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TITLE THE DETECTION AND QUANTIFICATION OF CANNABINOIDS AND THEIR METABOLITES IN BODY FLUIDS

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THE DETECTION AND QUANTIFICATION OF CANNABINOIDS
AND THEIR METABOLITES IN BODY FLUIDS

by

PETER LOUIS WILLIAMS, B.Sc.

A thesis submitted for the degree of
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### CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>SUMMARY</th>
<th>SECTION 1: INTRODUCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(A) GENERAL INTRODUCTION</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(B) THE BOTANY OF CANNABIS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C) THE PHARMACOLOGY OF CANNABIS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(D) THE ISOLATION AND STRUCTURAL ELUCIDATION OF NATURALLY OCCURRING CANNABINOIDs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(E) THE METABOLISM OF THE CANNABINOIDs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(F) CANNABINOID DETECTION AND QUANTIFICATION IN BODY FLUIDS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SECTION 2: PLASMA ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) RADIOIMMUNOASSAY</td>
</tr>
<tr>
<td>(B) HIGH PRESSURE LIQUID CHROMATOGRAPHY-RADIOIMMUNOASSAY</td>
</tr>
<tr>
<td>(C) HIGH PRESSURE LIQUID CHROMATOGRAPHY-GAS CHROMATOGRAPHY-MASS SPECTROMETRY</td>
</tr>
<tr>
<td>(D) CONCLUSION</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SECTION 3: URINE ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) RADIOIMMUNOASSAY</td>
</tr>
<tr>
<td>(B) HIGH PRESSURE LIQUID CHROMATOGRAPHY-RADIOIMMUNOASSAY OF URINE SAMPLES</td>
</tr>
<tr>
<td>(C) THE IDENTIFICATION OF URINARY CROSS-REACTING COMPOUNDS</td>
</tr>
<tr>
<td>(D) THE DETECTION OF CANNABINOIDs IN BODY FLUIDS BY THEIR PHOTOCHEMICAL CONVERSION TO FLUORESCENT PRODUCTS</td>
</tr>
<tr>
<td>(E) CONCLUSION</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SECTION 4: THE ANALYSIS OF SAMPLES SUBMITTED FOR FORENSIC EXAMINATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) INTRODUCTION</td>
</tr>
<tr>
<td>(B) CASE EXAMPLES</td>
</tr>
<tr>
<td>(C) GENERAL CONCLUSION</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>REFERENCES</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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</table>
SUMMARY

This thesis describes the evaluation of methods based on radioimmunoassay (RIA) and gas chromatography-mass spectrometry (GC-MS) and the development and subsequent evaluation of combined high pressure liquid chromatography and radioimmunoassay (HPLC-RIA) and HPLC-fluorescence methods for the detection and quantification of cannabinoids in body fluids. RIA was used to measure levels of cross-reaction in plasma and urine samples obtained from subjects after they had smoked a moderate dose of Δ⁹-THC. It was possible to distinguish these from blank samples. The GC-MS method was capable of quantifying Δ⁹-THC in plasma at concentrations down to 1 ng/ml. This method was used to measure Δ⁹-THC concentrations in plasma samples obtained from the Δ⁹-THC smokers. The HPLC-RIA method was able to quantify several individual cross-reacting cannabinoids in one analysis in either plasma or urine at concentrations as low as 0.1 ng/ml. This method was used to measure concentrations of Δ⁹-THC and its metabolites in both plasma and urine samples obtained from the Δ⁹-THC smokers. The HPLC-fluorescence method could detect less than 1 ng of cannabinol but has not been developed to the stage where other cannabinoids could be detected.

Forensic case samples requiring examination for cannabinoid content were analysed with the RIA, GC-MS and HPLC-RIA methods. RIA and HPLC-RIA were found to be suitable methods for the routine analyses of plasma and urine samples.
SECTION 1

INTRODUCTION
(A) GENERAL INTRODUCTION

This thesis describes the evaluation and development of methods for identifying and quantifying cannabinoid compounds in body fluids. These were investigated primarily for use in Forensic Science Laboratories to help fill a requirement for rapid methods which provide an unequivocal result for the confirmation of the presence of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, the psychoactive constituent of cannabis) and its metabolites in plasma and urine samples. Due to the gravity of the situation that a forensic scientist encounters when giving evidence concerning the illegal possession of cannabis in a court of law, he requires a detailed knowledge of many aspects of the subject. Accordingly this introduction contains general background information of relevance to the forensic scientist involved in the analysis of cannabinoid material.
(B) THE BOTANY OF CANNABIS

Cannabis is one of man's oldest cultivated plants, its fibre has been used for hempen cloth manufacture for at least 6000 years and was used for this purpose in regions as far apart as Eastern Europe and China. Knowledge of its use as a drug also extends back from the present by several millennia. The Scythians grew hemp in the Volga region 3000 years ago and burnt the cannabis seeds (presumably contaminated with resin) to produce an intoxicating smoke. In the Indian sub-continent medical writings compiled before 1000BC report the use of cannabis resin as a medicine. Hemp was introduced into Western Europe at about 1500BC, possibly by Scythian invaders, and was subsequently spread further west by Teutonic peoples.

The name Cannabis goes back to Roman times, but the starting point for its botanical nomenclature (Cannabis sativa) dates from Linnaeus's Species Planatarum of 1753. Cannabis is probably a monotypic genus (a genus with one species) that has diversified into a large number of ecotypes and cultivated races. Cannabis indica has been used to denote a race native to India with a high concentration of intoxicating principles, but to catalogue Cannabis sativa into its different races is complicated by the fact that the plant changes in some way to adapt to its environment. Plants grown in England and France from seed imported from India have changed over several generations to plants morphologically indistinguishable from varieties long acclimatised to European conditions. There are, however, considerable differences in the habit and properties of the
varieties, some of which have undoubtedly been caused by selection by man for a particular property of the plant.

*Cannabis sativa* is a rapidly growing erect annual which completes its life cycle within a few months. It is classified as allied to *Humulus*, the genus of the hop plant. The plant grows from 3 to 15 feet in height, with an angular sometimes hollow stem and long narrow leaves (Figure 1). The flowers are produced in great abundance on the upper part of the plant which is usually unisexual (normally the plant bears only one kind of flower and is either male or female, but sometimes the plant does have both characteristics). The fibres of the stem are used to produce hemp, while oils from the plant and seeds are used in the paint and soap industries and the seed is used to feed poultry and cagebirds. The pharmacological properties of cannabis are found in the resin produced in glandular hairs on the leaves, stem and inflorescences of the plant. The largest quantities of resin are found on the bracts (part of the flower) of the female plant during the flowering or fruiting period of development. The history and botany of *Cannabis* has been reviewed (Stearn, 1970 and Schultes, 1970).

The pharmacological effects of cannabis are usually obtained by smoking the plant material or an extract, though the drug may also be eaten or drunk (as a tea). Cannabis is used in several forms. Herbal cannabis is normally the dried upper part of the plant harvested during the fruiting and flowering period. Kif is prepared by finely chopping the plant material and removing large particles with a sieve. This preparation may also be referred to as herbal cannabis. Cannabis resin is
Figure 1. Cannabis sativa. Drawn by D Erasmus, the British Museum (Natural History), London.
obtained by compressing the dried plant material in gauze to extrude the resin. A modern form of the drug known as liquid cannabis, hash oil or THC, is prepared by extracting the plant material with an organic solvent to give a concentrated cannabinoid preparation. Because of the high concentration of THC in this preparation, it is one of the most potent forms of the drug. There are many names for preparations of cannabis. The dried plant material may be termed marihuana, grass or dagga; the resin may be called hashish, or charras and there is also an extensive and changing vocabulary relating to cannabis and its effects.
The major psychoactive constituent of the drug, $\Delta^9$-THC, acts upon the central nervous system. Its effects are dependent on the dose, the person taking it, his cultural background and to some extent the environment in which it is taken. The response to cannabis will also vary according to the method by which it is taken. When it is smoked the effects normally come within half an hour and last for two or three hours. Where it is taken by mouth the onset of effects are delayed sometimes up to two or three hours and may last as long as six hours. Because of the relatively rapid onset when the drug is smoked, experienced smokers can adjust their dosage to achieve the effect that they seek. Taking cannabis does not normally result in any obvious physical effects except that of redness of the eyes. When the drug is smoked there may be some initial rawness of the throat and tightness in the chest, sometimes this may be accompanied by nausea or a headache. The apparent effects of the drug in moderate amounts are predominantly psychological. They generally begin with a sense of excitement or tension, sometimes with apprehension or hilarity, followed by a sense of heightened awareness; colour, sounds and social intercourse appear more intense and meaningful and time may appear to pass more slowly. A sense of well being is then usual and after this a phase of tranquility and of passive enjoyment of the environment (the high) normally follows until, after a few hours, fatigue sets in and the subject may sleep. Once the effects of the drug have worn off there may be an increase in appetite and even ravenous hunger. The less
obvious physical effects of the drug include a raised pulse rate and blood pressure, dilated pupils and rapid shallow breathing. Cannabis also has a detrimental effect on manipulative ability, including driving performance. The pharmacological properties of cannabis have been summarised (Wootton Committee, 1968).

It is estimated that cannabis is consumed by between 200 to 300 million people in the world; 26 million citizens of the USA have tried the drug (Petersen, 1976) and cannabis users in the UK may number several hundreds of thousands (Wootton Committee, 1968).

It is of some importance therefore that any long term effects produced by the drug should be identified. The nature and degree of severity of such effects are at the moment the subject of some scientific controversy. While it is generally agreed that low consumption of the drug will probably cause no harmful effects it has been suggested that moderate or high consumption may cause irreversible brain damage, sharp personality changes, severe debilitation of the lungs and bronchial tract, affect hormone levels, cause chromosome damage and produce a disruption of cellular metabolism including synthesis of DNA (Maugh, 1974). As long as the possibility of such effects exist many authorities regard it as necessary to control the availability of cannabis. A report by the Wooten Committee (1968) concluded that because of the limits of present knowledge concerning the physical effects and social consequences of cannabis use, it was necessary in the interest of public health to maintain restrictions on the availability of the drug.

The sale of cannabis has been subject to control in the
UK since the Dangerous Drugs Act (1925). Restrictions concerning the possession and cultivation of cannabis were introduced in the Dangerous Drugs Act 1965. Control of cannabinoids was extended in the Misuse of Drugs Act 1971 to include cannabinol and its derivatives (class A drugs) as well as cannabis and cannabis resin (class B drugs).
During the last century a large part of chemical research was directed towards the isolation of pharmacologically active naturally occurring compounds. Three of the drugs which were successfully isolated, characterised and subsequently used in medicine during this period, were the alkaloids, morphine, cocaine and strychnine. This type of compound was, however, simple to isolate and purify relative to the naturally occurring terpenoids. These were usually present as complex mixtures of components whose separation was difficult to achieve with the techniques available at the time. The isolation of the active constituents of cannabis involved the separation of such a mixture of terpenes.

The first chemists to attempt the analysis of cannabis were Tscheepe (1821), Schlesinger (1840), Bohlig (1840), Savory (1843), Robertson (1847), Gastinel (1848), Smith and Smith (1847, 1848) and Smith (1885). Most of this work consisted of attempts to isolate an active cannabinoid by either the extraction of plant material with an organic solvent or the distillation of plant material to obtain the volatile oils. In many cases these workers were able to obtain a concentrated preparation containing the active constituent of cannabis, but a pure cannabinoid was not obtained. Wood et al. (1896) achieved a considerable purification of the components of cannabis by fractional distillation of an ethereal extract of the resin. A high boiling, viscous red oil was obtained which was assumed to be a single substance and was named cannabinol (CBN). The oil was extremely active, 20mg being sufficient to induce intoxication, and was regarded as the active constituent of the plant. Soon afterwards Dunstan and Henry
homogeneous and purified it further. After acetylation a crystalline acetate was obtained which upon hydrolysis yielded a resinous cryptophenol that analysed as $C_{21}H_{26}O_2$ (MW 310). The name of cannabinol was transferred to this compound which was the first cannabinoid to be obtained in a pure form. No indication was given at this time whether cannabinol was pharmacologically active.

Numerous workers over the following 30 years attempted to repeat this work without success, until the reinvestigation of the subject by Cahn (1930, 1931, 1932, 1933). Using the method of Wood et al. (1899) he obtained crystalline acetyl cannabinol with a melting point identical to that obtained previously. He conducted a series of complex degradative reactions upon cannabinol some of which had been used by Wood et al. (1899), in order to identify sections of the molecule. He was finally able to suggest a partial structure for cannabinol, in which the positions of the phenolic hydroxy and pentyl side chain groups were not fully determined (Figure 2).

![Cannabinol Partial Structure](image)

Figure 2 The partial structure of cannabinol elucidated by Cahn (1933)
The complete structure of cannabinol was finally established by the groups of Todd (Jacob and Todd, 1940<sup>a</sup> and 1940<sup>b</sup>, Simonsen and Todd, 1942, Leaf et al., 1942 and Todd 1946 in Britain and Adams et al. (1940<sup>a</sup>, 1940<sup>b</sup>, 1940<sup>c</sup>, 1941, 1942) in the United States. They synthesised the isomers of cannabinol suggested by Cahn, compared these with the natural compound and established which isomer was cannabinol (Figure 4). They also suggested that the active constituents of cannabis were probably a mixture of some of the isomers of THC.

![Formal numbering](image1)

![Monoterpenoid numbering](image2)

**Figure 3.** Numbering systems for Δ<sup>9</sup>-THC (Δ<sup>1</sup>-THC)

![Cannabinol and Δ<sup>10a</sup>-THC](image3)

**Figure 4.** Cannabinol and Δ<sup>10a</sup>-THC (Δ<sup>2</sup>-THC)

This work also produced a further important development in cannabinoid chemistry when both groups independently prepared pure samples of Δ<sup>10a</sup>-THC, (Figure 4) as an
intermediate in the cannabinol synthesis (the formal numbering system, Figure 3, has been used throughout this thesis). The compound was found to cause the characteristic effects of cannabis and because pure, natural THC was not available for a further 20 years it was subsequently used as a standard for biological work. Adams (1940b) also isolated cannabidiol (CBD) from cannabis, but did not determine its structure.

Wollner, et al. (1942), using the distillation and acetylation methods employed for the isolation of cannabinol, isolated a THC isomer from cannabis which was not identical to the synthesised Δ^{10a}THC. Wollner, et al. (1942) did not however determine the exact position of the double bond.

Further cannabinoid structural elucidation was not accomplished until the techniques of column chromatography, countercurrent distribution and nuclear magnetic resonance spectroscopy (NMR) were established. de Ropp (1960) separated cannabinoids on a florisil column and identified cannabinol and cannabidiol by comparison with the authentic samples from the work of Adams 20 years earlier. A third pharmacologically active component presumed to be a THC isomer was also isolated, but no structural work on this compound was reported. Korte and Sieper (1960) also isolated cannabidiol from cannabis using countercurrent distribution. Mechoulam and Shvo (1963) then determined the total structure of cannabidiol (Figure 5) using the existing data and further information obtained from its NMR spectrum.

Gaoni and Mechoulam (1964) chromatographed a hexane extract of cannabis resin on a florisil column and separated several cannabinoid components including a THC isomer. The ultra-violet spectrum of this component indicated that the double bond was not conjugated with the aromatic ring. From a
Figure 5. Cannabidiol

study of the NMR spectrum Gaoni and Mechoulam proposed that the double bond was in the $\Delta^9$ position. This was confirmed by the total synthesis of $\Delta^9$-THC by Mechoulam and Gaoni (1965a). The synthetic route included the preparation of cannabidiol as an intermediate. The relative stereochemistry of the two chiral centres of cannabidiol and hence $\Delta^9$-THC were established as trans, from NMR data by Mechoulam and Shvo (1963). The total structures of the three major components of cannabis had now been determined, and these confirmed by their synthesis. Details of the structural conformation of $\Delta^9$-THC (Figure 6) was reported by Archer et al. (1970).

The position of the double bond in a second naturally occurring THC isomer, $\Delta^8$-THC, a minor cannabis component, was established with NMR and mass spectral data by Budzikiewicz et al. (1965), Gaoni and Mechoulam (1966) and Hively (1966).

The structure of a further, often the major, component of cannabis, $\Delta^9$-THC acid (A) (Figure 7) was established by Korte et al. (1965), and that of the $\Delta^9$-THC acid (B) (Figure 7) by Mechoulam and Yagen (1969) on the basis of their NMR spectra.
The $\Delta^9$-THC acids are both decarboxylated to $\Delta^9$-THC by heat.

![Figure 6. Conformation of $\Delta^9$-THC](image)

Figure 6. Conformation of $\Delta^9$-THC

![Figure 7. $\Delta^9$-THC acid (A) and $\Delta^9$-THC acid (B)](image)

Figure 7. $\Delta^9$-THC acid (A) and $\Delta^9$-THC acid (B)

The structures of cannabidiolic acid and cannabinolic acid were established from NMR data by Mechoulam and Gaoni (1965). These compounds have a carboxylic acid group in the same position (2) as the $\Delta^9$-THC acid (A).

The propyl homologues (instead of pentyl) of $\Delta^9$-THC, cannabinol and cannabidiol, which were named $\Delta^9$-tetrahydrocannabinol, cannabivarol and cannabidivarol respectively were isolated and structurally identified by
Vollner et al. (1969), Vree et al. (1971), and Gill (1971) using NMR and mass spectrometry.

The structure of a minor cannabis component cannabigerol, (Figure 8) was established by Gaoni and Mechoulam (1964b, 1971) with UV and NMR, and confirmed by its synthesis.

![Figure 8. Cannabigerol](image)

Two groups, Claussen and Korte (1966) and Gaoni and Mechoulam (1966, 1971) independently isolated and established the structure of cannabichromene (Figure 9) with UV and NMR, both assigning the same name to this cannabinoid.

![Figure 9. Cannabichromene](image)

Cannabicyclol, a further minor cannabis component was
first isolated by Korte and Sieper (1964). Crombie and Ponsford (1968, 1971) proposed its structure from NMR data and confirmed this by synthesis (Figure 10).

![Cannabicyclol](image)

**Figure 10. Cannabicyclol**

The elucidation of the structures of the major cannabinoid components in cannabis would now appear to be essentially complete. This information together with an availability of pure compounds has permitted a study of cannabinoid metabolism.
Cannabinoids are extensively metabolised in mammals and a knowledge of this transformation may be of some help with the development of methods for their analysis in body fluids. The general metabolic pathway proceeds via an initial allylic or pentyl side chain hydroxylation, followed by other reactions of either further hydroxylation or further oxidation at the carbon atom containing the existing hydroxyl group. A final stage may be conjugation with glucuronic acid.

The excretion of metabolites in humans after the administration of radiolabelled $\Delta^9$-THC was measured by Lemberger et al. (1971). This group found that a lower proportion of metabolites were excreted in the urine relative to the faeces, and that the extent of the excretion in the urine over a seven day period represented approximately 25% of the original dose.

THC is a lipid soluble compound which is suitable for metabolic transformation by the hepatic microsomal enzyme system. The system requires as co-factors, reduced nicotinamide adenine dinucleotide phosphate and molecular oxygen and catalyses a variety of reactions, many being oxidations in which cytochrome P-450 serves as the terminal oxidase. Several laboratories throughout the world have examined the metabolic fate of THC with liver microsomal enzyme preparations. Nilsson et al. (1970) and Wall et al. (1970) incubated radiolabelled $\Delta^9$-THC with 10,000g liver supernatant from rabbits and rats and isolated a mono-hydroxylated $\Delta^9$-THC metabolite. This was identified by both groups using mass spectrometry and NMR as 11-hydroxy-$\Delta^9$-THC, (Figure 11).
Simultaneously Burstein et al. (1970) and Foltz et al. (1970) characterised the structure of a $\Delta^8$-THC metabolite, 11-hydroxy-$\Delta^8$-THC. Christensen et al. (1971) incubated $\Delta^9$-THC with human liver preparations obtained post-mortem and again the 11-hydroxy-$\Delta^9$-THC metabolite was identified.

\[ \text{Figure 11. 11-hydroxy-} \Delta^9 \text{-THC} \]

Lemberger et al. (1971), using radiolabelled $\Delta^9$-THC, found that in man 11-hydroxy-$\Delta^9$-THC was excreted mainly in the faeces and only 3% of the radioactivity was present as this metabolite in urine hydrolysed with $\beta$-glucuronidase. 11-hydroxy-$\Delta^9$-THC was identified by Wall et al. (1972) in a pooled plasma sample (1200ml) from five subjects who took $\Delta^9$-THC orally. Woodhouse (1972) examined the pooled urine of several cannabis smokers and identified this metabolite in the urine after its hydrolysis with $\beta$-glucuronidase. A GC-MS method for the measurement of this metabolite in plasma has been described by Wall et al. (1976). Plasma concentrations of the order of 1ng/ml were found by this method in several subjects that had been given 4 to 5 mg of $\Delta^9$-THC intravenously. The metabolite is known to have a similar pharmacological activity to $\Delta^9$-THC but these low concentrations in plasma could suggest that the compound is not responsible for the total
effects observed with $\Delta^9$-THC.

Other monohydroxylated THC metabolites have been isolated from liver incubates. Wall et al. (1970, 1971) identified pharmacologically active 8-β-hydroxy-$\Delta^9$-THC and Quarles et al. (1973) isolated the 8-α-hydroxy-$\Delta^9$-THC metabolite. Wall et al. (1974) detected both of these metabolites in human plasma after the administration of radiolabelled $\Delta^9$-THC. Maynard et al. (1971) using a dog liver microsomal preparation reported the occurrence of hydroxylation in the 1' and 3' positions of the side chain of $\Delta^8$-THC. Widman et al. (1975), using an isolated perfused dog lung to study $\Delta^9$-THC metabolism, isolated the 3'-hydroxy-$\Delta^9$-THC and 4'-hydroxy-$\Delta^9$-THC metabolites.

Several other simple oxidation products of $\Delta^9$-THC have been observed with incubates of microsomal preparations. Jones et al. (1974) isolated 8-oxo-$\Delta^9$-THC (Figure 12) from a mouse liver incubate of $\Delta^9$-THC.

![Figure 12. 8-oxo-$\Delta^9$-THC](image)

Gurny et al. (1972) identified 9,10-epoxyhexahydrocannabinol in a monkey liver incubate (Figure 13).

Ben-Zvi and Burstein (1974) obtained a small yield of
11-oxo-$\Delta^9$-THC (Figure 14) in a rat liver microsomal incubate of $\Delta^9$-THC. This aldehyde was postulated as an intermediate in the metabolic conversion of 11-hydroxy-$\Delta^9$-THC to the carboxylic acid metabolite.

![Figure 13. 9,10-epoxyhexahydrocannabinol](image)

The existence of a THC acid metabolite was originally reported by Nilsson et al. (1973). This group injected radiolabelled $\Delta^8$-THC into rabbits and identified $\Delta^8$-THC-11-oic acid in the urine. Wall et al. (1974) administered radiolabelled $\Delta^9$-THC to volunteers and observed that $\Delta^9$-THC-11-oic acid (Figure 15) was a major metabolite, present in the plasma, urine and faeces.

![Figure 14. 11-oxo-$\Delta^9$-THC](image)
The compounds described above are comparatively non-polar having only one polar group more than THC. They represent only a small proportion of the metabolites observed with in vivo studies and are formed as part of the initial metabolic process. Other more polar metabolites have been observed. For example, a pharmacologically inactive dihydroxy metabolite was isolated and identified as 8,11-dihydroxy-Δ⁹-THC (Figure 16) in a rat liver homogenate by Wall et al. (1971). Wall et al. (1974) detected this metabolite in human plasma and faeces after the administration of radiolabelled Δ⁹-THC.

An acidic metabolite was isolated by Burstein and Rosenfeld (1971) from rabbit urine after the administration of
radiolabelled Δ⁹-THC and identified it as 2'-hydroxy-Δ⁹-THC-11-oic acid (Figure 17).

A second acidic metabolite was reported by Burstein et al. (1972) in rabbit urine and identified as 1'-hydroxy-Δ⁹-THC-11-oic acid. Nordqvist et al. (1974) also administered radiolabelled Δ⁹-THC to rabbits and reported the identification of a dicarboxylic acid metabolite 4',5'-bisnor Δ⁹-THC-11,3'-dioic acid (Figure 18). With this metabolite two carbon atoms have been removed from the pentyl side chain.

A similar loss of side chain carbon atoms has been observed by Martin et al. (1976). Guinea pigs, rabbits and mice were given Δ⁹-THC intravenously and the metabolites 5'-nor-Δ⁹-THC-4'-oic acid, 4',5'-bisnor-Δ⁹-THC-3'-oic acid and 3',4',5'-trisnor-Δ⁹-THC-2'-oic acid were isolated from liver and identified with mass spectrometry. More polar metabolites present in mouse liver after Δ⁹-THC administration have been identified using mass spectrometry by Harvey and Paton (1976). These were 3',8α-dihydroxy-Δ⁹-THC-11-oic acid, 2',8α-dihydroxy-Δ⁹-THC-11-oic acid, 2'-hydroxy-Δ⁹-THC-11-oic acid, 3'-hydroxy-Δ⁹-THC-11-oic acid and 8α-hydroxy-Δ⁹-THC-11-oic acid.

![Figure 17. 2'-hydroxy-Δ⁹-THC-11-oic acid](image)

The metabolism of CBN has also been studied and found to resemble that of THC. Widman et al. (1971) and Wall et al. (1971) examined the transformation of CBN in liver microsomal
preparations and found that 11-hydroxy-CBN was the major metabolite. Wall et al. (1971) also identified a metabolite hydroxylated in the 2' position of the pentyl side chain. Wall et al. (1974) administered radiolabelled CBN (18mg) intravenously to volunteers and found that the most prominent single metabolite in plasma was CBN-11-oic acid and the major group of metabolites were polar and acidic. Low concentrations of the 11-hydroxy-CBN metabolite were also observed and approximately 8% of the administered dose was found in the urine and 35% in the faeces after 72 hours. The major transformation products in the faeces were the monohydroxylated metabolites and CBN-11-oic acid.

Several metabolites of CBN have been found after the administration of Δ⁹-THC. Ben-Zvi et al. (1974) found CBN-11-oic acid in the urine after the administration of radiolabelled Δ⁹-THC to a rhesus monkey. Ben-Zvi and Burstein (1975) identified 5',4',3',2'-quadnor-CBN-1'-oic acid, (Figure 19) again in monkey urine after the administration of Δ⁹-THC. This may be the result of an initial conversion of either Δ⁹-THC or metabolically formed 11-hydroxy-Δ⁹-THC to CBN followed by metabolism of the CBN.
Evidence for the formation of glucuronide conjugates has been reported by Harvey et al. (1977). This group identified, with mass spectrometry, glucuronides of CBN, 11-hydroxy-CBN and CBN-11-oic acid in mouse liver samples.

CBD also shares some similarity of metabolism with the other cannabinoids. Nilsson et al. (1971) studied the conversion of CBD by rat liver microsomes and identified 11-hydroxy-CBD.

Wall et al. (1974) administered radiolabelled CBD (20mg) intravenously to volunteers and found 11-hydroxy-CBD and CBD-11-oic acid in plasma and urine. A substantial quantity of unchanged CBD was found in the faeces.

Glucuronic acid conjugates of CBD and 11-hydroxy-CBD have been identified by Harvey et al. (1977) in mouse liver samples after the administration of CBD.

The metabolism of the three major cannabinoids has now been substantially elucidated, but the relative proportions of these products present in body fluids has not yet been determined fully. The development of methods with sufficient sensitivity and specificity to permit the quantitation of the low concentrations of these metabolites present in body fluids...
should eventually provide further information concerning cannabinoid metabolic pathways.
Attempts to devise methods for the detection of cannabinoids in plasma and urine have been complicated by the fact that the ingestion of sufficient quantities of cannabis to produce its pharmacological effects result in only low levels of cannabinoids and their metabolites in body fluids. This factor requires that not only should a method have the sensitivity to detect these concentrations, but should also have the specificity to avoid interference from endogenous compounds present in plasma and urine. Initial efforts to develop such a method were based upon thin-layer chromatography (TLC) with Fast Blue B as a visualising agent with some specificity of reaction towards cannabinoids.

The first report of the detection of a cannabinoid in body fluids was made by da Silva (1967) who used a TLC method which was claimed to be capable of detecting cannabiol in the blood, urine and saliva from subjects intoxicated with cannabis. Cannabinoids have been detected in saliva from cannabis smokers with TLC by Just et al. (1972) and Hackel (1972) but no subsequent workers have confirmed the results obtained by da Silva with plasma and urine. Christiansen and Rafaelson (1969) described a TLC method for the detection of cannabinoids in urine. The urine, obtained from subjects after they had ingested cannabis resin, was hydrolysed with β-Glucuronidase. Several Fast Blue B positive compounds were observed, one component having the characteristics of cannabidiol. Kanter et al. (1971) also examined the urine of subjects who received $\Delta^9$-THC by this method, but failed to obtain a positive reaction. Andersen et al. (1971) reported a further method for cannabinoid
detection. Urine samples, obtained from subjects who had taken cannabis resin orally, were extracted with petroleum ether and treated with p-toluene sulphonic acid to dehydrate any mono-hydroxylated metabolites to CBN. CBN was then identified by TLC. Urine analysed by this method showed the presence of CBN for 6 hours after ingestion of cannabis resin. Salaschek et al. (1973) reviewed these TLC methods for cannabinoid detection. Fifty samples of human urine obtained after ingestion of cannabis resin were compared with twenty two control samples. Positive results were obtained with only four of the urine samples and with these there was still a possibility of misinterpretation. It was concluded that reliable, routine detection of cannabis ingestion was not provided with the existing TLC methods.

Another TLC method for the detection of THC in blood has been described recently by Vinson et al. (1977). Blood was extracted, treated with 2-p-chlorosulfophenyl-3-phenylindone (DIS-C1) and the reaction product investigated by TLC. The derivatised THC was visualised with an alkoxide spray which produced a fluorescent spot visible under ultraviolet light. The method was claimed to be sensitive to 0.2ng/ml of THC in a 5ml serum sample.

TLC has advantages in the speed and low cost of analysis, but early methods were not successful, and many workers turned to other techniques. Several methods based upon gas chromatography (GC) employing specific detectors have been devised. Garrett and Hunt (1973) described a GC method with electron capture detection (ECD) for the analysis of Δ⁹-THC in blood. The method consisted of an initial plasma extraction
and derivative formation with pentafluorobenzoyl chloride followed by GC-ECD of the fluorinated THC derivative. The method was claimed to be capable of detecting THC levels of 40-125ng/ml in plasma. Its use was only described for the quantification of THC in dog plasma and a clean-up stage was not included in the method. Routine analysis of human plasma using this procedure would probably involve considerable problems of interference from the endogenous substances present in plasma.

Fenimore et al. (1973) described a GC-ECD method which employed a clean-up stage. An internal standard hexahydrocannabinol was added to the plasma sample which was then extracted with an organic solvent. The extract was treated with heptafluorobutyric anhydride and the derivatised products injected onto a dual oven GC. This was constructed so that when the derivatised cannabinoids eluted from the first column they were directed onto a second capillary column where they were chromatographed with only small quantities of interfering compounds. The capillary column eluent was monitored with ECD. The method was claimed to be capable of detecting 0.1ng/ml of Δ⁹-THC in plasma. The use of this procedure for the quantification of Δ⁹-THC in human plasma after a subject had smoked a Δ⁹-THC-impregnated cigarette was described by Fenimore et al. (1976). The method has the disadvantage that a suitable dual oven chromatograph would have to be constructed before analysis. McCallum (1973) described a GC method employing flame photometric detection. This consisted of extracting a plasma sample with an organic solvent and treating this with diethyl phosphorochloridate to produce a Δ⁹-THC phosphate ester. This was then injected onto
a GC equipped with a flame photometric detector. The method was claimed to have a limit of sensitivity of 2ng/ml and was able to detect both THC and CBN in human plasma. McCallum et al. (1974 and 1975) described further use of the method for cannabinoid quantitation in human plasma.

Agurell et al. (1973) reported another successful GC method for Δ⁹-THC quantification in which a mass spectrometer (MS) was employed as a specific detector. A quantity of internal standard, di-deuterated Δ⁹-THC, was added to the plasma sample, which was then extracted with an organic solvent. Many of the endogenous interfering compounds present in the extract were removed by chromatography on a Sephadex LH-20 column. The refined extract was injected onto a GC with a mass spectrometer tuned to monitor ions 299 and 314 for Δ⁹-THC and 301 and 316 for Δ⁹-(1',2'-2H₂)THC. The Δ⁹-THC plasma concentration was calculated from the ratio of peak heights obtained from the deuterated and non-deuterated Δ⁹-THC. This procedure was suitable for the detection of less than 1ng/ml of Δ⁹-THC in plasma and was used to measure plasma concentrations in subjects who had smoked Δ⁹-THC.

Several other procedures have been described in which a mass spectrometer was used as a GC detector. Rosenfeld et al. (1974) reported a method for Δ⁹-THC quantification in which a deuterated methyl ether of Δ⁹-THC was used as internal standard and in which an extraction procedure was used in place of a chromatographic clean-up stage. An organic solvent extract of the plasma sample to be assayed was treated with a potassium hydroxide solution (0.1M) in order to extract Δ⁹-THC by way of
its phenolic properties. The extract, together with an internal standard $\Delta^9$-THC-OC$^2$H$_3$, was injected onto a GC in a solution of N,N,N,-trimethylanilinium hydroxide in methanol. $\Delta^9$-THC was converted on column to its methyl ether and peak heights from the mass spectrometer tuned to record ions 328 for $\Delta^9$-THC-OCH$_3$ and 331 for $\Delta^9$-THC-OCD$_3$ were used to calculate the plasma $\Delta^9$-THC concentration. The assay was used to measure $\Delta^9$-THC concentrations in the range 1-200ng/ml for plasma samples obtained from subjects who had previously smoked $\Delta^9$-THC-impregnated cigarettes.

A GC method for $\Delta^9$-THC quantification in plasma employing chemical ionisation-mass spectrometry (CI-MS) was described by Detrich and Foltz (1976). The use of chemical ionisation in place of electron impact mass spectrometry resulted in a reduction of background interference from endogenous material present in plasma and allowed the use of a simple extraction procedure prior to chromatography. Deuterated $\Delta^9$-THC was used as the internal standard, and both the deuterated and non-deuterated $\Delta^9$-THC were chromatographed as TMS derivatives. The method was claimed to be sensitive to 0.5ng/ml of $\Delta^9$-THC and was used with spiked plasma samples.

GC-MS has also been used for the quantification of $\Delta^9$-THC metabolites in biological fluids. Rosenfeld and Taguchi (1976) reported a modification of their GC-MS method for $\Delta^9$-THC quantitation which could be used for the measurement of 11-hydroxy-$\Delta^9$-THC. The procedure was, however, only capable of measurement down to 3ng/ml and quantitation of the metabolite in human plasma after the administration of $\Delta^9$-THC was not reported.

Wall et al. (1976) used GC-MS for quantification of
Δ⁹-THC, CBN and 11-hydroxy-Δ⁹-THC in human plasma samples obtained after the intravenous administration of 4-5 mg of Δ⁹-THC. The method employed a Sephadex LH-20 column for the removal of interfering plasma constituents prior to GC-MS. Deuterated cannabinoids were used as internal standards. The method was able to measure down to 0.2ng/ml and levels of these cannabinoids were determined in plasma over the 24 hour period after Δ⁹-THC administration.

Nordqvist et al. (1976) modified the GC-MS method described by Agurell et al. (1973) for the analysis of Δ⁹-THC-11-oic acid in plasma and urine. The metabolite was chromatographed as its methyl ester and TMS derivative. The sensitivity of the method was claimed to be in the range of a few ng/ml but the quantitation of the metabolite in human body fluids was not described.

The methods described above all utilise a chromatographic separation prior to detection. A further form of detection involving the immune reaction, radioimmunoassay (RIA) has sufficient specificity to allow it to be used as the basis of a non-chromatographic assay. Several RIA procedures have been developed for the detection of cannabinoids in body fluids. Teale et al. (1974^a, 1974^b, 1974^c and 1975) described the production of an antiserum which would cross-react with Δ⁹-THC and the use of this for cannabinoid detection in body fluids. The assay could measure quantities of Δ⁹-THC as small as 50pg and levels of cross-reaction of 1ng/ml in urine and 7.5ng/ml in plasma. The assay was used to measure cross-reaction in plasma and urine samples obtained from subjects after they had smoked cigarettes impregnated with 5mg of Δ⁹-THC. Levels of cross-reaction were between 0-70ng/ml for
the urine and plasma samples obtained from four subjects. Antibodies to $\Delta^9$-THC conjugated to bovine serum albumin via the phenolic group were raised in sheep. The specificity of the antiserum was investigated and twenty four non-cannabinoid drugs were found to give no cross-reaction, suggesting that the assay was specific for cannabinoid compounds. As well as $\Delta^9$-THC the antiserum was found to cross-react with other cannabinoids and $\Delta^9$-THC metabolites. Marks et al. (1975) reported results which were obtained after the application of this assay to the analysis of urine obtained from hospital patients suspected of taking drugs. Several of the urine samples examined were found to contain cannabinoid material and gave levels of cross-reaction >100ng/ml. The high levels of cross-reaction which have been found in urine suggests that the assay is measuring cannabinoid metabolites rather than $\Delta^9$-THC which has never been detected in urine.

Grant et al. (1972) and Gross et al. (1974) also described the use of an RIA procedure for cannabinoid detection. The antiserum was raised in rabbits after the administration of a $\Delta^9$-THC-azobenzoyl-keyhole limpet haemocyanin conjugate. The assay was used to measure levels of cross-reaction in plasma samples obtained from chronic users of cannabis and these were found to give levels of cross-reaction between 200-250ng/ml. The sensitivity of the method was limited to 25-50ng/ml which was sufficient to confirm the presence of cannabinoid material in all but weakly positive samples. Gross and Soares (1976) also described the use of RIA procedures specific for $\Delta^9$-THC and the $\Delta^9$-THC-11-oic acid metabolite and the measurement of concentrations of these metabolites in human plasma and urine samples. The $\Delta^9$-THC plasma concentrations obtained with the RIA were compared with results obtained for the same
samples using a GC-MS method for $\Delta^9$-THC quantification and the two sets of results were found to give good agreement. The $\Delta^9$-THC-11-oic acid RIA gave results which were higher than would be expected for concentrations of this metabolite in body fluids, suggesting that this assay may not be totally specific for the one metabolite.

Chase et al. (1976) described a cannabinoid RIA procedure for which antisera were raised in rabbits after the administration of $\Delta^9$-THC conjugated by its phenolic hydroxyl group to bovine serum albumin. The assay was used to measure levels of cross-reaction in human urine samples obtained after the oral administration of $\Delta^9$-THC and cannabis extracts.

Cook et al. (1976) described a RIA procedure in which antisera were raised after $\Delta^9$-THC, bound to bovine serum albumin by the pentyl side chain of THC, was intravenously administered to rabbits. The use of this type of conjugate for raising antibodies was designed to provide antiserum specific for THC measurement. The assay was used with plasma samples spiked with 5-100ng/ml of $\Delta^9$-THC.

A free radical immunoassay has been described for cannabinoid measurement by Cais et al. (1975). Antiserum was raised in rabbits by administering a $\Delta^9$-THC-bovine serum albumin or ovalbumin conjugate. $\Delta^9$-THC was conjugated with the protein at several points of the molecule in order to provide antisera of varying specificities. Spin labelled antigen was synthesised by coupling $\Delta^9$-THC to 3-amino-2,2,5,5 tetramethyl pyrrolidine-1-oxyl. The proportion of unbound spin labelled $\Delta^9$-THC in the immunoassay and equilibrium mixture was measured using electron spin resonance. The assay
was capable of detecting cross-reacting compounds in urine from 5-72 hours after subjects had smoked quantities of cannabis resin.

The methods described in this section do not provide a complete answer to the problem of the detection and quantification of cannabinoids in body fluids. The chromatographic methods are at present capable of detecting only a few cannabinoids and are time consuming. RIA does not provide information concerning the concentration of the individual cannabinoids in body fluids. This thesis will describe some further contributions to the problem of detecting cannabinoids in plasma and urine.
SECTION 2

PLASMA ANALYSIS
(A) RADIOIMMUNOASSAY (RIA)

INTRODUCTION

RIA is a technique well suited to the detection of cannabinoids in body fluids. It has sufficient sensitivity for the measurement of the low concentrations of cannabinoids present in body fluids and the specific nature of the immune-reaction involved in the assay avoids interference from endogenous compounds present in body fluids.

Some of the advantages of this technique compared with the chromatographic methods for cannabinoid analysis described in the previous section are; only a small sample volume is required, minimal sample preparation is needed, analysis time is usually short, and many samples may be analysed at the same time. RIA does have the disadvantage that whereas the chromatographic methods usually provide measurement of a specific cannabinoid, RIA may give a result which represents the sum of contributions from several cross-reacting cannabinoids or their metabolites.

RIA represents one of the more viable methods for cannabinoid analysis and as part of an investigation into methods for cannabinoid detection in body fluids, antiserum was obtained for the assay procedure described by Teale et al. (1974), and this method was then evaluated. The objectives of this evaluation of RIA for the analysis of cannabinoids in plasma were: (a) obtaining an indication of the levels of cross-reaction in human plasma after the administration of a known quantity of $\Delta^9$-THC (b) determining how well the assay would distinguish between positive and negative samples and (c) obtaining a measure of the specificity of the assay.
with both cannabinoid and non-cannabinoid compounds.

EXPERIMENTAL

Materials and Equipment

Antiserum (133Y/22/5) for the assay was obtained from Dr J D Teale, Department of Biochemistry, University of Surrey and $\Delta^9$-THC and other cannabinoid compounds were generously provided by the National Institute on Drug Abuse, Rockville, USA. $\Delta^9-(^{3}H)$-tetrahydrocannabinol was purchased from the Radiochemical Centre, Amersham, Bucks, polyvinylpyrrolidone-40 and charcoal (Norit A) from Sigma Chemicals Ltd., Dextran T70 from Pharmacia, and methanol (Analar) and all other chemicals and solvents were obtained from BDH Chemicals Ltd. An Intertechnique SL30 was used for liquid scintillation counting.

Method

The RIA method was based upon that described by Teale et al. (1975), but was modified by the replacement of the THC solubilising agent Triton X-405 with methanol and with the replacement of bovine $\gamma$-globulin in the assay buffer with polyvinylpyrrolidone-40. Antiserum (133Y/22/5) was stored as aliquots in buffer (0.1M phosphate buffer pH 7.5 containing 0.2% polyvinylpyrrolidone-40) at $-20^\circ$C and diluted to 1:300 before use. $\Delta^9$-THC solutions used to calibrate the assay were made up in aqueous methanol (50% v/v, pH 7.5) at concentrations ranging from 500pg/ml to 50ng/ml and stored at $-20^\circ$C. $\Delta^9-(^{3}H)$-THC was also stored at $-20^\circ$C at a concentration of 0.25$\mu$Ci/ml (12Ci/mmol) in aqueous methanol (50% v/v, pH 7.5) ready for use.

The plasma sample to be assayed was mixed with three volumes of methanol, vortexed and allowed to stand for 30
minutes. It was then centrifuged and the supernatant added directly to the assay tubes. Normal human plasma similarly treated was used in the total, non-specific binding and zero tubes and for assay dilutions. Using the protocol in Table 1, reagents were added to a series of assay tubes (duplicated); the antiserum was added last. These were then allowed to stand at room temperature for 1 hour. Dextran-coated charcoal (Teale et al. 1975, pH 9.5) was added, the tubes were centrifuged after 2 minutes contact time and 500μl of the supernatant from each tube was counted. The results obtained from serial dilutions of a sample were averaged to obtain a mean value.

Plasma samples taken after Δ⁹-THC were obtained from three volunteers who smoked tobacco cigarettes impregnated with 10mg (subject 1 and 2) and 8mg (subject 3) of Δ⁹-THC.

### TABLE 1

**RADIOIMMUNOASSAY PROTOCOL**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total counts tube</td>
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<tr>
<td>Δ⁹-THC (50% MeOH)</td>
<td>50</td>
</tr>
<tr>
<td>THC standard (50% MeOH)</td>
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</tr>
<tr>
<td>50% MeOH</td>
<td>100</td>
</tr>
<tr>
<td>Plasma sample extract</td>
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<td>Normal plasma extract</td>
<td>100</td>
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<tr>
<td>Diluent buffer</td>
<td>250</td>
</tr>
<tr>
<td>Antiserum</td>
<td>100</td>
</tr>
</tbody>
</table>

*Incubated for 1 hour at room temperature*

| Diluent buffer                         | 200         | -             | -         | -             | -           |
| Dextran-coated charcoal (2.5%)         | -           | 200           | 200       | 200           | 200         |

*Centrifuged and 500μl of supernatant counted*
over a 10 minute period. Blood samples (10ml) were taken at timed intervals after the subjects had finished smoking. Blank plasma samples were also obtained from twelve volunteers with no history of cannabis use.

Anticoagulant (K$_2$EDTA, 10mg) was added to the blood which was centrifuged to separate the plasma. This was stored at -20°C until analysis.

The cross-reactivity of the cannabinoids were assessed by determining the quantity required to give a 50% depression of binding of the radiolabelled Δ$_9$-THC with the normal assay conditions. The measurement was made by assaying each cannabinoid over a range of concentrations.

The non-cannabinoid drugs were assessed for any cross-reactivity by assaying each compound using the normal RIA conditions.

RESULTS AND DISCUSSION

The assay methodology was similar to that described by Teale et al. (1975), except that methanol replaced Triton X-405 as the THC solubilising agent. This modification increased the solubility of THC and reduced non-specific binding in the assay. A reduction of blank levels was obtained with normal plasma samples together with improved assay sensitivity.

Blank plasma levels for the twelve samples examined all gave a level of cross-reaction less than 2ng/ml (Table 2).

The levels of cross-reaction in plasma samples obtained from the three subjects who smoked THC impregnated cigarettes
are given in Table 3. The samples taken after Δ⁹-THC gave levels of cross-reaction between 5 and 67 ng/ml. These were significantly greater than the results obtained with blank plasma samples.

Subject 1 was a regular user of cannabis and the control sample contained cross-reacting material. This was probably due to the consumption of cannabinoid material at some time before the experiment. Subject 3 was not a cannabis user and his control sample contained no indication of cross-reacting material. Levels of cross-reaction were highest for those samples obtained immediately after smoking while values obtained for subsequent samples fell to a constant level for the remainder of the two hour experiment. The absence of a continuing decline of plasma cross-reacting cannabinoids over
TABLE 3
THE PLASMA CONCENTRATION OF $\Delta^9$-THC IN VOLUNTEERS WHO HAD SMOKED $\Delta^9$-THC DETERMINED BY RADIOIMMUNOASSAY, HIGH PRESSURE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY - MASS SPECTROMETRY

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time after smoking (min)</th>
<th>Plasma concentration of $\Delta^9$-THC (ng/ml)</th>
<th>Direct RIAa</th>
<th>RIA after separation by HPLC</th>
<th>GC - MS</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Control</td>
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<td>23$^b$</td>
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<td>120</td>
<td></td>
<td>6</td>
<td>0.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

a These values include a contribution from THC metabolites
b Subjects 1 and 2 were both cannabis users and cannabinoid material may have been present in the plasma before the experiment
c A control sample was taken for subject 2 but this was lost during centrifugation
ND Not determined

The course of the experiment could not be fully explained without a greater understanding of the changes of plasma cannabinoid concentrations.

Teale et al. (1975) investigated antiserum specificity
and found no cross-reaction with twenty four non-cannabinoid drugs. Forty three non-cannabinoid drugs chosen as representative of a wide range of drugs were examined in this study (Table 4) at a concentration equivalent to 400μg/ml plasma and again no cross-reaction was observed with any of the compounds. Antiserum specificity to cannabinoid compounds using the modified RIA procedure was examined (Table 5). Results were similar to those obtained by Teale et al. (1975) in that the antiserum cross-reacted with the same avidity to THC as it did to some of the closely related THC metabolites.

### Table 4

**MISCELLANEOUS DRUGS TESTED FOR CROSS-REACTIVITY IN THE RADIOIMMUNOASSAY**

| Adrenaline | Methaqualone hydrochloride |
| Amtriptyline hydrochloride | Morphine sulphate |
| Amphetamine sulphate | Nicotine hydrogen tartrate |
| Ascorbic acid | Nitrazepam |
| Aspirin | Oestradiol |
| Barbitone | Papaverine hydrochloride |
| Bromodiphenhydramine hydrochloride | Paracetamol |
| Caffeine | Pentobarbitone |
| Codeine phosphate | Phenobarbitone |
| Cocaine hydrochloride | Phenmetrazine theoclate |
| Chlordiazepoxide | Phenylbutazone |
| Chlorpromazine hydrochloride | Progesterone |
| N,N-Dimethyltryptamine | Spironolactone |
| Diphenhydramine hydrochloride | Stilboestrol |
| Doxepin | Sulphadimidine |
| Ephedrine hydrochloride | Sulphamethoxyumpyridazine |
| Ergometrine maleate | Tetracycline hydrochloride |
| Ethylenediamine tetraacetic acid | Thiopeptone sodium |
| Imipramine | Trifluoperazine |
| Lignocaine | Tryptamine hydrochloride |
| Mescaline sulphate | Tyramine hydrochloride |
| Methadone hydrochloride | [43](#) |
The above results demonstrate that the assay is able to distinguish samples obtained after a moderate dose of Δ⁹-THC from blank samples and some indication that the assay is specific for cannabinoid compounds is provided by the absence of reaction with non-cannabinoid drugs. The results therefore substantiate the view that the assay provides a viable means of detecting the presence of cannabinoid material in plasma.
The assay antiserum was shown in part A of this section to cross-react with some of the metabolites of Δ⁹-THC as well as the parent compound. This suggests that Δ⁹-THC metabolites provide some contribution to the level of cross-reaction given by a plasma sample and this may explain why the level of cross-reaction in plasma decreases very slowly over a two hour period after Δ⁹-THC smoking (Table 3). Because of this slow elimination of cross-reacting material, plasma RIA data gives no information concerning the time the sample was taken after Δ⁹-THC smoking unless further data concerning the levels of the individual cross-reacting components are also available. Such information is often important in forensic science where a knowledge of whether a person was experiencing the effects of a drug may be significant. This disadvantage of the RIA technique for cannabinoid analysis may however be removed by combining the assay with a chromatographic technique such as HPLC so that the RIA acts as a selective detector for the liquid chromatograph. The individual cross-reacting cannabinoid components in a plasma sample may then be separated by HPLC and quantified with the RIA. The identity of each component would be indicated by its HPLC retention volume.

The objectives of the work described here are concerned with obtaining an assessment of the viability of an HPLC-RIA method as a means of quantifying Δ⁹-THC and some of its metabolites in plasma.
EXPERIMENTAL

Materials and Equipment

A constant flow pump (Waters Assoc. M-6000A) was used to deliver a methanol/water eluent to a stainless steel HPLC column (10cm length x 4.6mm id) slurry-packed with Spherisorb-5-ODS (Phase Separations). Samples were introduced onto the HPLC column with a six port injection valve (Specac) fitted with a 10ml injection loop. Column eluate was either monitored with an ultraviolet detector at 280nm (Cecil CB212) or collected with a Struers Samplomat fraction collector (Camlab). A freeze dryer (Chemlab Instruments, model SB4) was used to remove solvents.

Method

Plasma (0.2-1ml) was mixed with three volumes of methanol, vortexed, left to stand for 30 minutes and centrifuged. The supernatant was removed, the residue mixed with methanol and the sample again centrifuged. Water was added to the combined supernatants to give a methanol concentration of 50% v/v. The HPLC pump, sample loop and column were flushed with aqueous methanol (50% v/v) and the sample solution injected onto the ODS column. A stepped solvent elution programme was used: 10ml of 50% methanol/50% water, 10ml of 62.5% methanol/37.5% water, 20ml of 72.5% methanol/27.5% water (1ml/min) and eluent fractions were taken every 30 seconds. Solutions containing the appropriate THC standards and the HPLC fractions were freeze dried. The freeze drier was flushed with argon before and after drying to prevent atmospheric oxidation of sensitive metabolites. A solution of \( \Delta^9-(3H)-THC \) (0.025\( \mu \)Ci in 500\( \mu \)l of 30% methanol/70% diluent buffer) was added to each of the dried tubes.
with a solution of antiserum in diluent buffer (100μl of 1:300 antiserum solution). The non-specific binding tubes received diluent buffer in place of antiserum solution. Subsequent RIA procedure was the same as that described above.

Cannabinoid retention volumes were determined using the pure compounds by monitoring the eluate with either RIA, for cross-reacting compounds, or UV absorption (280nm), for non-cross-reacting compounds. UV detection required μg quantities compared with the ng quantities used with RIA detection.

RESULTS AND DISCUSSION

The plasma samples taken during the Δ⁹-THC smoking experiments and assayed for RIA cross-reactivity as described in part A of this section, were all examined using the HPLC-RIA system. Chromatograms representing the elution of cross-reacting compounds from the HPLC column were constructed for each plasma sample (figures 20 to 36) by plotting the RIA results obtained for consecutive HPLC eluent fractions against their retention volumes.

A comparison of cannabinoid retention volumes (Table 6) with the HPLC-RIA chromatograms for plasma samples obtained after Δ⁹-THC smoking showed the presence of cross-reacting compounds in the plasma samples with retention volumes corresponding to those of Δ⁹-THC, CBN, mono-hydroxylated metabolites (11-hydroxy-Δ⁹-THC, 8α-hydroxy-Δ⁹-THC and 11-hydroxy CBN all have the same retention volume) and di-hydroxylated metabolites (8α,11-dihydroxy-Δ⁹-THC and 8β,11-dihydroxy-Δ⁹-THC have similar retention volumes). Control plasma samples were found to contain none of
Figure 20: The HPLC-RIA chromatogram of a plasma sample from subject 1 taken 2 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

Figure 21: The HPLC-RIA chromatogram of a plasma sample from subject 1 taken 12 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and dihydroxylated metabolites are marked.
Figure 22 The HPLC-RIA chromatogram of a plasma sample from subject 1 taken 24 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

Figure 23 The HPLC-RIA chromatogram of a plasma sample from subject 1 taken 34 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.
Figure 24 The HPLC-RIA chromatogram of a plasma sample from subject 1 taken 64 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

Figure 25 The HPLC-RIA chromatogram of a plasma sample from subject 1 taken 126 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.
Retention volume (ml)

Figure 26 The HPLC-RIA chromatogram of a plasma sample from subject 2 taken 3 minutes after smoking A9-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

Cross-reaction (ng/ml)

Figure 27 The HPLC-RIA chromatogram of a plasma sample from subject 2 taken 13 minutes after smoking A9-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.
Figure 28 The HPLC-RIA chromatogram of a plasma sample from subject 2 taken 24 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

Figure 29 The HPLC-RIA chromatogram of a plasma sample from subject 2 taken 34 minutes after smoking $\Delta^9$-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.
Figure 30 The HPLC-RIA chromatogram of a plasma sample from subject 2 taken 64 minutes after smoking Δ⁹-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

Figure 31 The HPLC-RIA chromatogram of a plasma sample from subject 2 taken 124 minutes after smoking Δ⁹-THC (10mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.
The HPLC-RIA chromatogram of a plasma sample from subject 3 taken 2 minutes after smoking Δ⁹-THC (8mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

The HPLC-RIA chromatogram of a plasma sample from subject 3 taken 22 minutes after smoking Δ⁹-THC (8mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.
Figure 34 The HPLC-RIA chromatogram of a plasma sample from subject 3 taken 31 minutes after smoking Δ⁹-THC (8mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

Figure 35 The HPLC-RIA chromatogram of a plasma sample from subject 3 taken 60 minutes after smoking Δ⁹-THC (8mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.
Cross-reaction (ng/ml)

Figure 36 The HPLC-RIA chromatogram of a plasma sample from subject 3 taken 120 minutes after smoking $\Delta^9$-THC (8mg). The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

TABLE 6
CANNABINOID HPLC RETENTION VOLUMES

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention volume (ml)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta^9$-THC</td>
<td>31.5</td>
</tr>
<tr>
<td>$\Delta^8$-THC</td>
<td>31.0</td>
</tr>
<tr>
<td>CBN</td>
<td>30.0</td>
</tr>
<tr>
<td>11-hydroxy-$\Delta^9$-THC</td>
<td>25.5</td>
</tr>
<tr>
<td>$\Delta^9$-THC-11-oic acid</td>
<td>10.0</td>
</tr>
<tr>
<td>11-hydroxy-CBN</td>
<td>25.5</td>
</tr>
<tr>
<td>CBN-11-oic acid</td>
<td>10.0</td>
</tr>
<tr>
<td>8a-hydroxy-$\Delta^9$-THC</td>
<td>25.5</td>
</tr>
<tr>
<td>8β-hydroxy-$\Delta^9$-THC</td>
<td>20.5</td>
</tr>
<tr>
<td>8a,11-dihydroxy-$\Delta^9$-THC</td>
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</tr>
<tr>
<td>8β,11-dihydroxy-$\Delta^9$-THC</td>
<td>16.5</td>
</tr>
<tr>
<td>1'-oxo-CBN</td>
<td>29.0</td>
</tr>
<tr>
<td>1'-hydroxy-CBN</td>
<td>22.5</td>
</tr>
</tbody>
</table>

$^a$ For the system described in the text
these cannabinoids. Individual components were quantified by summing the data points for each area of cross-reaction (in the chromatograms Figures 20-36) and relating these to the original volume of plasma used.

The concentration of a cross-reacting component with retention volume equivalent to $\Delta^9$-THC was determined by this method for each plasma sample (Table 3) and plotted against the time the sample was taken to obtain a $\Delta^9$-THC elimination curve for each subject (figures 37, 38 and 39). These indicated a biphasic elimination curve for this compound with the three subjects. The results for subjects 1 and 2, the regular users of cannabis who each smoked 10mg$s$ of $\Delta^9$-THC, indicated an initial $\Delta^9$-THC plasma concentration

![Figure 37](image)

Figure 37 The elimination from plasma of cannabinoids with retention volumes equivalent to $\Delta^9$-THC ($\square$--$\square$), CBN ($\Delta$--$\Delta$) and mono-hydroxylated metabolites ($\circ$--$\circ$) for subject 1.
of approximately 50ng/ml decreasing rapidly during the first 30min (the distribution phase) to approximately 5ng/ml. Results for subsequent samples indicated a slower rate of decrease of the $\Delta^9$-THC concentration (the elimination phase) falling to 1 or 2 ng/ml at the conclusion of the experiment two hours after smoking. The results for subject 3, the non-cannabis user who smoked 8mg of $\Delta^9$-THC indicated a similar elimination pattern but lower $\Delta^9$-THC plasma concentrations. The initial $\Delta^9$-THC concentration was found to be 37ng/ml decreasing to less than 1ng/ml at the end of the two hour smoking experiment. These results are consistent
Figure 39  The elimination from plasma of cannabinoids with retention volumes equivalent to $\Delta^9$-THC (□—□), CBN (Δ—Δ) and mono-hydroxylated metabolites (○—○) for subject 3.

with the $\Delta^9$-THC plasma concentrations measured after the administration of $\Delta^9$-THC to human subjects by Agurell et al. (1973) and Wall et al. (1976).

Plasma concentrations of the cross-reacting compound with retention volume equivalent to CBN were also found to follow a biphasic elimination pattern for the three subjects (Figures 37, 38 and 39). The levels for samples taken immediately after smoking $\Delta^9$-THC were between 5 and 9ng/ml (Table 7) falling to concentrations well below 1ng/ml two hours later. CBN has been reported present in plasma samples
### TABLE 7

THE PLASMA CONCENTRATIONS OF CROSS-REACTING CANNABINOIDS OTHER THAN Δ⁹-THC IN VOLUNTEERS WHO HAD SMOKED Δ⁹-THC DETERMINED BY HIGH PRESSURE LIQUID CHROMATOGRAPHY - RADIOIMMUNOASSAY

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time after smoking (mins)</th>
<th>Plasma concentration as determined by radioimmunoassay and expressed as ng/ml of cross-reacting cannabinoid</th>
<th>Peak elution at the position of mono-hydroxylated metabolites</th>
<th>Peak elution at the position of CBN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
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<td>0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.6</td>
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<td>12</td>
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<td>1.2</td>
<td>1.4</td>
</tr>
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<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
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<td>34</td>
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<td>0.5</td>
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<td></td>
<td>64</td>
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<td>126</td>
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<td>0.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1.0</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
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<td>24</td>
<td>1.4</td>
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<td>1.7</td>
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<td></td>
<td>64</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>124</td>
<td>0.4</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
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<td>0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.3</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.3</td>
<td>0.3</td>
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<td>31</td>
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<td>0.3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

after Δ⁹-THC administration by McCallum et al. (1975) who suggested that CBN was a metabolic product of Δ⁹-THC. However Wall et al. (1976) found almost no CBN in plasma after the intravenous administration of Δ⁹-THC to human subjects and concluded that CBN was not a Δ⁹-THC metabolite. Because the elimination mode of the compound with the retention volume equivalent to CBN was biphasic, these results suggest that a metabolic explanation for the presence of CBN in the samples is unlikely.

One of the other areas of cross-reaction observed with
the HPLC-RIA data had a retention volume of 25.5ml. The metabolites 11-hydroxy-$\Delta^9$-THC, 8α-hydroxy-$\Delta^9$-THC and 11-hydroxy-CBN all have this retention volume with the HPLC system used and it is possible that the observed level of cross-reaction may represent a contribution from any of these metabolites. Levels of cross-reaction at this retention volume reached a maximum shortly after $\Delta^9$-THC smoking for the three subjects, falling slowly over the remainder of the experiment (Figures 37, 38 and 39). The concentrations and elimination curves for this area of cross-reaction are similar to those of 11-hydroxy-$\Delta^9$-THC measured by Wall et al. (1976) in plasma samples obtained from subjects receiving intravenously administered $\Delta^9$-THC.

An area of cross-reaction at the retention volumes corresponding to the di-hydroxylated metabolites 8β,11-dihydroxy- $\Delta^9$-THC and 8α,11-dihydroxy $\Delta^9$-THC was also observed with the HPLC-RIA data (Figures 20 to 36). These metabolites were not well resolved with the chromatographic system used (Table 6) and do not cross-react in the RIA to the same extent as each other or to $\Delta^9$-THC (Table 5). Quantitative results for these compounds were not therefore, obtained from the HPLC-RIA data.

A further feature of the HPLC-RIA data was the presence of an area of cross-reaction eluting from the HPLC column at a retention volume of 10ml (Figures 20 to 36). This corresponds to the retention volume of metabolite conjugates observed in human urine samples obtained after $\Delta^9$-THC administration as well as to the mono-carboxylated metabolites $\Delta^9$-THC-11-oic acid and CBN-11-oic acid (Table 6). This
area of cross reaction may therefore represent a mixture of cross-reacting metabolite conjugates and the mono-carboxylated metabolites. The contribution of this area of cross-reaction to the total RIA result increased as the THC concentration decreased so that the total RIA result remained approximately constant for the two hour period after THC administration (Table 3). The absolute identification of the structure of cross-reacting compounds would require the isolation of sufficient quantities of material to obtain mass spectral data.

HPLC-RIA has been found to provide a convenient method for separating, presumptively identifying and quantifying THC and some of its metabolites in plasma. It has the advantage over the previously reported methods of using a small volume of sample to simultaneously quantify THC and some of its metabolites. The provision of this additional data has the advantage that they may be used to justify a greater confidence in the validity of a result than RIA alone. Some additional conclusions may also be made from a knowledge of the Δ⁹-THC plasma concentration. The psychoactive effects of Δ⁹-THC usually continue for several hours after taking the drug (Wootton Committee, 1968) while the plasma concentration has been found to decrease rapidly to a level of the order of one ng/ml several hours after smoking. The presence of ng/ml concentrations of Δ⁹-THC in a plasma sample would therefore suggest that Δ⁹-THC had been taken within a period of a few hours before the sample was taken and this could help to substantiate other evidence suggesting cannabis intoxication.
INTRODUCTION

The GC-MS technique has been one of the more successful methods for cannabinoid analysis in plasma. Its application to the quantification of $\Delta^9$-THC in plasma was first described by Agurell et al. (1973). The method consisted of adding deuterated $\Delta^9$-THC as internal standard to a plasma sample, extracting this, removing interfering material from the extract with column chromatography and analysing the refined extract with GC-MS. The mass spectrometer was used as a sensitive and selective detector by tuning the instrument to record the intensities of two of the electron impact fragmentation ions of both $\Delta^9$-THC and the deuterated $\Delta^9$-THC. The method was capable of measuring ng/ml concentrations of $\Delta^9$-THC in plasma.

The measurement of $\Delta^9$-THC in plasma is one of the more important aspects of cannabinoid analysis and a method modified in the isolation stage by the replacement of liquid chromatography with HPLC was investigated using the plasma samples described previously. The results obtained with this method for $\Delta^9$-THC measurement were then compared with those obtained using the HPLC-RIA method.

EXPERIMENTAL

Materials and Equipment

HPLC equipment was the same as that described in part B of this section. GC-MS was conducted using a Pye 104 GC equipped with an OV17 column (Gas Chrom Q, 80-100 mesh) interfaced to a VG Micromass 16F Mass Spectrometer. The internal standard (5'-$^2$H$_3$)-$\Delta^9$-THC was kindly supplied by Dr S Agurell, Stockholm.
The GC-MS method used was similar to that described by Agurell et al. (1973). (5'-2H₃)-Δ⁹-THC (10ng) in methanol was added to the plasma sample (1ml) and the mixture extracted with methanol as described previously. The plasma extract was chromatographed using a 10cm ODS column (4.6mm id) and 67.5% methanol/32.5% water eluent. The Δ⁹-THC fraction (retention volume approximately 20ml) was collected, freeze dried and the residue dissolved in hexane. This was gas chromatographed with a mass spectrometer in the MID mode tuned to ions of m/e 299 and 314 for Δ⁹-THC and 302 and 317 for deuterated Δ⁹-THC. The Δ⁹-THC retention time was 4 minutes with a helium flow of 20ml/min at 190°. The Δ⁹-THC plasma concentration was calculated from the ratio of ion intensities for Δ⁹-THC and deuterated Δ⁹-THC using a previously prepared calibration graph.

RESULTS AND DISCUSSION

The Δ⁹-THC concentrations obtained by this method (Table 3) were found to be similar to, but consistently higher than those obtained by the HPLC-RIA method. The results from both methods showed similar biphasic elimination curves. The accuracy of the GC-MS method which incorporates an internal standard would be expected to be greater than that of the HPLC-RIA method in which no account of possible losses was made. The discrepancy between results could not however be fully explained by losses occurring during the plasma extraction and chromatography stages of the HPLC-RIA procedure which were found to be approximately 10%. The HPLC-RIA results for subjects 1 and 2 were obtained approximately six months after the GC-MS analyses were
performed. It is possible that decomposition of the $\Delta^9$-THC had occurred during this period.

The GC-MS method was found to be a viable method for the measurement of $\Delta^9$-THC concentrations in plasma. Its accuracy was probably greater than that obtained using HPLC-RIA. The method did have the disadvantages relative to HPLC-RIA that the analysis time was longer and that measurement of the concentration of only one cannabinoid component in a plasma sample was obtained.
(D) CONCLUSION

An ideal analytical method for cannabinoid detection in plasma, suitable for use in a forensic science laboratory would have the following features:

(a) a low operational cost,
(b) a short analysis time,
(c) provide quantitative results preferably for several of the cannabinoid components,
(d) require a small sample volume,
(e) provide an unequivocal result.

The methods described in section 1 are able to satisfy some but not all of these conditions.

The thin-layer chromatography method described by Vinson et al. (1976) provides a qualitative result for one cannabinoid (THC) at low cost with moderate analysis time but uses a relatively large sample volume (5ml). This method does have the serious limitation that non-cannabinoid material present in plasma may be labelled with the fluorescent reagent as well as $\Delta^9$-THC and this might lead to an erroneous result.

The GC methods employing electron capture or flame photometric detection provide a quantitative result for THC and sometimes CBN at moderate cost with a moderate analysis time using different sample volumes (1-10ml). The major limitation of these methods again results from the possibility of error due to the presence of interfering material in plasma.

Most of the GC methods employing a mass spectrometer as detector provide results which are derived from both the
The RIA procedure involves moderate cost, relatively short analysis time, especially where a large number of samples are to be analysed and requires a small sample volume. The method does not however provide a totally unequivocal result since this is derived from the measurement of only one parameter, i.e. the proportion of radioactive label bound to the antiserum. The result could be affected by simple error, or the presence in a sample of an unknown non-cannabinoid compound capable of influencing antiserum binding.

In order to obtain a high degree of confidence in the validity of a result with the methods described above, a sample would have to be assayed either by using two of the described methods for the analysis or by using a single method capable of quantifying at least two of the cannabinoids present in plasma after cannabis smoking. The former alternative though quite feasible has the disadvantage that the total analysis time required for two different procedures would be large and this might affect the practicality of this approach for its use on a routine basis. The latter alternative however might offer a possibility of reducing analysis time.

This could be achieved by using either RIA or one of the chromatographic methods. Two or more RIA procedures specific for different cannabinoids could be used to provide this, though the production of suitable antisera with the
required specificities would probably involve some difficulties. Most of the chromatographic methods may be modified to procedures capable of the detection of different cannabinoids, though the majority of these would probably fail to provide the sensitivity required for the detection of metabolite concentrations of 1ng/ml and below. Of the chromatographic methods GC/MS is the only technique reported capable of the measurement of levels of a $\Delta^9$-THC metabolite (11-hydroxy-$\Delta^9$-THC) in plasma (Wall et al., 1976). Measurement of $\Delta^9$-THC and 11-hydroxy-$\Delta^9$-THC by this method might require several days work and would therefore be unsuitable for use in routine analysis. The published methods described above, therefore have the main disadvantage that they are unable to give an unequivocal result in a time suitable for routine analysis.

The work described in the experimental part of this section has affected this situation. The development of the HPLC-RIA method has provided a means of quantifying $\Delta^9$-THC and other cross-reacting cannabinoids in a plasma sample in one analysis with moderate analysis time and cost, and with a relatively small plasma sample volume ($\leq$ 1ml). Retention volumes for as many as five cross-reacting components are obtained from an analysis using this method, as well as the quantitative data. A high degree of confidence may therefore be placed in the validity of a result obtained by this method. HPLC-RIA therefore appears at present to be the most suitable method for routine analysis.
cannabinoid analysis in plasma for use in a forensic science context where a high degree of confidence in the validity of a result is required. RIA alone is a valuable method for screening plasma to identify positive samples for subsequent analysis with HPLC-RIA.
SECTION 3

URINE ANALYSIS
(A) RADIOIMMUNOASSAY

INTRODUCTION

RIA is at present the only technique reported capable of providing a successful method for the routine analysis of cannabinoids in urine. The application of several thin-layer chromatography methods to the analysis of cannabinoids in urine have been described in the literature but these were all found to be unreliable and the results obtained capable of misinterpretation (Salaschek et al., 1973). A GC-MS method for the measurement of $\Delta^9$-THC-11-oic acid was described by Nordqvist et al. (1976) but the application of the method to the measurement of this metabolite in urine has not yet been reported (see Section 1).

Though methods other than RIA may be developed for cannabinoid analysis in urine this technique would probably still provide the most useful means of screening samples for cannabinoid content because of its short analysis time and moderate cost. Because of the advantages of RIA the procedure described previously for the analysis of plasma was adapted for use in the analysis of urine samples. This modified method was then evaluated, with the objectives of determining what magnitude the levels of cross-reaction would be in urine samples obtained from subjects after they had smoked $\Delta^9$-THC and whether these samples could be distinguished from blank urine samples.

EXPERIMENTAL
Materials and Equipment

The equipment and most of the materials were the same as those used for plasma analysis (see section 2A). Urine
samples taken over a 24 hour period after $\Delta^9$-THC smoking (10mg for subjects 1 and 2 and 8mg for subject 3) were taken during the same experiment in which the plasma samples were obtained. These were received from Professor J W Thompson (University of Newcastle Upon Tyne). Blank urine samples were obtained from twenty five volunteers with no experience of cannabis use.

**Method**

The RIA method used for the analysis of urine was similar to that described for plasma analysis (see section 2A). The only modifications of the assay protocol (Table 8) were adjustment of the solution volumes added to the assay equilibrium mixture. This allowed for the methanol which had been added to the assay solutions as part of the plasma

### TABLE 8

**RADIOIMMUNOASSAY PROTOCOL FOR URINE ANALYSIS**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Total counts tube</th>
<th>Non-specific binding tube</th>
<th>Zero tube</th>
<th>Standard tube</th>
<th>Sample tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-THC (50% MeOH)</td>
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<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
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<tr>
<td>THC standard</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>50% MeOH</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Urine sample extract</td>
<td></td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Diluent buffer</td>
<td></td>
<td>200</td>
<td>300</td>
<td>200</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Antiserum</td>
<td></td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Incubated for 1 hour at room temperature**

| Diluent buffer | 200 | - | - | - | - | - |
| Dextran-coated charcoal (2.5%) | - | 200 | 200 | 200 | 200 |

**Centrifuged and 500μl of supernatant counted**
extraction. The same methanol concentration (25%) in the assay was thus used for both plasma and urine analysis.

RESULTS AND DISCUSSION

The urine samples from subjects 1 and 2, the regular users of cannabis, gave levels of cross-reaction between 15 and 145ng/ml with the RIA (Table 9). Levels obtained from the urine samples of subject 3, the non-cannabis user, were lower than for these two subjects and were between 3 and 7.3ng/ml. Control urine samples from subjects 1 and 2 also contained

| TABLE 9 |
| LEVELS OF RIA CROSS-REACTION IN URINE SAMPLES OBTAINED FROM VOLUNTEERS WHO HAD SMOKED Δ9-THC |

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time sample was taken after smoking(h)</th>
<th>Level of cross-reaction (ng/ml)</th>
<th>Urine Volume (ml)</th>
<th>Total cross-reacting material (μg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>35</td>
<td>132</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>117</td>
<td>111</td>
<td>13.0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>142</td>
<td>34</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>125</td>
<td>56</td>
<td>7.0</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>145</td>
<td>44</td>
<td>6.3</td>
</tr>
<tr>
<td>6</td>
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<td>135</td>
<td>38</td>
<td>2.6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>78</td>
<td>113</td>
<td>4.4</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>49</td>
<td>600</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>113</td>
<td>95</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
<td></td>
<td>67</td>
<td>51</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>42</td>
<td>110</td>
<td>4.5</td>
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<tr>
<td>5</td>
<td></td>
<td>68</td>
<td>66</td>
<td>4.5</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>15</td>
<td>206</td>
<td>0.8</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>56</td>
<td>109</td>
<td>0.4</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.2</td>
<td>82</td>
<td>0.26</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>7.3</td>
<td>45</td>
<td>0.33</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>6.6</td>
<td>50</td>
<td>0.33</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>3.8</td>
<td>110</td>
<td>0.42</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>6.2</td>
<td>65</td>
<td>0.20</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>3.0</td>
<td>166</td>
<td>0.25</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>3.4</td>
<td>673</td>
<td>0.14</td>
</tr>
</tbody>
</table>
cross-reacting material which was most probably due to the consumption of cannabinoid material at some time prior to the experiment. The presence of this additional cannabinoid material contributed to the levels of cross-reaction observed in the samples taken after $\Delta^9$-THC smoking from subjects 1 and 2. The quantities of cross-reacting material excreted in the urine (Table 9) by subjects 1 and 2, without this contribution, might have been closer to that observed in the urine samples from subject 3. The rate of excretion of cross-reacting material for the three subjects was higher at the beginning of the experiment than towards its end. A totally consistent trend in the excretion rate was not observed between these times and the point at which the excretion rate was at a maximum could not be accurately determined from these data. Levels obtained from the urines of subject 3 suggest that the RIA is capable of detecting cross-reacting material

<table>
<thead>
<tr>
<th>TABLE 10</th>
</tr>
</thead>
</table>

LEVELS OF RIA CROSS-REACTION IN NORMAL CONTROL URINE SAMPLES

<table>
<thead>
<tr>
<th>Subject</th>
<th>Level of cross-reaction (ng/ml)</th>
<th>Subject</th>
<th>Level of cross-reaction (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0.3</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.2</td>
<td>22</td>
<td>0.2</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
in urine samples which have been taken during a period of at least 1 hour and up to 8 hours after $\Delta^9$-THC smoking. Levels of cross-reaction for the normal control urine samples (Table 10) were all less than 1ng/ml, which demonstrates that the RIA is capable of distinguishing these samples from those obtained during a period after the consumption of a moderate quantity of $\Delta^9$-THC.
HPLC-RIA is a method potentially capable of measuring the individual cannabinoid metabolites in urine. There is however a problem associated with cannabinoid analysis in urine which does not exist with plasma analysis. This is, that the type of $\Delta^9$-THC metabolites present in urine differ from those in plasma. Wall et al. (1974) found that the majority of cannabinoid metabolites in urine were present as water soluble conjugates. It is therefore probable that a proportion of the RIA cross-reacting compounds present in urine are metabolite conjugates. For the identical HPLC-RIA system used for plasma analysis to be applied to urine a suitable hydrolysis procedure capable of liberating metabolites from their polar conjugates might therefore be required.

The experimental objectives of the work described in this section are concerned with the development of a suitable procedure for the analysis of urine samples by the HPLC-RIA method and obtaining an indication of the suitability of the method for use in routine analysis.

**EXPERIMENTAL**

**Materials and Equipment**

These were the same as those described in Section 2B. $(3',5'-\text{14C})\Delta^9$-THC was purchased from the Radiochemical Centre, Amersham, and $\beta$-glucuronidase and sulphatase were purchased from Sigma Chemicals Ltd.

**Method**

Initial experiments designed to investigate the HPLC retention characteristics of urinary metabolites and devise a
suitable procedure for conjugate hydrolysis were conducted using rabbit urine containing radiolabelled Δ⁹-THC metabolites. A New Zealand white rabbit (4kg), from which a control urine sample had been taken, was injected with 9μCi(90μg) of (3',5'-¹⁴C)Δ⁹-THC (in 0.1ml ethanol and 0.4ml of saline containing 2%v/v Tween 80) into one of its ear veins. Urine samples were collected from the rabbit after 10 minutes, 5 hours, 48 hours and 72 hours. The radioactivity of each urine sample was measured by liquid scintillation counting and corrected for quenching by the channels ratio method. The molar concentrations of radiolabelled metabolites were subsequently calculated from the specific activity of the (3',5'-¹⁴C)Δ⁹-THC.

The HPLC system used in the investigation of hydrolysis conditions differed from that described previously. It consisted of two 10cm ODS columns in series, eluted with 20ml of 50% methanol/50% water, 20ml of 65% methanol/35% water and 20ml of 80% methanol/20% water. Eluate fractions (1ml) were taken at one minute intervals and mixed with scintillation fluid (4ml) for counting. The HPLC conditions for all the work employing HPLC-RIA were the same as those described for plasma analysis.

The treatment of urine samples with enzymes was investigated as a potential method for conjugate hydrolysis. Urine samples (1ml) were incubated with β-glucuronidase (2000 units of Sigma Type B1) and sulphatase (1000 units of Sigma Type H1) at pH5 and at 37° over a 24 hour period. Three volumes of methanol were added and the samples were centrifuged. The supernatant was diluted to a 50% aqueous
methanol solution and chromatographed. Alkaline conditions (0.5M sodium hydroxide) were also used in an attempt to hydrolyse metabolite conjugates. The effect of different incubation temperatures (4°-37°C) and incubation times (4-30 min) were examined in order to identify the optimum hydrolysis conditions.

The procedure finally chosen and subsequently used for the hydrolysis of human urine samples was as follows: an equal volume of methanolic sodium hydroxide (1M) was added to the urine; the sample vessel was evacuated in order to exclude oxygen and left at room temperature for 30 minutes. The pH of each sample was then adjusted with acetic acid.

For the initial investigation of basic hydrolysis conditions the rabbit urine samples were adjusted after hydrolysis to pH 4 and for the later work involving the RIA of HPLC eluate fractions the samples were adjusted to pH 5.5.

The stability of cannabinoids to acidic and basic conditions was investigated by incubating the compounds Δ⁹-THC, CBN, 11-hydroxy-Δ⁹-THC, 8α-hydroxy-Δ⁹-THC, 8β-hydroxy-Δ⁹-THC, 8β,11-dihydroxy-Δ⁹-THC and Δ⁹-THC-11-oic acid at approximately 5µg/ml concentrations in solutions of either sodium hydroxide (0.5M, 50% aqueous methanol) or hydrochloric acid (0.1M, 50% aqueous methanol) for two hours at temperatures between 20°C and 80°C. Changes in metabolite concentrations were measured using HPLC with 80% MeOH/20% water eluent and a 10cm ODS column. The column eluate was monitored using ultra-violet detection (280nm).

RESULTS AND DISCUSSION

The rabbit treated with radiolabelled Δ⁹-THC excreted 48% of the injected dose, based on radioactivity and a quantity
equivalent to 6.5 µg of THC cross-reacting material in the urine, over the 72 hour period after administration (Table 11).

**TABLE 11**

**LEVELS OF $^{14}$C RADIOACTIVITY AND CROSS-REACTION IN RABBIT URINE OBTAINED AFTER INTRA-VENOUS ADMINISTRATION OF 9 µCi $^{14}$C-$\Delta^{9}$-THC (90 µg)**

<table>
<thead>
<tr>
<th>Urine sample</th>
<th>Urine Volume (ml)</th>
<th>DPM/ml</th>
<th>Total DPM</th>
<th>RIA cross-reaction (ng/ml)</th>
<th>Total cross-reaction (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 10 min</td>
<td>23</td>
<td>42,300</td>
<td>973,000</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 min - 24' hr</td>
<td>20</td>
<td>220,000</td>
<td>4,400,000</td>
<td>25</td>
<td>496</td>
</tr>
<tr>
<td>24 hr - 48 hr</td>
<td>75</td>
<td>42,000</td>
<td>3,157,000</td>
<td>77</td>
<td>5,790</td>
</tr>
<tr>
<td>48 hr - 72hr</td>
<td>410</td>
<td>2,840</td>
<td>934,000</td>
<td>0.4</td>
<td>164</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9,500,000</strong></td>
<td><strong>9,500,000</strong></td>
<td><strong>6,450</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Equivalent to 48% of the administered dose.

The urine was used to investigate the retention characteristics of urinary cannabinoid metabolites. Urine samples (adjusted to pH 7) were chromatographed with the described HPLC system. The radioactivity present in the urine was found to elute as a single peak from the ODS column at a low retention volume. Urine samples acidified with acetic acid (pH 4) produced a similar elution pattern except that several components eluted at longer retention volumes (Figure 40a). These results suggested that the $\Delta^{9}$-THC metabolites present in the rabbit urine were relatively polar, since compounds are eluted from the ODS column used in this study in order of decreasing
polarity. The metabolites whose HPLC retention characteristics were affected by the addition of acetic acid to the sample are possibly weak acids.

Urine samples were hydrolysed enzymically as previously described in order to attempt the conversion of the polar conjugated metabolites into less polar compounds. Enzymically
treated rabbit urine samples were found to give HPLC elution patterns in which several components were present with greater retention volumes than those observed with the untreated samples (Figure 40b), suggesting that some hydrolysis of metabolite conjugates had occurred. It was considered that the total hydrolysis of conjugates had not been achieved since no radioactive peak was present at a retention volume corresponding to that of the expected major $\Delta^9$-THC metabolite, 11-hydroxy-$\Delta^9$-THC. The basic hydrolysis of metabolite conjugates was therefore examined.

The HPLC elution pattern constructed after chromatographing rabbit urine treated with sodium hydroxide (Figure 40c) demonstrated that material of low polarity was present. This had not been found in the enzymically hydrolysed sample and it was concluded that basic conditions provided a more effective means of conjugate hydrolysis. The conditions necessary for optimum conjugate hydrolysis were investigated. Variations of reaction temperature produced no differences in the chromatographic elution patterns but the reduction of incubation time to 4 minutes did reduce the amount of low polarity material observed. It was concluded from these results that the reaction was essentially complete after incubation for 30 min. at room temperature and these hydrolysis conditions were subsequently used.

The effect of sodium hydroxide on metabolite stability was also considered. No significant losses of the cannabinoids were observed over the temperature range 20°-80°C provided that oxygen was excluded from the reaction by evacuating the sample vessel. Losses of the cannabinoids, $\Delta^9$-THC, 11-hydroxy-
\(\Delta^9\)-THC and 8β,11-dihydroxy-\(\Delta^9\)-THC (5ng of each) were measured for the complete hydrolysis and HPLC-RIA procedure and were found to be no more than 10% when oxygen was excluded from the HPLC solvents. The pH of the cannabinoid solutions was adjusted to pH5.5 after treatment with sodium hydroxide, unlike the rabbit urine experiment where it was adjusted to pH4. These less acidic sample conditions were used to reduce the possibility of a pH change occurring in the assay buffer solution which might adversely affect the RIA result. These pH conditions were used subsequently for all the urine analyses involving the RIA of HPLC eluate fractions. The weak acidity of the sample solution (6ml of 50% aqueous methanol) did however affect the retention volumes of the acid metabolites \(\Delta^9\)-THC-11-oic acid and CBN-11-oic acid. These metabolites were eluted together at retention volume 10ml with the sample conditions used for plasma analysis (Section 2B, the pH of the plasma sample extract was normally between pH 7.5 to 8) but at increased retention volumes with the conditions used for urine analysis. The two metabolites were now well separated (Table 12). The combined hydrolysis

### TABLE 12

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Volume (ml)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta^9)-THC-11-oic acid</td>
<td>16.5</td>
</tr>
<tr>
<td>CBN-11-oic acid</td>
<td>15.0</td>
</tr>
</tbody>
</table>

² For the system described in the text
and HPLC-RIA procedure had now been developed to the stage that was considered suitable for the quantification of cross-reacting cannabinoid metabolites in urine.

The use of acidic conditions for metabolite conjugate hydrolysis was also considered, but the 11-hydroxy-Δ⁹-THC metabolite was found to be unstable in a dilute hydrochloric acid solution and this approach was not pursued further.

The HPLC elution pattern of radiolabelled Δ⁹-THC metabolites in rabbit urine had now been established but the extent to which these metabolites cross-reacted in the RIA was not known. A sample of the rabbit urine was therefore hydrolysed and chromatographed and the eluate fractions monitored for both radioactivity and RIA cross-reactivity.

Two chromatograms were constructed from the experimental data (Figure 41), one representing the total metabolite elution pattern and the other the elution pattern of cross-reacting metabolites. The chromatograms both showed similar concentrations of metabolites eluting at the retention volumes 16.5ml and 30ml. The equivalent quantitative result obtained by the two methods suggested that these metabolites cross-reacted in the RIA with the same avidity as the Δ⁹-THC used to calibrate the assay. The differences between the two chromatograms, were the presence of metabolite peaks with the chromatogram representing the elution of radioactive compounds (at retention volumes of 19.5ml and less than 15ml) which were not present in the chromatogram of cross-reacting metabolites. These compounds appear to be metabolites which do not cross-react in the RIA. This experiment has provided an indication of the proportion and retention volumes of cannabinoid metabolites in the rabbit urine samples which are able to cross-react in the RIA.
Figure 41  The HPLC elution patterns of radiolabelled \( \Delta^9 \)-THC metabolites and cross-reacting \( \Delta^9 \)-THC metabolites present in a hydrolysed rabbit urine sample.

The combined hydrolysis and HPLC-RIA procedure had now been developed to the stage where it could be applied to the analysis of human urine. The human urine samples described in part A of this section were examined by this method and the chromatograms of eluting cross-reactivity constructed. A representative sample of those chromatograms is given in Figures 42-47. All the samples which had given a positive RIA result with direct analysis of urine gave chromatograms which had areas of cross-reaction at the retention volumes 16.5 ml and 30 ml observed with the rabbit urine sample. The identification of these cross-reacting components required
Figure 42  The HPLC-RIA chromatogram of a urine sample from subject 1 for the period between 1 and 2 hours after smoking $\Delta^9$-THC (10mg).

Figure 43  The HPLC-RIA chromatogram of a urine sample from subject 1 for the period between 8 and 24 hours after smoking $\Delta^9$-THC (10mg).
Figure 44 The HPLC-RIA chromatogram of a urine sample from subject 2 for the period between 1 and 2 hours after smoking $\Delta^9$-THC (10mg).

Figure 45 The HPLC-RIA chromatogram of a urine sample from subject 2 for the period between 8 and 24 hours after smoking $\Delta^9$-THC (10mg).
Figure 46  The HPLC-RIA chromatogram of a urine sample from subject 3 for the period between 1 and 2 hours after smoking $\Delta^9$-THC (8mg).

Figure 47  The HPLC-RIA chromatogram of a urine sample from subject 3 for the period between 8 and 24 hours after smoking $\Delta^9$-THC (8mg).
their mass-spectral analyses (see section 3C). Two of the components do however have retention volumes equivalent to Δ⁹-THC-11-oic acid (16.5ml) and CBN (30ml).

Human urine samples obtained after Δ⁹-THC smoking have been analysed by the described method and cross-reacting components have been observed in these samples with retention volumes corresponding to those of radiolabelled metabolites present in rabbit urine. The method can be used for the routine analysis of urine samples and should provide a higher degree of confidence in the validity of a result than with RIA alone.
(C) THE IDENTIFICATION OF URINARY RIA CROSS-REACTING COMPOUNDS

INTRODUCTION

The HPLC-RIA procedure described in Section 3B was used to detect cross-reacting cannabinoids in urine. A result obtained by this method would however have a greater significance if the structures of these compounds were known. The work described here was concerned with the isolation and mass spectral identification of two of these urinary cross-reacting components.

EXPERIMENTAL

Materials and Equipment

The RIA and HPLC equipment was the same as that described in Section 2. GC-MS was conducted with a 3%OV17 column (2mm id., 0.3m length) interfaced by a jet separator to a VG Micromass 16F mass spectrometer. A urine sample (Case 2, see Section 4) found to contain a high level of cross-reacting material (680ng/ml) was used as the source of cannabinoid metabolites. N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane was purchased from Phase Separations Ltd.

Method

The urine sample (3ml) was hydrolysed and chromatographed with the HPLC system described in Section 2B. Aliquots (5µl) of the eluate fractions were assayed by RIA to identify those fractions containing cross-reacting material. Two metabolites designated A and B (Figure 56), having retention volumes of 30ml and 16.5ml respectively, were isolated by this method and re-chromatographed using an ODS column (10cm) and 67.5% methanol/32.5% water solvent. The HPLC eluate was again monitored with RIA (5µl
aliquots) and the solvent from the fractions containing cross-reacting compounds A and B (retention volumes 28ml and 8ml respectively) removed by freeze drying. A part of each of the metabolite samples was silanised with BSTFA (10μl) in dry pyridine (30μl) at 80°C. A further portion of the more polar metabolite B was methylated with an ethereal diazomethane solution (100μl) at room temperature. The metabolite or metabolite derivative solutions (10μl of an approximately 5ng/μl methanol solution, or for TMS derivatives, pyridine solution) were gas chromatographed at 220° with a helium flow rate of 20ml/min. The mass spectrometer source temperature was adjusted to 250° and the accelerating voltage to 4kv. Mass spectra were recorded when compounds were eluted from the GC column.

RESULTS AND DISCUSSION

The mass spectrum of the TMS derivative of the more polar metabolite B (GC retention time = 2.5min) gave major ion peaks at m/e 488, 473 (loss of -CH₃) and 371 (loss of -COOTMS) and the methylated derivative of B, (GC retention time = 5 min) at m/e, 358, 343 (loss of -CH₃) and 299 (loss of -COOCH₃). It was concluded from these data that the metabolite B reacted with diazomethane to give a derivative with one methyl group (CH₃ = 15) and with BSTFA to give a derivative with two TMS groups (2xTMS = 146) suggesting a molecular weight for the underivatised compound of 344. This corresponds to the molecular weight of Δ⁹-THC-11-oic acid, the metabolite which has the same HPLC retention volume (16.5ml) as metabolite B. The mass spectra of the methyl ester and TMS derivatives of Δ⁹-THC-11-oic acid were found to be identical to
those obtained with the corresponding derivative of this compound. It was concluded that the urinary cannabinoid metabolite B, with HPLC retention volume 16.5ml, was Δ⁹-THC-11-oic acid.

The mass spectrum of the less polar, underivatised, RIA cross-reacting compound (GC retention time = 5 min) gave major ions at m/e 358, 343 (loss of -CH₃) and 299 (loss of -COOCH₃) identical to that of methylated Δ⁹-THC-11-oic acid. The mass spectrum of the TMS derivative (GC retention time = 2 min) had major ions at m/e 430, 415 (loss of -CH₃) and 371 (loss of -COOCH₃) and was found to be identical to that of the TMS derivative of the methyl ester of Δ⁹-THC-11-oic acid. It was concluded that compound A was the methyl ester of Δ⁹-THC-11-oic acid.

This was an unexpected structure for a naturally occurring cannabinoid metabolite and the possibility that methylation might have occurred during hydrolysis was investigated. A solution of Δ⁹-THC-11-oic acid (5µg/ml) was subjected to the urine hydrolysis conditions and possible conversion of the acid metabolite to its methyl ester was measured with HPLC (10cm ODS column, 80% methanol/20% water solvent, UV detection at 280nm). No esterification of the Δ⁹-THC-11-oic acid was observed.

The possibility that urinary metabolite conjugates had been converted to the methyl ester was investigated and a sample of the urine was hydrolysed using ethanolic sodium hydroxide solution (1M) in place of the methanolic solution used previously. A compound was isolated as described previously and the mass spectral data obtained. This was found to have major ions at m/e 372, 357 (loss of -CH₃) and 299 (loss of
-COOC₂H₅), a spectrum expected to be given by the ethyl ester of Δ⁹-THC-11-oic acid. It was concluded that the hydrolysis conditions used were responsible for the formation of the methyl (or ethyl) ester of the Δ⁹-THC-11-oic acid metabolite. The two apparently conflicting experimental results might be explained if the basic methanolic hydrolysis conditions used were unable to esterify the free acid, but were able to effect a transesterification of a Δ⁹-THC-11-oic acid conjugate. The ability of the basic methanolic conditions to effect such a transesterification reaction could be dependent on the type of metabolite conjugate. If a urine sample contained more than one type of conjugate (possibly a sulphate conjugate of the phenol group or glucuronic acid conjugates) both the free and methylated Δ⁹-THC-11-oic acid compounds might be observed.

The work in this section has identified Δ⁹-THC-11-oic acid and its methyl ester as cross-reacting components in the hydrolysed urine. The acid metabolite may now be quantified in urine by the HPLC-RIA method.
INTRODUCTION

Bowd et al. (1971) observed that although Δ⁹-THC and CBN had only a weak intrinsic fluorescence they could be converted to a highly fluorescent compound by irradiating a cannabinoid solution with UV light at 280nm. The CBN photoproduct produced by this method was postulated to be a substituted phenanthrene (Figure 48) by Bowd et al. (1975).

![Figure 48](Conversion of CBN to a fluorescent photoproduct)

It was considered that the above reaction could be used in a procedure for detecting the low levels of cannabinoids present in body fluids. A suitable method would require a chromatographic separation stage in order to avoid interference from endogenous fluorescent material present in body fluids and in order to separate the cannabinoids from each other.

Such a procedure might provide an additional method for detecting cannabinoid compounds in body fluids. It could have the advantages of high specificity of detection, low analysis time and moderate analysis cost. The objectives of the work described in this section were therefore to examine the
EXPERIMENTAL

Materials and Equipment

The final design of a photochemical reactor, incorporating a medium pressure mercury arc lamp, is illustrated in Figure 49.

Figure 49  A sectional drawing of the photochemical reactor for HPLC. A - lamp, B - fused silica tube, C - coolant jacket, D - end plates, E - coolant connections, F - fused silica irradiation coil, G - 1/16" Swagelok connections.

The mercury arc lamp (A) was mounted co-axially within a fused-silica tube (B), (8.5cm x 2.1cm od., 1.8cm id). This was enclosed by an aluminium alloy cylindrical coolant jacket (C) (6.5cm x 4.7cm od) fitted with stainless steel end-plates (D) which were sealed to the tube (B) with rubber O-rings.
The inside surface of the coolant jacket was polished to form a reflective surface. Inlet and outlet connections for coolant were provided (E). Inside the coolant space was placed the quartz irradiation tube (F) (od 2mm, id 0.25mm and length 70cm). The ends of this tube were secured with epoxy resin to 1/16" Swagelok fittings (G) (100-C Phase Separations) brazed onto the end plate (D).

The photochemical reactor is the subject of British Patents Applications (Twitchett and Williams, 1977a, 1977b).

The quartz tubing used in the construction of the photochemical reactor was purchased from Heraeus Quartz Fused Products Ltd., and the medium pressure mercury arc lamp (100w) and power source from Engelhard Hanovia Lamps Ltd.

Samples were injected onto the HPLC column with a U6K valve injector (Waters Assoc). A Waters M-6000 HPLC pump was used to deliver eluent to a column (10cm x 4.6mm id) slurry-packed with Spherisorb-5-ODS.

The eluate was monitored with either a variable-wavelength UV detector (CE - 212, Cecil Instruments) or a fluorimeter (MPF 2A, Perkin Elmer) equipped with a flow cell.

**Method**

The conversion of cannabinoids to fluorescent photoproduct was initially investigated by irradiating methanol solutions of \( \Delta^9 \)-THC (5\( \mu \)g/ml) and CBN (0.5\( \mu \)g/ml) with UV light (280nm) in the MPF 2A fluorimeter for a period of one hour. Changes in the composition of these solutions were observed using HPLC with ODS column and an 80% methanol/20% water eluent, monitored for both UV absorbance and fluorescence.
The rate of formation of the photoproduct in methanol and 80% methanol/20% water which were either acidic (0.1M, sulphuric acid), basic (0.1M, ammonium hydroxide) or neutral, was investigated by irradiating the appropriate cannabinoid solution (10μg/ml) with UV light (280nm) and recording the intensity of fluorescent emission at 365nm.

The photochemical reactor was coupled between the HPLC column and fluorescence detector (Figure 50) by stainless steel tubing (0.01"id) using the connectors (G). The HPLC flow rate for optimum sensitivity was determined by repeatedly chromatographing 20ng of CBN at different flow rates with 80% methanol/20% water eluent and recording the photoproduct peak height.

The ability of the technique to detect CBN in urine was investigated by hydrolysing a urine sample containing CBN (20ng/ml) with an equal volume of methanolic sodium hydroxide, as described in section 3B. The hydrolysed urine was diluted with an equal volume of water and the CBN extracted with hexane. The CBN was back extracted into 80% methanol/20% water, diluted
to 50% aqueous methanol solution and injected onto the HPLC-photochemical reactor. A similar volume of control urine was similarly extracted and chromatographed.

RESULTS AND DISCUSSION

Conversion of $\Delta^9$-THC and CBN to the fluorescent photoproduct was performed as described above and the extent of reaction observed with HPLC using both UV and fluorescent detection. After irradiation for one hour a small proportion of the $\Delta^9$-THC and most of the CBN were converted to a product highly sensitive to fluorescence detection (Figure 51). This

![Figure 51](image)

Figure 51 HPLC chromatograms of (a)$\Delta^9$-THC and (b) CBN, after irradiation of the cannabinoid solutions with UV light (280nm) for 1 hour in an MPF-2A fluorimeter (excitation slit 40nm). The HPLC column eluate was monitored for UV absorbance (280nm) and fluorescence ($\lambda_{ex}$ 258nm, $\lambda_{em}$ 362nm, slits 10nm, sensitivity x4). Column: S-5-ODS, Eluent: methanol (80%)/water (20%).

gave a fluorescent spectrum (Figure 52). Since CBN was more effectively converted to the photoproduct this compound was used for subsequent experiments.
The rate of photoproduct formation was examined using CBN in different solvents and at different pH conditions. The fastest reaction rate was found to take place in neutral methanol/water solvent, the reaction being complete in less than one minute.

The conversion of a cannabinoid to the photoproduct could be conducted either before chromatography as a separate derivative formation stage, or after chromatography, where derivative formation could be achieved by continuously irradiating the HPLC column eluate before it passed into the fluorescence detector.

This approach has the advantages that derivative formation and detection are achieved in one process and that the retention volumes of the cannabinoids themselves, not that of the photoproduct, are measured. Such a procedure could also provide additional confirmation of cannabinoid detection (a) by re-chromatographing the cannabinoid without UV irradiation,
in which case the photoproduct would not be observed and (b) by trapping the photoproduct in the fluorometric detector and recording the total fluorescent spectrum characteristic of this compound (Figure 52).

Initial attempts to produce on-line photochemical reaction were conducted by winding a quartz irradiation coil (through which the solvent from the HPLC column would pass) round the fluorimeter UV source (high pressure Xenon) so that the transmission of radiation into the fluorimeter was not affected. With a very low HPLC solvent flow rate (0.2ml/min) the dwell time in the irradiation coil was found to be sufficient for almost total conversion to the photoproduct and the complete system was capable of detecting 1ng of CBN. It was considered however that the procedure would be more practical if a higher HPLC solvent flow rate could be used in order to reduce the analysis time. The rate of the photoreaction was increased by using a 100W medium pressure mercury arc UV source which had several times greater photon flux at 280nm and the photochemical reactor (Figure 49) designed for maximum irradiation of the HPLC solvent coil, with cooling, was constructed. This supplied sufficient radiation so that the optimum HPLC solvent flow rate for maximum conversion of CBN to the photoproduct was between 1.5 and 2ml/min, equivalent to a dwell time in the reactor of less than two seconds. The complete HPLC-reactor-fluorimeter system was capable of detecting less than 1ng of CBN (Figure 53). The system was also capable of detecting CBN, in a sample of hydrolysed control urine to which 20ng/ml of CBN had been added and
Figure 53 The detection of 1ng CBN using the photochemical reactor (a) with reactor UV lamp on (b) with reactor UV lamp off.
Column: S-5-ODS, Eluent: methanol (80%)/water (20%): Detector MPF-2 fluorimeter
λex 258nm, λem 362nm, slits 10nm, sensitivity x6.

distinguishing this from the endogenous fluorescent material in the urine (Figure 54).

The system was not able to detect Δ⁹-THC, 11-hydroxy-Δ⁹-THC or Δ⁹-THC-11-oic acid. CBN has not been detected in urine obtained after Δ⁹-THC smoking and the concentrations of this cannabinoid in plasma are extremely low (Figures 37, 38 and 39). It is probable therefore that the system does not have a practical value as a means of detecting CBN alone. The development of the method is however at an early stage and adaptation of the technique for the measurement of Δ⁹-THC and possibly its metabolites might be practical. Δ⁹-THC can be converted to CBN with dehydrogenating agents such as
Figure 54 HPLC chromatograms obtained using the photochemical reactor and fluorescence detection (λex 258nm, λem 362nm) from urine extracts (equivalent to 1ml of urine) of (a) a normal control urine sample containing 20ng/ml of CBN and (b) a normal control urine sample.

chloranil (Wollner et al. 1942) 2,3-dichloro-5,6-dicyanobenzo-quinone (DDQ,Cardillo et al., 1968) and possibly this reaction could be employed so that cannabinoids other than CBN could be detected in body fluids with the high sensitivity and specificity provided by the described system.
CONCLUSION

The RIA is capable of distinguishing blank urine samples from those obtained after Δ⁹-THC smoking and is considered to be a useful method for screening urine samples for cannabinoid content. In combination with HPLC, it will quantify some of the individual cannabinoid metabolites in urine with high sensitivity and specificity. The Δ⁹-THC-11-oic acid metabolite has been identified as one of the urinary components detected by this procedure and this metabolite may now be quantified using the method.

The work described in this section also contained an account of the development of a photochemical method for detecting low concentrations of CBN. This procedure is at an early stage of development and may have a potential application as a means of detecting low concentrations of Δ⁹-THC or its metabolites in body fluids with high sensitivity and specificity.
SECTION 4

THE ANALYSES OF SAMPLES SUBMITTED FOR FORENSIC EXAMINATION
It was noted in the previous sections that of the methods available for cannabinoid analysis in body fluids, RIA was a good screening procedure and HPLC-RIA a suitable highly specific method for routine analysis. These methods and the GC-MS method have been applied to samples submitted for forensic analysis. Analysis of samples for cannabinoid content may be required either by the police, in order to help substantiate a possible charge of cannabis possession or to help them with other enquiries (see case 9), or to assist a Coroner in determining the cause of death in cases where there is evidence to suggest that use of cannabis may be involved in a fatal road accident or sudden death. Some examples of these analyses are described in this section.
Case History

A 20 year old male who fell from his motor-cycle was run over by a following car and died in hospital 3 hours later. Cannabis was found in the subjects pocket. Analysis for cannabinoid material was required in order to determine if the drug was a contributing factor to the accident.

Samples

Within 30 minutes of the accident a blood sample was taken. A second blood sample was taken after a blood transfusion (10 units) and a sample of liver was taken during the post mortem examination.

RIA

Plasma from the pre-transfusion blood gave cross-reaction equivalent to 11ng THC/ml, the post-transfusion blood, 6ng/ml and the liver 72ng/g (the liver was macerated with methanol, the mixture centrifuged and the methanolic supernatant added directly to the assay).

HPLC-RIA

Plasma from the pre-transfusion blood was analysed using the method described in section 2B. A chromatogram was obtained (Figure 55) which had similarities to those obtained from the plasma samples of subjects 1, 2, and 3 (Figures 20-30) and which demonstrated that Δ⁹-THC (4.4ng/ml) and its metabolites were present in the sample.

Conclusion

The samples were found to contain cannabinoid material. The HPLC-RIA procedure showed that the pre-transfusion sample...
The HPLC-RIA chromatogram of the pre-transfusion plasma sample from Case 1 contained $\Delta^9$-THC and this indicated that the drug had been taken within a period of a few hours before the accident (section 2B).
Case History

A young male was observed by the police to be in a state of intoxication and admitted smoking cannabis the previous day.

Sample

A sample of urine was obtained.

RIA

The urine gave 680ng/ml cross-reaction a very high level compared with previous results (section 3A).

HPLC-RIA

The urine sample was analysed by the method described in Section 3B. The chromatogram (Figure 56) had

![Image](image-url)

Figure 56 The HPLC-RIA chromatogram of the urine sample from Case 2.
similarities to those obtained from the urine samples of subjects 1, 2, and 3 (Figures 42-47). The areas of cross-reaction eluting at retention volumes 16.5 ml and 30 ml were isolated and identified by mass-spectrometry as $\Delta^9$-THC-11-oic acid and $\Delta^9$-THC-11-oic acid methyl ester respectively (Section 3C).

Conclusion

The above results indicated that the subject had smoked a large quantity of cannabis.
Case History

The police were following a car which was being driven in an erratic manner and saw a package thrown from the car onto the road. This was later found to contain 15g of cannabis resin. The car driver gave a negative result with an Alcotest breath test.

Sample

A sample of blood was obtained from the car driver. This was found to contain 42 mg of ethanol in 100 ml of blood.

RIA

Plasma from the blood sample gave 20 ng/ml cross-reaction.

GC-MS

The plasma sample was analysed by the method described in Section 2C. A $\Delta^9$-THC concentration of 6 ng/ml was found.

Conclusion

The above results show that cannabinoid material was present in the blood. The presence of $\Delta^9$-THC in the sample suggests that the drug was taken within a few hours prior to the sample being taken.
Case 4

Case History

A young man died as a result of inhaling vomit. There was evidence to suggest that prior to death he had been using cannabis and it was considered possible that this may have impaired his reflexes.

Samples

Samples of blood and urine were obtained during the post mortem examination. The blood sample appeared to have undergone decomposition and smelled strongly of hydrogen sulphide.

RIA

The plasma was found to contain 80ng/ml of cross-reacting material and the urine 23ng/ml.

HPLC-RIA

The plasma sample was analysed by this method. The chromatogram obtained (Figure 57) was consistent with the

![Chromatogram](chart.png)

**Figure 57** The HPLC-RIA chromatogram of the plasma sample from Case 4.
presence of cannabinoid material in the sample, but no $\Delta^9$-THC was observed.

Conclusion

The above results show that cannabinoid material was present in the blood and urine samples. The absence of $\Delta^9$-THC in the blood sample does not prove conclusively that the drug had not been taken within a few hours of death since the sample was in a state of decomposition and any $\Delta^9$-THC may have been lost.
Case 5

Case History

A man was observed by the police to be smoking a cannabis cigarette. He denied this.

Sample

A sample of urine was obtained.

RIA

The urine was found to contain 16ng/ml of cross-reacting material.

HPLC-RIA

The chromatogram (Figure 58) showed that the urine

![Figure 58](image)

The HPLC-RIA chromatogram of the urine sample from Case 5.
contained $\Delta^9$-THC metabolites. As well as areas of cross-
reaction at retention volumes corresponding to $\Delta^9$-THC-11-oic
acid and $\Delta^9$-THC-11-oic acid methyl ester the chromatogram
also had a major peak at the same retention volume as the mono-
hydroxylated metabolites.

**Conclusion**

The above result indicated that cannabis had been taken.
Case History

A man was observed to swallow a piece of cannabis resin when being questioned by the police. Small quantities of cannabis resin were found on a ring worn by the subject.

Sample

A sample of urine was obtained.

RIA

The urine was found to contain 15ng/ml of cross-reacting material.

HPLC-RIA

The chromatogram (Figure 59) contained areas of cross-reaction corresponding to $\Delta^9$-THC-11-oic acid and $\Delta^9$-THC-11-...
Conclusion

The above result indicated that cannabis had been taken.
Case History

A 19 year old male motor-cyclist died after a collision on a bend. No alcohol was detected.

Sample

A blood sample was obtained.

RIA

Plasma from the blood sample was found to contain 10ng/ml cross-reacting material.

HPLC/RIA

The chromatogram (Figure 60) showed that the plasma contained Δ⁹-THC (1.5ng/ml) and Δ⁹-THC metabolites.

Conclusion

The above result indicated that cannabis had been consumed within a few hours prior to the sample being taken.
Case 8

Case History

A 23 year old male car driver died after an accident. The blood alcohol level was 343mg/100ml.

Sample

A sample of blood was obtained.

RIA

Plasma from the blood was found to contain 19ng/ml cross-reacting material.

HPLC-RIA

The chromatogram (Figure 61) showed that the plasma contained $\Delta^9$-THC (2.3ng/ml) and $\Delta^9$-THC metabolites.

![Figure 61 The HPLC-RIA chromatogram of the plasma sample from Case 8.](image)

Conclusion

The above result indicated that cannabis had been consumed within a few hours prior to death. The blood alcohol level however would probably be the major factor contributing to the accident.
Case 9

Case History

A female murder victim had cannabis in her possession. The police required to know if she had been using the drug immediately prior to death. This could have been of assistance in determining the circumstances of death.

Samples

Samples of blood and urine were obtained.

RIA

The blood was negative and the urine contained 4ng/ml cross-reacting material.

Conclusion

The above results are not consistent with cannabis having been taken immediately prior to death. The presence of a low level of cross-reacting material in the urine may indicate that the drug was possibly taken a considerable time before death.
Case 10

Case History

A man was observed by the police to be in a 'drugged condition'. He had previous convictions for the possession of cannabis.

Sample

A urine sample was obtained.

RIA

The urine was found to contain 8ng/ml cross-reacting material.

Conclusion

The above result indicated that cannabis had at some time been taken. The level of cross-reaction was low however and this suggests that cannabis was probably not the cause of the state of intoxication.
Case 11

Case History

A 20 year old man was interviewed by the police after causing a disturbance in a public house. During the interview he admitted that he had been smoking cannabis.

Sample

A sample of urine was obtained.

RIA

The urine was found to contain 9ng/ml cross-reacting material.

Conclusion

The above result indicated that cannabis had at some time been taken.
The RIA and HPLC-RIA methods have been successfully applied to the analyses of cannabinoids in both plasma and urine case samples. RIA was used as a screening procedure in order to select positive samples for subsequent analysis with HPLC-RIA. This latter method gave quantitative data for individual cannabinoids in body fluids and was found to provide a high degree of confidence in the validity of a result. The GC-MS method was found to be less suitable for routine analysis since the analysis time was longer than for HPLC-RIA and it only provided data for $\Delta^9$-THC. Information concerning the concentration of $\Delta^9$-THC in plasma has been used to obtain some indication of the length of time between consumption of the drug and the taking of the sample.
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PHOTOCHEMICAL DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO CANNABINOID ANALYSIS

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SUMMARY

A novel technique of on-line photochemical derivatization is described which can enhance considerably both the sensitivity and specificity of detection in high-performance liquid chromatography (HPLC). Material eluting from the column is irradiated with a high flux of UV light, which may induce a reaction to form fluorescent or highly UV-absorbing products. The irradiated eluent then passes into a suitable detector. The photochemical reactor has a negligible effect on resolution, and reaction is achieved in 1–5 sec.

An example of the use of this technique is in the detection of cannabinol (CBN), a component of cannabis, which is converted into a highly fluorescent compound on irradiation with UV light. Thus, if a sample containing CBN is chromatographed and the column eluent irradiated, CBN can be detected (as the fluorescent photoproduct) with a sensitivity of less than 1 ng. If the chromatogram is then repeated without UV irradiation, only naturally fluorescent products are detected. A comparison of the two chromatograms allows these to be eliminated and leads to a very high specificity for the method.

This approach is being developed as the basis of a rapid, sensitive and specific method for the detection of cannabinoids in body fluids. It is expected, however, that photochemical derivatization will extend the use of HPLC to many substances that cannot be satisfactorily detected at present.

INTRODUCTION

An important requirement in high-performance liquid chromatography (HPLC) is for a detector which is both sensitive and specific. Ultraviolet (UV) monitors and fluorescence detectors are commonly used, but it frequently happens that a substance of interest does not possess a suitable chromophore or fluorophore. This has led to the use of derivatization procedures which render the substance more readily detectable by chemical reaction either before (pre-column) or after chromatography.

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TABLE I
DERIVATIZATION TECHNIQUES IN HPLC

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-column derivatization</td>
<td>Reaction conditions are not restricted.</td>
<td>Each sample requires individual preparation.</td>
</tr>
<tr>
<td></td>
<td>Eluent choice is unrestricted.</td>
<td>Reproducibility may be low. In quantitative work an internal standard is necessary for the derivatization.</td>
</tr>
<tr>
<td>Post-column derivatization</td>
<td>Sample preparation is minimal.</td>
<td>Eluent must be a suitable solvent and reaction medium for the derivatization.</td>
</tr>
<tr>
<td></td>
<td>Derivatization is automated and quantitative precision can be high.</td>
<td>Reaction must be rapid. Resolution is usually adversely affected. Mixing and pumping pulses may affect sensitivity.</td>
</tr>
</tbody>
</table>

graphy (post-column). Conventional derivatization methods such as these suffer a number of disadvantages\(^1,2\), which are summarized in Table I.

This paper describes a novel method of derivatization which combines many of the advantages of conventional methods, but with few of the disadvantages. The technique involves a photochemical reaction which takes place within a few seconds as the eluent leaves the column before passing into the detector.

This method of derivatization has been applied to the detection of cannabinol (CBN), a component of cannabis, with high sensitivity and specificity. Cannabis is a widely used illicit drug and its detection in body fluids is of considerable forensic importance. The complex metabolism of the drug\(^3\) and the low levels found in body fluids have meant that no simple, rapid and specific method is yet available to the forensic toxicologist. Although a radioimmunoassay\(^4\) has been developed for screening purposes and a technique based on gas chromatography–mass fragmentography\(^5\) is available, the latter method requires extensive sample preparation and is not suitable for the analysis of large numbers of samples.

The application of photochemical detection to cannabinoid analysis is based on observations by Bowd et al.\(^6\) that such substances, while having only a weak intrinsic fluorescence can be converted into highly fluorescent compounds on irradiation with UV light at 280 nm for up to 4\(\frac{1}{2}\) h. For CBN (I), the photoproduct was postulated to be the substituted phenanthrene (II)\(^7\).

\[\text{CBN} \rightarrow \text{II}\]

The present study was intended as an investigation of the feasibility of on-line photochemical derivatization in HPLC, primarily for the detection of cannabinol in
body fluids. The use of the technique to enhance the specificity of detection of other substances is also briefly discussed.

EXPERIMENTAL

High-performance liquid chromatography

A Waters M-6000 pump (Waters Assoc., Northwich, Great Britain) was used to deliver eluent to a column of dimensions 10 cm × 4.6 mm I.D. slurry-packed with Spherisorb 5 ODS (Phase Separations, Queensferry, Great Britain), or 25 cm × 4.6 mm I.D. packed with Partisil 10 PAC (Whatman Ltd., Maidstone, Great Britain).

Samples were introduced via a U6K valve injector (Waters Assoc.) and eluted components were detected by a variable-wavelength UV monitor (CE-212, Cecil Instruments, Cambridge, Great Britain) or, for CBN, a fluorimeter (MPF-2A, Perkin-Elmer, Beaconsfield, Great Britain) equipped with a home-made flow cell. The eluent for the reversed-phase (ODS) column was prepared from methanol and aqueous (0.025 \( M \)) disodium hydrogen orthophosphate and adjusted to pH 8 before use. For the normal-phase column (Partisil PAC), eluents consisting of iso-octane and dioxane were used.

Photochemical reactor

The photochemical reactor (PCR), which was designed and constructed at the Home Office Central Research Establishment, is illustrated in Fig. 1. A 100-W medium-pressure mercury arc (A) (Engelhard Hanovia Lamps, Slough, Great Britain) was placed inside a fused-silica tube (B) and protected by a coolant jacket (C). The reactor was enclosed in a cylinder (D) with coolant connections (E). The lamp was operated with a high-pressure head (not shown) and the reactor was connected to a high-pressure system. The irradiation coil (F) was made of fused-silica and was located in the middle of the reactor. The flow cell (G) was made of 1/16-in. Swagelok connectors.

Fig. 1. Sectional drawing of the photochemical reactor for HPLC. A = lamp; B = fused silica tube; C = coolant jacket; D = end-plates; E = coolant connections; F = fused-silica irradiation coil; G = 1/16-in. Swagelok connectors.
Britain) was mounted co-axially within a fused-silica tube (B) (8.5 cm × 2.1 cm O.D., 1.8 cm I.D., Heraeus Quartz Fused Products Ltd., Byfleet, Great Britain). This tube was surrounded by an aluminium alloy cylindrical coolant jacket (C) (6.5 cm × 4.7 cm O.D.) fitted with alloy or stainless-steel end-plates (D), which sealed to tube (B) with O-ring seals. The inside surface of the coolant jacket was polished to form a reflective surface, and inlet and outlet connections for coolant were provided (E). Immersed in the annular coolant space was a convoluted irradiation tube of fused silica (F), O.D. 2 mm, I.D. 0.25 mm; length 70 cm (Heraeus Quartz Fused Products). The ends of the tube were attached by epoxy resin into drilled-out 1/16-in. Swagelok fittings (G) (Type 100-C, Phase Separations), which were brazed on to the end-plate (D).

The photochemical reactor was coupled between the HPLC column and detector (Fig. 2) using conventional 1/16-in. O.D. capillary-bore tubing attached to the connectors (G). Samples were chromatographed twice, both with and without UV irradiation. A single chromatogram would suffice if an additional detector were coupled between the column and the photochemical reactor and traces from the two detectors were compared.

The photochemical reactor is the subject of British Patent Applications 8, 9.

![Fig. 2. HPLC with photochemical derivatization.](image)

**Extraction of urine samples**

As no samples were available from subjects who had smoked or ingested cannabis or CBN, urines from non-drug users were collected and spiked by addition of 20 ng·ml⁻¹ of CBN. It was expected that CBN would be present in the urine of cannabis users as a glucuronide or sulphate conjugate, and urines (10 ml) were therefore hydrolysed by treatment with an equal volume of cold methanolic sodium hydroxide (1.0 M) for 5 min. The urine was then diluted with water (10 ml) and extracted into n-hexane (10 ml; BDH, Poole, Great Britain, spectroscopic grade). Aliquots of the extract were injected directly on to the Partisil PAC column.

**RESULTS AND DISCUSSION**

**Detection of cannabinol by photochemical derivatization**

The photochemical detection of cannabinol is shown in Fig. 3, which illustrates the specificity of the method: CBN was detected only after irradiation of the eluent. A high sensitivity was achieved and the technique has been found to be suitable for the detection and quantitation of CBN in the range 0.5–500 ng injected on-column.

The value of this specificity for the analysis of biological samples is shown in the chromatogram of urine extracts (Fig. 4). Although several endogenous urinary components are naturally fluorescent, only CBN is photolabile and forms a fluorescent...
Fig. 3. Photochemical detection of 10 ng of CBN: (a) without UV irradiation; (b) with UV irradiation. Column, Partisil PAC; eluent, isooctane–dioxane (3:2); flow-rate, 1 ml/min⁻¹; detector, MPF-2 fluorimeter, $\lambda_{ex} = 258$ nm, $\lambda_{em} = 362$ nm, slits 12 nm, sensitivity $\times 4$.

Fig. 4. Photochemical detection of CBN in urine extracts. (a) Extract (500 μl) of urine containing 20 ng·ml⁻¹ of CBN; (b) extract of blank urine; (c) and (d), as (a) and (b), respectively, but chromatographed with UV irradiation. Column, Partisil PAC; eluent, isooctane–dioxane (82.5:17.5); flow-rate, 1 ml·min⁻¹; detector, MPF-2A fluorimeter, $\lambda_{ex} = 258$ nm, $\lambda_{em} = 362$ nm, slits 12 and 15 nm, sensitivity $\times 5$. 
product, and it can be readily detected by comparison of the chromatograms with and without UV irradiation. In contrast, the chromatograms of blank urine extracts are identical, with and without irradiation. Hence, by disconnecting or by-passing the photochemical reactor, each sample can be made to serve as its own blank.

Effect of the photochemical reactor on resolution

The efficiency of the chromatographic system (with the Partisil PAC column) was determined by measurement of the number of theoretical plates using the CBN photoproduct (II) as a convenient solute. Samples were chromatographed using the eluent and conditions given in Fig. 3 with the PCR connected but with the lamp switched off. The measurements were repeated with the column eluent flowing directly into the detector in the conventional fashion. The conventional chromatographic system gave an average of 4710 theoretical plates, which was reduced to 4650 plates when the PCR was incorporated into the system. Thus, for all practical purposes, the effect of the PCR on column resolution is negligible. This is thought to be due to the narrow bore of the irradiation coil (0.25 mm) and the absence of unswept dead volumes which would cause eluent mixing. In contrast, most conventional methods of post-column derivatization involve coils of wide (2 mm) bore tubing for mixing and reaction to take place before detection.

Effect of chromatographic parameters on the derivatization process

**Eluent flow-rate.** The eluent flow-rate has a fundamental effect on the yield of photoproduc (II), as the flow-rate regulates the dwell time of a chromatographic peak in the photochemical reactor. Using an irradiation coil of length 70 cm, irradiation times of 0.5–4.1 seconds were achieved by variation of the flow-rate from 4 to 0.5 ml·min⁻¹. The optimal sensitivity of detection for CBN was achieved at flow-rates between 0.5 and 1.0 ml·min⁻¹. Presumably, at higher flow-rates, the sample receives insufficient irradiation, while at flow-rates below 0.5 ml·min⁻¹, the photoproduct itself may undergo photodecomposition to a non-fluorescent product. Thus although the PCR can be used at flow-rates above 1 ml·min⁻¹, the sensitivity of detection for CBN is reduced unless an irradiation coil longer than 70 cm is fitted. At a flow-rate of 2 ml·min⁻¹, for example, the sensitivity for CBN was 60% of that found at 1 ml·min⁻¹ when a 70-cm irradiation coil was used.

**Eluent composition** The eluent composition had only a small effect on the efficiency of the photochemical derivatization of CBN, and both normal- and reversed-phase eluents have been used successfully. Some variation in the sensitivity of detection is to be expected, however, as factors such as the eluent polarity, pH, hydrogen bonding and proton-donor ability may influence the rate and course of the photochemical reaction and the fluorescence quantum efficiency of the photoproduct. Eluents with a UV absorbance above 250 nm also act as an “inner filter” in both the derivatization and detection stages of the process.

Other applications of the photochemical reactor

The PCR can be used to enhance the specificity of detection other than by the formation of fluorescent derivatives. A shift in the wavelength of UV absorbance can be advantageous, as interferences due to extraneous UV-absorbing substances are generally minimized at longer wavelengths of detection. An example of this is the use
PHOTOCHEMICAL DETECTION IN HPLC

Fig. 5. Photochemical detection of CBN (180 ng) by UV absorbance at 360 nm. Conditions as in Fig. 3 except: flow-rate, 0.5 ml·min⁻¹; detector, Cecil CE-212, 360 nm, 0.02 a.u.f.s.

of a UV monitor in place of a fluorimeter for the detection of CBN, when the drug can be detected by its UV absorbance at 360 nm (Fig. 5).

The specificity of detection of the hallucinogen LSD may also be enhanced. Upon irradiation at 320 nm, the naturally fluorescent LSD is converted into the non-fluorescent lumi derivative¹⁰,¹¹ (Fig. 6), and HPLC with UV irradiation may be valuable in distinguishing the drug from other fluorescing material with similar chromatographic properties.

Photochemical derivatization may be applicable to the HPLC analysis of other drugs which are at present difficult to detect with the required sensitivity or specificity. An example is dextropropoxyphene, a widely prescribed analgesic which has been implicated in many drug overdose fatalities¹². The drug has only a weak UV absorbance, and detection by UV spectroscopy or HPLC is difficult. Irradiation of the drug with UV light leads to the formation of a photoproduct with an increased UV absorbance¹³, and preliminary results indicate that the use of the PCR described here leads to a 10-fold increase in the sensitivity of detection by HPLC using a UV monitor. Further work on this aspect is in progress.

It is envisaged that the application of the technique can be further extended by the use of derivatization reagents which photoreact with the substance of interest. Such reagents can conveniently be added to the eluent prior to chromatography.

CONCLUSION

HPLC with photochemical derivatization and fluorimetric detection has been shown to provide a highly specific means of detection of nanogram amounts of
cannabinol. A general method based on this principle for the detection of cannabinoids in body fluids is under development. The photochemical technique is also applicable to the specific detection of several other drug substances, such as dextropropoxyphene, for which conventional HPLC with UV detection gives poor sensitivity.

Photochemical derivatization offers several advantages over other pre- and post-column derivatization techniques. Time-consuming sample preparation procedures associated with pre-column methods are eliminated, and the retention volume measured is that of the parent substance rather than that of a derivative. In contrast with conventional post-column derivatization methods, photochemical derivatization has a negligible effect on the resolution of the chromatographic system and imposes few practical restrictions on the eluent composition. As no reagents need to be added to the eluent stream, problems of mixing and pulsating flow do not arise. The principal advantage of photochemical derivatization is, however, the specificity of detection that it provides, and the simple means by which the substance of interest can be differentiated from non-photolabile interfering compounds. It is expected that photochemical derivatization will form a valuable addition to available derivatization methods owing to its simplicity, speed of operation and very high specificity.

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The Incidence of Cannabinoids in Fatally Injured Drivers: An Investigation by Radioimmunoassay and High Pressure Liquid Chromatography

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A survey of cannabinoid levels in post-mortem blood from 66 fatally injured drivers showed, by radioimmunoassay alone, cannabis use in 6 cases. Further examination of 3 of the specimens by a combined system of high pressure liquid chromatography and radioimmunoassay showed typical patterns of separated cross-reacting cannabinoids and gave a specific measurement of THC levels. The total cross-reacting cannabinoid levels in the positive samples were low compared with the levels detected in earlier cases of intoxication or in volunteers smoking moderate doses of pure THC. An assessment of the effect on driving impairment is given.

Introduction

The effects of drugs on driving capability are being examined more closely as prescribed drug-taking by the public increases. The involvement of drugs in fatal road accidents may be more important than has been realised. In cases involving drugs, the substance found most often is alcohol, but it is becoming increasingly apparent that medically prescribed drugs can also be effective in impairing judgement and co-ordination. Particularly dangerous is the intake of drugs in combination with alcohol. Although the blood alcohol level may be measured and found to be low, other undetected drugs may be present, resulting in serious impairment of driving ability.

The incidence of involvement of groups of drugs has been the subject of a recent survey in the U.S.A. (Glauz and Blackburn, 1975). In a comparison of the incidence of drugs in living and fatally injured drivers the authors estimated that the use of stimulants for example, increased the risk of involvement in a fatal accident 14 times above control. Other groups of drugs, such as sedatives (5 times) and antihistamines (5 times) were less of a risk, whilst tranquillisers and nicotine produced no increase in risk. Cannabis increased the risk 3½ times above control.

From experimental studies of driving skills there is evidence that administration of tetrahydrocannabinol (THC) causes, for example, impairment of tracking ability and perceptual performance and it is concluded that cannabis use will increase the probability of driving accidents (Moskowitz, 1976). However, direct evidence of the involvement of cannabis use in accidents is needed. Whilst the assessment of cannabis use in the survey by Glauz and Blackburn is an attempt to produce such evidence, the results are based on a detection system of uncertain reliability involving swab testing of mouth and fingers. A sensitive technique for the analysis of blood specimens is required and a radioimmunoassay (RIA) method has been developed (Teale et al., 1975)
permitting the rapid routine screening of samples in this type of epidemiological study.

RIA has been used already for the confirmation of the involvement of cannabis use in a fatal car accident (Teale and Marks, 1976). With the cooperation of the coroners and pathologists throughout England and Wales, an attempt has been made to assess the incidence of cannabis involvement in fatal road accidents by screening blood samples from fatally injured vehicle drivers.

**Methods and Materials**

A circular letter was sent to all coroners in England and Wales requesting their assistance in a survey of cannabis use in fatally injured drivers. It was suggested that the request, if acceptable, be passed on to the pathologists carrying out post-mortem examinations of such cases and, where appropriate, that they send specimens of blood for screening. The use of anticoagulant or preservative was not important, since none are known to affect the RIA method. On receipt, blood samples were centrifuged and the supernatants separated for storage at —20°C. One volume of each blood supernatant was mixed with two volumes of methanol. The methanolic supernatant was separated from the precipitated proteins and stored at —20°C.

Aliquots (50μl) of methanolic supernatant were assayed initially by direct addition to the RIA system by a similar method to that previously published (Teale et al., 1975). The same volume of methanolic supernatant extract of normal serum was added to all other assay tubes. Results are expressed in THC cross-reacting cannabinoids (THC-CRCs) since THC was used as the standard and the antiserum cross-reacts with THC and several of its metabolites.

Blood samples regarded as positive for the presence of cannabinoids were examined further by the use of high pressure liquid chromatography (HPLC) combined with the RIA (Williams et al., 1977). Methanolic supernatant extracts were subjected to HPLC separation on a reverse-phase column and by elution with a step-wise gradient of methanol-water mixtures. Column fractions were assayed by direct addition of aliquots to the RIA system. THC was identified and quantified by this method.

**Results**

Between July, 1976 and April, 1977, 65 blood specimens from fatally injured drivers were received at the Department of Biochemistry, University of Surrey: 54 from car drivers and 11 from motor-cyclists. One other specimen (number 86), a sample from a motor-cyclist suspected of cannabis use prior to his accident, was received at the Home Office Central Research Establishment.

Figure 1 shows the ratio (B/Bo) of the amount of radioactive label bound to antibody in the presence of an aliquot of each sample extract (B) to the amount of label antibody-bound in the presence of the same volume of normal serum extract (Bo). Samples which produced a decrease in binding to below a ratio of 0.65 were regarded as positive for cannabinoids. From Figure 1 it can be seen that six samples, viz. numbers 8, 15, 21, 42, 53 and 86 were positive for THC-CRCs using this criterion. The histories for these cases are given below:

*Sample 8*
A 22-year-old male died at 23.05 after being involved in an accident at 21.30 when his sports car, containing 3 passengers failed to take a corner. No alcohol was detected.

*Sample 15*
A 17-year-old male, with a passenger who was also killed, was involved in an accident on his motor cycle at 10.30. Blood alcohol was negative.

*Sample 21*
A 27-year-old driver died at 23.00, soon after his car had collided with a lamp-post whilst overtaking. Alcohol was not detected.
Figure 1. Antibody-bound \(^{4}\)H-THC in the presence of methanolic supernatant extracts of each of 66 post-mortem "serum" specimens (B) compared with antibody-bound \(^{4}\)H-THC in the presence of methanolic supernatant extract of normal serum (Bo). Specimens producing a decrease in \(^{4}\)H-THC binding greater than 35\% (B/Bo < 0.65) were regarded as containing cannabinoids.

Sample 42

A 19-year-old male motor-cyclist died at 18.50 after a head-on collision on a bend at 17.00. No alcohol was detected.

Sample 53

A 23-year-old male car driver died at 18.45 after an accident. His blood alcohol was 343mg/100ml blood.

Sample 86

A 20-year-old male fell off his motor-cycle and was run over by a following car. The accident happened at 20.05 and within 30 minutes a blood sample was taken after hospitalisation. The subject died at 23.20. No alcohol or other drugs were detected in the blood. Cannabis was found in the subject’s pocket.

Figure 2 shows the samples grouped according to the age of the victim. The shaded areas represent the number of samples regarded as positive for THC-CRCs, as determined by direct RIA. As might be expected, the incidence of cannabis use is apparently confined to the younger drivers.

Samples 8 and 15 were not investigated further. Aliquots of the other specimens were re-assayed using different conditions (Williams et al., 1977). The level of THC-CRCs in sample 21 was considered to be too low (5ng/ml) to attempt an HPLC separation. However, the levels in samples 42, 53 and 86
were 10, 19 and 11ng/ml respectively and separation of the cannabinoids in each sample was performed. Figure 3 shows the resultant radioimmuno-chromatograms. By peak area integration the concentration of THC in sample 42 was estimated as 1.5ng/ml, in sample 53, 2.3ng/ml and in sample 86, 4.4ng/ml.

Discussion

The use of RIA, based on anti-THC antibodies, is of proven value in the sensitive and rapid detection of cannabis use (Marks et al., 1975). When coupled to an HPLC separation system, the capacity of the RIA for the measurement of THC and its cross-reacting metabolites confers greater specificity on the technique, one application of which is illustrated by the present study.

The patterns of cross-reacting cannabinoid peaks, observed by HPLC/RIA analysis of the post-mortem blood specimens regarded as positive for cannabinoids by direct RIA, may be compared with the patterns produced by similar analyses of samples collected from a volunteer following the smoking of a cigarette impregnated with 10mg of pure THC (Teale et al., 1977). With the reverse-phase HPLC system used, the RIA cross-reacting metabolites elute from the column in decreasing order of polarity. Major features of the chromatograms include an initial very polar peak (probably due to metabolite conjugates) which is followed by peaks due to other metabolites. The last peak is that for THC. The current system therefore provides a measurement of THC, confirms cannabinoid detection by direct RIA and will ultimately permit identification of the other isolated peaks of cross-reacting metabolites.

From Figure 1 it is apparent that direct addition of post-mortem serum extract to the RIA system often causes marked decrease or increase in 3H-THC binding to antibody. For this reason a relatively low cut-off level was selected (B/Bo = 0.65). Any sample causing a depression of label binding below this
level was regarded as containing cannabinoids. Under normal conditions samples from subjects causing a decrease in binding greater than 10% (B/Bo< 0.90) would be taken as containing cannabinoids. It is possible, therefore, that some of the post-mortem specimens classified as negative for cannabinoids by direct RIA but producing a decrease in label binding between 10% and 35%, may contain low levels of cannabinoids. Although this sensitive detection of cannabinoids should be strictly interpreted as further incidence of cannabis involvement in fatal accidents, it is unlikely that such very low blood levels of THC-CRC would be associated with a degree of intoxication likely to cause the impairment of driving ability. The THC-CRC levels obtained by direct RIA analysis of all 6 positive samples may be regarded as moderate compared with the limited data previously available. For example, in the first road accident case examined by RIA (Teale and Marks, 1976) the blood THC-CRC level was found to be 350ng/ml. Cannabis use by the driver had been clearly indicated by discovery of the substance in the car and, similarly, this was the
situation in one of the present cases (86), although the blood THC-CRC level, measured under the same assay conditions, was relatively low. Even though cannabis use was virtually certain in both these cases, and samples of the material consumed were available, there was no information on the amount consumed or the time of consumption. For this reason it is difficult to relate the THC-CRC level with a degree of intoxication. Recently, in a case of cannabis intoxication resulting in coma, a blood THC-CRC level of 180ng/ml was recorded in a sample taken during the comatose state, approximately 8 hours after the smoking of an unknown amount of material (Garrett et al., 1977). Data from volunteers smoking cigarettes impregnated with 5mg pure THC showed a maximum blood THC-CRC level of 72ng/ml in one volunteer immediately after smoking and at a time when mild euphoria was experienced (Teale et al., 1974).

On the basis of these findings, the moderate THC-CRC levels (10–30ng/ml) in the other road accident cases (8, 15, 21, 42 and 53), in which cannabis use was not initially implicated, would not at first sight appear to be the probable cause of driving impairment. In one case (53) a high blood alcohol level was more likely to be the reason. Nevertheless, the blood level at the time of the accident may have been higher than that detected in a sample taken, perhaps, several hours afterwards and, for example, after a blood transfusion. The picture is further complicated by variation in psychoactive cannabinoid composition in similar total THC-CRC levels. Intoxication may be related to the THC component of blood cannabinoids. In sample 86 THC comprised 40% of the total THC-CRCs detected, whilst in samples 42 and 53 the THC fraction was less (15% and 12%) even though the total THC-CRC levels in the latter samples were higher than that in sample 86. Therefore, the relatively low THC-CRC level may be more intoxicating than a higher level by virtue of its higher THC content. The level of THC may also be a reflection of the time the blood sample was collected. Sample 86 was taken soon after the accident, and before death, whereas all the other specimens were collected at post-mortem examination.

If the moderate blood THC-CRC levels detected in the majority of the positive cases are associated with a degree of intoxication likely to be the cause of driving impairment, a much broader survey of cannabis involvement in all accidents, or even in all road-users, should be undertaken to ascertain the extent of its use. With more potent preparations of cannabis extract becoming popular (Lopez et al., 1974; Garrett et al., 1977), degrees of intoxication may exceed those previously experienced and in an increasing number of users. This development may be reflected in cannabis becoming a more important factor in the causation of road accidents.

The minimum apparent incidence of cannabis involvement in 66 fatal road accidents was 9%. The 66 specimens analysed cannot, however, be considered a representative group for several reasons. For example, only 8 specimens were received from the most highly populated area of the country—the South East of England, and nearly half came from another—the Midlands. It will also be noted (Figure 2) that 70% of the specimens came from subjects under 33 years of age. This suggests that some of the pathologists involved in sending samples selected only subjects most likely to have used cannabis. Since all the positive cases fell within this age-group the deliberate selection of such samples will result in a correspondingly higher incidence of cannabis use. The relatively high number of samples from motor-cyclists (16%) may be a reflection of age selection and also the apparently high incidence of cannabis use amongst that group (18%).

The present survey constitutes an example of the type of study which can readily be carried out using an RIA as an initial screening method followed by closer examination of drug-containing samples by a specific HPLC/RIA combined technique.
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References

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