blind spikes and repeat analysis of approximately 30% of the samples.

3.4.3.2 Results

A total of 8 volunteers, 3 males and 5 females with an average age of 31 years, completed the trial successfully and one volunteer withdrew because of influenza. The results are shown in table 19, and the overall mean erythrocyte sorbitol was found to be 1.03 with a standard deviation of ± 0.3 μg/ml. The overall mean erythrocyte myo-inositol was found to be 9.3 with a standard deviation of ± 1.7 μg/ml. These mean values are equivalent to 5.7 and 51 nmol/ml for sorbitol and myo-inositol respectively. The normal packed cell haemoglobin concentration is approximately 0.3 g/ml so that the mean sorbitol and myo-inositol concentrations referenced to their haemoglobin

166
content are 19 and 170 nmol/gHb respectively. An example separation, obtained by the capillary GLC using the PTV injector programmed to dump solvent, is shown in figure 16 and it is noticeable that there is considerable loss of the earlier eluting components (sample discrimination).

Table 19. Daily Variation in Normal Non-diabetic Erythrocyte Sorbitol and Myo-inositol

<table>
<thead>
<tr>
<th>VOLUNTEER</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>J</th>
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</thead>
<tbody>
<tr>
<td>male/female</td>
<td>m</td>
<td>f</td>
<td>m</td>
<td>m</td>
<td>f</td>
<td>f</td>
<td>f</td>
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</table>

**SORBITOL**

<table>
<thead>
<tr>
<th>DAY</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>MEAN</th>
<th>SD</th>
<th>C.V.%</th>
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<td>0.9</td>
<td>1.1</td>
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<td>1.0</td>
<td>0.8</td>
<td>0.92</td>
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<td>1.0</td>
<td>0.93</td>
<td>0.15</td>
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<tr>
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<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
<td>0.97</td>
<td>0.05</td>
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<td>0.8</td>
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**MYO-INOSITOL**

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<th>MEAN</th>
<th>SD</th>
<th>C.V.%</th>
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<tbody>
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<tr>
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<td>8.0</td>
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<td>9.8</td>
<td>10.7</td>
<td>8.4</td>
<td>1.6</td>
<td>19</td>
</tr>
</tbody>
</table>

All results are μg/ml red blood cells.
Detector Response

Key:
1 d(+)-Glucose
2 Unknown
3 Myo-inositol
4 d-Mannitol
5 Sorbitol
6 Galactitol (internal standard)

Conditions:
Column, 25 m X 0.32 mm (ID) immobilised OV1, 1 μ phase (S.A.C. Ltd.) programmed from 110°C for 1.5 min at 30°C/min to 220°C then at 1.5°C/min to 225°C hold for 10 min with helium carrier gas at 0.7 ml/min. Injector PTV programmed from 100°C for 0.5 min then split closed and temperature to 300°C with split vent opened 1.5 min after injection. GC: Perkin Elmer 8320 with FID detection (300°C) and GP2000 printer plotter at 10 mm/min.

Figure 16. Capillary GLC Separation of Polyols from the Blood of a Normal Non-diabetic Subject, Volunteer D Day 6.

Sorbitol equivalent to 1.0 μg/ml erythrocyte.
[Note: Chromatogram reduced by x 0.75]
The samples were analysed over 5 separate days and the standard curves, obtained from the sorbitol calibration standards on each of these occasions, gave correlation coefficients > 0.99 with the C.V. of the slopes equal to 5.6%. The erythrocyte sorbitol blind spikes, generated by another operator, consisted of samples prepared at levels corresponding to endogenous, middle and high calibration points. The precision of the blind spikes at these levels were found to be 11, 9 and 9% respectively. This precision is slightly poorer compared to an average 6% precision obtained at similar levels during the validation of this method. However, the validation study was performed using splitless with solvent effect injection without programmable temperature vaporisation.

Replicate analysis was carried out on a different day for approximately 30% of the samples and the mean % difference from the mean was found to be 10% for sorbitol. Out of the 14 samples that were repeated at random 4 of them had values greater than 10%. However, the actual concentrations determined in all cases (average 1.0 μg/ml) are low approaching the limit of quantitation, at or near the endogenous level, where the precision was found to be 11%.

By comparison, an overall % difference from the mean of 36% was found for myo-inositol concentrations in the replicate samples. The actual levels determined are high (average 9 μg/ml), where detection is not a problem, so that the lack of reproducibility may be attributed to daily variations in the response factor standards and storage effects. Analysis of the response factor standards show that they vary from day to day by 18% and by 15% within day so that this variation accounts for approximately half of the imprecision. Storage effects for erythrocyte myo-inositol have not been studied and contributions of this effect on imprecision, if any, are not known.
The reproducibility of myo-inositol determinations was previously found to be 18% (for the % difference from the mean, second Sorbinil trial) where calibration was obtained by myo-inositol standards spiked into the matrix; which in addition, gave very good recovery and precision for blind spikes. The generation of many supposedly identical replicate response standards, although possibly easier, has little advantage over creating calibration standards in the matrix. However, the advantage of using response standards is that several components can be quantified by this method. Therefore, if response standards are to be used for quantitation several must be generated and their variation determined for each analysis with every batch of samples.

3.4.3.3 Discussion

The daily variation of erythrocyte sorbitol and myo-inositol concentrations in normal non-diabetic subjects has not been previously investigated. This study was primarily designed to determine the daily variation both in individuals and in the group as a whole. In individuals there is little variation in the daily sorbitol levels with the average daily variation being 15% from the mean or 1 ± 0.15 μg/ml. The individual variation for myo-inositol levels averaged 13%; equivalent to 9 ± 1.2 μg/ml. The overall group range (mean ± SEM) for sorbitol and myo-inositol levels were found to be 1.03 ± 0.04 and 9.3 ± 0.3 μg/ml respectively.

There was no pattern between the sorbitol and myo-inositol levels and any of the subjective observations made by the volunteers. The subjective observations of diet, exercise and well being showed that out of the 8 subjects, 3 females had a vegetarian diet. The rest had a mixed diet with one female taking multivitamin tablets. Five out
of 8 subjects led sedentary lives with little or no exercise and 3 subjects reported feeling unwell with cold symptoms at some time during the two week period. The most erratic day to day sorbitol levels was found in the one individual who smoked (volunteer F, C.V. 30.7%). However, from this study no conclusions can be made concerning the daily variation in individual polyol levels or the factors affecting them. A systematic study would have to be designed to investigate factors such as smoking, diet or stress.

Considering the relatively narrow daily sorbitol range found in these normal subjects it can be reasonably expected that for diabetics to behave in a similar way the levels would be comparable. This is clearly not the case when just the placebo values for all diabetics (days 0, 7, 28 and 35 obtained from the second Sorbinil trial, table 16) are compared with the erythrocyte sorbitol levels found in normal non-diabetic subjects. These levels were found to average 26.7 and 5.5 nmol/ml in diabetics and normals respectively. By comparison the average placebo erythrocyte sorbitol values in diabetics, when analysed by the enzyme method (in the first Sorbinil trial) were found to be even higher at 43 nmol/ml. Consequently, the erythrocyte sorbitol levels in diabetic subjects compared to healthy non-diabetic subjects are raised by a factor of approximately five.

To be able to state that Sorbinil treatment lowered erythrocyte sorbitol in diabetics to within the normal range requires knowing that range and its daily variation and this has now been established. The second Sorbinil trial sorbitol levels, especially during the second week of therapy (days 11-18) are within, and very similar in variation to, the normal non-diabetic range found in this study. This result is very encouraging showing that Sorbinil therapy can normalise high sorbitol concentrations in diabetics to the levels encountered in normal subjects. Moreover, this reversal to normal is
consistent during the therapy period but upon cessation of treatment diabetic sorbitol levels are seen to revert to increased concentrations.

The situation with myo-inositol has been found to be different. The erythrocyte myo-inositol in normal subjects was found to be greater in normal than diabetic subjects (average 170nmol/g haemoglobin with a daily variation of ± 5 and in diabetics 138 ± 3nmol/gHb). Therefore, the erythrocyte polyol pattern would appear to be raised sorbitol and lower myo-inositol in diabetics (before Sorbinil treatment) compared to the reverse in normal subjects. This pattern is consistent with the hypothesis that the erythrocyte models the polyol levels in non accessible tissues. However, the exception is that myo-inositol levels in diabetics when treated with Sorbinil were not increased towards normal.

One way that may prove useful for examining erythrocyte polyol changes, and the factors affecting them, is by incubating red blood cells with glucose. The aim of such an experiment would be to try to mimic hyperglycaemic conditions encountered in diabetes. Sorbitol, polyols, and other sugars, could then be determined by the enzyme and capillary GLC assays in an attempt to determine these changes. In addition, such studies may help to elucidate the role of AR in the fate of glucose through the polyol pathway.
3.5 **In-Vitro Aldose Reductase Assay**

The Sorbinil clinical trials showed that a drop in erythrocyte sorbitol concentrations was observed in all diabetics treated with this inhibitor. This effect is believed to be due to inhibition of the erythrocyte AR enzyme by the drug. An in-vitro live cell assay for erythrocyte AR may be a suitable system to investigate the role of this enzyme in the intracellular accumulation of sorbitol. Moreover, the intracellular sorbitol accumulation in some diabetics compared to normals could be due to an increased AR activity. Therefore, the aim is to develop such an assay and determine if there is any difference in AR activity between healthy, normal non-diabetic and diabetic subjects. Furthermore, it would be especially useful to determine whether there is a correlation between increased AR activity and subjects diagnosed as suffering from an increased degree of diabetic complication. If such a correlation does exist then there is the possibility of using an assay for AR activity as a diagnostic tool. The eventual aim would be to produce a diagnostic assay in kit form which would be readily available for use in routine hospital laboratories. This is especially needed because no such diagnostic kit is currently available.

One possible approach to assay AR activity is by incubating erythrocytes in a suitable medium, substituted with or without glucose, and follow the production of sorbitol with time. In the first instance, for pilot studies, it is more feasible to determine the erythrocyte sorbitol levels using the enzyme assay; which is a cheaper and more rapid method. However, should the in-vitro incubation system be shown to work, further confirmatory studies could be conducted using the more specific capillary GLC assay.
3.5.1 Sample collection

Blood samples (10 ml) were taken from diabetic patients, chosen at random, attending the out patients clinic at St. Luke's Hospital, Guildford. In addition, samples of whole blood were taken by venepuncture from five healthy, normal non-diabetic volunteers and the erythrocytes washed and pooled as already described. The blood was immediately prepared for incubation studies as described in the methods (chapter 2). A group of diabetic subjects with various degrees of neuropathy was selected from those attending the out patients clinic of Dr. P. Wise at the Charing Cross Hospital Medical School, London. Blood was taken from these patients, stored on ice and immediately transported back to the Robens Institute, where the erythrocytes were prepared for incubation studies. The assessment of neuropathy in these patients was performed at Charing Cross Hospital and the degree of severity of diabetic complication, or any other clinical information, was not made available to the analyst until after the analysis stage of the study was completed.

3.5.2 Results

Figure 17 shows the results of an incubation with 50 mM glucose of pooled normal erythrocytes, with sampling points at 0, 0.5, 1, 2 and 3 hr. Sorbitol concentration in the incubated erythrocytes can be seen to rise with time. The increase in sorbitol after two hours is inhibited by 70% (in the same pooled erythrocytes) when incubated in the presence of 10 μg/ml Sorbinil. There is no assay interference between Sorbinil and the SDH enzyme method. Therefore, the in-vitro lowering of erythrocyte sorbitol levels is due to inhibition of AR by Sorbinil. This can be compared to the result of the
second clinical trial where an average 5 µg/ml plateau Sorbinil plasma concentration reduced the sorbitol concentration by 60% (figure 14).

Figure 17. Erythrocyte Sorbitol Levels in Response to In-vitro Incubation with 50 mM Glucose

Each result is the mean of two separate determinations and the % difference for each sample was 5% or less. The incubation was carried out in washed, pooled erythrocytes collected from 5 non-diabetic volunteers.
A comparison was carried out using pooled erythrocytes from both a group of diabetics and normals. These results are shown in table 20. Pooled erythrocytes from diabetics averaged a 33% greater accumulation of sorbitol than normal, non-diabetic pooled red blood cells.

Table 20. In-vitro Incubation of Erythrocytes with 50 mM Glucose

<table>
<thead>
<tr>
<th>TIME (Hr.)</th>
<th>NORMAL</th>
<th>CONTROL</th>
<th>DIABETIC</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>11.0</td>
<td>7.8</td>
<td>15.8</td>
<td>10.8</td>
</tr>
<tr>
<td>.5</td>
<td>25.6</td>
<td>8.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>40.2</td>
<td>7.4</td>
<td>49.9</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
<td>59.2</td>
<td>5.4</td>
<td>87.2</td>
<td>15.7</td>
</tr>
<tr>
<td>3</td>
<td>71.5</td>
<td>3.9</td>
<td>93.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Diff</td>
<td></td>
<td>3.2</td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>.5</td>
<td></td>
<td>17.2</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>32.8</td>
<td></td>
<td>39.2</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>53.8</td>
<td></td>
<td>71.5</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>67.6</td>
<td></td>
<td>88.8</td>
</tr>
</tbody>
</table>

All results are nMol/ml. Diff = incubated - control
* Slope for incubated normal = 20
# Slope for incubated diabetic = 27

Erythrocytes were washed and pooled from 5 non-diabetic and 10 diabetic subjects.

The in-vitro incubation system was used to determine the increase in sorbitol in blood samples from individual diabetics. The results are shown in table 21 and at least two subjects appeared to accumulate sorbitol to a considerably greater extent than the rest (shown by *).
Table 21. Erythrocytes from Individual Diabetics Incubated with 50 mM Glucose for 2 hours

<table>
<thead>
<tr>
<th>SEX</th>
<th>TREATMENT</th>
<th>BASAL</th>
<th>+ 50 mM GLUCOSE</th>
<th>% INCREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Insulin</td>
<td>15.6</td>
<td>73.7</td>
<td>372</td>
</tr>
<tr>
<td>M</td>
<td>Insulin</td>
<td>8.2</td>
<td>60.7</td>
<td>640</td>
</tr>
<tr>
<td>M</td>
<td>Insulin</td>
<td>36.3</td>
<td>123.5</td>
<td>240</td>
</tr>
<tr>
<td>M</td>
<td>Insulin</td>
<td>8.2</td>
<td>66.0</td>
<td>*705</td>
</tr>
<tr>
<td>M</td>
<td>Oral</td>
<td>18.1</td>
<td>81.2</td>
<td>349</td>
</tr>
<tr>
<td>F</td>
<td>Oral</td>
<td>10.4</td>
<td>73.3</td>
<td>605</td>
</tr>
<tr>
<td>F</td>
<td>Oral</td>
<td>11.1</td>
<td>65.2</td>
<td>487</td>
</tr>
<tr>
<td>F</td>
<td>Oral</td>
<td>10.2</td>
<td>79.4</td>
<td>678</td>
</tr>
<tr>
<td>F</td>
<td>Oral</td>
<td>15.4</td>
<td>77.1</td>
<td>400</td>
</tr>
<tr>
<td>F</td>
<td>Insulin</td>
<td>16.1</td>
<td>134.2</td>
<td>*734</td>
</tr>
</tbody>
</table>

Basal and incubated values are nMol/ml. * ? High
% Increase = \[\frac{\text{Incubated} - \text{Basal}}{\text{Basal}} \times 100\]

In collaboration with Dr. P. Wise and his colleagues at Charing Cross Hospital Medical School, London, blood samples were taken from two groups of age matched diabetics. One group with diabetic complications, assessed by degree of retinopathy, and the other group without any obvious sign of diabetic complications. The glucose concentration chosen for this experiment was 25 mM rather than 50 mM because it was thought to be a concentration that would not disrupt the viability of the red blood cells during incubation. In addition, 25 mM glucose was thought to be a concentration that may be more usually encountered in diabetics. The results are shown in table 22 and there appears to be no correlation between the degree of severity of diabetic complication and erythrocyte aldose reductase activity.
Table 22. Incubation of Erythrocytes from Diabetics with Varying Degrees of Neuropathy Incubation with 25 mM Glucose for 2 hours

<table>
<thead>
<tr>
<th>BASAL</th>
<th>+ 25 mM GLUCOSE</th>
<th>% INCREASE</th>
<th>DEGREE OF COMPLICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.5</td>
<td>11.2</td>
<td>7</td>
<td>NONE</td>
</tr>
<tr>
<td>12.2</td>
<td>22.6</td>
<td>85</td>
<td>NONE</td>
</tr>
<tr>
<td>18.7</td>
<td>38.3</td>
<td>105</td>
<td>MAJOR</td>
</tr>
<tr>
<td>14.1</td>
<td>29.5</td>
<td>109</td>
<td>NONE</td>
</tr>
<tr>
<td>15.3</td>
<td>36.9</td>
<td>141</td>
<td>MINOR</td>
</tr>
<tr>
<td>8.8</td>
<td>22.6</td>
<td>157</td>
<td>MINOR</td>
</tr>
<tr>
<td>7.3</td>
<td>19.9</td>
<td>173</td>
<td>MAJOR</td>
</tr>
<tr>
<td>9.1</td>
<td>25.6</td>
<td>181</td>
<td>MINOR</td>
</tr>
<tr>
<td>5.0</td>
<td>14.3</td>
<td>186</td>
<td>MINOR</td>
</tr>
<tr>
<td>12.7</td>
<td>39.1</td>
<td>208</td>
<td>NONE</td>
</tr>
<tr>
<td>8.4</td>
<td>28.3</td>
<td>237</td>
<td>MINOR</td>
</tr>
<tr>
<td>12.8</td>
<td>43.5</td>
<td>240</td>
<td>MINOR</td>
</tr>
<tr>
<td>11.4</td>
<td>39.1</td>
<td>243</td>
<td>MAJOR</td>
</tr>
<tr>
<td>6.4</td>
<td>23.3</td>
<td>264</td>
<td>NONE</td>
</tr>
<tr>
<td>5.2</td>
<td>21.8</td>
<td>319</td>
<td>MINOR</td>
</tr>
<tr>
<td>10.5</td>
<td>45.7</td>
<td>335</td>
<td>MINOR</td>
</tr>
<tr>
<td>5.5</td>
<td>32.1</td>
<td>484</td>
<td>MAJOR</td>
</tr>
</tbody>
</table>

All results are nmol/ml.

The % increase in normal pooled non diabetic erythrocytes at 25 mM glucose for 2 hours is approx 250%.

The capillary GLC method for polyol analysis was used to analyse normal erythrocyte samples incubated for 2 hours with 25 mM glucose. The purpose was to confirm the sorbitol increase found using the enzyme method and to assess any other qualitative changes that may be observed from the chromatogram. An example of the capillary chromatogram obtained is shown in figure 18. Two peaks which elute after the galactitol are quantitatively doubled (using galactitol as internal standard), from their basal level, after 2 hours incubation with glucose. One of the peaks (number 7 in figure 18) has been initially assigned as glucosamine by its retention. However, mass-spectrometry is needed to confirm the true identities of these peaks.
Figure 17. Capillary GLC Separation of Normal Erythrocytes Incubated with 25 mM Glucose for 2 Hours

The erythrocyte sorbitol concentration corresponds to 4 μg/ml equivalent to 21.8 nmol/ml.

Conditions: Column 50 m x 0.32 mm (ID) BP1, 2 μ (S.G.E. Ltd.) programmed from 120°C for 1 min at 30°C/min to 230°C hold for 35 min with helium carrier gas at ≈1.5 ml/min. Injector 275°C, FID detection (300°C) x1, x16 with JJ Lloyd chart recorder at 5 mm/min. GC: Sigma 3B. [Note: Chromatogram reduced x 0.75]
3.5.3 Discussion

In-vitro incubation of erythrocytes was first reported by Travis et al., (1971) who demonstrated increased sorbitol accumulation in blood obtained from healthy males incubated with 50 mM glucose. Sorbitol was found to accumulate to a concentration of 90 nmol/ml after 2 hours incubation. Sorbitol accumulation in erythrocytes from diabetics was not investigated by them. By comparison the present study found sorbitol to accumulate to approximately 54 nmol/ml in pooled erythrocytes from healthy volunteers and by 33% more, to 72 nmol/ml, in blood from diabetics (table 20).

Sorbinil at a 10 μg/ml concentration was seen to inhibit the in-vitro sorbitol accumulation by 70% in pooled erythrocytes obtained from 5 healthy subjects (figure 16). This is a similar inhibition to that seen in the second Sorbinil clinical trial. The in-vitro Sorbinil inhibition, however, does not lower the sorbitol level completely to pre-incubation basal levels although it would appear to do so in the clinical trial (table 16). This is probably because of the falsely high glucose concentration used for the in-vitro incubation. This is not seen in the Sorbinil clinical trials because the diabetic subjects still received their normal therapy to control the blood glucose level. This has to be the case because if hyperglycaemia were allowed to occur in diabetics they would risk severe acute ketoacidosis, shock and coma. Consequently, the only ethical way of investigating hyperglycaemia in diabetics is by in-vitro techniques. This was the reason for studying pooled and individual erythrocytes from diabetic subjects.

The erythrocytes from both normal and diabetic subjects when incubated with various glucose concentrations appear to remain viable. There was no evidence of haemolysis at up to 50 mM glucose concentrations for 3 hours. Bareford et al., (1986) have
confirmed this by showing no appreciable change in erythrocyte mean cell volume, haemoglobin concentration or morphology when incubated with 50 mM glucose. In addition, Travis et al., have demonstrated that the glucose utilisation by erythrocytes is unchanged from normal glucose levels (5 mM) when incubated in hyperglycaemic conditions up to 50 mM. However, as a precaution, for the assessment of AR activity in erythrocytes from diabetics 25 mM glucose concentrations were used in later studies.

Initially, in-vitro assessment of AR activity was carried out on a few individual samples from diabetics to determine the feasibility of the technique and any individual variations in them (table 21). Approximately half of the diabetics accumulated sorbitol with a percentage change from basal of 600% or more. The percentage change from basal in normals was found to be approximately 440% (table 20) and the remaining diabetics were within this range. As some of the diabetics appeared to accumulate sorbitol to a greater extent than others the question was whether this was correlated with clinical evidence for increased symptoms of neuropathy. This was investigated in a blind trial using diabetic patients drawn from the very extensive case histories kept at the outpatients clinic at Charing Cross Hospital Medical School, London. A total of 17 diabetic subjects, with long term complications varying from none to minor to severe, were assessed for their AR activity. There was no evidence of any correlation between the degree of severity of complication and AR activity (table 22).

This is the first time that attempts have been made to correlate the degree of severity of neuropathy with a biochemical marker. The failure to show any such correlation, although disappointing, can be partially explained. Firstly, the assay as such was subjected to considerable logistic difficulties (not least the delay in transporting the samples back to the laboratory). The assay requires extensive washing of the
erythrocytes both before and after incubation. There is at least two hours incubation with glucose followed by the time required for the sorbitol assay by the enzyme method. This would total probably 5 to 6 hours. If the capillary GLC with freeze drying method was used, instead of the more rapid enzymatic assay, then the whole procedure would take two days to complete. As a consequence the AR assay, whichever method is used to determine sorbitol, is long and tedious and certainly not a rapid method readily applicable for use in a routine hospital laboratory. A possible alternative would be to assess erythrocyte AR activity in haemolysed erythrocyte samples which could result in a more rapid and simple assay. Such methods already exist for the determination of other enzymes in haemolysate for screening purposes, for example, the determination of erythrocyte glucose-6-phosphate dehydrogenase deficiency, as described by Beutler (1983). It is recommended that such an approach is investigated because there is a real need for some sort of test which may be of diagnostic value.

Secondly, the accumulation of sorbitol must be a reflection of both the in-vivo AR activity and the metabolism of sorbitol by the enzyme sorbitol dehydrogenase (SDH). The existence of AR has been reported to be increased (as the activated form of the enzyme) when partially purified from the clear lens of human diabetics compared to non-diabetic, Das and Srivastava (1985a). It is not known whether this is the case with erythrocyte AR. Therefore, it would be worth attempting to examine the activity of purified erythrocyte AR using their methods. Furthermore, as attempted in this study, correlations would have to be performed between activity and degree of severity of diabetic complication. However, considerable expertise is required in enzyme purification and the resulting method would be expensive and not suitable for routine clinical diagnostic use.
Thirdly, the sorbitol flux through the polyol pathway depends upon the activity of SDH. This activity is almost completely unknown and has not been investigated in relation to diabetes and the associated long term complications. It is possible that the accumulation of sorbitol is controlled by the next step in the polyol pathway; metabolism by the enzyme SDH. One possibility, therefore, is to determine the activity of SDH in normal and diabetic subjects in a manner analogous to that attempted herein for aldose reductase. There is the possibility that the activity of SDH is lowered in diabetics compared to normals thus favouring accumulation of sorbitol. Furthermore, that the SDH activity is lowered more so in diabetics and correlated with neuropathic symptoms. If this is not the case then no such correlation will exist as already found for AR. This question is fundamental to the understanding of the polyol pathway in relation to the long term complications of diabetes and thus far has been completely overlooked. Thus, if neither enzyme is implicated then some alternative biochemical mechanism for the aetiology of neuropathy (possibly still involving sorbitol) may exist.

The problem of predicting which diabetics are most prone to the long term complications has been found clinically to be virtually insoluble. The results obtained in this study typify the problem. For example, some diabetics have no evidence of diabetic complication but appear to accumulate sorbitol to a great extent. Similarly very poorly controlled diabetics that may be expected to develop complications clinically never do so. The reverse is also seen both in the clinic and in our assessment of AR activity; with high levels of sorbitol accumulation in some individuals who do not show signs of developing the complications. The results of the work of this thesis and the methods developed to study the role of polyols in the management of diabetes are further discussed in chapter 4.
CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

Diabetes is classified into two broad categories according to treatment. These categories are type I, insulin dependent diabetes mellitus, (IDDM) and type II, non-insulin dependent diabetes mellitus (NIDDM). Type I diabetes requires daily injections of insulin to help control the blood glucose level. Therapy is usually self administered before meals. Type II diabetes does not require insulin but may be treated by oral antihyperglycaemic agents, diet control, and sometimes insulin, self administered and controlled by the patient. In both cases the co-operation of the patient, in coming to terms with, and continuing to regulate their daily activities and diet, is fundamental to the medical management of their condition. Needless to say such imposed self therapy is widely differing in success. The factors affecting this success are very diverse and include; duration of diabetes before and after clinical diagnosis, insulin and drug intolerance, psychological and socio-economic considerations, and the appearance of complications. The latter factor, long term complications, are a very painfull and debilitating series of conditions, manifested in a variety of ways, all of which require extensive clinical management.

The long term complications of diabetes appear with equal frequency in both diabetic types. There is little evidence of any genetic predisposition to the long term complications developing in certain types of diabetic (Leslie et al., 1989 and Taylor
1989). However, studies in twins and long term familial retrospective surveys may help to clarify any genetic markers and or, tendencies towards the development of complications.

The elevated glycosylated haemoglobin and blood glucose levels have been found to be strongly associated with diabetic neuropathy (Boulton et al., 1984). Furthermore, the duration of diabetes is considered to be a major risk factor in the development of the long term complications. Consequently hyperglycaemia and glycosylation, a direct effect of the duration of diabetes, predominate even in the presence of daily temporary measures to control them. It is this predominance along with the increased activity of the polyol pathway that points to the aetiology of diabetic complications being metabolic in origin. The early detection of the onset of these complications gives the physician the best chance of managing the condition in an effort to prolong the life of the patient. This work has attempted to establish analytical methods that may be of use in investigating some of these metabolic interactions with an ultimate aim of obtaining a simplified assay that could be of diagnostic value.

In the first instance for any pathological change to be attributed to a causative agent requires the latters' reliable quantitation in the affected tissue. In the case of diabetic complications sorbitol is believed to be a major factor in the pathogenesis of neuropathy. The increases in sorbitol that occur in the less accessible tissues affected by neuropathy are directly correlated with similar changes in the red blood cell. Consequently much of the analytical methodology of this work has centered on the analysis of sorbitol in erythrocytes.
Ideally, a reliable method should produce accurate determinations which are reproducible, within day and from day to day, with good linearity and the required detector sensitivity. Furthermore, the method should be robust, easily used by other operators, and capable of some degree of automation to enhance its cost effective use. In addition, a method should be capable of a certain degree of selectivity so that potentially interfering components are avoided; by either sample preparation procedures or the use of selective detectors. Of special relevance to the routine hospital laboratory a method, as far as can be ascertained, should be free from any drug test interaction. Rarely do all these factors combine together in any one method.

This work has shown that the sorbitol enzyme assay meets most of the above criteria. The assay was accurate to within $\pm$ 5% for blind spikes compared to calibration standards. Reproducibility, by repeat sample analysis on a different day, was found to give a result generally within 10% of the previous determination. The assay was found to be consistently linear over the calibration range with linear regression correlation coefficients greater than 0.993. The limit of quantitation was found to be approximately 0.5 $\mu$g/ml, detection below this level is restricted because of high fluorescence values for sample blanks. The method is robust in the sense that it was unaffected by different batches of enzyme preparation. Therefore, the assay should yield similar results in different laboratories if the same commercial preparation of the SDH enzyme and analytical conditions are employed.

With appropriate autosamplers or slight modifications to the procedure the method could become semi-automated. No interference was observed for the method when Sorbiniil was present in the assay mix. Other drug test interactions were not tested for and cannot be ruled out. However, no sample interference was observed in any of the
Sorbinil trial diabetic patients receiving oral antihyperglycaemic agents such as Tolbutamide.

There are reservations regarding the specificity of the method and sample storage. Xylitol acts as a substrate for this particular preparation of SDH. Xylitol is metabolised in the erythrocyte to a small extent (Travis et al., 1971) and it is possible that this accounts for the small change in sorbitol levels observed upon prolonged storage of erythrocyte samples. The more specific capillary GLC assay would be very useful to measure erythrocyte xylitol levels and any storage effects. In addition, the apparent lack of specificity may be overcome by the use of a new purified SDH preparation, free of polyol dehydrogenase, which has just become commercially available. This assumes that the new SDH preparation is highly specific for sorbitol and that other polyols and sugars do not interfere or act as substrates. It is recommended that this new enzyme preparation should be substituted in the assay and compared with the results already obtained in this study.

In contrast to the enzyme assay the capillary GLC method also meets many of the analytical requirements mentioned above. The capillary method has the added advantage that other polyols and sugars can be determined. The method has been continually modified as the factors affecting its performance have been investigated. These factors include the nature of the stationary phase, injection technique, sample preparation and derivatisation conditions, and methods of calibration.

The best capillary method for sorbitol obtained in our laboratory thus far appears to be freeze drying of the sample containing galactitol as internal standard, followed by acetate derivatisation, extraction into chloroform with sample concentration and
injection using splitless with solvent effect onto a capillary column with an immobilised non-polar phase with flame ionisation detection. Moreover, neutralisation of the perchlorate extract is critical, prior to the recovery of polyols as their acetate derivatives, which was found to be maximal at a pH of 9 to 9.5. Additionally, the method of generating calibration standards and blind spikes in bulk, in conjunction with the above conditions, has produced consistent linear calibrations and excellent performance data. Using this method the accuracy for sorbitol determination was ± 2.0% with a between day precision for replicate determinations of ± 10%.

The determination of myo-inositol was found to be less reliable, particularly in diabetic samples, where shoulders were seen on either side of the myo-inositol peak (figure 15). This effect was less obvious in chromatograms from normal non-diabetic subjects and could indicate qualitative differences in the polyol profile between the two groups; assuming the interference is due to similar chemical species. However, the capillary method developed in this study was able to estimate erythrocyte myo-inositol concentrations and in so doing blind spikes were found to be accurate to within ± 2%. Reproducibility, determined as repeat samples assayed on different days, gave results generally within ± 10% of each other (for diabetic samples from the second Sorbinil trial). In contrast poor reproducibility for myo-inositol was found when the samples were injected using the PTV injector and this technique is not recommended for such determinations.

It is possible that the interference around the myo-inositol retention could be due to very closely related inositol isomers. Therefore, for myo-inositol to be accurately determined it is recommended that methods capable of separating the closely related inositol isomers are investigated. For example; the method of Leavitt and Sherman
(1982) may be very useful because these authors have successfully resolved D- and L- chiro-inositol on a chiral capillary column. If a method such as theirs is used it remains to be determined whether differences in inositol isomer composition occur between diabetics and normals. Furthermore, if such differences do occur their relationship to diabetes will open the possibility for yet further alternative metabolic pathways which have not yet been fully appreciated. Evidence that this may be the case is seen in the results of capillary GLC analysis of erythrocytes incubated with glucose discussed in more detail below.

The capillary GLC method was found to be robust in the sense that the same separation could be obtained using equivalent columns (length, diameter and phase thickness) supplied from different manufacturers. Additionally, the resuspended hexitol acetates are stable for up to three months which facilitates their re-injection if any part of the GLC system fails. In practice the only routine maintenance that was regularly required was daily changing of the injector septum.

Consistent FID responses to both extracted polyol and unextracted SHA standards was also a feature of this method and either calibrants could be used to test the GLC analytical conditions or to investigate problems with different stages of the assay procedure. Considering the consistent separations and detector response the method may be transferable from one laboratory to another. However, the relative cost of the equipment and time required for the assay does not make it readily available; especially to routine hospital laboratories.

One time saving feature is the use of an automated injector; which allows large numbers of samples to be run unattended. Such a system is currently undergoing
assessment in our laboratory using splitless with solvent effect injection. The C.V. of the peak height of the internal standard (galactitol) for a run of 40 samples has been found to be 10% which indicates a very consistent recovery reflected throughout all stages of the assay. A further time saving feature is the possible use of 4-dimethylaminopyridine (DMAP) as the catalyst for the acetylation procedure. Initial investigations with this catalyst in place of pyridine were found to be promising with linear calibrations obtained for standards spiked into both erythrocytes and plasma. It is recommended that a full method validation is performed to establish the suitability of DMAP in the capillary assay.

The enzymatic and capillary methods were compared by assaying a series of blood samples from diabetics using the two techniques. Taking the results overall for assay on both the same and different aliquots gave a correlation coefficient of 0.87 when subjected to linear regression analysis. The slope of the line was 1.17 (with enzyme as y and capillary as x) indicating that the enzyme generally gave a 17% higher value than the capillary. Part of this difference may be accounted for by the presence of xylitol in the sample which acts as a substrate for the SDH enzyme. Further stability studies using a validated method specific for xylitol may help to clarify the contribution this polyol makes to the enzyme result. A method comparison between the capillary and the newly available purer SDH would also be useful in elucidating the latter's suitability of the latter for routine use in the sorbitol enzymatic assay.

Apart from the enzyme method for sorbitol analysis the question remains as to what improvements can be made to the GLC assay and what alternative methods are available? Most analytical procedures can be roughly divided into three or more stages which consist of sample collection, sample preparation and the final analytical
detection often proceeded by a chromatographic separation step. Techniques which combine several if not all of these stages together in one step would be the method of choice.

In the ideal world there would be available a single specific detector for every single component known to exist. Thus, a liquid mixture, such as plasma, whole blood or urine, could be collected and a probe, for example ultraspecific for sorbitol, inserted and a suitable readout obtained. Another probe specific for myo-inositol can be used and so on.

There are probes in existence for the determination of ions in solution such as the ion selective electrodes used for serum electrolyte determinations in clinical chemistry (Blijenberg et al., 1989). The advantages of this sort of approach are that several probes can be inserted in turn into relatively small sample volumes in a very rapid automated fashion. However, as yet such systems are not available for the enormous variety of compounds encountered in biological matrices but there is much current interest in the development of biosensors for their use in analysis. One example is the pilot studies of Takei et al., (1985) using a membrane bound alcohol dehydrogenase enzyme to measure serum ethanol. The final visualisation is obtained by spectrophotometric determination of a coloured dye which presumably eventually will be calibrated to obtain readouts on a hand held chart. By analogy there may in the future be the possibility of achieving blood sorbitol determinations using immobilised SDH but whether this will be specific and sensitive enough remains to be determined.

With highly specific biosensors the need for extensive sample preparation or separation steps would be negated although there is still the requirement to maintain the integrity
of the sample upon collection. This latter point is one that can often be overlooked when, for example, whole blood is improperly drawn by venepuncture and on arrival back in the laboratory is found to be haemolysed and unsuitable for assay. Therefore, to a certain extent the analysis begins at the stage of sample collection. Furthermore, blood samples generally still require some preparation such as centrifugation to obtain the required fraction. And where erythrocytes are concerned their washing in saline is obligatory to remove white blood cells and the lipids associated with them all of which can be potential sources of interference.

The following comments concerning alternative sample workup procedures are primarily directed at chromatographic assays. However, they could equally apply to any analytical system (including the sorbitol enzyme assay) used to determine water soluble components in biological matrices; such as amino acids, carbohydrates, polar drugs and their metabolites, and polyols.

Polyols are highly water soluble, neutral compounds which are ionised at a pH of about 13 or above. The problem is to isolate them from an aqueous environment for which they have complete affinity while leaving behind proteins, lipophilic compounds and a multitude of other components. Protein precipitation of biological matrices is the most commonly employed method to denature proteinaceous material in the sample. A very simple method would be to precipitate the proteins in strong alkali and inject the sample directly onto an ion exchange HPLC with amperometric detection such as used by Ohsawa et al., (1986) for the determination of xylitol in plasma and saliva. However, this method requires filtering before injection, has not yet been attempted on red blood cells, and is costly not making it a readily available technique. We have discussed with Dionex (the manufacturers of the above ion exchange columns and
HPLC equipment) the possibility of comparing the capillary GLC analysis for erythrocyte polyols with their alternative method but as yet nothing has been forthcoming.

An alternative sample preparation procedure is to precipitate the proteins with alcohol, methanol or ethanol being a common choice. However, it has to be borne in mind that some polyols are only slightly soluble in alcohol for example, erythritol and ribitol. Sorbitol while soluble in methanol is sparingly soluble in cold ethanol. Precipitation with methanol opens up the possibility of using solid phase sample preparation as the next clean up step in the procedure. This has been attempted by Valdez and Reier (1987) who used C18 solid phase cartridges to obtain glycols, polyols and hydroxyamines in a non-aqueous environment suitable for derivatisation. The C18 phase was found to be inappropriate for erythritol with recoveries of only 50% which is because polyols are not well retained on hydrophobic phases. However, hydrophobic phases such as C18 could be used in the reverse situation. This is where the aqueous/alcoholic extract is passed through the conditioned column which would retain lipophilic components while the polyols would be eluted in the original aqueous volume thus providing a simple sample cleanup step. This was attempted in a pilot study in our laboratory and the recoveries of an aqueous mix of glucose and polyols was found to be 100% there being no retention for them on a C18 cartridge.

It is often stated that if the HPLC conditions are known for a given compound then to a certain extent the conditions for solid phase sample preparation are already at hand. By analogy the above example of ion exchange HPLC of polyols gives a clue to the possible conditions for their solid phase sample preparation. As the polyols are ionised at high pH, by for example 0.15 M sodium hydroxide, this solution could be
used to precipitate proteins and then passed through a solid phase cartridge containing a strong anion exchange porous resin. The polyols would be retained, while other material was not, and could then be eluted by using a buffer, neutral pH solvent such as water or possibly methanol. The usefulness of this sample preparation step should be examined for both the GLC and enzymatic assays. Applied to the enzyme assay this sample preparation step would avoid the necessity to neutralise the perchloric acid. Furthermore, the polyols could be eluted from the ion exchange column with the buffer (pH 9) used in the enzyme assay the only problem being strict control of the elution volume.

Polyols are insoluble in ether and non-polar solvents so that if solid phase sample preparation is not available then straight forward non-polar extraction can be used to remove lipophilic substances from the sample. Some workers use combinations of immiscible polar and non-polar solvents (such as methanol precipitation followed by hexane extraction) to obtain a cleaner sample with the polyols remaining in methanol which is then taken to dryness under OFN (Roboz et al., 1984, Jansen et al., 1986 and Johnson and Mayersohn, 1984).

The next major problem is to obtain very dry polyol extracts if they are to be derivatised which is necessary for GLC and a usual requirement for HPLC detection. Drying down under OFN has already been mentioned and two alternatives were tried in the current study. The first of these involving vortex evaporation at 70°C under vacuum is rapid but Willars et al., (1987) have reported variable loss of myo-inositol upon oven drying which could be a problem with vortex evaporation using elevated temperatures.
Freeze drying provides maximum sample integrity (Leenheer 1984) and although very time consuming may have to be the compromise between a more rapid but less reliable alternative. It is at this stage that the analyst has to make a critical decision as to either drying down for derivatisation or turning to an alternative HPLC technique that does not require a derivatisation step for detection.

There are alternative HPLC analysis for sugars and polyols such as capillary HPLC with laser refractive index (RI) detection (Bornhop et al., 1987) or affinity HPLC (Honda et al., 1987). In the former, Bornhop et al., have demonstrated that increased sensitivity for sugars can be achieved down to 100 ng amounts for glucose. The column consisted of a capillary tube about 0.6 metres long by 0.4 mm internal diameter packed with 5 \( \mu m \) diameter C18 spheres. The RI detector in this example is specially designed to have an extremely low volume which is achieved by its optical path passing through the bottom of the capillary column where the outer polyimide coating is removed. This technique while not commercially available at present has enormous possibilities for many types of analysis. Not least is the universal nature of the RI detector which to date has been limited by its insensitivity. And in addition, the wide selectivity of phases and eluent modifications used in HPLC are readily applicable to such a system. As yet the system has not been applied to complex biological mixtures or polyols derived from biological matrices but there may be combinations of capillary columns alone or packed that could achieve the desired separations with the required sensitivity. The future for this technique is very promising.

Affinity chromatography had not been applied to the analysis of carbohydrates of small molecular weight until Honda et al., demonstrated such separations using affinity phases consisting of carbohydrates immobilised on ion exchange resins. The particular
immobilised carbohydrates employed were glycamines; such as glucamine (1-amino-deoxy-D-sorbitol) and lactamine (4-O-β-D-galactopyranosyl-1-amino-1-deoxy-D-sorbitol). The method used post column derivatisation to visualise the carbohydrates but the glucamine phase was only able to separate classes of carbohydrates such as pentoses from hexoses. However, the versatility of this form of separation is in the choice of affinity phase and there are a multitude of different carbohydrates or lectins to be investigated as possible alternative phases.

The most useful application of the affinity chromatographic approach is probably as a sample separation step for classes of compounds. Although not applied to polyols as yet it may prove a useful and rapid sample cleanup procedure especially if the polyols can be separated from reducing sugars. This would be particularly useful because there is the requirement, especially with in-vitro incubation studies, of characterising just the polyol changes. Thus, reducing sugars could be removed and analysed separately to the polyols. Indeed using the capillary GLC separation it has become evident that there are many components in the sample which still require identification and the chances of achieving this are increased if interfering sugars are removed.

Alternatively, the use of an immobilised enzyme such as sorbitol dehydrogenase specific for sorbitol as a chromatographic cleanup procedure is possible. Although this would be of no benefit if the aim was to measure as many polyols as possible in the sample. In addition, there is still the problem of detection with either this or any of the above HPLC methods if the compounds do not possess a chromophore in the molecule. This is the case with polyols which require derivatisation when a sensitive RI detector is not available. Polyols do respond at low wavelength UV but our initial investigations with pure dilute aqueous solutions suggested that, for example, at 195 nm there would
be insufficient detector sensitivity above the background noise. Furthermore, many compounds absorb at low wavelength UV and unless they are completely removed from the sample interference is a likely possibility.

Polyols require derivatisation to make them volatile for GLC and any of the above sample preparation procedures could be applied for sample cleanup at various stages of the assay. As already discussed there are numerous possibilities for sample cleanup prior to derivatisation. Sample cleanup after derivatisation is a further possibility where the derivatives could be passed through, for example, a non-polar solid phase cartridge such as C8 or C18. The particular sample cleanup will depend upon the nature of the derivative and with polyol acetates the use of a semi-polar solid phase, may be possible. This is because acetate derivatives are slightly more polar than the TMS variety and may be better retained on mid-polar phases although as yet there is no evidence to support this.

The advantage of using solid phase sample preparation, or solvent extraction, after derivatisation for the GLC assay is the removal of non-volatile underivatised components or unwanted derivatisation by-products. These components if injected onto a column, or the injector liners used in capillary GLC, can have non-specific adsorption effects which can cause components to elute poorly with considerably tailing peaks. Consequently the cleaner the final sample the better for GLC analysis.

There are improvements that could be made to the capillary GLC operating conditions which may, in conjunction with various solid phase sample preparation procedures, confer an added degree of selectivity to the analysis. For example, there is the possibility of using thermal desorption as a sample injection technique. This injection
technique is most commonly used for the analysis of organic volatiles in air which are adsorbed on packings contained in stainless steel tubes. With the right choice of packing material large solvent volumes, i.e. 100 µl or possibly more, containing the derivatised polyol acetates could be spiked into the tubes and placed on the thermal desorption apparatus.

The reasoning behind this approach is the possibility of eradicating the carrier solvent through the desorption process while leaving the solutes to be sent to the analytical column. If the system were to work then it would also allow the quantitation of low molecular weight polyol derivatives such as glyceroltriacetate. In addition, the solutes arriving at the analytical column would be free to elute without hindrance from solvent flooding effects. It would be very interesting to conduct a small pilot study to determine the feasibility of this approach although ultimately thermal desorption is not a readily available technique.

As already indicated there are at least 30 unidentified peaks that elute from the capillary GLC column most of them before glucose. The possibility of their separation from each other may be increased with thermal desorption and slow temperature programming. However, some of these components are present in low concentrations and not easily detected by the FID. To overcome this detection problem an alternative is the possible use of column switching in conjunction with dual detection systems or the use of alternative detector orientated derivatisations.

The GLC detectors most commonly used in trace organic analysis are the mass-spectrometer, electron capture and FID. The practical range of these detectors vary from about 500 pg/ml to 10 µg/ml for MS, from 500 pg/ml to 100 ng/ml for ECD
and 0.1 μg/ml to 10 μg/ml for FID (de Silva 1978). When GC-MS is not available there is the potential of coupling in series ECD followed by FID. This is probably not the approach for the polyol analysis because large glucose peaks would swamp the ECD and certainly be in concentrations above the linear working range of the detector.

If column switching is available then the flow can be directed for earlier parts of the chromatogram to the ECD and diverted for the later eluting and more concentrated solutes to the FID. However, this requires considerable time and expertise to obtain the optimised conditions and ultimately may not be fully justified if alternatives are quicker and more reliable. Therefore, the analyst may turn to derivatisation with electron capturing reagents such as the halogenated alkyl anhydrides.

The trifluoroacetate derivatives of polyols are more volatile than the acetates and elute more quickly on non-polar GLC columns thus speeding up the analysis. They can be detected with FID and this alternative should be investigated because it is possible that enough sensitivity is achieved without having to resort to ECD. This approach has not yet been applied to the analysis of erythrocyte polyols but in doing so it would provide a very useful comparison with the existing methods.

There is then a considerable number of approaches that the analyst can adopt to improve an assay. Among those mentioned here probably the most useful for polyol analysis is at least an ion exchange sample cleanup prior to any further analytical procedure. When using the capillary GLC assay freeze drying of the biological extracts, prior to derivatisation, although time consuming is the method of choice. Derivatisation looks likely to be a requirement for some time to come until alternatives, such as the
capillary HPLC with laser RI detection, are fully developed and shown to be applicable.

The favoured derivatisation is the use of the DMAP catalyst not least because it gives rapid acetylations and the feasibility has been established with a biological matrix. Capillary GLC as opposed to packed column GLC gives the best polyol separations currently available with the required sensitivity for hexitols when using FID. However, further work is required to identify the earlier eluting components extracted from erythrocytes and because of their low concentration alternative derivatives to the acetates will have to be investigated. Ideally the use of mass spectrometry would be the detector of choice because it is both sensitive enough and highly specific. The considerable price of these detectors and the specialist expertise required to operate them do not make them readily available in many laboratories. However, the new 5890-II benchtop GC-MS (made by Hewlett-Packard) is priced at a level which is now just beginning to be affordable.

Furthermore, the bonded non-polar capillary columns used in these studies did not resolve components which were shoulders on the myo-inositol peak. Consequently this problem will have to be overcome by the use of alternative phases as discussed above. We tried using a Chirasil-Val capillary GLC column to investigate the separations of polyol acetates but this was without success. A possible explanation is that the hexitol acetates which were the components studied do not elute on non-polar columns below about 220°C and the maximum operating temperature of Chirasil-Val is about 200°C. The use of Chirasil-Val, or other capillary enantioselective phases, with more volatile polyol derivatives, such as the trifluoroacetates, should be investigated for their ability to resolve the inositol isomers.
Thus, the enormous range of sample preparation procedures in conjunction with various separation techniques and available detectors can result in different combinations to suit the particular analytical requirements. In terms of other applications of these techniques to the analysis of polyols there are many. These include the identification of polyols in food, either as their purity as food additives (Samarco 1977), or when food is adulterated by their addition to make unsuitable products taste better. For example, the clandestine adulteration of immature wines by the addition of excessive amounts of xylitol or sorbitol (Estrella et al., 1986). Also the identification of polyols originating from Rust fungus has enabled the quality of wheat crops to be monitored to guard against this commonly occurring infection (Maclean and Scott 1976).

In biochemical applications, for example, Kontrohr and Kocsis (1984) have used capillary GLC to identify rare aminohexoses originating from bacterial cell walls as their reduced aminoalditol acetate derivatives. This has allowed the rapid identification and classification of various pathogenic bacteria. Therefore, methods of polyol analysis have been used to investigate a wide range of problems and the particular applications that these methods are applied to will only be limited by the amount of available funding. The results obtained from the current study in relation to the particular problem of diabetes are now discussed.

The enzyme and capillary assays were used to determine erythrocyte sorbitol levels in diabetics in two separate clinical trials of Sorbinil. It was found that in both trials the levels of sorbitol were reduced upon Sorbinil treatment. This trend was reversed at the end of the Sorbinil treatment period, during the wash out phase, with erythrocyte sorbitol reverting to high levels.
The usefulness of a lower dose of Sorbinil (125mg daily compared to 250mg daily) was demonstrated for the first time in the second clinical trial where erythrocyte sorbitol levels were found to be greatly reduced by 60% within 24 hours after the first daily dose. This has important implications considering the known dose related Sorbinil intolerance in about 10% of subjects receiving this compound. This result indicates that there is the potential to administer a lower Sorbinil dose, which may help to minimise any side effects, while still achieving the desired therapeutic aim. This is especially required when the possibility is considered of using AR inhibitors for long term prophylactic use. Martyn et al., (1987) have since confirmed our erythrocyte sorbitol findings in a trial of diabetics receiving a low oral dose of Sorbinil (125mg daily) for 6 months. However, only a single sorbitol determination was made by them for each patient at the 3 month time point. Their results indicate that Sorbinil was well tolerated over an extended period of time and that long term therapy with this compound may be possible. Thus Sorbinil therapy may be particularly beneficial in patients who, when screened, do not shown signs of hypersensitivity to this drug.

In the second clinical trial (using the capillary GLC assay) erythrocyte myo-inositol levels in diabetics were found to be unchanged during either Sorbinil or placebo therapy but higher than the mean level found in non-diabetics. This finding does not fit with the view that in diabetic tissues, particularly animal models, sorbitol is raised while myo-inositol is concomitantly lowered. Therefore, it could be concluded that the results do not support the widely believed model that changes occurring in less accessible tissues are reflected by similar changes in the erythrocyte; where the levels of myo-inositol would be expected to be similarly reduced. And, moreover, that sorbitol in some way affects myo-inositol metabolism in nerve cells.
This view may have to be modified in the light of recent observations by Dyck et al., (1988) who used capillary GLC of polyol TMS derivatives derived from biopsy of human sural nerve. They found no evidence of deficiency in nerve (biopsy) myo-inositol levels or its relationship to the pathogenesis of neuropathy. Raised nerve sorbitol levels were, however, strongly associated with the severity of neuropathy. Unfortunately their study did not correlate the nerve sorbitol and myo-inositol levels with those of the erythrocyte which given the design of the study would have been extremely interesting. Furthermore, Sima et al., (1988), using a fluorescence enzymatic sorbitol assay, have independently corroborated that human sural nerve sorbitol content is raised in diabetics with neuropathy. In addition, both of the above studies showed beneficial effects of oral Sorbinil treatment for one year; with reduction in nerve sorbitol content and improvement of nerve morphology and physiological function.

Considering the above evidence and the trial results from the current work it is possible that the erythrocyte actually models the biochemical events that contribute to neuropathy. And that sorbitol alone, rather than in conjunction with myo-inositol, could be the primary causative agent. It is recommended that before the role of myo-inositol is dismissed completely alternative separations, such as that already mentioned for inositol isomer determination, are investigated for their potential application to both the erythrocyte model and other tissues affected by diabetes.

In normal non-diabetic subjects the erythrocyte myo-inositol concentrations were found to be fairly consistent averaging 9 µg/ml; equivalent to 50 nmol/ml erythrocyte, and is similar to the levels found in diabetics in the second Sorbinil trial. However, the interference observed for myo-inositol retentions in the diabetics may cause the levels
in this group to be overestimated. Thus, there may be a true difference in myo-inositol concentrations between the two groups. It would appear that the analytical conditions described herein results in estimates for myo-inositol determinations and underscores the need for a more specific assay for inositol isomers.

It was found that the sorbitol concentrations in healthy, non-diabetic volunteers were remarkably consistent averaging approximately 1.0 µg/ml (5.5 nmol/ml) with a variation of about ± 10%. Moreover, the sorbitol levels were consistent for any one individual from day to day over a two week period. This is a new finding and important in relation to the levels found in diabetics. The reason for this is that the consistent sorbitol levels in healthy, non-diabetic subjects could serve as a target to be achieved by diabetics. Ways of achieving this may be by very good glycaemic control or alternatively by therapy such as Sorbinil.

In diabetic subjects treated with Sorbinil the erythrocyte sorbitol levels fall and stay within this newly defined normal range. This suggests that Sorbinil helps diabetics in achieving consistent sorbitol levels similar to that observed in healthy, non-diabetic subjects. However, on one occasion the erythrocyte sorbitol level was seen to be spuriously high (for one patient in the first trial). This high level has already been explained on the basis of very poor glycaemic control but in addition there is the possibility that a preceding days dose of Sorbinil was not taken by that particular patient. Consequently AR inhibition may be very sensitive to small changes in the circulating plasma Sorbinil concentrations which is quickly reflected by the sorbitol levels. The speed with which the erythrocyte sorbitol reverts to former levels at the end of Sorbinil treatment in diabetics has not been established. It is unfortunate that in the second Sorbinil trial, where there was the opportunity to do so, this speed of reversion
was not investigated. As a consequence it is recommended that in future studies of AR inhibition the reversion of sorbitol levels should be closely examined. It is important that this be done because there is the possibility that certain therapies may suppress the sorbitol levels for prolonged periods of time. If this were the case there may be the facility for introducing dosing regimens, for example, on a once a week basis.

It is of interest that the concentration of Sorbinil required to inhibit the in-vitro accumulation of erythrocyte sorbitol was found to be similar to the plasma Sorbinil concentrations found in the clinical trials. It would have been useful to perform an in-vitro Sorbinil titration down to the lowest concentration which still gave maximal inhibition. This could have provided a simple model for indicating the lowest efficacious dose of Sorbinil for in-vivo use. Somewhat surprisingly the erythrocyte model for this application has not been investigated by others. It is recommended therefore that the in-vitro erythrocyte incubation system developed in this work could serve as the initial model for comparing the efficacy of aldose reductase inhibition by different compounds. One compound of particular current interest is ascorbic acid (vitamin C) which in man, who cannot synthesise it, is required as an essential vitamin.

A recent report by Vinson et al., (1989) has shown that vitamin C given as a dietary supplement to diabetics, 2 g/day for 3 weeks, lowered erythrocyte sorbitol levels by 45%. This finding opens up the possibility of an alternative therapy for treating neuropathy which bypasses problems of drug tolerance. However, the analytical methodology used by these workers, which was a micro scale enzymatic assay for erythrocyte sorbitol, is open to question because no validation data was reported by them. The effect needs confirmation by a second analytical technique such as the
capillary GLC assay. Furthermore, evidence of improvement in nerve morphology and physiological function need to be obtained. Additionally, the exact relationship between ascorbic acid and its modifying effect on sorbitol or the polyl pathway is not known. No doubt these questions are currently under investigation.

It was found that erythrocytes from diabetics accumulated sorbitol to a greater extent than erythrocytes from healthy non-diabetics when incubated in the presence of glucose. The increase was approximately 30% and could be explained by an increased aldose reductase activity in the diabetics compared to normals. Using the capillary GLC assay it was found that normal erythrocytes from non-diabetics when incubated with glucose appear to contain component peaks around the myo-inositol retention which are not normally present. The identity of these components has yet to be established. It would be very interesting to determine whether the components around the myo-inositol retention in normals are the same as already observed in this work for diabetics. If they are then there is the suggestion that yet a further metabolic pathway for glucose is active in diabetics but not in normals. In this respect myo-inositol has been demonstrated to be synthesised from glucose (Niwa et al., 1983). Therefore, what is possibly being observed is this conversion in the erythrocyte when subjected to enforced hyperglycaemia and that this conversion is occurring in diabetics and not in normals. It is recommended that any link between this phenomena and neuropathy is further investigated.

Attempts were made to correlate erythrocyte AR activity, by assessing the accumulation of sorbitol when incubated with glucose, with the degree of severity of neuropathy. The aim was to achieve a simple diagnostic test which would indicate which subjects had increased AR activity and therefore the possibility of increased
susceptibility to neuropathy. No such correlation was found and it could be concluded that the erythrocyte model is not a valid reflection of AR activity.

However, this particular investigation was subjected to both logistical difficulties and more importantly to probable errors in the experimental incubation conditions employed which did not allow for the full expression of AR activity. According to Das and Srivastava (1985b) the presence of 0.4M lithium sulphate in the assay mix is essential for the full expression of AR activity. Lithium sulphate was not added in the assay mix and therefore its effect, if any, on the outcome of the study is not known. The repeatability of the AR activity determination for individual diabetics with differing degrees of neuropathy is also not known. It would be useful to repeat the experiment, with the inclusion of lithium sulphate, before the erythrocyte AR activity model is rejected as a possible diagnostic test. Therefore, further work is required to establish the erythrocyte AR activity assay conditions and to determine its use as a diagnostic aid to neuropathy.

According to Ghahary et al., (1989) there is no convincing data demonstrating that AR activity is increased in diabetic tissues. However, they have demonstrated that renal AR activity in the streptozotocin-induced diabetic rat is increased and accompanied by increased messenger RNA levels coding for aldose reductase. Consequently, in the future there is the possibility of using gene probes, in appropriate cells or biopsy material, for diagnostic purposes which would replace assessment of AR activity by enzymatic assays.

In summary, the enzyme assay has been validated and found to be useful for determining the sorbitol levels in diabetic subjects. In particular the assay, at this stage
of its development, was sensitive enough to determine gross changes in the erythrocyte sorbitol levels both in-vivo and in-vitro. Considering the ability of the assay to achieve this it may now be of most use as a screening assay for routine hospital laboratory use. It would not be difficult to adapt the conditions of the assay to produce a kit that could be used under standard conditions. If the new preparation of SDH, mentioned above, is truly mono specific for sorbitol it would be the best choice for turning into a diagnostic kit. This would provide the clinician with a front line cost effective screening test for sorbitol levels as an indicator of aldose reductase activity until more refined biochemical diagnostic tests become available. The application of such a test would be best utilised at the early stage of diagnosis of diabetes with monitoring of individual erythrocyte sorbitol levels perhaps at yearly intervals.

Erythrocyte sorbitol determination used in conjunction with other tests already available, such as HbA1c determination, give the best possible chance of aiding the physician in detecting the early onset of diabetic complications. This is especially needed in the absence of obvious clinical signs of neuropathy. Indeed, when the clinical signs of overt neuropathy become apparent it is too late to change the course of medical management to avoid the condition. In the ideal world national screening programmes would be introduced to screen for AR activity but this is unlikely in the present economic climate. By contrast it is recommended that the capillary GLC method is the tool of choice for research into the polyol and sugar changes occurring in diabetes.

In conclusion, the results of this work support the hypothesis that erythrocyte sorbitol levels are raised in diabetics above normal. Therapy with Sorbinil does lower diabetic erythrocyte sorbitol levels to within the normal non-diabetic range and that in normals
this range is very consistent. Both good glycaemic control of diabetics and whatever therapy is currently available to them should be used to achieve consistent erythrocyte sorbitol levels similar to normals. It is recommended that sorbitol levels should be monitored at an early stage at the onset of initial diagnosis of diabetes. If this is done then the long term management of diabetes may help to safeguard those who are at most risk of developing the long term complications associated with this condition.
APPENDIX 1

REFERENCES


