IMMUNOLOGICAL DETECTION OF
HUMAN EXPOSURE TO AFLATOXINS

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ABSTRACT

There is considerable evidence indicating an association between aflatoxin ingestion and liver cancer in humans. The development of methods that would permit the monitoring of aflatoxin exposure in individuals would provide useful information in assessing human risk from this toxin. In this study, an ELISA technique, using a polyclonal antibody raised against aflatoxin B₁ in the rabbit, was developed for monitoring the levels of aflatoxin excreted in human urine samples. Urine could not be used directly in the assay because high blanks were observed from the presence of some aflatoxin-like substances. A 'clean-up' procedure using Sep-Pak C₁₃ cartridges and immunoaffinity columns was developed. The methods were validated using ^3H-AFB₁ in both buffer and presumed uncontaminated human urine samples from Europeans. As AFB₁ itself is unlikely to be found in human samples, urine from marmoset monkeys treated with ^1⁴C-AFB₁ were also used in the validation processes as they are likely to contain a spectrum of aflatoxin metabolites similar to those in human urine. The levels of AFB₁ equivalents were monitored by both radioactive counting and ELISA. The radioactive measurements demonstrated that the overall recovery of the ELISA method was approximately 50%. The failure to detect all of the aflatoxin contamination in the urine may be due to the inability of the antibody to detect most of the polar aflatoxin metabolites.

When the ELISA method was used in monitoring aflatoxin excreted in patients with or without liver disease from Thailand, a range of aflatoxin levels in the urine samples were obtained. Since in Thailand, liver fluke infection, caused by Opisthorchis viverrini is a serious health problem in the area where there is high contamination with aflatoxin in food and a high incidence of liver cancer, the interaction between liver fluke infection and aflatoxin ingestion was also studied using hamster as an animal model. The results indicated that liver fluke infection may alter the pattern of aflatoxin metabolites excreted in hamster urine, which in turn could affect the results obtained from the ELISA.

Measurements by the ELISA method of aflatoxin excreted in urine samples from Thai vegetarians; who are a population at high risk of exposure to aflatoxin suggested that this group of people excreted higher levels of aflatoxin, but a further study with a greater number of subjects would be necessary to confirm this finding.
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Finally, I owe my life to my parents who always give me their love and care, even when we are so far apart, and to whom this thesis is dedicated.
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<td>AFB₁</td>
<td>Aflatoxin B₁</td>
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<td>AFB₂</td>
<td>Aflatoxin B₂</td>
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<td>AFG₁</td>
<td>Aflatoxin G₁</td>
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<tr>
<td>AFG₂</td>
<td>Aflatoxin G₂</td>
</tr>
<tr>
<td>AFL</td>
<td>Aflatoxicol</td>
</tr>
<tr>
<td>AFM₁</td>
<td>Aflatoxin M₁</td>
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<td>AFP₁</td>
<td>Aflatoxin P₁</td>
</tr>
<tr>
<td>AFQ₁</td>
<td>Aflatoxin Q₁</td>
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<tr>
<td>AFB₁-Cys</td>
<td>8,9-Dihydro-8-(S-cysteiny1)-9-hydroxy aflatoxin B₁</td>
</tr>
<tr>
<td>AFB₁-Cys-Gly</td>
<td>8,9-Dihydro-8-(S-cysteiny1-glycyl)-9-hydroxy aflatoxin B₁</td>
</tr>
<tr>
<td>AFB₁-dhd</td>
<td>8,9-Dihydro-8,9-dihydroxy aflatoxin B₁</td>
</tr>
<tr>
<td>AFB₁-FAPy</td>
<td>8,9-Dihydro-8-(N⁵-formyl-2',5',6'-triamino-4'-oxo-N⁵-pyrimidy1)-9-hydroxy aflatoxin B₁</td>
</tr>
<tr>
<td>AFB₁-GSH</td>
<td>8,9-Dihydro-8-(S-glutathionyl)-9-hydroxy aflatoxin B₁</td>
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<td>AFB₁-Gua</td>
<td>8,9-Dihydro-8-(N⁷-guanyl)-9-hydroxy aflatoxin B₁</td>
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<tr>
<td>AFB₁-NAcCys</td>
<td>8,9-Dihydro-8-(S-Cysteinyl)-(N-acetyl)-9-hydroxy aflatoxin B₁</td>
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<tr>
<td>AFB₂a</td>
<td>8,9-Dihydro-8-hydroxy aflatoxin B₁</td>
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<td>APLH₁</td>
<td>Aflatoxicol of AFQ₁</td>
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<td>APLM₁</td>
<td>Aflatoxicol of AFM₁</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PHC</td>
<td>Primary hepatocellular carcinoma</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>UALS</td>
<td>Urinary aflatoxin-like substances</td>
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CHAPTER 1

General Introduction
Aflatoxins were first discovered in the early 1960's following the outbreak of 'Turkey X disease' in the South and East of England. This disease was characterized by subcutaneous haemorrhage, weight loss, lethargy and death in turkeys. Postmortem examination revealed acute hepatic necrosis with generalised bile duct proliferation. There were losses of other birds, including ducklings, and also swine, cattle and sheep. They all had similar symptoms to the turkeys. The common factor in the death of these animals was shown to be the use in animal feedstuffs of batches of groundnut meal which had been imported from Brazil (see Goldblatt, 1969). The groundnut meal was found to be heavily contaminated with a fungus which, using subsequently imported toxic groundnuts, was identified as Aspergillus flavus (Sargeant et al., 1961). A toxic substance was extracted which produced the same results in ducklings as the groundnut meal and this mycotoxin was given the name "aflatoxin".

When conditions of humidity and temperature permit, aflatoxins are formed on stored foodstuffs by species of Aspergillus (Stoloff, 1977). The aflatoxins are a group of secondary metabolites produced by some strains of Aspergillus flavus and Aspergillus parasiticus. Aflatoxins have been found as natural contaminants in many types of food, e.g. peanut, cottonseed meal, corn and rice (Wogan, 1968; Shank et al., 1972a,b), particularly in tropical countries.
Aflatoxins are highly substituted coumarins containing a fused dihydrofurofuran moiety. There are four main naturally occurring aflatoxins (Figure 1.1). AFB₁ and AFB₂ were so designated because of their strong blue fluorescence under UV light, whereas AFG₁ and AFG₂ fluoresced greenish yellow (Nesbitt et al., 1962; Asao et al., 1965). The B toxins can be characterized by the fusion of a cyclopentenone ring to the lactone ring of the coumarin structure; the G toxins contain an additional fused lactone ring instead. AFB₁ and, to a somewhat lesser extent, AFG₁ were responsible for the extreme biological potency of aflatoxin-contaminated meals and crude fractions derived from toxigenic A. flavus cultures. These two toxins possess an unsaturated bond at the 8,9-position on the terminal furan ring. The much less potent AFB₂ and AFG₂ are saturated at this position (Asao et al., 1965).

The aflatoxins are soluble in methanol, chloroform, dimethyl sulfoxide, dimethylformamide and other organic solvents. They are also soluble in ethanol and propylene glycol but less so and only sparingly soluble in water (10-30 μg/ml) (Busby and Wogan, 1984). Although aflatoxins are quite stable in food and feedstuffs, they are rapidly deactivated by extremes of pH (< 3 or > 10), oxidizing agents, or exposure to UV light in the presence of oxygen (Stoloff, 1977). A thorough review of the chemistry of aflatoxins is given by Heathcote and Hibbert (1978).
Figure 1.1 Structures of primary aflatoxins

AFB1

AFB2

AFG1

AFG2
1.2 CONTAMINATION OF FOOD BY AFLATOXINS

A great many different food have been shown to be contaminated by aflatoxins. Food most commonly implicated are peanuts, maize, cassava, dried fish, rice and some alcoholic beverages fermented from maize (see Busby and Wogan, 1984). Milk from some village dairies has been found to contain high levels of AFM$_1$ (Suzanger et al., 1976). In a study carried out in Sudan, it was found that some women excreted aflatoxins in breast-milk at levels similar to or higher than those considered safe in animal milk, for human consumption (Coulter et al., 1984).

Human populations may be exposed to aflatoxins by the consumption of commodities that have been directly contaminated by toxigenic strains of A. flavus or A. parasiticus during growth, harvest or storage. Secondary exposure may occur by the consumption of products, e.g. meat and other edible tissues, milk and dairy products and eggs, derived from animals that have consumed aflatoxin-contaminated feeds (Busby and Wogan, 1984). The risk of exposure to aflatoxins is apparently less in technologically developed countries than in developing ones. This may be because of inadequate food storage facilities and preservation techniques, combined with the naturally warm, humid weather, generally unhygienic environmental conditions and ignorance which provide an atmosphere conducive to the growth of fungus and consequently to the elaboration of aflatoxins on foodstuffs (see Denning, 1987).
Many ways of removing aflatoxins from food have been devised, either intentionally or by long traditional means of food handling (Sauer, 1978). The most effective means of lowering aflatoxin contamination in the developing countries is by preventing insect and chemical damage to crops, picking during the dry season (if possible) and ensuring adequate drying of crops and storage of crops in a cool, dry atmosphere protected from insect infestation.

1.3 METABOLISM OF AFLATOXINS

AFB₁ is actively metabolized in a variety of animal species. Figure 1.2 shows an overall scheme of AFB₁ metabolism. The relative importance of each individual pathway varies substantially depending on the animal species and the experimental conditions. Essentially, the initial metabolism of AFB₁ involves three principal types of reactions: (a) hydroxylation, (b) epoxidation and (c) ketoreduction. The former two reactions are believed to be carried out principally by a microsomal mixed-function oxidase system, the latter by a cytosolic NADPH-dependent reductase. In most animal species the hydroxylated AFB₁ metabolites may undergo phase II metabolism by conjugating with glucuronic acid or sulphate (see Busby and Wogan, 1984; Woo et al, 1988).

There have been a large number of studies on the metabolism of AFB₁ in different species (see Busby and Wogan, 1984; Neal, 1987). Unfortunately, many of the
Figure 1.2 Primary pathways of AFB$_1$ metabolism in animal systems
earlier investigations used species whose primary metabolic pathways have subsequently been found to be different from man's and are, therefore, of limited help in understanding the relationship between metabolism and toxicity in man (Hsieh et al., 1977). The monkey apparently possesses similar pathways of aflatoxin metabolism to the human and, therefore, information available from studies involving this species is relevant to the human situation. In the last decade, there has been a large number of studies of the metabolism of aflatoxins in in vitro systems using a variety of human tissue preparations. These have mainly involved the presumed target tissue, the liver, used in various forms from subcellular fractions to isolated hepatocytes and tissue explants. In many of these studies, assays using the corresponding animal tissues have been carried out, in parallel with the human studies, to enable the assessment of relative susceptibilities (see Neal, 1987).

The human hepatic metabolism of aflatoxins will largely depend on the cytochrome P<sub>450</sub> content, which is controlled by exogenous and endogenous factors. Amongst endogenous determinants, which may be viewed as analogous to the strain differences which occur within animal species, there is increasing evidence for polymorphism in the cytochrome P<sub>450</sub> content of human liver, in which the types of cytochrome present, or their activities, are genetically segregated into specific cohorts within general population (Idle and Smith, 1979; Ritchie and Idle, 1982).
In addition to the genetically determined factors related to the individual species of cytochromes which affect the susceptibility of humans to aflatoxins, there is the influence of environmental or exogenous factors. General dietary sufficiency or insufficiency is known to affect general cytochrome P<sub>450</sub> levels in animals and presumably would similarly affect humans. A comparison of the effects of low and sufficient protein diets on the type of aflatoxin-induced lesions in rats (Madhavan and Gopaland, 1965) could reflect, in part, the effects of these diets on the levels of drug metabolizing systems. Also, in humans the influence of dietary constituents, tobacco smoking and alcohol intake will modify the intrinsic capacity to metabolize environmental pollutants, including the aflatoxins.

In human microsomal systems, a frequently reported major route of AFB<sub>1</sub> metabolism is by hydroxylation in the cyclopentenone ring to form AFQ<sub>1</sub> (Masri et al., 1974; Roebuck and Wogan, 1977; Moss and Neal, 1985), although in one study, AFM<sub>1</sub> and AFP<sub>1</sub> were the principal metabolites observed (Merrill and Campbell, 1974). The high metabolic production of AFQ<sub>1</sub> in vitro appears to be a feature of primates, similar results having been obtained using hepatic microsome fractions from rhesus and marmoset monkeys (Masri et al., 1974; G.E. Neal, personal communication). Details of studies involving AFQ<sub>1</sub> are given in Chapter 3.

Another feature of the in vitro primary microsomal metabolism of AFB<sub>1</sub> by human liver is an overall high rate
of metabolic conversion which can be compared with the high rate of metabolism observed in avian species (Moss and Neal, 1985). Human microsomal AFBl metabolism results in relative levels of production of AFQ1 and AFBl-dhd of 60-90% and 10-20% respectively, whereas using avian microsomes the production of these metabolites is 0% and 85-95% respectively (Moss and Neal, 1985; Neal et al., 1986). With few exceptions, AFM1 has generally been reported to be a minor microsomal metabolite in human liver systems, although some individual variation in its production has been indicated (Moss and Neal, 1985). AFM1 probably comprises 1-4% of consumed AFBl (Campbell et al., 1970; Sun and Chu, 1984) but despite the low level of production, AFM1 appears to be the only primary metabolite of AFBl to be excreted in an unconjugated form and, therefore, it may be useful in assessing aflatoxin consumption (Denning, 1987).

Reports of the formation of AFP1, from AFBl by a mixed-function oxidase catalysed O-demethylation process, in in vitro human microsomal incubations have varied from AFP1 being a principal metabolite to its being a minor product, if present at all (Merrill and Campbell, 1974; Roebuck and Wogan, 1977; Moss and Neal, 1985). Even within individual studies, the level of formation of AFP1 relative to AFQ1 has varied between individual samples from 1:4 to 1:54 (Roebuck and Wogan, 1977). AFP1 is frequently found as the β-glucuronide or sulphate conjugates in the urine or bile of animals treated with AFBl, especially in rhesus
monkeys (Bassir and Emafo, 1970; Dalezios and Wogan, 1972). These conjugates are readily hydrolysed by β-glucuronidase or sulphatase because the conjugation is on the phenol ring (Martin et al., 1978). A significant excretion in the urine of AFP₁, either in free or conjugated by humans, would therefore be readily detectable.

The metabolism of AFP₁ to AFB₂a by human cytosols has also been reported but AFB₂a would appear to be a minor metabolite, and a misidentification of AFB₁-dhd cannot be excluded (Patterson and Roberts, 1972; Neal et al., 1981). The metabolism of AFB₁ to AFL by human cytosol fractions has also been demonstrated (Salhab and Edwards, 1977). It was also detected in the sera of children in the Sudan (Hendrickse et al., 1982). AFLH₁ has been reported to be a major metabolite of AFB₁ in in vitro systems when both microsomal hydroxylase and cytosolic reductase of human- or monkey-derived fractions are present (Salhab and Hsieh, 1975). AFL is metabolized to AFB₁ and AFLM₁ to AFM₁, in the presence of human microsomes and NADP (Salhab et al., 1977).

The AFB₁ activating capacity of human microsomal systems in vitro, assessed by the production of AFB₁-dhd (assayed by the Tris complex), indicated that 10-20% of the soluble AFB₁ metabolites are formed via epoxidation (Moss and Neal, 1985). However, it has been found that the rapid metabolism of AFB₁ by human microsomes with NADPH is accompanied by only a low recovery of AFB₁ as soluble metabolites, and it is possible either that Tris buffer
does not compete effectively with macromolecular binding for the AFB₁-dhd formed, or that a higher binding of activated AFB₁ to protein or RNA takes place via the AFB₁-epoxide in human microsomes than in microsomes from animal species (Moss and Neal, 1985). Binding of ³H-AFB₁ to ribosomal RNA added to in vitro human liver microsomal incubations have been reported (Swenson et al., 1974).

In in vivo experiments, the binding of labelled AFB₁ to nucleic acid via AFB₁-epoxide has a higher specific activity than binding to protein (Swenson et al., 1977), which indicates that the conversion of non-covalently bound epoxide to AFB₁-dhd, followed by Schiff base binding to protein, is limited by the high level of binding to nucleic acid at the stage of AFB₁-epoxide formation. If the lower recovery of soluble AFB₁ metabolites when human microsomes are incubated with AFB₁ is due to a high covalent binding of AFB₁-epoxide, then these incubations might more accurately reflect the in vivo (high nucleic acid binding) situation (Neal, 1987).

Factors affecting differences between in vitro and in vivo behaviour also presumably involve the cellular architecture itself. A considerable individual variation in AFB₁ activation capacity of human liver has been observed using fresh biopsy samples incubated with labelled AFB₁. Binding to DNA varies between 0.7 and 8.5 ng AFB₁ bound/mg DNA, which is intermediate between the levels found in the AFB₁ sensitive hamster and resistant mouse (Booth et al., 1981). AFB₁-epoxide which would appear to
be a highly reactive species has not been isolated successfully but it can be generated chemically (Martin and Garner, 1977). This AFB₁-epoxide can combine with guanine bases in DNA to produce alterations in DNA. DNA binding has been demonstrated in human epithelial lung cells using tissue culture (Wang and Cerutti, 1979) and in human bronchus and colon explants (Autrup et al., 1979). In a study of the capacity to form AFB₁-DNA adducts in cultured human organs when incubated with AFB₁, both the AFB₁-Gua adduct and the imidazole ring-opened, AFB₁-FAPy adduct, were found (Autrup and Harris, 1983). The ratios between these adducts varied in the different tissues and could indicate a role for enzymatic ring opening in addition to the non-enzymatic reaction. Recently the AFB₁-Gua adduct has been detected in urine among residents in Kenya (Autrup et al. 1985).

As can be seen in Figure 1.2 following the formation of the AFB₁-8,9-oxide, the only pathway of detoxification, other than possible macromolecular binding not involving a toxic response, is via glutathione conjugation (AFB₁-GSH). Recent experiments suggest that the human potential for this means of detoxification is small, as very little AFB₁-GSH is produced (Moss and Neal, 1985). The presence of AFB₁ mercapturate has not been reported in human systems. In this connection the reported low level of AFB₁-GSH conjugating capacity in human cytosols might be relevant. Despite a similar low level of AFB₁-GSH conjugating capacity in marmoset monkey liver cytosol
fractions, the presence of the mercapturate has been detected in the urine of these animals treated with AFB$_1$ (Moss et al., 1985).

Since most of the excreted AFB$_1$ is in the form of water-soluble non-primary microsomal metabolites, a knowledge of the conjugation of primary metabolites to secondary metabolites by human tissue systems is clearly of importance in terms of monitoring. But many of the in vitro studies have examined the primary metabolites of aflatoxins in which the metabolism leads to the production of chloroform-soluble rather than insoluble, unbound metabolites (Roebuck and Wogan, 1977). Despite the fact that, from microsomal studies, AFQ$_1$ would appear to be the major human hepatic AFB$_1$ metabolite, no conjugate of AFQ$_1$ has been rigorously characterized to date, or reported in urine samples.

1.4 Human Diseases Possibly Related to Exposure to Aflatoxins

Aflatoxins are among the few chemically identified and widely disseminated environmental carcinogens for which quantitative estimations of human exposure have been systematically sought. Significant differences in responsiveness are known to exist among animal species, but less is known about the human response. The character and intensity of the human response might vary depending on factors such as age, sex, nutritional status, concurrent...
exposure to other agents, genetic factors, concurrent illness (e.g. viral hepatitis or parasitic infestation), as well as the level and duration of exposure to aflatoxins (Wogan, 1975).

1.4.1 **Acute aflatoxicosis**

Acute aflatoxicosis is probably only recognized in connection with an outbreak of poisoning in specific communities and as such is rare. The evidence for acute aflatoxicosis has been reported from Taiwan (Ling et al., 1967), Uganda (Serck-Hanssen, 1970) and Kenya (Ngindu et al., 1982). The syndrome was characterized by vomiting, abdominal pain, pulmonary oedema, and fatty infiltration and necrosis of the liver. More extensive documentation of an outbreak of putative aflatoxin poisoning was provided in 1974 from Western India (Krishnamachari et al., 1975). The causative agent appears to have been a particular crop of maize which was contaminated by *Aspergillus flavus* due to wet storage conditions resulting from unseasonal heavy rains. The major symptom evident was jaundice. Males were affected twice as commonly as females. Out of a total of 397 patients, 106 died. Several samples of the maize had high levels of aflatoxin contamination (6.25 to 15.6 mg aflatoxin/kg maize) and it was calculated that consumption of aflatoxins could have been in the range of 2.6 mg daily for several weeks. The actual involvement of aflatoxin ingestion in the onset of disease was strongly supported by the pathological evidence. Liver pathology indicated the
presence of multinucleate giant cells with extensive bile duct proliferation, these lesions showing striking similarity to those induced in the livers of experimental animals exposed to aflatoxins (Butler, 1964). Death was due to gastrointestinal haemorrhage.

1.4.2 **Hepatocellular carcinoma**

There is a considerable body of evidence implicating aflatoxins as an important aetiologic factor in human liver cancer (see Busby and Wogan, 1984; Denning, 1987). Epidemiological studies established a positive association between the geographic distribution of areas of high liver cancer incidence and that of prevalence of aflatoxin contamination of foodstuffs. Although the evidence gives no information concerning the latent period and does not constitute definitive scientific proof of a principal aetiologic role for aflatoxins in human liver cancer, nevertheless, the evidence for involvement of aflatoxins appears to be strong.

Epidemiological data regarding aflatoxin contamination of food and the incidence of hepatocellular carcinoma as well as monitoring of aflatoxin and its metabolites in both urine and serum are now available from Uganda (Alpert et al., 1968a,b; 1971), Swaziland (Keen and Martin, 1971; Peers et al., 1976, 1987), Kenya (Peers and Linsell, 1973; Autrup et al., 1983, 1987), Mozambique (Prates and Torres, 1965; van Rensburg et al., 1974, 1985), Transkei (van Rensburg et al., 1985), Nigeria (Denning et al., 1988), the
Philippines (Campbell et al., 1970; Campbell and Salamat, 1971; Bulatao-Jayme et al., 1982), Thailand (Shank et al., 1972a-e), and China (Hu et al., 1983; Zhu et al., 1987; Gan et al., 1988).

A dose-response relationship between aflatoxin consumption and liver cancer incidence has been provided by Peers and Linsell (1977) by combining the data from four separate field studies in Kenya (Peers and Linsell, 1973), Thailand (Shank et al., 1972c-d), Mozambique (van Rensburg, 1974) and Swaziland (Peers et al., 1976). The summary of data shown in Table 1.1 demonstrated a high degree of positive correlation between estimated daily intake of aflatoxin [expressed as ng/kg body weight/day (X)] on one hand, and the adult incidence rate of liver cancer [expressed as cases per $10^5$ adult/year (Y)], on the other hand. The correlation equation was given as $Y = 7.6 \log X - 3.6$ (significant at $p < 0.001$).

These data provide strong circumstantial evidence of a putative causal relationship between aflatoxin ingestion and liver cancer incidence in humans. Although this evidence does not constitute proof that aflatoxins are the cause of human liver cell carcinoma, these data, together with the extensive animal data on aflatoxin carcinogenicity, are sufficient to associate exposure to the carcinogen with elevated risk of this form of cancer (Busby and Wogan, 1984).
<table>
<thead>
<tr>
<th>Country</th>
<th>Area</th>
<th>Aflatoxin intake (ng/kg/day)</th>
<th>Number of cases</th>
<th>Incidence (no/10^5persons/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kenya</td>
<td>High altitude</td>
<td>3.5</td>
<td>4</td>
<td>1.23</td>
</tr>
<tr>
<td>Thailand</td>
<td>Songkhla</td>
<td>5.0</td>
<td>2</td>
<td>2.00</td>
</tr>
<tr>
<td>Swaziland</td>
<td>High veld</td>
<td>5.2</td>
<td>11</td>
<td>2.18</td>
</tr>
<tr>
<td>Kenya</td>
<td>Middle altitude</td>
<td>5.9</td>
<td>33</td>
<td>2.51</td>
</tr>
<tr>
<td>Swaziland</td>
<td>Mid veld</td>
<td>8.9</td>
<td>29</td>
<td>3.83</td>
</tr>
<tr>
<td>Kenya</td>
<td>Low altitude</td>
<td>10.0</td>
<td>49</td>
<td>4.01</td>
</tr>
<tr>
<td>Swaziland</td>
<td>Lebombo</td>
<td>15.4</td>
<td>4</td>
<td>4.27</td>
</tr>
<tr>
<td>Thailand</td>
<td>Ratburi</td>
<td>45.0</td>
<td>6</td>
<td>6.00</td>
</tr>
<tr>
<td>Swaziland</td>
<td>Low veld</td>
<td>43.1</td>
<td>42</td>
<td>9.18</td>
</tr>
<tr>
<td>Mozambique</td>
<td>Inhambane</td>
<td>222.1</td>
<td>101</td>
<td>16.1-25.4</td>
</tr>
</tbody>
</table>

*a* Adapted from Peers and Linsell (1977). Periods covered were 1 year in Thailand, 3 years in Mozambique and 4 years each in Kenya and Swaziland

*b* Estimated average daily intake (excluding native beers) of aflatoxin by adults expressed as ng aflatoxin/kg body weight/day

*c* Incidence expressed as number of new case/10^5 population/year
1.4.3 **Reye's syndrome**

Reye's syndrome is an acute, often fatal disease affecting infants and young children typified by acute encephalopathy accompanied by fatty degeneration of the viscera (Reye et al., 1963). The disease usually progresses from a mild prodromal viral illness to severe cerebral involvement with coma. It is thought that Reye's syndrome results from a number of interacting factors, of which exposure to aflatoxins is possibly one. Clustering of Reye's syndrome has been observed in Northeast Thailand in predominantly rural areas, geographically and seasonally related to heavy contamination of market food samples with aflatoxins (Olson et al., 1971; Shank et al., 1971; Bourgeois, 1975). Trace amounts of aflatoxins were detected in tissues, body fluids or gastrointestinal contents of 22 out of 23 Thai patients who died from Reye's syndrome (Shank et al., 1971). The presence of aflatoxins was also demonstrated in the liver specimens of a number of Reye's syndrome patients in New Zealand (Becroft, 1966; Becroft and Webster, 1972), Czechoslovakia (Dvorackova et al., 1977), and the United States (Chaves-Carballo et al., 1976; Hogan et al., 1978); however, a dietary source of aflatoxin was not identified in any of these cases. It should be noted that not all Reye's syndrome patients had detectable amounts of aflatoxins in their tissues or body fluids (Ryan et al., 1979; Nelson et al., 1980; Rogan, 1985). Moreover, no unusual clustering of Reye's syndrome has been found in other countries with high frequencies of food contamination with aflatoxins.
1.4.4 Kwashiorkor

Protein energy malnutrition is the dominant form of malnutrition in childhood in certain Third World areas and has a wide spectrum of associated clinical disorders that embraces both marasmus and kwashiorkor. Nutritional marasmus is the childhood equivalent of chronic starvation. Kwashiorkor, in contrast, occurs mainly in recently weaned children, and is typical by oedema accompanied by hypoalbuminaemia (Williams, 1933, 1935; Alleyne et al., 1976). Skin disorders are frequently present and an accompanying immune deficiency leads to a susceptibility to infection (Frank et al., 1975). The condition is almost invariably accompanied by a fatty liver. Suggestions have been made that the condition is associated with a protein deficiency in the presence of an energy sufficiency in the diet but this theory is no longer widely supported (Gopaland, 1968). It has been demonstrated that there are no essential differences in the protein/energy ratios in the diets of children who develop marasmus or kwashiorkor. It is postulated that kwashiorkor results from a 'dysadaptation' of biochemical mechanisms that protect essential organs such as the liver at the expense of less essential tissues such as muscle during protein energy malnutrition. The cause of this postulated dysadaptation remains obscure (Gopaland, 1968). Epidemiological evidence has indicated that the geographical distribution and seasonal variation in the incidence of kwashiorkor parallels aflatoxin contamination of local
foodstuffs (Hendrickse, 1984). Evidence has also been obtained that aflatoxins are detected more frequently, and at higher levels, in sera from kwashiorkor children than in marasmus or adequately nourished children from the same areas (Hendrickse et al., 1982; Coulter et al., 1986a; De Vries et al., 1987). Aflatoxins have been reported to be present in biopsy or autopsy specimens of liver from kwashiorkor patients, in contrast to specimens from non-kwashiorkor children (Hendrickse et al., 1983; Coulter et al., 1986b; Apeaguei et al., 1986). Urinary excretion of aflatoxins in kwashiorkor appears to be less than in other groups (Hendrickse et al., 1983; De Vries et al., 1987). Although these results undoubtedly show that the kwashiorkor children were more at risk from the toxin, it is not possible to conclude, at present, whether the relationship exists because aflatoxins cause kwashiorkor or because children with kwashiorkor are unable to metabolize and excrete these substances (Editorial, 1984; De Vries et al., 1987).

1.4.5 Indian childhood cirrhosis syndrome

A direct involvement of aflatoxin ingestion in human hepatic cirrhosis has been proposed in the case of Indian childhood cirrhosis syndrome (Robinson, 1967). This is a disease which affects children in the Indian sub-continent, usually between the ages of 6 months and 6 years. A role for aflatoxins in the onset of this disease has been suggested based on the frequent contamination of local
foodstuffs with the toxin, and the reported presence of aflatoxins in the urine of affected children and in the milk of the mothers (Robinson, 1967; Amla et al., 1970, 1974). It has also been reported that the accidental feeding of aflatoxin-contaminated protein to infants with kwashiorkor has led to a syndrome resembling Indian childhood cirrhosis, both clinically and histologically (Amla et al., 1971). The presence of highly fluorescent compounds in the urine of Indian childhood cirrhosis patients has been confirmed, but to date these have not been identified as any known aflatoxin metabolite (Yadgin et al., 1970). At present, therefore, the role of aflatoxin ingestion in the incidence of this disease remains uncertain.

1.4.6 Impaired immune function

In experimental animal model systems, aflatoxin ingestion has been found to modify the immune system in many ways, including depressive effects on antibody formation, complement and phagocytosis, as well as cell-mediated immunity (Pier and Heddleston, 1970; Richard and Thurston, 1975; Pier et al., 1977). The implications of this in terms of an effect on human health are clearly very considerable. An impaired immune function could be a factor in the high incidence of hepatocellular carcinoma in areas of high viral hepatitis infection and consumption of aflatoxins (Prince et al., 1975; Tabor et al., 1977). An efficient cell-mediated immune system is required for the elimination of hepatitis B virus. If the immune system
becomes impaired by aflatoxin ingestion, this could result in the prolonged presence of hepatitis B with the consequent development of the cirrhotic condition, which in turn could facilitate the subsequent carcinogenic change.

An immunosuppressive effect due to the presence of aflatoxins could influence the pattern of infection with many infectious diseases (Denning, 1987) including the human immunodeficiency virus (Hendrickse and Maxwell, 1988). This area of research interest demands further investigation.

1.5 AETIOLOGICAL FACTORS IN HEPATOCELULAR CARCINOMA

The incidence of primary hepatocellular carcinoma (PHC) is highest in Africa and Asia (Waterhouse et al., 1982; Doll, 1986). Crude estimates indicate that probably more than 200,000 people die of this cancer each year (Sun and Wang, 1983). It appears to be a global health problem. Studies have narrowed down the search for causative factors and have identified the hepatitis B virus (HBV) (Blumberg and London, 1985) and mycotoxins, especially AFB₁ (Linsell, 1984), to be the major candidates as aetiological agents for human liver cancer.

Attention has been focused on HBV because (a) prospective epidemiological studies have shown a high incidence of PHC among carriers in HBV-endemic areas (Beasley et al., 1981; Sakuma et al., 1982; Lu et al.,
1983); (b) of the clinical observation that most of the patients with PHC are carriers of hepatitis B surface antigen (HBsAg) and have chronic active hepatitis (Blumberg and London, 1981; Sakuma et al., 1982; Lu et al., 1983; Hino et al., 1984a); and (c) recently, integrated HBV sequences have been found in the hepatocyte genome in patients with chronic hepatitis, with hepatocellular carcinoma and HBV carriers (Chakraborty et al., 1980; Shafritz et al., 1981; Brechot et al., 1982; Hino et al., 1984a,b).

PHC is less common and occurs at an older age in urban populations than in rural populations. HBV infection at an early age in rural areas is considered to be the explanation for these observations. However, recent evidence suggests that other environmental factors are important (Harris and Sun, 1986). For example, a case control study of PHC patients of whom all were born and reared in rural areas but half had then moved to an urban setting was conducted by Kew et al. (1983). They concluded that HBV status, which was similar in both groups, was not responsible for the differences in incidence and age at onset of PHC in rural and urban populations. Similar conclusions have been arrived at based on data from China (Sun et al., 1980).

The additional environmental factors most likely to increase the risk of PHC include exposure to $\text{AFB}_1$ (IARC, 1976; Wogan, 1976; van Rensburg, 1977; Linsell, 1979; van Rensburg et al., 1985; Hsieh, 1986; Stora and Dvorackova,
consumption of alcoholic beverages (Lieber et al., 1979; Martini, 1980; Ohnishi et al., 1982; Bulatao-Jayme et al., 1982; Yu et al., 1983; Austin et al., 1986), contaminated water (Su, 1979), low selenium levels (Yu et al., 1985), occupation (Stemhagen et al., 1983), tobacco smoking (Trichopoulos et al., 1980; Lam et al., 1982; Yu et al., 1983; Kew et al., 1985; Austin et al., 1986), and androgen therapy (Westaby et al., 1983). The detail of aetiological factors in hepatocellular carcinoma was thoroughly reviewed by Bassendine (1987).

Consumption of alcoholic beverages has long been considered to be a major aetiological factor in the pathogenesis of PHC in Western countries (Tuyns and Obradovic, 1975; Keller, 1978; Martini, 1980; Bassendine, 1986). A recent case-control study has also suggested that the consumption of alcoholic beverages may be an important factor of PHC in the Philippines (Bulatao-Jayme et al., 1982). Individuals who consumed daily more than 21g of alcohol and more than 4 µg of AFB₁ in contaminated-food (estimated) had a 35-fold increased relative risk of PHC. The results indicated that the effects of alcohol intake and dietary mycotoxin intake were synergistic.

Case-control studied in Greece (Trichopoulos et al., 1980), Hong Kong (Lam et al., 1982) and the USA (Yu et al., 1983) have demonstrated an increased risk of PHC among cigarette smokers, but a recent study has failed to confirm this effect (Austin et al., 1986).
In the People's Republic of China, the correlation between PHC incidence and estimated dietary mycotoxin intake is statistically higher than the correlation between PHC incidence and the geographical distribution of HBV infection (Wang et al., 1983). Whereas the incidence of HBV infection does not vary among people living in the low- and high-altitude areas of Kenya, both the incidence of food contamination by AFB\textsubscript{1} produced by \textit{Aspergillus flavus} and the incidence of PHC are higher in the low-lying regions (van Rensburg, 1977). Further indirect evidence of an interactive effect between HBV infection and chemical carcinogens comes from a study of Eskimos in Greenland, an area of presumed low dietary consumption of AFB\textsubscript{1}, but which has a high prevalence of HBV carriers. A low incidence of PHC was reported for this area (Melbye et al., 1984).

The precise roles of HBV infection and aflatoxin intake in the development of PHC remain to be elucidated. The existence of regions of low incidence for PHC, despite an elevated prevalence of HBV infection, may indicate that HBV infection acting alone has limited carcinogenic effect but that it needs to be potentiated by other factors. The clear geographical association between aflatoxin contamination and PHC incidence in parts of Africa and Asia, and the lack of an association with HBV prevalence at higher levels of incidence for PHC suggests that in these regions aflatoxin is a major determinant of risk (van Rensburg et al., 1985). It is of interest that areas where a moderate to high prevalence of HBV is accompanied by a low incidence of PHC tend to be either too dry, as in
Botswana, or too cold, as in Greenland, for the growth of Aspergillus flavus. An aflatoxin survey in Egypt, which has a hot and dry climate, and a reportedly low incidence of PHC, revealed exceedingly low levels of contamination (Girgis et al., 1977).

However, epidemiological studies have firmly established HBV infection as, at least, a probable prerequisite for the development of PHC in the majority of cases. The viral carrier status is acquired during early childhood, and carries a relative risk for the development of the tumour of over 200. Integration of HBV DNA probably acts as a genotoxic initiator in the multistep process of hepatocarcinogenesis, although the precise mechanisms involved have not been determined. Aflatoxin ingestion may also have an aetiological role in some areas, probably as a genotoxic or epigenetic promotor to HBV-initiated carcinogenesis (Kew, 1986).

From the evidence being accumulated, it is postulated that hepatocellular carcinoma is multifactorial in origin, and the pattern of its aetiological associations differs between populations at high and low risk (Harris and Sun, 1984; Kew, 1986). This multifactorial aetiological hypothesis is rapidly gaining support from recent findings of molecular genetic investigations on the mechanism of hepatocarcinogenesis. It now appears highly likely that there are synergistic effects between AFB\textsubscript{1} and HBV in causing PHC (Ayoola, 1984; Harris and Sun, 1984) but more evidence is required to determine which one is the initiator and which one is the promotor.
The primary goal of biochemical and molecular epidemiology is to identify individuals at high cancer risk by obtaining evidence of (a) high exposure to carcinogens, leading to pathobiological lesions in target cells, and/or (b) increased oncogenic susceptibility due to either inherited or acquired host factors. This emerging and multidisciplinary area of cancer research combines epidemiological and laboratory approaches (Perera and Weinstein, 1982; Harris et al., 1985).

Progress has been made in identifying chemical carcinogens in human body fluids and tissue samples. For example, both AFB$_1$ metabolites (e.g. AFM$_1$) (Campbell et al., 1970; Sun et al., 1983; Wu et al., 1984; Groopman et al., 1985; Sun et al., 1986), and nucleic acid repair products (Autrup et al., 1983, 1985, 1987; Groopman et al., 1985) can now be measured in body fluids and by both immunological and chemical methods, and these products have been detected in urine samples from individuals ingesting contaminated food. Urinary excretion of AFM$_1$ from local inhabitants in areas of high PHC prevalence in the People's Republic of China was found to be significantly elevated, up to 40-fold or more, over that found in people in areas of lower PHC incidence (Sun et al., 1983; Wu et al., 1984). During the rainy season, in the high PHC incidence area, the amount of AFM$_1$ excreted in the urine may increase to a level of 100-fold more than that excreted in the low
incidence area. By measurement of AFB\textsubscript{1} and AFM\textsubscript{1}, the conversion ratio in humans has been calculated to be 1.5-5\% (Sun et al., 1983; Sun et al., 1986; Zhu et al., 1987). On the basis of these data, it has been calculated that the AFB\textsubscript{1} intake in approximately 10\% of adults living in the high incidence area exceeds 1 mg per year. The primary source of contamination is corn and rice; the second is the local undistilled alcoholic beverage obtained from these grains. Since men generally eat more food and also drink more alcoholic beverages than women, both the 3-fold or more male preponderance of PHC and the age peak in the fourth and fifth decades of life in areas of prevalence (facts difficult to explain on the basis of viral aetiology alone) can at least partly be attributed to AFB\textsubscript{1} ingestion and/or alcohol consumption (Harris and Sun, 1986).

Food samples collected in Murang'a District, Kenya, have been demonstrated to be contaminated with AFB\textsubscript{1}, and also one of its putative nucleic acid repair products, AFB\textsubscript{1}-Gua, has been detected in human urine collected in this region of high cancer risk (Autrup et al., 1985, 1987). Recent findings from this continuing biochemical epidemiological investigation have also shown a seasonal variation in that the urinary AFB\textsubscript{1}-adducts are found more frequently during the months of high contamination of food by AFB\textsubscript{1} (Autrup et al., 1985). The aflatoxin-DNA adduct was also found in urine samples from the Guangxi Province of China (Groopman et al., 1985). The presence of this AFB\textsubscript{1}-DNA base adduct in the urine is an indication not
only of exposure to AFB₁, but also of its metabolic activation and interaction between the ultimate carcinogenic form of AFB₁ and cellular nucleic acid in vivo. This further supports the hypothesis that AFB₁ may have an important role in the aetiology of human liver cancer (Harris and Sun, 1986).

AFB₁ is a procarcinogen, i.e. it requires enzymatic activation to an electrophilic metabolite that binds covalently to DNA. Interindividual differences (more than 10-fold) have been found both in the metabolic activation of AFB₁ to form DNA adducts in cultured human hepatic explants (Autrup et al., 1984) and for hepatic microsomes to cause mutations in the Ames Salmonella assay (Harries et al., 1986). It would be of considerable interest to compare the rates of metabolic activation of AFB₁ in individuals with normal livers with those in patients with chronic active hepatitis, a predisposing condition for hepatocellular carcinoma. AFB₁ and its metabolites have also been found in human sera and various human tissues, including liver (see Garner et al., 1985). Promising methodology to measure AFB₁ binding to serum albumin is being developed (Wild et al., 1986; Sabbioni et al., 1987) and that should provide another means of monitoring AFB₁ exposure.

Measurement of AFB₁ modified DNA by enzyme immunoassays (Haugen et al., 1981; Groopman et al., 1982; Hsieh et al., 1988) and synchronous scanning fluorimeter (Harris et al., 1986) in liver samples from males and females will
be of particular interest in regard to the male preponderance of PHC. It will also allow the critical evaluation of the quantitative relationship, if one exists, between adduct levels and the risk of developing PHC.
CHAPTER 2

Development of a Method to Monitor Aflatoxins

Excreted in Human Urine Samples
The aflatoxins and several other mycotoxins may present health hazards that are far more extensive and serious than had been realised due to the extreme potency of many of these compounds. Growing concern with aflatoxin poisoning and their potentially hazardous effects as carcinogens at doses insufficient to manifest the symptoms of aflatoxicosis has led to a concerted effort to devise analytic methods for the accurate determination of aflatoxin in food, physiologic fluids and tissues (Langone and van Vunakis, 1976).

Common methods used to analyse samples for the presence of aflatoxins include TLC or HPLC of extracted samples with confirmation of identity by physicochemical methods such as fluorescence photon counting or mass spectrometry with selective ion monitoring. The methodology for measuring aflatoxin levels in food by TLC or HPLC is well established and there have been many reports on the use of these two methods (Seitz, 1975; van Edgmond and Paulsch, 1986). However, these methods are not specific for the aflatoxin structure and extensive preliminary 'clean-up' procedures are necessary when examining complex samples which can be time consuming and costly, and require technical skill and expensive instruments. For these reasons, these methods are decreasingly being used in measurements of aflatoxin metabolites in human body fluids in epidemiological studies (Garner et al., 1985).
The recent development of immunological techniques (Langone and van Vunakis, 1976; Poirier, 1981; Müller et al., 1982) that use antibodies with a high degree of sensitivity and specificity against certain carcinogens including AFB1 provides the possibility of determining individual exposure in relatively large human populations. RIA has been applied to the quantitative determination of circulating substances, such as hormones, drugs and infectious agents. The high specificity of antibodies and the sensitivity afford by radiolabelled tracers often permit the use of RIA to measure picomole levels of the target compound in unprocessed physiological fluids or other samples. However, RIA has certain practical limitations. The short half-life of the isotopes used limits the shelf life of the reagents. In addition, the need for γ-emitting isotope subjects the users of RIA to a radiation hazard.

Enzyme immunoassay which is also known as ELISA has been developed in an attempt to overcome those problems of RIA. ELISA is similar in design to solid-phase RIA except that an enzyme is used as the immunoglobulin marker instead of a γ-emitting isotope. It is based on the reaction between an antigenic compound and an antibody with specificity for a particular part of the antigenic molecule. After binding of the primary antibody to the immobilized antigen, a second antibody (with conjugated enzyme) having specificity for the bound antibody is allowed to bind to the primary antibody. On adding a
suitable chromogenic substrate for the bound enzyme and assaying the colour produced, quantitation of the bound antigen is achieved. There are two types of ELISA, one is the "non-competitive" assay in which the colour developed is proportional to the amount of immobilized antigen present on the plate (exactly as described above), while the other type is the "competitive" assay in which the colour developed is inversely related to the amount of antigen present in the sample. This competitive ELISA is used throughout these studies and is described in Section 2.2.3.1. The fact that a single molecule of enzyme is capable of reacting with a large number of substrate molecules provides for amplification and, thus, a high degree of sensitivity. In the sandwich type ELISA technique, there are two stages of amplification; the first one is when secondary antibody is bound to primary antibody, and the second stage is when substrates are bound to the enzymes as shown in Figure 2.1. Because stable enzymes such as alkaline phosphatase or peroxidase can be used, ELISA also has the advantage of using reagents with a long shelf life. In practice, however, the sensitivity of the ELISA system is at least as sensitive as, if not more sensitive than, RIA. ELISA methods should be concentrated on for routine monitoring work.

A number of reports have appeared on the generation of antibodies against AFB$_1$ and its metabolites (Garner et al., 1985). The characteristics of these antibodies very much depend on the specific immunogen used. Polyclonal
Figure 2.1 The amplification provided by the two antibodies (sandwich system) used in ELISA plates

- Primary antibody with specificity for antigen
- Secondary antibody with specificity for primary antibody
antibodies appear to be particularly suitable for measurements of exposure since various aflatoxin-derived materials can be assayed, thus giving a more representative picture of the exposure. In contrast, a battery of monoclonal antibodies might be advantageous in determining at an individual level the pattern of aflatoxin metabolites present in the samples.

These antibodies can be used in conjunction with other chemical analytical techniques as non-invasive screening methods to monitor human exposure to aflatoxins. Useful screening methods will require the ability to quantify aflatoxin and its metabolites in readily accessible compartments, such as serum and urine. Earlier studies on aflatoxin derivatives in human samples have used techniques such as TLC, HPLC and immunological assay to prepare and analyse aflatoxin-contaminated samples (Campbell et al., 1970; Hendrickse et al., 1982; Autrup et al., 1983; Hu et al., 1984; Martin et al., 1984; Groopman et al., 1984; Tsuboi et al., 1984; Groopman et al., 1985; Wild et al., 1987). However, when attempting to apply immunological assays especially ELISA to human urine samples, despite the specific nature of the antibody reaction, the presence of interfering substances often gave apparent positive results for aflatoxins. These substances which are also known as urinary aflatoxin-like substances (Dragsted et al., 1988) can be removed by extraction, dilution or concentration of the urine samples on Sep-Pak C18 cartridges and immuno-affinity columns (Martin et al., 1984; Groopman et al., 1984).
The objective of this study was to develop the procedures necessary to monitor aflatoxin levels in human urine samples using an ELISA technique. A polyclonal antibody against AFB_1 was used in order to give a more representative picture of the exposure. Uncontaminated urine samples from Western European people which were presumed to be free of aflatoxin contamination were used as control samples. They were spiked with known concentrations of AFB_1, or ^3H-AFB_1, to validate the methods being developed.

2.2 MATERIALS AND METHODS

2.2.1 Materials

2.2.1.1. Aflatoxins

AFB_1 was purchased from Makor Chemicals Ltd. (Jerusalem, Israel). AFB_2, AFG_1, AFG_2, AFM_1, AFP_1, AFQ_1, and AFL, were purchased from Sigma Chemicals Company (Poole, Dorset). AFB_1-Gua and AFB_1-FAPy were prepared by activating AFB_1 \textit{in vitro} followed by reaction with calf thymus DNA. AFB_1-GSH was prepared using activated AFB_1 with mouse cytosolic GSH-S-transferase (Moss and Neal, 1985). AFB_1-Cys-Gly, AFB_1-Cys and AFB_1-NAC-Cys were prepared \textit{in vitro} as the degradation products of AFB_1-GSH using isolated rat kidney cells. ^14C-AFB_1 and ^3H-AFB_1 were purchased from Morevak Biochemicals (Brea, California, USA).
2.2.1.2 Chemicals

BSA, Tween 20, dithiothreitol, sodium cholate, 3,3',5,5'-tetramethylbenzidine and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were obtained from Sigma Chemicals Company (Poole, Dorset). DMSO of Spectrosol grade and gelatine were purchased from BDH Chemicals Ltd. (Poole, Dorset). PBS was obtained from Oxoid Ltd. (Basingstoke, Hants). All other chemicals and organic solvents used in all experiments were of analytical or HPLC grade.

2.2.2 Preparation of all the components necessary for analysing aflatoxins by ELISA

2.2.2.1 Preparation of the immunogen

AFB₁ bound to quail liver microsomal protein by metabolic activation was used as the antigen. The protein was solubilized from microsomal membranes and partially purified by technique used by Elshourbagy and Guzelian (1980). Quail liver microsomal pellets containing bound AFB₁ were suspended in 50 mM Tris buffer (pH 7.4) containing 25% glycerol (v/v), 5 mM MgCl₂, 0.1 mM EDTA, and 0.5 mM dithiothreitol to have the concentration of 30 mg protein/ml, sonication being used to aid dissolution of the pellets. Then 50 mM potassium phosphate (pH 7.4) buffer containing 0.1 mM EDTA and 30% glycerol (v/v) was added to yield a final concentration of 7 mg protein/ml buffer. A 10% solution of sodium cholate was added dropwise, with stirring, at 0-5°C to give a final concentration of 3 mg
cholate/mg protein. After these additions, the solution was stirred for 20 min, a 50% (w/v) polyethylene glycol solution was added dropwise to achieve a final polyethylene glycol concentration of 10%, and stirring was continued for an additional 20 min. The mixture was centrifuged at 40,000 x g for 30 min. The supernatant was discarded and pellets were redissolved in 3 ml PBS. The protein content of the immunogen was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.2.2.2 Preparation of the rabbit AFB1 antiserum

Sandy half-lop female rabbits were immunized with AFB1 conjugate by injecting 500 ug protein of immunogen mixed 50/50 (v/v) with Freund's complete adjuvant intramuscularly into front and hind limbs of each animal. Subsequent injections employed the solubilized protein antigen (500 µg protein) mixed with an equal volume of Freund's incomplete adjuvant. They were injected four weeks apart on three occasions. Ten days after the third injection, blood samples (30 ml) were obtained from each animal via an ear vein. The samples were then left at 4°C for 3 h to allow the complete coagulation process to take place; the serum was then separated by centrifugation of the clotted blood at 1000 x g for 20 min at room temperature. The antibody titre of the serum obtained was evaluated using competitive ELISA binding assay (see Section 2.2.3.1). A booster dose of 500 ug protein of immunogen mixed with equal volume of
Freund's incomplete adjuvant was given to the rabbits every 6 months. The serum obtained had to be tested for antibody titre.

2.2.2.3 Preparation of the BSA-\(\text{AFB}_1\) conjugate

BSA-\(\text{AFB}_1\) for use in the ELISA technique was prepared according to Sizaret et al. (1982). The conjugate was synthesized by dissolving \(\text{AFB}_1\) (6.5 mg) in methylene chloride (3 ml) in which any traces of water that might have been present were removed with molecular sieve beads type 3A (BDH Chemicals Ltd., Poole, Dorset). Chlorine gas was bubbled through the solution until a bright yellow colour appeared. The yellow colour was free chlorine in the solution. After drying the \(\text{AFB}_1\text{Cl}_2\) solution under vacuum, BSA (9.3 mg) in 0.01 M sodium phosphate buffer, pH 7.4 (4.5 ml) was quickly added; immediately thereafter, DMSO (0.5 ml) was added dropwise using magnetic stirring to dissolve \(\text{AFB}_1\text{Cl}_2\). The mixture was kept for 30 min at 37°C in a shaking water bath. The insoluble material was removed by centrifugation at 1000-1200 x g for 15 min at 4°C. The modified BSA was then precipitated by sequential addition of NaCl (43 mg), ethyl acetate (6 ml) and acetone (12 ml) under magnetic stirring. The mixture was kept on ice for 30 min before being centrifuged at 1000-1200 x g, for 15 min at 4°C. The precipitated BSA-\(\text{AFB}_1\) conjugate was washed with ethanol (20 ml x 6) to remove unbound \(\text{AFB}_1\). The BSA-\(\text{AFB}_1\) was dissolved in PBS (pH 7.4, 10 ml), sonication being used to aid dissolution of this
conjugate. The BSA-AFB$_1$ solution was then stored at -40°C. The protein content of the conjugate was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. The number of molecules of AFB$_1$ bound to 1 molecule of BSA was determined by spectrophotometry (UVIKON 860; Kontron Instruments, Watford, Herts) at 405 nm using samples of the stock BSA-AFB$_1$ solution (250 µl) diluted with PBS to 1 ml.

2.2.2.4 Preparation of the ELISA plates

Flat-bottomed polystyrene microtitre plates (model M129E, Dynatech Laboratories Inc., Virginia, USA) were coated with BSA-AFB$_1$ (5 ng) in PBS (50 µl) in each well. The outer rows of wells were not coated because in preliminary studies it had been found that they showed a much greater variation in the assay. The plates were left to dry overnight at 37°C. They were stored at -40°C until used. Coated plates could be kept at this temperature for extended periods without apparent loss of binding capacity. When plates were to be used for assay, they were removed from the -40°C freezer and washed 4 times with 0.05% Tween 20 in PBS (PBS-Tween 20). Non-specific binding sites were blocked with 100 µl of 0.25% gelatine in PBS (PBS-gelatine) for 60 min at room temperature. The plates were then washed twice with PBS-Tween 20 before used.

2.2.2.5 Preparation of the immunoaffinity gel

An immunoaffinity column was prepared by the procedure
described by Pharmacia. Freeze-dried powder (6 g) of AH-Sepharose 4B (Pharmacia, Milton Keynes, Herts) was suspended in 0.5 M NaCl (200 ml NaCl/g freeze-dried powder) for 30 min at room temperature. The gel was washed with distilled water (1200 ml) on a sintered glass filter (porosity G3) to remove NaCl. Water was added to 6 ml undiluted rabbit anti-AFB serum to a final volume of 18 ml before adding to the gel. The pH of the gel was adjusted to between 4.5 and 6.0 with 0.1 M HCl. The mixture was left stirring at room temperature using slow-moving propeller stirring. A solution (2 ml) of 1 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added dropwise and the pH was maintained between 4.5-6.0 for 1 h. Changes observed in pH after 1 h were small. The reaction was allowed to proceed for 24 h at 4°C. The gel was washed alternately with 0.1 M sodium acetate/0.5 M NaCl (pH 4.6; 1000 ml) and 0.1 M sodium hydrogen carbonate/0.5 M NaCl (pH 8.3; 1000 ml) with water (1000 ml) in between each buffer. The gel was washed by each buffer for 3 times before suspension in PBS with 0.02% sodium azide (20 ml) to prevent bacterial growth. The gel was stored at 4°C until required.

2.2.3 General procedures used in the analysis of aflatoxins

The methods described in this section were used in all experiments unless stated otherwise.
2.2.3.1 **Competitive ELISA binding assay**

Competitive binding assays were performed in glass tubes by adding an aliquot (200 μl) of test solution, diluted in PBS, to rabbit anti-AFB₁ serum diluted 1:10,000 in PBS-gelatine (200 μl) and incubating on a shaking plate at 37°C for 60 min. Aliquots (50 μl) of these mixtures were dispensed into each well of washed microtitre plates in 6 replicates. The plates were sealed with plate sealing tape (Titertek, Flow Laboratories, Rickmansworth, Herts.) to prevent evaporation, and incubated at room temperature with continuous shaking on Vari-Shaker (Dynatech Laboratories Ltd., Billingshurst, Sussex) for 90 min. Plates were washed 5 times with PBS-Tween 20 by complete immersion of the plates in the washing solution. Aliquots (50 μl) of anti-rabbit IgG-peroxidase conjugate (I.C.N. Biochemicals Ltd., High Wycombe, Bucks.) diluted 1:5000 in PBS-gelatine were added to each well and sealed plates were further incubated at room temperature for 90 min with continuous shaking. The plates were washed 5 times with PBS-Tween 20 followed by a single distilled water wash. The bound peroxidase was detected by adding aliquots (50 μl) of 3,3',5,5'-tetramethylbenzidine 0.1 mg/ml in 0.1 M sodium acetate buffer (pH 6.0) with 0.002% H₂O₂ to each well and the plates were left at room temperature for 30 min. The reaction was stopped with 2 M H₂SO₄ (50 μl). Absorbance at 450 nm was read on a Titertek Multiskan MCC (Flow Laboratories, Rickmansworth, Herts.).

Inhibition was calculated as a percentage of the
absorbance where no inhibition was present in the test solution. Values of AFB₁ equivalents present in the test samples were calculated from a standard curve of AFB₁ concentrations ranging from 0.01-100 ng/ml.

To check on interplate variation, on every microtitre plate used was included one column (6 wells) of blank or non-inhibited samples and one column of internal standard (1 ng AFB₁/ml). This internal standard was then used to correct for the variation between the plates. The correction applied was to normalise the standard curve for the inhibition observed for 1 ng AFB₁/ml for each individual plate.

2.2.3.2 Sep-Pak C₁₈ cartridge

Sep-Pak C₁₈ cartridges (Waters Associates, Millipore UK, Harrow, Middx.) were pre-wetted with methanol (10 ml) to activate the cartridges followed by distilled water (10 ml) to dilute the concentration of methanol. After the sample had been loaded onto the cartridge, it was washed with distilled water (3 ml). The retained materials were eluted with methanol (5ml). The cartridge was reactivated with methanol (10 ml) followed by distilled water (10 ml). The cartridge was reusable up to 4 times.

2.2.3.3 Immunoaffinity column

The column was packed with affinity gel, prepared as described in Section 2.2.2.5, in a 2 ml disposable plastic syringe giving a 1 ml bed volume as shown in Figure 2.2.
Figure 2.2 Immunoaffinity column

A. Disposable syringe for loading samples onto column and applying washing and eluting solutions.

B. Tap for switching flow from column to solvent reservoir to enable syringe A to be filled.

C. Locking screws allowing PTFE plunger (F) to be damped at different heights enabling different bed volumes of affinity gel to be used.

D. PTFE holder clamping top of disposable syringe containing affinity gel column.

E. Disposable syringe (2 ml) containing affinity gel.

F. Rubber seal on end of hollow PTFE plunger, through which sample and eluting solvents are introduced onto column.

G. Affinity gel.

H. Glass fibre disc support for affinity gel.

I. Solvent reservoir.
The column was then washed with PBS (20 ml) to remove sodium azide added to the gel to prevent bacterial growth. The sample was loaded on the column and then washed with PBS (15 ml) before elution with 85% methanol in PBS (15 ml). Preliminary studies demonstrated that more than 95% of AFB₁ or aflatoxin metabolites retained on the affinity column were eluted with the first 10 ml of methanol. This was a convenient volume for the subsequent evaporation in one tube of the Speedvac Concentrator and therefore facilitated one tube per sample. The column was then washed with PBS (20 ml) before the next sample was loaded. When urine samples were used, it was found that the columns could only be used for two samples and were then discarded.

2.2.3.4 Evaporation process

The purpose of evaporation in these experiments was to reduce the volume of methanol present in the samples. The process was carried out in the Savant Speedvac Concentrator (Stratech Scientific Ltd., London) at 40°C. In preliminary experiments it was found that the evaporation of the samples to dryness followed by reconstitution led to variable recoveries. In order to avoid this problem, a small volume of water (0.5 ml) was always added into each sample which was in 100% methanol. This provided for a residual aqueous solution after the rapid removal of methanol by evaporation in the Speedvac.

2.2.3.5 HPLC

Samples to be used in the HPLC were dissolved in
methanol/water (1:1, v/v) and clarified by centrifugation. Samples were analysed using Models 6000 pumps and 660 solvent programmer (Waters Associates, Millipore U.K., Harrow, Middx.) and were introduced by a Rheodyne 7120 injector valve (Berkeley, California, USA). The column (100 x 4.6 mm) was laboratory-packed with Magnusphere 5 ODS reverse phase protected by a guard column (65 x 1.2 mm) of CO:Pell ODS (Whatman, Maidstone, Kent). The column was eluted with a linear 15-40% methanol/water gradient containing 0.01% phosphoric acid and 8% acetonitrile at a flow rate of 1.2 ml/min. The gradient elution took 8 min followed by maintenance of 40% methanol for another 4 min and then the gradient was reversed to 15% methanol in 1 min. The machine was left to re-equilibrate at 15% methanol for 5 min before the next injection.

U.V. absorption was monitored at 365 nm using an LDC Spectromonitor III (Laboratory Data Control, Florida, USA). Fluorescence was also monitored by using a Fluorescence Detector Model 420 (Waters Associates, Millipore U.K., Harrow, Middx.) with the excitation band pass filter centred on 365 nm and long pass emission filter starting at 425 nm.

Fractions (1 min/1.2 ml) from the HPLC were collected in 5 ml plastic scintillation minivials using an LKB Redirac Fraction Collector (Bromma, Sweden). Normally 18 fractions per sample were collected.

2.2.3.6. Radioactive counting

All radioactive determinations were carried out in 5
ml plastic scintillation minivials using aliquots (1 ml) of sample and scintillant (4 ml) (Monofluor, National Diagnostic, Aylesbury, Bucks) followed by counting in a liquid scintillation counter (Packard Model 460, Pangbourne, Berks).

2.2.3.7 Creatinine assay

All human urine samples were analysed for creatinine concentration using "Merckotest Creatinine 3385" (E. Merck, Darmstadt, West Germany). Samples were diluted 1:100 as described in the kit. The reaction is based on the principle that creatinine forms a yellow-orange compound in alkaline solution with picric acid. All the assays were carried out at 25°C. The absorbance at 492 nm was read in a Kontron Spectrophotometer (Model UVIKON 860, Kontron Instruments Ltd., Watford, Herts).

2.2.4 General procedures adopted for analysing aflatoxins in human urine samples

Human urine samples were centrifuged at 1000 x g for 10 min at room temperature to remove any precipitate present in the samples. They were then subjected to the procedure summarized in Figure 2.3.

2.3 Experimental protocols

2.3.1 Antibody recognition of structural analogues to AFB1

Several structural analogues and metabolites of AFB1
Figure 2.3 Diagram showing the procedure analysing level of aflatoxins in human urine samples

Human urine sample (1 ml)

\[ \downarrow \]

pass through pre-wetted Sep-Pak C\textsubscript{18} cartridge

\[ \downarrow \]

wash with distilled water (3 ml)
elute with 100% methanol (5 ml)

\[ \downarrow \]

collected methanol eluate and add water (0.5 ml)

\[ \downarrow \]

evaporate to 0.5 ml and reconstitute with PBS (5 ml)

\[ \downarrow \]

load onto immunoaffinity column (1 ml bed volume)

\[ \downarrow \]

wash with PBS (15 ml)
elute with 85% methanol in PBS (15 ml)

\[ \downarrow \]

collect first 10 ml methanol eluate

\[ \downarrow \]

evaporate to 0.5 ml and reconstitute to 1 ml with PBS

\[ \downarrow \]

use appropriate volume in ELISA
were tested in the ELISA, including AFB₂, AFG₁, AFG₂, AFM₁, AFP₁, APQ₁, AFL, AFB₁-Gua, AFB₁-FAPy, AFB₁-GSH, AFB₁-Cys-Gly, AFB₁-Cys and AFB₁-NACys. The structures of these compounds are shown in Figure 2.4. They were tested against rabbit anti-AFB₁ serum in the standard competitive ELISA (Section 2.2.3.1). The percentage inhibition of antibody binding to the coating antigen, BSA-AFB₁, was determined for concentrations ranging from 0.01 to 100 ng aflatoxin analogue/ml. Inhibition curves were drawn and 50% inhibition values for each aflatoxin analogue were determined.

2.3.2 Standard AFB₁ solutions in PBS and in urine sample

2.3.2.1 Analysis of standard AFB₁ solutions without 'clean-up' procedures

Human urine samples from West Germany which were presumed to be free of contamination with aflatoxins were used as control samples. Standard concentrations of AFB₁ ranging from 0.01 to 100 ng/ml were prepared in both PBS and these urine samples, using a stock solution of 10 µg AFB₁/ml. Competitive ELISA were performed (as in Section 2.2.3.1) using the 'spiked' PBS, 'spiked' and 'unspiked' urine samples and the percentage inhibitions obtained were calculated with reference to the PBS control.

2.3.2.2 Analysis of standard AFB₁ solutions with 'clean-up' procedures

Standard concentrations of AFB₁ ranging from 0.01 to 100 ng/ml in both PBS and the uncontaminated Western urine
Figure 2.4 Structures of aflatoxins and aflatoxin metabolites
samples were prepared as in Section 2.3.2.1. Four different urine samples were used. They were processed by a Sep-Pak and an affinity column using the general procedures for 'clean-up' of urine samples (see Section 2.2.4). Samples were reconstituted to 1 ml PBS before analysing for AFB\textsubscript{1} by ELISA. Also one uncontaminated urine sample was spiked with the same range of AFB\textsubscript{1} concentrations after the sample had been passed through a Sep-Pak and an affinity column. Competitive binding assays were performed (see Section 2.2.3.1) and the percentage inhibitions were calculated by comparing with both PBS control and unspiked urine samples.

2.3.3 Methanol concentrations and ELISA

Standard concentrations of AFB\textsubscript{1} ranging from 0.01 to 100 ng AFB\textsubscript{1}/ml were prepared in PBS, 50% methanol in PBS and 85% methanol in PBS using the stock solution of 10 ug AFB\textsubscript{1}/ml in PBS. Competitive ELISA were performed and the percentage inhibitions of each solution were calculated from PBS control.

2.3.4 Development of a method to monitor aflatoxins excreted in human urine samples

2.3.4.1 Sep-Pak C\textsubscript{18} cartridge

A. Capacity of Sep-Pak C\textsubscript{18} cartridge

A range of concentrations, 0.1, 1.0, 10.0 and 100 ng \textsuperscript{3}H-AFB\textsubscript{1}/ml (100,000 dpm/ml) was prepared in PBS and in uncontaminated urine sample. A sample (1 ml) of each
concentration was loaded onto a Sep-Pak C\textsubscript{18} cartridge, washed with distilled water (3 ml) and the retained materials eluted with 100% methanol (5 ml). All fractions were collected and aliquots (1 ml) of each fraction in duplicate were taken to count for radioactivity. The percentage of the total radioactivity which was present in each fraction was calculated.

B. Evaporation of methanol eluate and HPLC fractions obtained from Sep-Pak C\textsubscript{18} cartridge

The solution containing 20 ng \textsuperscript{3}H-AFB\textsubscript{1}/ml (10,000 dpm/ml) was prepared in 20 ml uncontaminated urine sample. A sample (10 ml) was loaded onto a Sep-Pak C\textsubscript{18} cartridge washed with distilled water (3 ml) and eluted with 100% methanol (5 ml). All fractions were collected and aliquots (1 ml) of each fraction were counted for radioactivity. When the nature of the labelled material which was not retained by the Sep-Pak was examined by HPLC, the unretained (2 ml) and the aqueous washing fractions (2 ml) from the Sep-Pak were combined and methanol (4 ml) was added to this combined fraction giving a total concentration of methanol 1:1 (v/v). In the case of the methanol fraction from the Sep-Pak, a sample (2 ml) was taken and water (2 ml) was added giving a methanol concentration of 1:1 (v/v). Each sample (500 \mu l) was injected into the HPLC. Fractions (1 min/1.2 ml) from the HPLC were collected and radioactivity was counted. In order to compare the distribution of radioactivity in the
HPLC fractions obtained from the Sep-Pak, a sample with the original $^3$H-AFB$_1$ solution, 200 ng $^3$H-AFB$_1$/ml (50,000 dpm/ml), was prepared in uncontaminated urine and the methanol (1 volume) was added before aliquots (500 μl) were injected into the HPLC as reference.

Aliquots (2 ml) of methanol elute from the Sep-Pak cartridge were added with water (200 μl) before evaporation to 0.5 ml and reconstitution with PBS to 2 ml. The sample (500 μl) was counted for radioactivity and aliquots (500 μl) were injected into the HPLC and fractions were collected and also counted for radioactivity.

2.3.4.2 Immunoaffinity column

A. Capacity of affinity gel

Affinity gels were prepared by using immune and non-immune serum (see Section 2.2.2.5). The columns were packed with gel suspension (0.8 ml) in 2 ml disposable plastic syringes giving a 0.5 ml bed volume. Two columns were packed; one with the immune gel, the other one with non-immune gel. Columns were washed with PBS (20 ml) to remove sodium azide. The columns were loaded with 1 ng $^3$H-AFB$_1$ in PBS (1 ml) containing $10^5$ dpm, and then washed with PBS (10 ml). Retained materials were eluted with 80% methanol in PBS (15 ml). Fractions (1 ml) were collected throughout the loading, washing and elution procedures for radioactive counting.

B. Characteristics of the immunoaffinity columns

Affinity gel was packed in a 2 ml disposable plastic
syringe to a bed volume of 0.5 ml. Standard concentrations of 0.1, 1.0, 10.0 and 100 mg AFB$_1$/ml were prepared in both PBS and uncontaminated urine samples. $^3$H-AFB$_1$ was added to each sample to give a radioactive level of 50,000 dpm/ml. The samples were loaded onto the columns in different volumes containing different amounts of $^3$H-AFB$_1$. The volumes ranged from 0.2 to 10 ml and the amounts from 1 to 100 ng $^3$H-AFB$_1$. The columns were washed and eluted by the standard procedures described in Section 2.2.3.3. Each column was used twice to determine if the column was reusable. All fractions were collected and aliquots (1 ml) were counted for radioactivity in duplicate.

C. Evaporation of methanol fractions eluted from the affinity column

The columns were packed with affinity gel to a bed volume of 0.5 ml. Two sets of samples were loaded; one with 2 ng $^3$H-AFB$_1$ in uncontaminated urine (2 ml) the other with 5 ng $^3$H-AFB$_1$ in uncontaminated urine (5 ml). Columns were washed and eluted by the standard affinity column procedures (see Section 2.2.3.3). Radioactivity in each fraction was counted. From methanol fractions, samples (500 ul) were injected directly into the HPLC and the radioactivity present in the AFB$_1$ peak (retention time of 12 min) was determined. Then aliquots (7ml) of these methanol fractions were evaporated to 0.5 ml and reconstituted to 7 ml with PBS before samples (500 µl) were injected into the HPLC. Radioactivity present in the HPLC fractions was determined.
D. Routine testing of the capacity of the affinity gel

The columns were packed with affinity gel suspension (1.5 ml) in 2 ml disposable plastic syringes giving a 1 ml bed volume. Two columns were used; one for standard $^3$H-AFB$_1$ 10 and 20 ng/ml in PBS containing $10^6$ dpm/ml; the other for standard $^3$H-AFB$_1$ 1 and 5 ng/ml in uncontaminated urine ($10^6$ dpm/ml). The column was loaded with 20 ng $^3$H-AFB$_1$/ml in PBS (1 ml) and the standard affinity column elution procedures followed (see Section 2.2.3.3), before the column was reloaded with 10 ng $^3$H-AFB$_1$/ml in PBS (1 ml). Fractions from each loading were collected and duplicate samples (1 ml) were counted for radioactivity. Total dpm in each fraction was calculated and the percentage distributions of radioactivity between the fractions were determined. In the case of the urine samples, the procedures adopted were essentially the same as for PBS samples except that the column was loaded with 5 ng $^3$H-AFB$_1$ (1 ml) followed by 1 ng $^3$H-AFB$_1$ (1 ml). All fractions were counted for radioactivity.

2.3.4.3 Evaluation of overall clean-up procedures

A. Monitoring of $^3$H-AFB$_1$ in uncontaminated urine samples

Urine sample (10 ml), spiked with 20 ng $^3$H-AFB$_1$/ml was loaded onto a Sep-Pak C$_{18}$ cartridge and the $^3$H-AFB$_1$ eluted with methanol (see Section 2.2.3.2). The same Sep-Pak cartridge was then reloaded with a further 10 ml aliquot of the original urine sample and the elution procedure repeated. The comparable fractions from the two Sep-Pak
loadings were combined and counted for radioactivity. Aliquots (500 \mu l) of the methanol fraction were directly injected into the HPLC to determine radioactivity associated with AFB\textsubscript{1} peak. Then the methanol fraction (7 ml), with water (0.5 ml) added, was evaporated to 0.5 ml and reconstituted to 7 ml with PBS. Recovery after evaporation was determined by radioactive counting before the samples (500 \mu l) were injected into the HPLC to check radioactivity associated with AFB\textsubscript{1} peak. Then 0.1, 0.2 and 0.5 ml of this fraction were diluted to 5 ml with PBS before loading onto the affinity columns. Unretained, washing and methanol fractions from the affinity columns were collected and counted for radioactivity. In this experiment the methanol eluate from the affinity column was collected in 2 different fractions; an initial 10 ml and a subsequent 5 ml fraction. The overall recovery of the procedure was calculated.

B. Monitoring of AFB\textsubscript{1} in spiked urine samples using ELISA

Urine samples, spiked with 0.1, 0.5 and 1 ng/ml of AFB\textsubscript{1} were prepared using a stock solution of 10 \mu g AFB\textsubscript{1}/ml in PBS. The samples (10 ml) were loaded on the Sep-Pak cartridge. Fractions of the methanol eluates were collected and aliquots (4 ml) evaporated and reconstituted to 5 ml with PBS before loading on the affinity columns with 1 ml bed volume and elution with methanol. The first 10 ml of the methanol eluates were collected and aliquots were evaporated and then reconstituted in 2 ml PBS. Samples (100 \mu l) were analysed for AFB\textsubscript{1} by ELISA using
five replicate assays with six wells per assay. The results were expressed in terms of ng AFB\(_1\)/ml. An 'unspiked' urine sample was included in the experiment to determine the background level or UALS in the sample. It was subjected to the same procedure as those applied to the 'spiked' urine samples.

2.3.5 **Urinary aflatoxin-like substances (UALS)**

2.3.5.1 **Validation of the use of the 'clean-up' procedures**

Uncontaminated urine samples from 3 Western male subjects were used. Each sample (1 ml) was loaded onto a Sep-Pak C\(_{18}\) cartridge and the methanol eluates after evaporation and reconstitution in PBS (see Section 2.2.4) were loaded onto the affinity columns. The unretained and washing fractions from the affinity columns were combined and desalted by reloading onto a Sep-Pak cartridge. Possible inhibitory substances (UALS) present in the fraction were then eluted from the Sep-Pak with methanol. Methanol eluates from the Sep-Pak were collected and water (0.5 ml) added, followed by evaporation to 0.5 ml and reconstitution to 1 ml with PBS. Methanol eluates from the affinity column were evaporated to 0.5 ml, without passing through Sep-Pak, and reconstituted in 1 ml with PBS. The samples (100 \(\mu\)l) were diluted 1:1 with PBS before using in ELISA. Three replicate assays were performed per sample.

2.3.5.2 **Attempts to fractionate UALS by HPLC**

Uncontaminated urine samples (1 ml) from 3 Western
people were evaporated to dryness using the Speedvac Concentrator and redissolved in 50% methanol in water (100 μl). The samples were then centrifuged for 10 min and aliquots (20 μl) of supernatant were injected into the HPLC. Fractions (1 min/1.2 ml) were collected and evaporated to dryness before reconstitution with PBS (1 ml). The apparent AFB₁ in each fraction was analysed in duplicate by ELISA.

2.3.5.3 Procedures adopted for reduction of UALS prior to ELISA

Urine samples from West Germany, 5 males and 5 females were analysed for apparent AFB₁ concentration by ELISA using undiluted samples and the dilutions in PBS of 1:25, 1:100 and 1:250.

When the 'clean-up' procedures were used, undiluted urine sample (1 ml) were passed through the Sep-Pak cartridges and the affinity columns using the standard procedure (see Section 2.2.4). The samples were finally reconstituted to 1 ml with PBS before analysing by ELISA in triplicate.

The creatinine level in each sample was also analysed using method described in Section 2.2.3.7.

2.4 RESULTS AND DISCUSSION

2.4.1 Rabbit anti-AFB₁ serum

Rabbit serum samples, diluted 1:5,000 and 1:10,000
with PBS-gelatine, were tested for the presence of anti-AFB₁ antibody using standard AFB₁ concentrations (0.01-100 ng/ml) in the competitive ELISA. The results are shown in Figure 2.5. When using a dilution of 1:5,000, the competition curve obtained exhibited no region of "steep slope" over any concentration of AFB₁, and, therefore, was not suitable for precise quantitation. In contrast, at the dilution of 1:10,000 the curve exhibited a "steep slope" over the range 0.03-1.00 ng AFB₁/ml and, therefore, this dilution was used in further experiments.

When fresh serum was required, a booster dose of immunogen was injected into the rabbit, a small sample of serum was removed and tested by using a 1:10,000 dilution and a concentration range of 0.01 to 100 ng AFB₁/ml. The competition curve observed was compared with that obtained from a previous "standard" serum sample.

2.4.2 BSA-AFB₁ conjugate

The presumed structure of BSA-AFB₁ conjugate is shown in Figure 2.6. Theoretically, 1 mole of AFB₁ in 1 litre solution would have the absorption of 20,000 at 405 nm; i.e. 1 n mole of AFB₁ in 1 ml solution would have the absorption of 0.02. From spectrum shown in Figure 2.7, the absorption was 0.153 which was equivalent to 7.65 n moles AFB₁. This spectrum came from 250 μl solution, hence, the total n moles of AFB₁ in 1 ml solution was 30.6 n moles. The protein content of the conjugate was found to be 300 ug/ml. Molecular weight of BSA is 66,200 so the protein content in 1 ml solution was equivalent to 4.5 n moles.
Figure 2.5  Standard AFB₁ ELISA competition curves

Dilution of the serum 1:5000 (▲——▲) and 1:10,000 (●——●)
Figure 2.6  Presumed structure of BSA-AFB_1 conjugate

(From Sizaret et al., 1982)
Figure 2.7  UV-visible absorption spectrum of BSA-AFC1 conjugate.

Absorbance

Wavelength (nm)

200 270 350 430 500
Therefore the ratio of AFB₁ molecule : BSA molecule was 7:1.

2.4.3 Antibody recognition of structural analogues to AFB₁

It was shown from the results in Figures 2.8 and 2.9 that rabbit anti-AFB₁ serum could detect various metabolites of AFB₁ with different sensitivities. The concentration of each analogue at 50% inhibition was determined and the ratio of sensitivity relative to AFB₁ was calculated as shown in Table 2.1. The data indicated that rabbit anti-AFB₁ serum could detect AFB₁-NAcCys, AFB₁-GSH, AFB₁-FAPy, AFB₁-Cys, APM₁, AFB₁-CysGly and AFB₂ with approximately the same sensitivity as it detected AFB₁, which gave 50% inhibition of the ELISA at 0.25 ng/ml. The 50% inhibition concentrations for these analogues were 0.14, 0.32, 0.36, 0.42, 0.44, 0.69 and 0.70 ng/ml respectively. This antibody could also detect AFL, AFB₁ and AFB₂ with lower sensitivity. The 50% inhibition concentrations were 7.50, 8.40 and 12.80 ng/ml respectively. AFB₁-Gua could be detected with the sensitivity intermediate between these two groups with 50% inhibition at 2.45 ng/ml. AFG₁ and AFG₂ were detected with much lower sensitivity with 50% inhibition of 50ng/ml. These data clearly indicate that the major epitope for antibody recognition of aflatoxin lies in the coumarin and cyclopentenone rings of the aflatoxin molecule. From this hypothesis, it could be predicted that the antibody would detect AFB₁-Gua and AFB₁-FAPy at a similar sensitivity but
Figure 2.6  ELISA competition curves for aflatoxins and some aflatoxin metabolites
Figure 2.9  ELISA competition curves for some aflatoxin metabolites

![Graph showing ELISA competition curves for some aflatoxin metabolites.](image-url)
### Table 2.1

Concentration of aflatoxin metabolites which showed 50% inhibition in ELISA

<table>
<thead>
<tr>
<th>Aflatoxins</th>
<th>Concentration for 50% inhibition (ng/ml)</th>
<th>Ratio of sensitivity relative to AFB₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB₁</td>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td>AFB₂</td>
<td>0.70</td>
<td>2.9</td>
</tr>
<tr>
<td>AFG₁</td>
<td>50.00</td>
<td>204.1</td>
</tr>
<tr>
<td>AFG₂</td>
<td>50.00</td>
<td>204.1</td>
</tr>
<tr>
<td>AFM₁</td>
<td>0.44</td>
<td>1.8</td>
</tr>
<tr>
<td>AFP₁</td>
<td>8.40</td>
<td>34.3</td>
</tr>
<tr>
<td>AFQ₁</td>
<td>12.80</td>
<td>52.2</td>
</tr>
<tr>
<td>AFL</td>
<td>7.50</td>
<td>30.6</td>
</tr>
<tr>
<td>AFB₁-Gua</td>
<td>2.45</td>
<td>10.0</td>
</tr>
<tr>
<td>AFB₁-FAPy</td>
<td>0.36</td>
<td>1.5</td>
</tr>
<tr>
<td>AFB₁-NAcCys</td>
<td>0.14</td>
<td>0.6</td>
</tr>
<tr>
<td>AFB₁-GSH</td>
<td>0.32</td>
<td>1.3</td>
</tr>
<tr>
<td>AFB₁-Cys-Gly</td>
<td>0.69</td>
<td>2.8</td>
</tr>
<tr>
<td>AFB₁-Cys</td>
<td>0.42</td>
<td>1.7</td>
</tr>
</tbody>
</table>
it was found that the 50% inhibition values for AFB₁-Gua and AFB₁-FAPy were 2.45 and 0.36 ng/ml respectively. These data indicate that the antibody is about 7 times more sensitive in detecting the AFB₁-FAPy adduct than the AFB₁-Gua adduct. This finding was unanticipated since the aflatoxin moiety is identical in these adducts when compared to the parent molecule (Figure 2.4). These observations suggest that the epitope for the antibody may be partially obstructed in these DNA adducts due to the stereochemistry of covalent binding of aflatoxin to guanine.

When this polyclonal rabbit anti-AFB₁ serum was compared with the monoclonal antibody raised by Groopman et al. (1984) against AFB₁-bovine gamma globulin and having the major epitope for aflatoxin recognition in the coumarin and cyclopentenone rings of the aflatoxin molecule, it was found that both antibodies could detect AFB₁, AFB₂ and AFM₁ much more sensitively than AFG₁, AFG₂ and AFQ₁. The sensitivities of both antibodies against AFB₁-FAPy were much higher than that of AFB₁-Gua. The results indicate that as the major epitope for antibody recognition is in the same area of the antigen molecule for both the monoclonal and polyclonal antibodies, they would detect the range of aflatoxin metabolites with a similar order of sensitivities.

2.4.4 Standard AFB₁ inhibition curves in PBS and in urine samples

Since human urine was the sample of interest, standard
ELISA competition curves for AFB₁ in PBS and in spiked urine samples were compared. It was found that the percentage inhibition of an unspiked control urine sample compared with control PBS was 72% (Table 2.2). This was equivalent to 1 ng AFB₁/ml in PBS. When the percentage inhibitions at the same concentrations of AFB₁ in PBS and in 'spiked' control urine sample were compared, the values obtained from 'spiked' urine sample were consistently higher than those obtained from PBS. This was due to the fact that unspiked urine samples already had high percentage inhibition which was the result of the presence of interfering substances in the urine.

The data in Table 2.3 indicate that when an uncontaminated urine sample (urine 1) was passed through the 'clean-up' procedures before spiking with known concentrations of AFB₁, similar percentage inhibitions to 'spiked' PBS were obtained. When urine samples were spiked with known concentrations of AFB₁ before passing through the 'clean-up' procedures (urine samples 2-4), the percentage inhibitions at the lower concentration of AFB₁ (0.01 ng/ml) were higher than the corresponding one in PBS solution. There were no differences in the percentage inhibitions when using higher concentrations of AFB₁ in PBS and in urine samples.

When the percentage inhibitions of the 'spiked' urine samples were calculated by comparison with the corresponding 'unspiked' urine samples (Table 2.4), the percentage inhibitions at each concentration of AFB₁ in
Table 2.2

Percentage inhibition of various concentrations of AFB$_1$ in PBS and in undiluted control urine sample in ELISA

<table>
<thead>
<tr>
<th>AFB$_1$ concentration (ng/ml)</th>
<th>% Inhibition in the ELISA</th>
<th>PBS</th>
<th>urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>71.7</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>15.8</td>
<td>73.3</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>43.1</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>70.9</td>
<td>85.8</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>82.4</td>
<td>91.9</td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td>84.6</td>
<td>92.6</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3

Percentage inhibitions of various concentrations of AFB\textsubscript{1} as compared to PBS in uncontaminated human urine samples after passing through the clean-up procedures

<table>
<thead>
<tr>
<th>AFB\textsubscript{1} concentration (ng/ml)</th>
<th>% Inhibition in the ELISA compared with control PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>0.01</td>
<td>3.3</td>
</tr>
<tr>
<td>0.10</td>
<td>41.5</td>
</tr>
<tr>
<td>1.0</td>
<td>72.5</td>
</tr>
<tr>
<td>10.0</td>
<td>87.2</td>
</tr>
<tr>
<td>100.0</td>
<td>91.9</td>
</tr>
</tbody>
</table>

N.B. - Urine 1 was passed through the clean-up procedures before spiking with known concentrations of AFB\textsubscript{1}.

- Urine 2-5 were spiked with known concentrations of AFB\textsubscript{1} before passing through the 'clean-up' procedures.
Table 2.4

Percentage inhibitions of ELISA in PBS and in urine samples

<table>
<thead>
<tr>
<th>AFB₁ concentration (ng/ml)</th>
<th>PBS*</th>
<th>urine 1⁺</th>
<th>urine 2⁺</th>
<th>urine 3⁺</th>
<th>urine 4⁺</th>
<th>urine 5⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>3.3</td>
<td>2.6</td>
<td>3.0</td>
<td>16.5</td>
<td>13.4</td>
<td>11.2</td>
</tr>
<tr>
<td>0.10</td>
<td>41.5</td>
<td>49.1</td>
<td>8.1</td>
<td>43.4</td>
<td>45.5</td>
<td>35.1</td>
</tr>
<tr>
<td>1.0</td>
<td>72.5</td>
<td>76.4</td>
<td>63.7</td>
<td>58.4</td>
<td>54.5</td>
<td>61.7</td>
</tr>
<tr>
<td>10.0</td>
<td>87.2</td>
<td>85.0</td>
<td>84.0</td>
<td>82.8</td>
<td>79.4</td>
<td>79.0</td>
</tr>
<tr>
<td>100.0</td>
<td>91.9</td>
<td>87.8</td>
<td>84.6</td>
<td>85.4</td>
<td>83.6</td>
<td>85.4</td>
</tr>
</tbody>
</table>

N.B. - Urine 1 was passed through the 'clean-up' procedures before spiking with known concentrations of AFB₁

- Urine 2-5 were spiked with known concentrations of AFB₁ before passing through the 'clean-up' procedures

* Percentage inhibition in PBS was calculated by reference to control PBS

⁺ Percentage inhibition in 'spiked' urine samples were calculated by reference to the corresponding unspiked urine samples
urine samples were similar to those observed in the PBS samples. The values obtained were slightly higher at 0.01 AFB₁/ml than those seen in the PBS samples. The pattern of the standard inhibition curves, however, was similar.

Although in these preliminary spiking experiments, a range of AFB₁ concentrations up to 100 ng/ml was used, a limiting factor in the subsequent assays was the capacity of the affinity column (20ng AFB₁) using a 1 ml bed volume (see Section 2.4.6.3). When samples containing 100 ng AFB₁ were loaded onto the columns, the columns were overloaded and only 10-20% of AFB₁ loaded was recovered in the methanol eluates. But in the subsequent ELISA, even giving this lower level of recovery from the column, the inhibition obtained was in the plateau part of the curves. Therefore, in order to obtain ELISA inhibitions in the proportionate (steep) part of the curves which is in the range of 50% inhibition, it is desirable to start with concentration of AFB₁ in the sample of not more than 20 ng/ml. Anything in excess of this would be in the non-proportionate (plateau) part of the inhibition curves.

When uncontaminated urine samples were directly assayed for apparent AFB₁ concentration, there were some inhibitory substances present in the samples which reacted with rabbit anti-AFB₁ serum in ELISA giving high percentage inhibitions. These inhibitions could be reduced by passing the samples through the preliminary 'clean-up' procedures (Sep-Pak and affinity column). These substances were referred to in one study as urinary aflatoxin-like
substances (UALS) (Dragsted et al., 1988). It is widely accepted that even in the Western urine samples, which are presumed to be free of aflatoxin contamination, from France (Martin et al., 1984), USA (Groopman et al., 1984) and Denmark (Dragsted et al., 1988) there are these UALS which often gave positive results for aflatoxins. These substances can be reduced by using Sep-Pak and immunoaffinity column (Groopman et al., 1984) but they cannot be totally removed from the samples as shown in Table 2.3. Due to the presence of these UALS in the uncontaminated urine samples, it is possible that when these samples were 'spiked' with AFB₁, the percentage inhibitions would be higher than those obtained in PBS. All these percentage inhibitions were calculated by comparing with 'unspiked' PBS which was free of any inhibitory substances. When the percentage inhibitions of 'spiked' urine samples in ELISA were calculated from the corresponding 'unspiked' urine samples, similar percentage inhibitions to those seen from comparing 'spiked' with 'unspiked' PBS were obtained (Table 2.4).

In practice, it is not possible to achieve the ideal situation in which the percentage inhibitions are calculated by comparing contaminated samples with uncontaminated samples from the same individual because one cannot find uncontaminated urine samples to serve as controls before obtaining another sample from the same source which is contaminated with aflatoxins. However, the protocol adopted in which urine samples from a presumably
unexposed population such as Western people are used to serve as controls and then to monitor the exposure in people from a different part of the world is not entirely satisfactory. It was observed that not only did the control Western urine samples contain inhibitory substances (UALS), but these varied between individuals (Table 2.16). There is evidence that the UALS present in the urine samples of Western people vary according to the food being eaten (Dragsted et al., 1988), so it is not appropriate to compare people from different parts of the world because the dietary pattern varies from country to country.

One theoretical possibility would be to establish standard UALS inhibitory level for each population to be studied. However, this could only be achieved using large numbers of samples, and even then only a range of inhibitory level would be established. So a compromise solution for monitoring aflatoxin exposure is to calculate the percentage inhibitions in urine samples by reference to PBS controls.

2.4.5 The effect of methanol on ELISA

Since all the procedures used in the preliminary 'clean-up' of urine samples involved the use of methanol, it was of interest to study the effect of this solvent on the competitive ELISA. In 50% methanol solution, the standard ELISA curve of AFB₁ was less steep than the one obtained using PBS (Figure 2.10). In the presence of 50% methanol, the percentage inhibitions at AFB₁ concentrations
Figure 2.10  Effect of methanol on the AFB₁ competition assay
less than 0.1 ng/ml were higher than those seen in PBS, while at the higher concentrations the percentage inhibitions were lower. In 85% methanol solution, there was a 'flat' competition ELISA curve and even at the lowest concentration of AFB₁ (0.01 ng/ml) the percentage inhibition was 60% compared with the PBS control.

From these results, it can be seen that methanol solutions of 50% or more were not suitable for ELISA because they not only gave high percentage inhibition at low concentrations of AFB₁, but also the competition curves were "shallow" when compared with the curves obtained in PBS.

It had already been shown that uncontaminated urine samples could give high percentage inhibition in competitive ELISA due to UALS. If these two factors (methanol and UALS) acted additively in the ELISA, high percentage inhibitions compared with PBS could be obtained. As a result of this experiment, it was decided that methanol would be removed from the samples prior to their assays by ELISA.

2.4.6 Methods developed to monitor aflatoxins excreted in human urine samples

It had been a common experience of people working in this field that monitoring of aflatoxin levels in human urine samples was not straightforward (Groopman et al., 1985, 1986; Garner et al., 1985). The samples could not be used directly in ELISA because they always had high and variable background levels of inhibitory substances. The
suggestion had been made to use some 'clean-up' procedures to remove non-aflatoxin substances or UALS that might react with the antibody in ELISA. The use of Sep-Pak C\textsubscript{18} cartridges and immunoaffinity columns had been suggested (Groopman \textit{et al.}, 1984). In order to establish a suitable method for use in monitoring aflatoxin levels in human urine samples, all the 'clean-up' procedures had to be validated. They were divided into each individual step and were tested separately.

2.4.6.1 \textbf{Capacity of Sep-Pak C}_{18} \textbf{Cartridge}

The data given in Table 2.5 clearly show that when 1 ml of a solution containing up to 100 ng of $^{3}$H-AFB\textsubscript{1} in either PBS or 'spiked' urine sample was loaded onto a Sep-Pak cartridge, more than 90\% of the $^{3}$H-AFB\textsubscript{1} was retained on the cartridge and could subsequently be eluted with methanol.

Table 2.6 shows that when up to 200 ng of $^{3}$H-AFB\textsubscript{1} in urine was loaded onto a Sep-Pak cartridge, 90\% of the radioactivity was retained and subsequently could be eluted with methanol. About 9\% of the radioactivity was in the unretained and the washing fractions. When these two fractions were combined and injected into the HPLC, most of the radioactivity eluted in the first few fractions. When the methanol fractions from the Sep-Pak cartridge were subjected to the HPLC, peaks which corresponded to those obtained by injection of standard $^{3}$H-AFB\textsubscript{1} were observed (Figure 2.11).
Table 2.5

Radioactivity in fractions from Sep-Pak C\textsubscript{18} cartridge after loading with various concentrations of $^3$H-AFB\textsubscript{1} in PBS and in urine sample

<table>
<thead>
<tr>
<th>$^3$H-AFB\textsubscript{1} (ng/ml)</th>
<th>Radioactivity in fractions</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unretained</td>
<td>washing</td>
<td>methanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dpm</td>
<td>%*</td>
<td>dpm</td>
<td>%*</td>
</tr>
<tr>
<td>in PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>280</td>
<td>1.9</td>
<td>830</td>
<td>5.7</td>
</tr>
<tr>
<td>1.0</td>
<td>560</td>
<td>4.0</td>
<td>550</td>
<td>3.9</td>
</tr>
<tr>
<td>10.0</td>
<td>270</td>
<td>1.9</td>
<td>630</td>
<td>4.4</td>
</tr>
<tr>
<td>100.0</td>
<td>260</td>
<td>1.8</td>
<td>800</td>
<td>5.5</td>
</tr>
<tr>
<td>in urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>380</td>
<td>3.0</td>
<td>980</td>
<td>7.6</td>
</tr>
<tr>
<td>1.0</td>
<td>550</td>
<td>3.4</td>
<td>880</td>
<td>5.4</td>
</tr>
<tr>
<td>10.0</td>
<td>370</td>
<td>2.6</td>
<td>800</td>
<td>5.5</td>
</tr>
<tr>
<td>100.0</td>
<td>360</td>
<td>2.7</td>
<td>1040</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Percentage of total radioactivity recovered in all fractions

80
Table 2.6
Radioactivity in fractions from Sep-Pak C\textsubscript{18} cartridge after loading with 10 ml of urine sample containing 200 ng $^3$H-AFB\textsubscript{1}

<table>
<thead>
<tr>
<th>Fraction from Sep-Pak</th>
<th>Radioactivity total dpm</th>
<th>%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>unretained</td>
<td>48,210</td>
<td>4.9</td>
</tr>
<tr>
<td>washing</td>
<td>36,735</td>
<td>3.7</td>
</tr>
<tr>
<td>methanol</td>
<td>896,630</td>
<td>91.4</td>
</tr>
</tbody>
</table>

* Percentage of total radioactivity recovered in all fractions
Figure 2.11  The examination of various HPLC fractions obtained from Sep-Pak C_{18} cartridge, for the coincidence of radiolabel with AFB_{1}

- Standard
- Unretained
- Methanol

Sep-Pak C_{18} cartridge fractions; unretained (–•–); methanol eluate (—■—); Standard AFB_{1} (▲▲▲)

* Percentage of total radioactivity recovered in all fractions
When the methanol samples eluted from the Sep-Pak were evaporated and reconstituted to the original volume in PBS, the percentage recovery of radioactivity was 92%. A similar percentage of the radioactivity in the AFB$_1$-associated HPLC peak was observed using the methanol fraction either directly or after evaporation and reconstitution in PBS.

From the data shown in Tables 2.5 and 2.6 it could be concluded that when $^3$H-AFB$_1$ was loaded onto a Sep-Pak C$_{18}$ cartridge up to the amount of 200 ng, 90% of the radioactivity was retained on the cartridge and subsequently eluted with methanol but this corresponded with 100% retention of AFB$_1$. It was demonstrated by the correspondence with the AFB$_1$-associated peak in the HPLC fractions of the label present in the methanol eluate from the Sep-Pak, in contrast to the radioactivity in the unretained and the washing fractions which eluted early from the HPLC. The label present in the unretained and the washing fractions of the Sep-Pak represented tritium exchange from the AFB$_1$. Therefore, these unretained and washing fractions from Sep-Pak were subsequently discarded and only the methanol eluate was used in further analysis. The evaporation process to remove methanol was proved to be effective and more than 90% of the radioactivity could be recovered following this process.

However, it must be pointed out that these experiments represented an early stage in the development of monitoring protocol and subsequently when actual biological samples
containing AFB₁ metabolites were to be examined, the
distribution of these metabolites among the Sep-Pak
fractions would need to be reassessed.

2.4.6.2 Capacity of affinity gel

The results from a non-immune column loaded with 1 ml
of 1 ng ³H-AFB₁ in PBS (Table 2.7) showed that the column
did not retain ³H-AFB₁. All the radioactivity came out in
the unretained and the PBS washing fractions. The slight
retardation observed in the emergence of the ³H-AFB₁ was
presumably the result of normal chromatographic action.

When the immune column was loaded with 1 ml of PBS
containing 1 ng ³H-AFB₁, the results indicated that the
column strongly retained ³H-AFB₁. Only 18% of the
radioactivity was eluted in the unretained and the washing
fractions and as had already been demonstrated by the HPLC,
these samples of ³H-AFB₁ contained at least 10% of tritium
exchange. The data indicated that with 0.5 ml bed volume,
the immune column was capable of retaining 1 ng ³H-AFB₁ in
PBS and the immunoaffinity column had potential for
"cleaning-up" the samples prior to ELISA.

2.4.6.3 Characteristics of the immunoaffinity columns

Immunoaffinity columns with 0.5 ml bed volume were
tested. The results shown in Table 2.8 are divided into
two parts depending on the nature of the samples used (PBS
or urine).

When using standard concentrations of ³H-AFB₁ in PBS,
Table 2.7
Radioactivity in fractions from immune and non-immune affinity column loaded with 1 ng $^{3}$H-\text{AFB}_1

<table>
<thead>
<tr>
<th>Fraction from affinity column</th>
<th>Radioactivity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>non-immune</td>
<td>immune</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>total dpm</td>
<td>column</td>
<td>total dpm</td>
<td>%*</td>
</tr>
<tr>
<td>unretained</td>
<td>2435</td>
<td>587</td>
<td>19.0</td>
<td>5.1</td>
</tr>
<tr>
<td>washing</td>
<td>10244</td>
<td>1452</td>
<td>79.9</td>
<td>12.7</td>
</tr>
<tr>
<td>methanol</td>
<td>142</td>
<td>9393</td>
<td>1.1</td>
<td>82.6</td>
</tr>
<tr>
<td>total dpm</td>
<td>12821</td>
<td>11432</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Percentage of total radioactivity recovered in all fractions
the volumes loaded varied from 0.2 ml to 10.0 ml and the amounts of \(^3\text{H-}\text{AFB}_1\) loaded were 1 to 100 ng. The percentages of radioactivity in the eluted methanol fractions following replicate loadings of \(^3\text{H-}\text{AFB}_1\) were: from 1 ng \text{AFB}_1 loaded, 84, 84 and 75%; from 7 ng, 82%; from 20 ng 67, and 69%; from 75 ng, 22 and 10%; and from 100 ng, 18%.

When urine samples 'spiked' with various concentrations of \(^3\text{H-}\text{AFB}_1\) were used, the volumes loaded did not exceed 2 ml. The percentages of radioactivity in the methanol fractions from 1 ng \text{AFB}_1 loaded were 72% in two replicates; from 10 ng, 68%; and from 20 ng, 59 and 69%. It should be noted that when samples containing 20 ng \(^3\text{H-}\text{AFB}_1\) were used, the samples volumes loaded differed by a factor of 10 times (0.2 and 2 ml). This difference in the sample volumes loaded resulted in differing retentions of the radioactivity on the column.

It was clearly shown from these results that in using PBS solution, the capacity of the column was 20 ng \text{AFB}_1 with 0.5 ml bed volume and the column could be reused. The volumes of samples loaded did not affect the capacity of the column, as when 2.0 and 0.2 ml of PBS containing the same amount of \(^3\text{H-}\text{AFB}_1\) (20 ng) were loaded onto the column, the amounts of radioactivity retained on the column were the same. And when 1 and 10 ml of PBS containing 1 ng of \(^3\text{H-}\text{AFB}_1\) were loaded, similar percentages of radioactivity were retained on the column, and it was also shown that the column was reusable.
### Table 2.8

Characteristics of the immunoaffinity columns using 0.5 ml bed volume

<table>
<thead>
<tr>
<th>Nature of sample</th>
<th>PBS</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>column number</td>
<td>1  2  2  3  3  4  4  5  5  6  7  7  8  8</td>
<td></td>
</tr>
<tr>
<td>volume loaded (ml)</td>
<td>0.7  0.75  0.75  10  1  1  1  2  0.2  1  1  1  2  0.2</td>
<td></td>
</tr>
<tr>
<td>amount of AFB₁ (ng)</td>
<td>7  75  75  1  1  100  1  20  20  1  1  10  20  20</td>
<td></td>
</tr>
</tbody>
</table>

% of $^3$H-AFB₁ in fraction

- unretained
  | 5  4  5  14  5  12  11  15  13  15  14  9  28  18 |
- washing
  | 13  74  85  2  11  70  14  18  18  13  14  23  13  13 |
- methanol
  | 82  22  10  84  84  18  75  67  69  72  72  68  59  69 |

% recovery from column

| 92  86  86  83  84  87  87  84  92  86  91  86  85  69 |
When urine samples were used, less radioactive AFB₁ was retained on the column when compared with loading the same amount of ³H-AFB₁ in PBS. The amount of ³H-AFB₁ loaded should not exceed 20 ng using 0.2 ml urine samples with a column bed volume of 0.5 ml. The capacity of the column was lower than when PBS solutions were used. The volume of urine samples used affected the capacity of the column; when small volumes of urine samples were used, the column was capable of retaining more ³H-AFB₁. The possibility of reusing the columns depended on the volume of urine loaded.

From these preliminary studies it was evident that loading higher amounts of AFB₁ in either PBS or urine samples resulted in decreasing percentages of the samples being retained. Comparison of loading 1 ng of AFB₁ in PBS or in urine indicated that more radioactivity was retained on the column when AFB₁ was in PBS. The percentage of tritium found in the unretained and the washing fractions when samples were applied in PBS, and presumably due to exchange was around 16-25% but in the urine samples, the exchange was higher. It is also possible that there are some substance(s) in the urine samples that might interfere with the binding of ³H-AFB₁ onto the column.

These results applied only to sample loading which did not exceed the AFB₁ capacity of the column (20 ng AFB₁ for a 0.5 ml bed volume). When higher sample loadings were attempted, radioactivity in the unretained and the washing fraction were due to both tritium exchange and also unretained AFB₁.
When 2 ng $^3$H-AFB$_1$ were loaded onto the column in a 2 ml urine sample, 60% of the radioactivity was retained on the column, while when 5 ng $^3$H-AFB$_1$ were loaded in a 5 ml urine sample only 27% was retained (Table 2.9). The percentage radioactivity in the unretained fraction following 5 ng AFB$_1$ loading was nearly twice that found in the comparable fraction following 2 ng loading. Since this difference is not due to tritium exchange it can be concluded that the capacity of the column (20 ng AFB$_1$ in PBS) is effectively reduced when samples are applied in urine. This could be due to substance(s) in the urine which prevented the binding of AFB$_1$ onto the column.

When the samples from the methanol eluates were injected into the HPLC either directly or after evaporation, the percentage radioactivity in the AFB$_1$-associated peak was more than 80% as shown in Table 2.10. And the recoveries of radioactivity after evaporation of methanol column eluates following 2 and 5 ng loadings were 55 and 102% respectively. The low 55% recovery might be due to the fact that this sample was taken down to dryness. In order to clarify this point, a further experiment was carried out in which 2 ng of $^3$H-AFB$_1$ in 2 ml urine sample was loaded onto an affinity column and the whole procedures repeated except that the sample was not evaporated to dryness but only to a low volume (0.5 ml) before reconstitution. In this case 97.5% of the radioactivity was recovered.

The data demonstrate that recovery of $^3$H-AFB$_1$ after
Table 2.9

Radioactivity in fractions from affinity columns after loading with 2 and 5 ng $^3$H-AF$\beta_1$ in 2 and 5 ml urine respectively

<table>
<thead>
<tr>
<th>Fraction from affinity column</th>
<th>% Radioactivity</th>
<th>2 ng loaded</th>
<th>5 ng loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>unretained</td>
<td></td>
<td>36.9</td>
<td>64.8</td>
</tr>
<tr>
<td>washing</td>
<td></td>
<td>3.1</td>
<td>8.3</td>
</tr>
<tr>
<td>methanol</td>
<td></td>
<td>60.0</td>
<td>26.9</td>
</tr>
</tbody>
</table>

Overall % recovery of radioactivity loaded$^+$  
106.1  
100.3  

$^+$ Essentially all recoveries approximately 100%
Variation due to radioactive counting using 1 ml aliquots of samples.
Table 2.10

Radioactivity in the AFB$_1$-associated peak on HPLC after injection of methanol fractions from affinity column loaded with 2 and 5ng $^3$H-AFB$_1$

<table>
<thead>
<tr>
<th>HPLC fraction</th>
<th>% Radioactivity in the AFB$_1$-associated peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 ng loaded</td>
</tr>
<tr>
<td>directly injected</td>
<td>81</td>
</tr>
<tr>
<td>after evaporation</td>
<td>90</td>
</tr>
</tbody>
</table>

% recovery after evaporation  55  102
evaporation of the methanol fractions from affinity column exceeded 90% and were similar to the percentage recovery of radioactivity found using methanol eluates from the Sep-Pak C₁₈ cartridges. It was also shown that methanol samples should not be evaporated to dryness because this reduced the recovery of ³H-AFB₁.

2.4.6.4 Routine testing results of the capacity of the affinity gel

The routine standard testing of the capacity of the affinity gel was developed in order to test each new batch of gel prepared using different samples of antisera, either from the same or from different rabbits. The results had to be in the range of the data shown in Table 2.11, otherwise the gel was classified as not suitable for further analysis. With 1 ml column bed volume, the retention of ³H-AFB₁ on the column was approximately 93% with the capacity of up to 20 ng AFB₁ in PBS. Whereas using urine samples the radioactivity retained on the column was approximately 90%, with the capacity in the order of 5 ng AFB₁. This may be due to the presence of some interfering substances which could prevent the binding of AFB₁ on the column. In order to avoid possible problems associated with this factor when using samples of unknown AFB₁ content, it was decided to restrict the volume of the sample to 1 ml and to increase the column bed volume to 1 ml.

It had to be borne in mind that the urine samples used
Table 2.11
Radioactivity in fractions from affinity column in routine testing with standard AFB1 concentration in both PBS and urine sample

<table>
<thead>
<tr>
<th>3H-AFB1 concentration (ng/ml)</th>
<th>Unretained dpm</th>
<th>%*</th>
<th>Washing dpm</th>
<th>%*</th>
<th>Methanol dpm</th>
<th>%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3,310</td>
<td>0.5</td>
<td>39,945</td>
<td>6.4</td>
<td>579,840</td>
<td>93.1</td>
</tr>
<tr>
<td>10</td>
<td>4,466</td>
<td>0.7</td>
<td>50,430</td>
<td>8.0</td>
<td>572,445</td>
<td>91.3</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5,522</td>
<td>0.9</td>
<td>51,315</td>
<td>8.5</td>
<td>548,805</td>
<td>90.6</td>
</tr>
<tr>
<td>1</td>
<td>12,968</td>
<td>2.7</td>
<td>49,215</td>
<td>10.1</td>
<td>426,495</td>
<td>87.3</td>
</tr>
</tbody>
</table>

* Percentage of total radioactivity recovered
in the column evaluation studies were untreated samples which meant they were not subjected to any previous 'clean-up' procedures. So it was anticipated that when samples were subjected to prior 'clean-up' procedures and 1 ml of these samples were subsequently loaded onto 1 ml bed volume of the affinity column, it would be possible for amounts of $\text{AFB}_1$ in excess of 5 ng $\text{AFB}_1$ to be retained onto the column.

When each batch of affinity gel was prepared, these routine tests were carried out. If the results of the tests were not in the acceptable range of the gel capacity, the decision had to be made whether the gel was suitable for use or not. If the gel retained $^3\text{H}-\text{AFB}_1$ but a little less than anticipated, by adjusting the column bed volume the capacity of the column used could be in the acceptable range. But if the retention was much lower than it should be, the gel was discarded.

2.4.6.5 Recovery of the overall 'clean-up' procedures

The study was aimed to evaluate the recovery of $^3\text{H}-\text{AFB}_1$ in urine samples after passing through the Sep-Pak C$_{18}$ cartridge and the immunoaffinity column by monitoring radioactivity and using ELISA techniques to check the levels of $\text{AFB}_1$ in the samples.

When 20 ml of urine sample containing 20 ng $^3\text{H}-\text{AFB}_1$/ml was loaded on a Sep-Pak cartridge (a total loading of 400 ng $^3\text{H}-\text{AFB}_1$), 89% of the radioactivity was retained on the cartridge (Table 2.12). When the methanol
Table 2.12

Radioactivity in fractions from Sep-Pak C<sub>18</sub> cartridge after loading with 20 ml of urine containing 20 ng $^{3}$H-AFB<sub>1</sub>/ml

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>% Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>unretained</td>
<td>20</td>
<td>10.0</td>
</tr>
<tr>
<td>washing</td>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>methanol</td>
<td>10</td>
<td>88.9</td>
</tr>
</tbody>
</table>

Overall % recovery of radioactivity loaded* 109.3

* Essentially all recoveries approximately 100%
Variation due to radioactive counting using 1 ml aliquots of samples
eluate from the Sep-Pak was directly injected into the HPLC, 93% of the radioactivity was in the AFB₁-associated peak. After evaporation (not to dryness), the radioactive recovery was 92% and on subsequent HPLC chromatography the radioactivity in the AFB₁-associated peak was 90%. So the overall recovery from the Sep-Pak procedure and subsequent reconstitution to 5 ml with PBS was 82%, hence the amount of \(^3\text{H}-\text{AFB}_1\) in the solution according to radioactivity should be 324 ng. When 0.1, 0.2 and 0.5 ml aliquots of this methanol fraction from the Sep-Pak were loaded onto the affinity column with 1 ml bed volume, the amount of AFB₁ loaded should have been 3.24, 6.48 and 16.20 ng respectively.

The methanol eluate from the affinity column was collected in two fractions; the first fraction was 10 ml of the eluate and the second fraction was 5 ml eluate. As shown in Table 2.13, most of the radioactivity in the methanol fractions was in the first 10 ml and that the radioactivity in this fraction was more than 80% of the total radioactive present in all the affinity column fractions, hence this elution volume was sufficient for subsequent use in ELISA. A total methanol eluate volume of 10 ml was very convenient for the subsequent evaporation in the Speedvac Concentrator which had tubes of 10 ml capacity.

The average recovery from the affinity column was 86%. Therefore, the overall recovery of radioactivity using \(^3\text{H}-\text{AFB}_1\) in the complete 'clean-up' procedures of a
Table 2.13

Radioactivity in fractions obtained from affinity columns following loading with different volumes of a 'spiked' urine sample pre-purified by Sep-Pak C18 cartridge*

<table>
<thead>
<tr>
<th>Fraction from affinity column</th>
<th>% Radioactivity 0.1 ml</th>
<th>% Radioactivity 0.2 ml</th>
<th>% Radioactivity 0.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>unretained</td>
<td>2.5</td>
<td>5.7</td>
<td>9.0</td>
</tr>
<tr>
<td>washing</td>
<td>0.1</td>
<td>3.8</td>
<td>6.2</td>
</tr>
<tr>
<td>10 ml methanol</td>
<td>92.1</td>
<td>85.9</td>
<td>80.6</td>
</tr>
<tr>
<td>5 ml methanol</td>
<td>5.3</td>
<td>4.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Overall % recovery of radioactivity loaded+</td>
<td>115.1</td>
<td>94.4</td>
<td>103.1</td>
</tr>
</tbody>
</table>

* 20 ml of a urine sample 'spiked' with 400 ng \(^3\text{H}\)-AFB\(_1\) was loaded onto Sep-Pak C18 cartridge, washed and eluted with 10 ml methanol.\(^x\) Sample (7 ml) was evaporated (not to dryness) and reconstituted in 7 ml PBS. Different volumes of this sample were then applied to the affinity column.

+ Essentially all recoveries approximately 100%

Variation due to radioactive counting using 1 ml aliquots of samples

\(^x\) See Table 2.12
Sep-Pak C_{18} cartridge followed by an affinity column was 71%. This recovery was deemed to be of an acceptable level.

From Table 2.13, although the percentage radioactivity recoveries using the 3 sample volumes did not differ greatly, nevertheless, there was a trend for a lower recovery, in the first 10 ml methanol eluate, the larger the sample volume applied. This suggested that the volume of urine samples applied to the column should be kept as low as possible, consistent with obtaining significant results in the subsequent ELISA.

An experiment was carried out in which 'unspiked' or AFB_{1}-spiked urine samples were subjected to the 'clean-up' protocols and finally the AFB_{1} content assayed by ELISA. The data given in Table 2.14 show that for a 10 ml unspiked control urine sample, an apparent AFB_{1} content of 1.77 ng (0.177 ng/ml) was found. This level (0.177 ng apparent AFB_{1}/ml) was consistent with other experiments carried out in this study using unspiked urine samples with the 'clean-up' procedures (see Table 2.16).

In the AFB_{1}-spiked urine samples, the amounts of AFB_{1} determined by ELISA were 2.95, 4.34 and 9.47 ng for the 1, 5 and 10 ng AFB_{1} level respectively. The results were reproducible in 5 separate assays. The amounts of AFB_{1} obtained in the ELISA were similar to the known amounts of AFB_{1} added to the samples. But the apparent level of 1.77 ng AFB_{1} in 10 ml unspiked urine sample should be subtracted from the values obtained using the 'spiked' samples.
Table 2.14

AFB₁ concentrations from ELISA of samples after passing through Sep-Pak C₁₈ cartridge and affinity column procedures

<table>
<thead>
<tr>
<th>Amount of AFB₁ loaded (ng)</th>
<th>AFB₁ from ELISA (ng/ml)</th>
<th>Mean</th>
<th>S.D.</th>
<th>Amount of AFB₁ in original sample* (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.43    0.94  0.31  0.56  0.28</td>
<td>0.51</td>
<td>0.27</td>
<td>1.77</td>
</tr>
<tr>
<td>1</td>
<td>1.05    1.00  0.74  0.86  0.62</td>
<td>0.85</td>
<td>0.18</td>
<td>2.95</td>
</tr>
<tr>
<td>5</td>
<td>1.30    1.70  1.00  1.20  1.04</td>
<td>1.25</td>
<td>0.28</td>
<td>4.34</td>
</tr>
<tr>
<td>10</td>
<td>3.00    2.50  2.56  3.20  2.40</td>
<td>2.73</td>
<td>0.35</td>
<td>9.47</td>
</tr>
</tbody>
</table>

* Not all of the original 10 ml sample loaded onto Sep-Pak C₁₈ cartridge was subjected to all the subsequent procedures up to ELISA. It was calculated that the factor of 3.47 had to be used to multiply the values obtained from ELISA to have the amount of AFB₁ in original samples. This factor does not take account of the losses during the 'clean-up' procedures.
Following this correction, the amounts of AFB₁ present in each sample would be 1.18, 2.57 and 7.70 ng AFB₁ respectively.

When using $^3$H-AFB₁ in the 'clean-up' procedures, the results presented in Tables 2.12 and 2.13 show an overall radioactive recovery for the procedures of approximately 71%. Therefore, the results of the ELISA and radioactive assays for the 'clean-up' procedures are essentially in agreement.

2.4.7 Urinary aflatoxin-like substance (UALS)

2.4.7.1 Apparent AFB₁ concentrations in uncontaminated human urine samples

When uncontaminated human urine samples were loaded onto the affinity columns, the apparent AFB₁ concentrations were higher in the combined unretained and washing fractions from the affinity column than in the methanol eluate fractions (Table 2.15). The values in the washing fractions were 0.70, 1.06 and 1.88 ng apparent AFB₁/ml, with the average of 1.21 ng/ml. While in the methanol fractions, the apparent AFB₁ values were 0.24, 0.40 and 0.78 ng/ml, with the average of 0.47 ng/ml. These results indicate that the apparent AFB₁ concentration in the washing fractions of the affinity column was nearly 3 times higher than that obtained using the methanol fractions. Since the urine samples used were presumed to be free of aflatoxin contamination, the level of aflatoxin found in the samples should represent the background level
### Table 2.15

Apparent AFB₁ concentrations in 3 human urine samples in fractions from affinity columns

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Fraction</th>
<th>Apparent AFB₁ from ELISA (ng/ml)</th>
<th>Mean</th>
<th>SD</th>
<th>Apparent AFB₁ in original sample (ng)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>washing</td>
<td>0.32</td>
<td>0.41</td>
<td>0.32</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>methanol</td>
<td>0.13</td>
<td>0.15</td>
<td>0.09</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>washing</td>
<td>0.57</td>
<td>0.57</td>
<td>0.46</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>methanol</td>
<td>0.24</td>
<td>0.20</td>
<td>0.17</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>washing</td>
<td>0.72</td>
<td>0.93</td>
<td>1.18</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>methanol</td>
<td>0.40</td>
<td>0.50</td>
<td>0.26</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* These values obtained are based on the mean of three replicate assays which involved a dilution of 1:1 before being analysed for apparent AFB₁ by ELISA
or the interfering substances that could react with the antibody used in the ELISA. Therefore, it appeared that the methanol eluate from the affinity column was the appropriate fraction to use in ELISA to detect aflatoxins in human urine samples.

When the uncontaminated urine samples were fractionated by HPLC before subjecting to the ELISA, the apparent \( \text{AFB}_1 \) was very high in fraction 2 of the HPLC (Figure 2.12). All the other fractions nearly had the same apparent \( \text{AFB}_1 \) concentration of 0.2 ng/fraction. The results also show that the substances present in the uncontaminated human urine samples which were reactive in the ELISA system could be separated chromatographically and were eluted in the first 4 fractions. Even if the level of interfering substances was as low as 0.2 ng/fraction it would be desirable to remove them before analysing the actual potentially contaminated samples. This is the point why the 'clean-up' procedures should be used. It had already been demonstrated that the background level in the methanol fraction of the affinity column was much lower than in the washing fraction (Table 2.15). This meant that the majority of the reactive substances had already been removed by the washing procedure. However there was still some reactive material present in the methanol fraction which gave a background reading in the ELISA.

2.4.7.2 Reduction of UALS in uncontaminated human urine samples

From the previous experiment it was found that the
An examination of HPLC fractions for the apparent AFB$_1$ content of uncontaminated human urine samples (B, C and D) by ELISA

* AFB$_1$ would be eluted in fraction 12
interference in the uncontaminated urine sample could be reduced by using a Sep-Pak C₁₈ cartridge and an immuno-affinity column. Comparisons of the apparent AFB₁ concentrations in the samples before and after the 'clean-up' procedures were made.

When undiluted urine samples were used directly in ELISA, they gave very high inhibitions of approximately 90% and when the 1:250 dilutions were used they gave very low inhibitions of approximately 10%. The appropriate dilution of human urine samples to be used directly in ELISA was 1:25 because this dilution gave inhibitions compatible with the proportional range of ELISA. The results show that the very high inhibitions of neat urine samples could be reduced by dilution, but this would not be a practical solution when using potentially contaminated samples since aflatoxins in the samples would be diluted as well.

When human urine samples diluted 1:25 were analysed by ELISA, the apparent AFB₁ content of the samples ranged from 1 to 12 ng/ml (Table 2.16). The variation in apparent background concentration between individual samples might be due in part to differences in the urine concentrations. Creatinine concentration can be used as a reference point to correct for these differences, and apparent AFB₁ can be expressed as ng AFB₁/mg creatinine; in this case, they ranged from 1.6 to 10.7 ng/mg.

When the samples were passed through the 'clean-up' procedures, the background or apparent AFB₁ levels were reduced. They ranged from 0.08 to 0.44 ng/ml or 0.04 to
<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Creatinine (mg/ml)</th>
<th>Apparent AFB1 (ng/ml) untreated*</th>
<th>AFB1/creatinine (ng/ng) untreated*</th>
<th>% reduction of interference untreated*</th>
<th>AFB1/creatinine (ng/ng) treated*</th>
<th>% reduction of interference treated*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.66</td>
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</tr>
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<td>96.8</td>
</tr>
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<td>5</td>
<td>1.27</td>
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<td>0.17</td>
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</tr>
<tr>
<td>6</td>
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<td>6.88</td>
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<td>11.63</td>
<td>0.24</td>
<td>11.88</td>
<td>0.11</td>
<td>97.9</td>
</tr>
<tr>
<td>10</td>
<td>2.00</td>
<td>6.48</td>
<td>0.18</td>
<td>6.66</td>
<td>0.14</td>
<td>96.6</td>
</tr>
<tr>
<td>Mean</td>
<td>1.37</td>
<td>6.48</td>
<td>0.18</td>
<td>6.66</td>
<td>0.14</td>
<td>96.6</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.49</td>
<td>2.74</td>
<td>0.11</td>
<td>2.36</td>
<td>0.07</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* urine samples diluted 1:25 analysing without 'clean-up' procedures
+ urine samples (undiluted) analysing with 'clean-up' procedures

Urinary aflatoxin-like substances (UALS) in ELISA before and after the 'clean-up' procedures.
0.24 ng/mg creatinine. Therefore, the interference could be reduced by 97% (Table 2.16). From these data using 10 subjects the average apparent AFB₁ was 0.14 ng/mg creatinine with a standard deviation of 0.07. If the mean background level plus one standard deviation is considered to be the lower limit of significant detection for the presence of aflatoxins assayed by this system, this gives a value of 0.2 ng AFB₁/mg creatinine. If the samples being analysed had the level of AFB₁ equivalent lower than 0.2 ng/mg creatinine, no firm conclusion could be made as to the presence of aflatoxins in these samples. Since the number of subjects used in determining this lower limit of significant detection was small, it would be possible that if more subjects were used, this limit of detection would be changed. But from all the data obtained from uncontaminated urine samples analysed so far it appears that this limit is an appropriate one to use.

2.5 SUMMARY

A method to monitor aflatoxins excreted in human urine samples has been developed using the ELISA technique. It was found that urine samples cannot be used directly in ELISA because of a high interference due to unknown substances (UALS) present in the urine. These UALS can be reduced by the 'clean-up' procedures of a Sep-Pak C₁₈ cartridge and an immunoaffinity column. When the presumed uncontaminated human urine samples were spiked with ³H-AFB₁
and the 'clean-up' procedures were carried out, the overall percentage recovery of the radiolabelled AFB₁ was 71%.

The antibody used in the preparation of the immunoaffinity columns and in the ELISA is polyclonal and has as its major epitope for antibody recognition, the coumarin and cyclopentenone rings of the aflatoxin molecule. This antibody can be diluted 10,000 times before using in ELISA analysis. When immunoaffinity columns with 1 ml bed volume were prepared, their capacity was 20 ng AFB₁/ml PBS or 5 ng AFB₁/ml urine (without any 'clean-up' of the samples). It is suggested that UALS may obstruct the binding of aflatoxin onto the column. Therefore, the volume of urine loaded should be kept as low as possible, but consistent with obtaining significant results in the subsequent ELISA.

The general procedures for analysing aflatoxins excreted in human urine samples developed in this chapter should be further validated using the actual aflatoxin metabolites excreted in the appropriate animal model system.
CHAPTER 3

Marmoset Monkey as an Animal Model to Validate the Methods Developed for Monitoring Human Exposure to Aflatoxins
3.1 INTRODUCTION

In order to monitor the levels of aflatoxin exposure using human urine samples, methods employing an ELISA technique with preliminary 'clean-up' procedures on Sep-Pak C\textsubscript{18} cartridge and immunoaffinity column have been developed. These methods have been validated step by step using 'spiked' \textsuperscript{3}H-AFB\textsubscript{1} in either PBS or uncontaminated human urine samples. Only AFB\textsubscript{1} was used in developing these monitoring procedures, however, AFB\textsubscript{1} is unlikely to be found in human urine samples because it will be metabolized in the body by microsomal enzyme system to various metabolites (see Section 1.3). In view of this, it would have been desirable to validate the methods using known contaminated human urine samples. Since it is not possible to conduct this type of study in human beings, an appropriate animal model which has similar pathways of AFB\textsubscript{1} metabolism to humans should be used.

Primates are considered to be the animal species which have metabolic pathways of many xenobiotic compounds similar to man. Several studies have suggested that AFQ\textsubscript{1} is the major metabolites of AFB\textsubscript{1} in the monkey and man (Büchi \textit{et al.}, 1974; Hsieh \textit{et al.}, 1977; Moss and Neal, 1985; Yourtee \textit{et al.}, 1987). AFQ\textsubscript{1} is identified as an hydroxylated product of AFB\textsubscript{1} with the hydroxyl on the carbon atom β to the carbonyl of cyclopentenone ring (Figure 2.4) (Masri \textit{et al.}, 1974). In one study, 33% of AFB\textsubscript{1} was metabolized by human liver microsomal fractions in
75 min to a variety of metabolites, of which AFQ₁ made up 8.5% of the total, while using monkey liver microsomes 48% of AFB₁ was metabolized and 10.1% was AFQ₁ (Hsieh et al., 1977). In a recent study using human liver microsomes, AFQ₁ comprised 70-90% of soluble, detectable metabolites and 15-20% of the added AFB₁ (Moss and Neal, 1985). It is thought that AFQ₁ is metabolized further to water-soluble conjugates such as glucuronides, which are difficult to detect (Wei et al., 1985).

The formation of AFQ₁ is considered to be a detoxifying step, although the fate of the AFQ₁ produced is unclear (Moss and Neal, 1985). It was not detected in the urine of monkeys dosed with AFB₁ (Dalezois and Wogan, 1972; Dalezois et al., 1973) nor in the urine of humans ingesting aflatoxin-contaminated diet (Campbell et al., 1970). In both instances, however, the analytical methods employed would have detected only unconjugated derivatives, because the chloroform soluble fraction of the urine was examined, and it is possible therefore that conjugated AFQ₁ may, in fact, have been present.

Marmoset monkey is the animal of choice because preliminary in vitro studies in this laboratory using the liver microsomal fraction from the animals showed that the major metabolite obtained (AFQ₁) is similar to that obtained from human liver microsomes (Moss and Neal, 1985). AFQ₁ accounted for at least 30% of metabolites produced from both marmoset monkey and man (G.E. Neal, D.J. Judah and E.J. Moss, personal communication).
In competitive ELISA using polyclonal rabbit anti-AFB\textsubscript{1} serum, AFQ\textsubscript{1} is detected with a sensitivity 52.2 times less than that of AFB\textsubscript{1} (Table 2.1). It is most likely that in both human and monkey, AFQ\textsubscript{1} is excreted mainly in the conjugated form. Standards of these AFQ\textsubscript{1} conjugates are not available to be tested, but it is expected that they would inhibit even less effectively than AFQ\textsubscript{1}. As stated in Section 2.4.3, it was found that modification in the cyclopentenone ring (AFQ\textsubscript{1}) substantially decreased the sensitivity of detection by the polyclonal antibody used in this study and so additional modification at this position by the conjugating moiety would further reduce the sensitivity. Due to the problems being outlined, it was necessary to validate the methods developed for monitoring the level of aflatoxin exposure in man using the marmoset monkey.

The objective of this study was to use marmoset urine samples to validate the methods proposed for use in monitoring aflatoxin exposure in humans. Male and female marmosets were treated with \textsuperscript{14}C-AFB\textsubscript{1} to avoid the complication due to exchange when \textsuperscript{3}H-AFB\textsubscript{1} was used (see Section 2.4.6.1). Marmoset urine samples which contained a spectrum of metabolites assumed to be representative of that expected to be found in human urine samples were collected. The AFB\textsubscript{1} equivalent concentrations in the samples were determined by using both radioactive counting and the ELISA technique.
3.2 MATERIAL AND METHODS

3.2.1 Animals

One male and one female marmoset (*Saguinus oedipomidas*), 2-year old, were made available by the Ministry of Defence, Porton Down, Salisbury, and the animal procedures were carried out by personnel from that unit at the Porton Down site. The body weights of the male and female marmosets were 305 and 350 g respectively. They were placed in separate metabolism cages in which $^{14}$C-CO$_2$ could be trapped. Animals were allowed to have food and water *ad libitum*. Urine samples were collected over 24 h before dosing the animals with $^{14}$C-AFB$_1$ to serve as control samples.

3.2.2 Chemicals

Chemicals used in this study were of analytical or HPLC grade.

3.2.3 Preparation of $^{14}$C-AFB$_1$ solution for injection

$^{14}$C-AFB$_1$ for injection was prepared from a stock unlabelled AFB$_1$ solution (2 mg AFB$_1$ in 1 ml DMSO) and stock $^{14}$C-AFB$_1$ (120 mCi/m mol in methanol). Stock $^{14}$C-AFB$_1$ (1 ml methanol containing 10 µCi) was blown with nitrogen gas to remove methanol and then redissolved in DMSO (380 µl). The injection solution was prepared by adding 130 µl of the unlabelled AFB$_1$ solution in DMSO (0.26 mg AFB$_1$) to the 380
μl of $^{14}$C-AFB$_1$ in DMSO (10 μCi). The actual specific activity of $^{14}$C-AFB$_1$ injection solution was determined directly using radioactive counting of the AFB$_1$ peak from the HPLC and the AFB$_1$ quantitated from the peak area of the UV absorption. It was found that the specific activity of $^{14}$C-AFB$_1$ injection solution was 115.4 dpm/μg AFB$_1$.

3.2.4 Dosing of the marmoset monkeys

Animals were injected with $^{14}$C-AFB$_1$ intraperitoneally. The male marmoset was injected with 200 μl DMSO solution containing 204 μg $^{14}$C-AFB$_1$ (2.3 x $10^7$ dpm) while in the case of the female, the injection volume was 250 μl containing 255 μg $^{14}$C-AFB$_1$ (2.9 x $10^7$ dpm). So the dose of $^{14}$C-AFB$_1$ received by male and female was 0.67 and 0.73 mg AFB$_1$/kg respectively. Two urine samples were then collected over the following 2 days; the first samples over the period 0-24 h (24 h sample), and the second over the period 24-48 h (48 h sample). Animals were sacrificed 48 h after dosing. Urine volumes were measured and the samples were divided into aliquots (1 ml) and stored at -70°C until used. Creatinine concentration was analysed as described in Section 2.2.3.7.

3.3 EXPERIMENTAL PROTOCOLS

3.3.1 An examination of $^{14}$C-AFB$_1$ metabolites excreted in marmoset urine samples

Aliquots (20 μl) of 24 and 48 h urine samples from
both male and female marmosets were injected into the HPLC in duplicate. The fractions (1 min) from the HPLC were collected and radioactivity in each fraction was counted.

3.3.2 Preliminary analysis of aflatoxins in marmoset urine samples

The urine samples from both male and female marmosets collected as 24 and 48 h samples after dosing with $^{14}$C-AFB$_1$ were analysed without any 'clean-up' procedures for the presence of aflatoxins both by radioactive monitoring and by competitive ELISA (see Section 2.2.3.1). Aliquots (50 μl) of each sample was counted for radioactivity and the estimated aflatoxin concentration was calculated from specific activity. In addition, the control urine samples (i.e. before dosing with $^{14}$C-AFB$_1$) were included in the study to determine the apparent AFB$_1$ content obtained in the ELISA. These samples had to be diluted in order to be in the inhibition range required for evaluation by the competitive ELISA curve. The aflatoxin concentrations are expressed as ng AFB$_1$ equivalent because AFB$_1$ was used in the construction of the standard curve.

3.3.3 Validation of the methods used in the 'clean-up' procedures of urine samples for aflatoxin analysis

3.3.3.1 Sep-Pak C$_{18}$ cartridge and immunoaffinity column

Urine samples (1 ml) from 24 and 48 h of both male and female marmosets were loaded onto Sep-Pak C$_{18}$ cartridges,
washed with water (3 ml) and eluted with 100% methanol (5 ml). Aliquots (500 μl) from each fraction were counted for radioactivity and the distribution of radioactivity between the fractions was calculated.

For 24 h urine sample, aliquots (1 ml) of the washing fractions and samples (50 μl) of the methanol fractions from the cartridges, diluted with PBS to 5 ml, were loaded on the affinity columns (1 ml bed volume). The columns were washed with PBS (15 ml) and the retained materials eluted with 85% methanol in PBS (15 ml). All samples from each fraction were evaporated down to 1 ml before counting for radioactivity. The distribution of radioactivity in each fraction was calculated.

For 48 h urine samples, aliquots (2.5 ml) of the washing fractions and samples (1 ml) of the methanol fractions from the Sep-Pak cartridges, diluted with PBS to 5 ml, were loaded on the affinity columns. The columns were washed and eluted in the same way as 24 h urine samples. Samples were evaporated to 1 ml before counting for radioactivity.

3.3.3.2 Radiolabelled metabolites of 14C-AFB1 present in fractions obtained from the affinity column using 24 h male marmoset urine sample

Male marmoset 24 h urine samples (1 ml) were loaded onto Sep-Pak C18 cartridges, washed with water (3 ml) and the retained materials were eluted with methanol (5 ml). In view of the high concentration of aflatoxin metabolites present in these methanol samples, as seen from their
radioactive content, it was not necessary to evaporate and reconstitute them in PBS. Instead, an aliquot (500 µl) of the methanol eluate was diluted with PBS (45 ml) which ensured that the affinity column was not overloaded with either methanol or aflatoxins. The radioactivity present in the fractions obtained from the affinity column was checked to ensure that there were sufficient counts available for the subsequent HPLC assay. It was found to be necessary to pool the fractions obtained from 8 individual loadings onto 8 affinity columns, using aliquots (5 ml) of the diluted methanol eluate from the Sep-Pak cartridge, to achieve the level of radioactivity required for the HPLC. The unretained and the washing fractions were bulked, giving a total volume of 160 ml, while the total volume of methanol eluate was 120 ml. Because of their large volumes and low aflatoxin concentrations, the washing fraction was freeze dried and redissolved in water (10 ml). The excess salt from PBS was removed by passing the sample onto a Sep-Pak cartridge and the methanol eluate was collected. The volume of this sample (5 ml) was reduced to 100 µl using a Speedvac Concentrator at 40°C before aliquots (20 µl) were injected into the HPLC.

For the methanol fraction from the affinity column, the sample was evaporated to dryness using a rotary evaporator at room temperature and then redissolved in water (10 ml). The sample was passed through a Sep-Pak cartridge and the methanol eluate (5 ml) was collected and the volume was reduced to 100 µl before injecting aliquots.
(20 µl) into the HPLC. Fractions (1 min/1.2 ml) from both the affinity column samples were collected from the HPLC and were counted for their radioactivity.

3.3.3.3 **Attempt to remove interfering substances from control marmoset urine samples**

Control marmoset urine samples (1 ml) from both male and female were subjected to the 'clean-up' procedures developed for using with human urine samples. The protocol was exactly the same as that described in Section 2.3.5.1 using human samples. The apparent AFB₁ concentrations in both the washing and methanol eluate fractions from the affinity column were analysed in triplicate by ELISA using 6 wells per assay (see Section 2.2.3.1).

3.3.3.4 **Extraction of 24 h marmoset urine samples and analysis of aflatoxin levels by ELISA**

Aliquots (600 µl) of 24 h urine samples from both male and female marmosets were made up to 2 ml with 0.2 M sodium acetate (pH 5.0) before being extracted with 2 volumes of chloroform. The samples were shaken vigorously for a couple of minutes before centrifugation at 1000 x g for 10 min at 4°C to separate chloroform and aqueous phases. The aqueous fractions were freeze dried, while the chloroform fractions were evaporated to dryness using a rotary evaporator. The samples were then redissolved in 50% methanol/water (100 µl), and aliquots (20 µl) were injected into the HPLC. Fractions (1 min/1.2 ml) were collected, and samples (600 µl) were taken to count for radioactivity.
while the remaining samples (600 μl) were evaporated to dryness, using a Speedvac Concentrator, before reconstitution in PBS (1 ml). Fractions 2 to 13 from the HPLC were analysed for AFB₁ equivalent concentrations using the ELISA.

3.3.4 Analysis of aflatoxin levels in marmoset urine samples using the 'clean-up' procedures

Urine samples (200 μl) from 24 and 48 h of both male and female marmosets treated with ¹⁴C-AFB₁ were loaded onto Sep-Pak C₁₈ cartridges, washed with water (3 ml), and the retained materials eluted with 100% methanol (5 ml). Aliquots of 150 and 500 μl were taken from the methanol eluates of 24 and 48 h urine samples respectively and diluted to 5 ml with PBS before loading onto the immunoaffinity columns. The general procedures for affinity column were followed (see Section 2.2.3.3). Methanol eluate fractions from the columns were collected, and 8 out of 15 ml of these samples were evaporated to 0.5 ml. For 24 h urine samples, they were further diluted 5 times with PBS while 48 h samples were used directly in the ELISA (see Section 2.2.3.1). AFB₁ equivalent levels were analysed with six and three replicates for 24 and 48 h urine samples respectively using 6 wells per assay.

3.4 RESULTS AND DISCUSSION

3.4.1 Urine volumes and creatinine concentrations

Urine samples from male and female marmosets were
collected for 24 h before and for 2 days after dosing with $^{14}$C-AFB$_1$. The volumes of male urine were 10 and 9 ml for the test 24 and 48 h samples respectively while those of the female were 33 and 26 ml (Table 3.1). Creatinine concentrations are also given in the Table 3.1.

The volume of urine samples from the male were less than those of the female even though the animals were allowed free access to water. Creatinine concentrations in male urine samples were higher than those of female. These may reflect the volume of urine excreted. This difference in urine volumes is one factor that has to be considered when calculating total excretion of aflatoxins in urine, particularly in those cases where the total urine volume is not known. Some correction for differences in total volume of urine excreted can be applied by expressing the concentration of aflatoxins in relation to the creatinine concentration, which acts as an internal reference in urine; i.e. the concentration of aflatoxins can be expressed as ng aflatoxin/mg creatinine. Since the purpose of this study was to use the marmoset urine samples, which contained a variety of aflatoxin metabolites, to validate the methods developed to monitor aflatoxin exposure in humans, most of the data in this chapter were still expressed as ng AFB$_1$ equivalent/ml.

3.4.2. Pattern of $^{14}$C-AFB$_1$ metabolites excreted in marmoset urine samples

When the distribution of radiolabelled aflatoxin metabolites in 24 h urine samples from both male and female
Table 3.1

Marmoset urine volumes and creatinine concentrations after dosing with $^{14}$C-\(\text{AFB}_{1}\)

<table>
<thead>
<tr>
<th>Urine sample</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>volume (ml)</td>
<td>creatinine (mg/ml)</td>
</tr>
<tr>
<td>control</td>
<td>NM*</td>
<td>0.44</td>
</tr>
<tr>
<td>24 h</td>
<td>10</td>
<td>0.90</td>
</tr>
<tr>
<td>48 h</td>
<td>9</td>
<td>1.28</td>
</tr>
</tbody>
</table>

* not measured
marmosets was compared (Figure 3.1A), the pattern of distribution was similar in that there were major peaks in fraction 9 of the HPLC in both male and female. However, in the male sample there was an additional minor peak in fraction 5. In the 48 h urine samples, there were at least 3 major peaks which were in fractions 6, 9 and 11 (Figure 3.1B).

The results also indicate that there was no significant difference in the pattern of metabolites excreted in both sexes at the same time point. However, the major feature was the differences in the patterns of radioactivity between the 24 and 48 h samples for both the male and female. These results are not conclusive because only one male and one female marmosets were used, but in order to validate the methods used in monitoring exposure to aflatoxins, these two animals would be sufficient to serve the purpose. The identification of all the metabolites excreted in the urine samples was beyond the scope of this thesis.

The prominent feature of the 24 h urine sample was the major radioactive peak in fraction 9, which, although still present in the 48 h sample, was much reduced. The retention time of this peak is consistent with AFM₁ and this is consistent with published data for urine excretion of AFM₁ shortly after exposure of human to AFB₁ (Campbell et al., 1970; Hu et al., 1983; Hu et al., 1984; Zhu et al., 1987). This again supports the validity of using marmoset as a model for human exposure to aflatoxins.
Figure 3.1  The distribution of radiolabelled aflatoxin metabolites in HPLC fractions of male and female marmoset urine samples

* Results are percentages in each fraction of the total radioactivity recovered during the HPLC assays
Aflatoxin levels in marmoset urine samples analysed without any 'clean-up' procedures

From the preliminary study using presumed uncontaminated human urine samples, these samples could not be used directly in the competitive ELISA (see Section 2.4.4). Similar results were observed using undiluted marmoset urine samples obtained prior to dosing with $^{14}C$-AFB$_1$. Different dilutions of the samples were tried. It was found that for these uncontaminated (control) urine samples, the appropriate dilution for ELISA with approximately 50% inhibition was 1:25. The apparent AFB$_1$ in both male and female urine samples were less than 1 ng/ml.

For the 24 and 48 h urine samples, the appropriate dilutions for the ELISA were 1:5000 and 1:2000 respectively. This was equivalent to an AFB$_1$ concentration for male and female 24 h urine samples of 420 and 675 ng AFB$_1$/ml while those of 48 h samples were 64 and 49 ng AFB$_1$/ml (Table 3.2).

By radioactive monitoring, the counts in 50 μl of male and female 24 h samples were 6483 and 7991 dpm respectively. Using the specific activity of 115.4 dpm/ng, these counts corresponded to aflatoxin concentrations of 1127 and 1384 ng AFB$_1$/ml respectively, or total aflatoxin contents of 11.26 and 47.75 μg. For the 48 h samples, the counts in 50 μl for male and female were 774 and 443 dpm which corresponded to concentrations of 129 and 77 ng
Table 3.2

A comparison on the concentrations of aflatoxins excreted in marmoset 24 and 48h urine samples assayed by radioactivity and ELISA

<table>
<thead>
<tr>
<th></th>
<th>AFB₁ equivalent (ng/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MALE</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>ELISA</td>
<td>420</td>
<td>64</td>
<td>675</td>
</tr>
<tr>
<td>Estimated by</td>
<td>1127</td>
<td>129</td>
<td>1384</td>
</tr>
<tr>
<td>radioactivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA as % of</td>
<td>37.3</td>
<td>49.6</td>
<td>48.7</td>
</tr>
<tr>
<td>amount</td>
<td>estimated by radioactivity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
AFB\textsubscript{1}/ml or total excretions of 1.16 and 1.99 \(\mu\)g respectively (Table 3.2).

The results given by both radioactive counting and ELISA indicate that most of the aflatoxin excretion by marmoset monkeys treated with \(^{14}\text{C}-\text{AFB}_1\) takes place in the first 24 h after dosing. About 50\% of the estimated aflatoxins excreted, calculated from radioactivity, was detected by ELISA (Table 3.2). This discrepancy may be due to the fact that a major aflatoxin metabolite in marmosets is \text{AFQ}_1 (G.E. Neal, D.J. Judah and E.J. Moss, personal communication). The antibody used in the ELISA can recognize \text{AFQ}_1 52.2 times less sensitivity than AFB\textsubscript{1} (see Table 2.1), hence, at the same percentage of inhibition from competitive ELISA, \text{AFQ}_1 will give a lower reading when expressed as AFB\textsubscript{1} equivalent units. With the additional fact that \text{AFQ}_1 may be excreted in conjugated forms, the recognition by the antibody will be even lower than \text{AFQ}_1 itself. With these points in mind, 50\% of the estimated AFB\textsubscript{1} from radioactivity detectable by ELISA is in the acceptable range.

When the creatinine concentration was taken into consideration, the ng AFB\textsubscript{1} equivalent(from ELISA)/mg creatinine for male and female, 24 and 48 h urine samples were 468, 1288, 50 and 127 respectively. The female excreted approximately 2.6 times more aflatoxins than the male. When the percentages of the doses of aflatoxin excreted in urine samples were calculated from radioactive assay, the percentages of aflatoxin excretion in the 24 h
samples of the male and female were 5.52 and 17.94% respectively, while those obtained in the 48 h samples were 0.57 and 0.78%. The differences in the levels of aflatoxins in urine samples may be due to a sex-related difference in metabolism. The different sexes may excrete different metabolites which are detected with differing sensitivities by the antibody. The metabolite profile in both sexes may not be the same. This topic will be dealt with in more detail in Section 3.4.4.4.

3.4.4. Validation of the methods used in the 'clean-up' procedures of urine samples for aflatoxin analysis

3.4.4.1 Capacities of the Sep-Pak C18 cartridge and immunoaffinity column

When samples (1 ml) of 24 and 48 h urines of both male and female marmosets were loaded onto Sep-Pak cartridges, more than 95% of the radiolabelled metabolites were retained on the cartridges and subsequently eluted with 100% methanol (Table 3.3).

When the washing fractions from the Sep-Pak cartridges were loaded onto the affinity columns, most of the radioactivity failed to bind. Less than 8% of the radiolabel was found in the methanol eluates from the columns. On the other hand, when the methanol eluates from the Sep-Pak cartridge were loaded onto the affinity columns, more than 50% of the radiolabel was bound onto the columns and subsequently eluted with 85% methanol (Table 3.4). The remainder of the radioactivity was evenly distributed.
Table 3.3

The distribution of radioactivity in fractions from Sep-Pak C\textsubscript{18} cartridge after loading with 24 and 48 h marmoset urine samples

<table>
<thead>
<tr>
<th>Fraction</th>
<th>MALE radioactivity (dpm)</th>
<th>% total radioactivity*</th>
<th>FEMALE radioactivity (dpm)</th>
<th>% total radioactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unretrained</td>
<td>1576</td>
<td>1.2</td>
<td>408</td>
<td>0.3</td>
</tr>
<tr>
<td>washing</td>
<td>2144</td>
<td>1.7</td>
<td>3328</td>
<td>2.9</td>
</tr>
<tr>
<td>methanol</td>
<td>124,920</td>
<td>97.1</td>
<td>111,760</td>
<td>96.8</td>
</tr>
<tr>
<td>48 h urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unretrained</td>
<td>48</td>
<td>0.4</td>
<td>130</td>
<td>1.4</td>
</tr>
<tr>
<td>washing</td>
<td>288</td>
<td>2.1</td>
<td>320</td>
<td>3.5</td>
</tr>
<tr>
<td>methanol</td>
<td>13,360</td>
<td>97.5</td>
<td>8,680</td>
<td>95.1</td>
</tr>
</tbody>
</table>

* Expressed as the percentage of radioactivity in each fraction when compared with the total counts present in all fractions.
Table 3.4

The distribution of radioactivity in affinity column analyses obtained by fractionation of samples after treatment of 24 and 48 h marmoset urine by Sep-Pak cartridge

<table>
<thead>
<tr>
<th>Affinity column fraction</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>washing&lt;sup&gt;b&lt;/sup&gt;</td>
<td>methanol&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>dpm</td>
<td>%</td>
</tr>
<tr>
<td>24 h urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unretained</td>
<td>130</td>
<td>20.0</td>
</tr>
<tr>
<td>washing</td>
<td>495</td>
<td>76.3</td>
</tr>
<tr>
<td>methanol</td>
<td>24</td>
<td>3.7</td>
</tr>
<tr>
<td>48 h urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unretained</td>
<td>107</td>
<td>48.9</td>
</tr>
<tr>
<td>washing</td>
<td>95</td>
<td>43.4</td>
</tr>
<tr>
<td>methanol</td>
<td>17</td>
<td>7.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Urine samples $\rightarrow$ Sep-Pak C<sub>18</sub> cartridge

<sup>b</sup> Fractions from Sep-Pak C<sub>18</sub> cartridge
between the unretained and the washing fractions of the affinity columns.

From these experiments, the 'clean-up' procedures using Sep-Pak C\textsubscript{18} cartridges did not lead to significant loss of radiolabelled aflatoxin metabolites from the urine samples because more than 95\% of the label was recovered from the cartridges in the methanol eluates. It is interesting to note that the reverse phase Sep-Pak C\textsubscript{18} cartridge could retain not only the non-polar aflatoxin metabolites but also the polar metabolites of AFB\textsubscript{1} which result from secondary metabolism. To further verify that the washing fractions from the Sep-Pak cartridges did not contain any significant amount of aflatoxin polar metabolites, these fractions were loaded onto the affinity columns. More than 92\% of the label present in these fractions (which comprised 5\% of the original radioactivity) was not recognized by the antibody. Therefore, the washing fractions from the Sep-Pak cartridge could be discarded and only the methanol eluates used for further analyses.

When the methanol eluate fractions from the Sep-Pak cartridges were subjected to the affinity columns, 50-60\% of the radiolabelled metabolites was retained and subsequently eluted from the columns using 85\% methanol. The rest of the radioactivity was in the unretained and the washing fractions of the affinity columns. The nature of the radioactive compounds present in these unretained and washing fractions was further examined by the HPLC.
In the experiments using 24 and 48 h urine samples from marmosets treated with $^{14}$C-AFB$_1$, the average recoveries of the radioactivity in the methanol eluate fractions of the Sep-Pak cartridges and the affinity columns were 96.6 and 54.9% respectively. Therefore, the overall recovery of the whole 'clean-up' procedure using marmoset urine containing a variety of aflatoxin metabolites was 53% which compared favourably with the 71% obtained from $^3$H-AFB$_1$ spiked in presumed uncontaminated human urine samples (see Section 2.4.6.5).

Using this information, one should be aware that by analysing aflatoxins in urine samples using ELISA technique with the pretreatment of the samples with Sep-Pak C$_{18}$ cartridge and immunoaffinity column, only 50% of the actual value would be observed. Therefore, the levels obtained in terms of AFB$_1$ equivalent in the samples would be underestimated.

3.4.4.2 The HPLC distribution of aflatoxin metabolites from the affinity column fractions

The distribution of radioactivity in the fractions obtained by HPLC of the washing and the methanol eluate fractions from the affinity column are shown in Figure 3.2. The radiolabelled metabolites in the washing fractions were more polar than those in the methanol eluates from the affinity column. However, the major peak present in the methanol eluate corresponded with the major peak of radioactivity obtained when the urine sample was directly
Figure 3.2 The HPLC distribution of radiolabelled aflatoxin metabolites obtained by chromatographing the affinity column fractions

* Results are percentages in each fraction of the total radioactivity recovered from either the washing or methanol fractions
injected into the HPLC (Figure 3.1A). The probable identity of this metabolite as AFM₁ has already been referred to (see Section 3.4.2). The results also implied that the antibody used in the immunoaffinity column failed to recognize or recognized the polar metabolites with only low affinity. Since the radioactive compound used in these experiments was ¹⁴C-AFB₁, in place of ³H-AFB₁ as used in the earlier experiments (see Chapter 2), there would not be any isotope exchange and the radioactivity in the polar region of the washing fraction from the affinity column should, therefore, be associated with actual aflatoxin metabolites. However, it is possible that ¹⁴C from the aflatoxins could be reincorporated into non-aflatoxin metabolites, e.g. O-demethylation of AFB₁ leads to the formation of the phenol AFP₁. It is not known in what form the methyl group is released but the label associated with ¹⁴CH₃ is believed to be largely expired as ¹⁴CO₂. However, it is possible that some of this one carbon unit is reincorporated and is accounted for by some of the radiolabel not recognized by the affinity column used in this study. But, despite all of the possible complications, the recovery of the radiolabelled materials after loading on the immunoaffinity column was 50% as shown in Table 3.4, which is an acceptable level for the monitoring assays proposed.

3.4.4.3 Apparent AFB₁ concentrations in control marmoset urine samples

The ELISA determinations (Table 3.5) showed that in both male and female marmoset urine samples, the apparent
Table 3.5

Apparent AFB₁ concentrations obtained by ELISA in control marmoset urine samples following fractionation on affinity columns

<table>
<thead>
<tr>
<th>Assay No.</th>
<th>Apparent AFB₁ (ng/ml)</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>washing</td>
<td>methanol</td>
</tr>
<tr>
<td>1</td>
<td>6.4</td>
<td>0.36</td>
<td>9.0</td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
<td>0.39</td>
<td>9.6</td>
</tr>
<tr>
<td>3</td>
<td>6.6</td>
<td>0.56</td>
<td>5.4</td>
</tr>
<tr>
<td>mean</td>
<td>7.0</td>
<td>0.44</td>
<td>8.0</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.9</td>
<td>0.11</td>
<td>2.3</td>
</tr>
</tbody>
</table>
AFB₁ concentrations were higher in the washing fractions than in the methanol fractions from the affinity column. The average level in the washing fractions was 7-8 ng/ml, while in the methanol fractions, it was less than 0.8 ng/ml. Since the samples being used were the control samples (from untreated animals), it was assumed that the apparent AFB₁ level was due to either the background level of the system or interfering substances present in the urine samples. These results, using control marmoset urine samples, were in agreement with those of uncontaminated human urine samples (Table 2.15) in which the interfering substances or UALS were in the washing fractions obtained from the affinity columns. Therefore, based on the results of these two systems, it is apparent that the methanol eluate fraction from the affinity column represents the appropriate fraction for subsequent analysis using ELISA. Although the unretained and washing fractions from the affinity column contain the same level of radiolabelled metabolites (approximately 50%) as that present in the methanol eluate, the presence of the inhibitory substances in the unretained and washing fractions means that analysing these fractions by ELISA, for samples of unknown contamination with aflatoxins, would be difficult to interpret.

3.4.4.4 A comparison of the estimations by ELISA and radioactivity of the aflatoxin present in marmoset urine samples extracted by chloroform

In order to further clarify the capacity of the antibody to detect aflatoxin metabolites, 24 h urine
samples from both male and female marmosets were extracted with chloroform to separate polar and non-polar metabolites of aflatoxin. The patterns of metabolites detected were compared using both radioactive counting and ELISA techniques.

From radioactive counting, the counts in the chloroform and aqueous phases of male urine sample were 1304 and 1900 dpm respectively, while those of female were 929 and 3173 dpm. Most of the radioactivity was in the aqueous phase, and the ratio of radioactivity in chloroform:aqueous phase in male and female were 1:1.5 and 1:3.4 respectively. It is suggested from these experiments, and the results shown in Section 3.4.3, that the pattern of metabolites in marmoset urine samples 24 h after dosing with \(^{14}\text{C-AFB}_1\) was different in the male and female marmoset monkeys. The female animal excreted more aflatoxin metabolites in the urine and a higher amount of polar metabolites when compared to the male sample. But these results were not conclusive since they come from only one animal of each sex. However, from Figure 3.1A, the patterns of radiolabelled metabolites obtained when urine samples from both sexes were injected into the HPLC were similar. In these HPLC analyses of total urine samples the greater excretion of polar metabolites by the female marmoset was not apparent (Figure 3.1A). However, when the chloroform and aqueous fractions of these urine samples were subjected to HPLC individually, the greater content of polar aflatoxin metabolites in the female urine sample was
clear (see also Figure 3.3A). Therefore, in order to have more conclusive evidence regarding metabolism of aflatoxins in different sexes of marmoset monkey, more animals would be needed. However, this was outside the scope of this study.

The distribution of radiolabelled metabolites in the fractions from HPLC (Figure 3.3) showed that in chloroform phases of both the male and female samples, there was one major peak in fraction 9 and a minor peak in fraction 11. In the aqueous phases, the major peaks were in fractions 2 and 7 but radioactivity was also distributed in fractions 5 to 11 without evidence of individual peaks. The pattern of distribution in both sexes was similar.

In parallel with the aflatoxin estimation of the HPLC fractions by radioactivity, aliquots of fractions 2-13 were also analysed by ELISA. The results given in Figure 3.4 showed that the total aflatoxin metabolites (expressed as AFB₁ equivalent/fraction) in the chloroform phases of urine samples from both sexes were appreciably greater than the values obtained in the aqueous phases. This result contrasted with the result from the radioactive counting from the HPLC. It was evident from the ELISA analysis of the HPLC fractions that good correlation between the ELISA and radioactive counting existed for the chloroform phase. This would be consistent with this fraction containing primary metabolites of aflatoxin, in which the epitope would have undergone the least modification compared with secondary conjugated metabolites. The peak observed in
Figure 3.3 The distribution of radiolabelled aflatoxin metabolites in chloroform and aqueous phases of male and female marmoset urine samples fractionated by HPLC.
Figure 3.4 The levels of AFB$_1$ equivalent by ELISA in the HPLC fractions of chloroform and aqueous phases obtained from male and female marmoset urine samples

A. Female

B. Male

- chloroform
- aqueous

Fraction no.
fraction 9 for both ELISA and radioactivity is consistent with this fraction being largely comprised of AFM₁, which was recognized as sensitively as AFB₁ (see Table 2.1). However, in the case of the aqueous phase metabolites, it is evident that the ELISA did not recognize these as sensitively as the radioactivity and, furthermore, for the most polar metabolites in fractions 1-4, the ELISA failed to detect the presence of any aflatoxin metabolites. These HPLC experiments were carried out using total urine samples and, therefore, if similar phenomena applied to the prepurification by immunoaffinity columns, it is likely that these polar metabolites would be lost during the 'clean-up' procedures. This is proved to be the case as demonstrated in Figure 3.2, where the polar metabolites are removed in the washing fraction of the affinity column, and only the more non-polar metabolites are retained and subsequently eluted with methanol.

3.4.5 Aflatoxin levels in marmoset urine samples after the 'clean-up' procedures

From the results of the previous experiments, it was concluded that the amounts of AFB₁ loaded onto the affinity column should not exceed 20 ng in 1 ml PBS or 5 ng in 1 ml urine samples (see Section 2.4.6.4). In order not to overload the column, 200 μl aliquots of the original marmoset urine samples were used. This level of loading was based on the results of the radioactivity assays shown in Table 3.2, and the dilution being calculated to result
in appropriate final amounts of aflatoxin loaded onto the column.

The results of aflatoxin levels in marmoset urine samples (Table 3.6) were expressed as ng AFB1 equivalent/ml sample. The concentrations of AFB1 equivalents in the original samples were calculated from the volumes being used in each step. The concentrations in the male 24 and 48 h urine samples were 281.7 and 40.5 ng/ml, while those of the female were 343.4 and 35.5 ng/ml respectively (Table 3.6).

The data indicate that the majority of aflatoxins in the urine samples were excreted in the first 24 h after dosing. When these results were compared with those obtained without any 'clean-up' procedures (Table 3.2), the values obtained using ELISA with the 'clean-up' procedures were approximately 36% lower. The explanation for these differences in AFB1 equivalents may be due to the fact that when the urine samples were analysed directly in ELISA, interfering substances (UALS) would react with the antibody giving a high level of AFB1 equivalents. Furthermore from the previous results (Section 3.4.4.1), the overall recovery from the 'clean-up' procedures assayed by radioactivity was 53%. In view of these complicating factors, the results obtained were considered to be in the acceptable range.

3.5 SUMMARY

The results obtained in this chapter indicate that for
Table 3.6

Calculation of aflatoxins present in 1 ml of original marmoset urine samples 24 and 48 h after treatment with \(^{14}\)C-\(\text{AFB}_1\)\(^*\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>ELISA result of samples (mean ± S.D.)</td>
<td>0.18 ± 0.07</td>
<td>0.43 ± 0.29</td>
</tr>
<tr>
<td>Concentration in 1 ml original sample</td>
<td>281.7</td>
<td>40.5</td>
</tr>
</tbody>
</table>

* * *

Sep-Pak methanol eluate

Volume loaded on affinity column

Affinity column methanol eluate

Volume taken for evaporation

Reconstitution volume in PBS

Volume taken for ELISA

24 h

5 ml

\(\rightarrow\) 150 \(\mu\)l

\(\rightarrow\) 15 ml

\(\rightarrow\) 8 ml

\(\rightarrow\) 1 ml

\(\rightarrow\) 200 \(\mu\)l (following 5 x dilution)

48 h

5 ml

\(\rightarrow\) 500 \(\mu\)l

\(\rightarrow\) 15 ml

\(\rightarrow\) 8 ml

\(\rightarrow\) 1 ml

\(\rightarrow\) 200 \(\mu\)l (undiluted)
the system developed, it is possible to detect aflatoxin metabolites in marmoset urine samples. In the 'clean-up' procedure the Sep-Pak C_{18} cartridge retained most of the metabolites excreted in the urine samples with more than 96% recovery of radioactivity. The reverse phase Sep-Pak C_{18} cartridge could retain not only the non-polar but also the polar metabolites of AFB_1. The capacity of the Sep-Pak cartridge in this study was not significantly different from that used with ^3H-AFB_1-spiked urine samples. The immunoaffinity column retained 50-60% of the radioabelled metabolites which subsequently could be eluted with methanol. The overall recovery of the 'clean-up' procedures was 53% compared to 71% when ^3H-AFB_1 was being used in uncontaminated human urine samples. The lower percentage recovery is in part, at least, due to the fact that the sensitivity of the antibody used for some metabolites and their conjugates is low, especially with AFQ_1 and its conjugate which are the most likely major metabolites excreted in marmoset urine samples. The antibody also has low sensitivity in recognizing all the polar metabolites in chloroform-insoluble fractions. However, if all these points are borne in mind, it is possible to conclude that the levels of aflatoxins obtained in the samples in term of AFB_1 equivalents using ELISA with these 'clean-up' procedures will be underestimated.
CHAPTER 4

Urinary Excretion of Aflatoxins in Hamsters
Infected with Liver Fluke,

Opisthorchis viverrini
Differences in liver cancer incidence have been reported in different parts of the world (Parkin et al., 1984). This is believed to be due to the presence or absence of environmental factors responsible for liver carcinogenesis. In Thailand, liver cancer is the most common fatal neoplasm and is ranked third as a cause of death (Srivatanakul et al., 1988). Liver cancer was the cancer with the highest incidence recorded by the Cancer Registry in 1980-1982 for males and third for females, after cervical and breast cancers. According to histological diagnoses, hepatocellular carcinoma was found more frequently than cholangiocarcinoma in both males and females. The geographical variations in the incidence of hepatocellular carcinoma in Thailand are rather small, in contrast to the frequency of cholangiocarcinoma which may vary as much as 14-fold between regions (Srivatanakul et al., 1988). The areas with high incidence of the latter cancer are also found to be heavily infested by the liver fluke, *Opisthorchis viverrini*.

Opisthorchiasis, a disease caused by the liver fluke, *Opisthorchis viverrini*, is endemic particular in Northeast Thailand (Viranuvatti and Stitnimankarn, 1972; Upatham et al., 1982). In 1980-1982, the prevalence in the North, Northeast, Centre and South of Thailand was 5.59, 34.60, 6.34 and 0.01% respectively (Harinasuta and Harinasuta, 1984). The clinical manifestations of the infection range
from asymptomatic to severe malnourishment, cirrhosis of the liver and neoplastic changes of the biliary system of the liver (Harinasuta et al., 1984; Upatham et al., 1984). The disease is considered to be a major public health problem in Thailand, since about 7 million Thai people are suffering from this disease according to a survey carried out a few years ago (Preuksaraj, 1984). In some endemic areas, prevalence reaches at least 90% of the population, and the disease produces clinical symptoms which can be correlated with infection intensity (Upatham et al., 1985).

Development of a complete life cycle of liver fluke requires two intermediate hosts. The first intermediate hosts are snails (Bithynia tentaculata, B. goniomphalus, B. funiculata and B. siamensis). The second intermediate hosts are freshwater fish that contain encysted metacercariae in their gills, flesh and skin. A wide variety of fish, e.g. Puritus orphoides, Hampala dispar and Cycloheilichthys siaja may be infected (Vichasri et al., 1982). The adult fluke is a common parasite of the dog, cat, fox and pig. Man is an accidental host (Harinasuta and Harinasuta, 1984; Sornmani, 1987). The parasites live in the distal biliary passage of the definitive hosts without invading the liver parenchyma, and occasionally in the pancreatic ducts and gall bladder. The worm contains both testis and ovary, so self-fertilization is the common means of reproduction. The ova are carried down the common bile duct and pass through the bowel (Viranuvatti and Stitnimankarn, 1972).
The life cycle of the fluke starts from eggs passing out in faeces (Figure 4.1). On reaching the water the eggs are eaten by snails, the first intermediate host. In the snail, the miracidia hatch and develop further to the stages of sporocysts, rediae and cercariae in six to eight weeks. The cercariae then leave the snail, and when the mature cercariae come into contact with a suitable species of fish, especially cyprinoid fish, they attach themselves to the fish scales, lose their tails, penetrate the tissue of the fish and encyst. Encysted cercariae mature in about six weeks after ingestion by the natural host or the accidental host which is man. Excystation takes place in the duodenum by the action of gastric and duodenal juices. The free metacercariae pass through the ampulla of Vater to the bile ducts, pancreatic ducts, gall bladder or bile duct tributaries and attach themselves to the epithelial lining. They become mature within four weeks and begin to produce eggs. The life span of the fluke is 25 to 30 years (Viranuvatti and Stitnimankarn, 1972; Harinasuta and Harinasuta, 1984; Sornmani, 1987). The flukes usually move about in the biliary canals of the hosts and result in chronic mechanical irritation of epithelial linings and finally in pathological changes of the biliary system. Possible toxic secretion by the worms may produce additional changes of the canals.

Most infection in man is acquired by eating raw fish or inadequately cooked fish containing viable infective metacercariae. Eating food by using contaminated hands
Figure 4.1  Life cycle of *Opisthorchis viverrini*

(From Sornmani, 1987)
after handling infected fish may also lead to infection. Infection can also occur by drinking contaminated unboiled or unfiltered water. The prevalence of liver fluke infection in the Northeast of Thailand is due to the fact that "Koi Pla" is the most popular raw fish there. This consists of raw flesh from small fish chopped with vegetables, garlic, lemon juice, fermented fish sauce or salt, chilli and roasted ground rice. It is eaten daily by villagers.

*O. viverrini* has also been implicated in the causation of cholangiocarcinoma (Chainuvati et al., 1976; Sonakul et al., 1978; Koomprochana et al., 1978; Schwartz, 1980; Kurathong et al., 1985). There were three separate studies of necropsy cases at the Siriraj Hospital in Bangkok (Bhamarapravati and Viranuva, 1966; Stitnimankarn, 1976; Koomprochana et al., 1978) which found that opisthorchiasis was closely associated with intrahepatic bile duct carcinoma. It is interesting that opisthorchiasis-related cancers occurred predominantly in the patients who came from the Northeast of Thailand. Koomprochana et al. (1978) also showed that patients having opisthorchiasis-related tumours were much younger than patients in Western countries having cholangiocarcinoma and suggested that chronic infection with the fluke might play a role in carcinogenesis.

It has also been postulated that opisthorchiasis infection in the presence of certain chemical carcinogens may have a synergistic effect in the induction of cholan-
gilocarcinoma as well as primary hepatocellular carcinoma (Flavell, 1981; Flavell and Lucas, 1982; Thamavit et al., 1987a). Cholangiocarcinoma was found to develop in 100% of golden Syrian hamsters experimentally treated with opisthorchis metacercariae and dimethylnitrosamine (Thamavit et al., 1978; Bhamarapravati and Thamavit, 1978). Animals which received either nitrosamine or liver fluke alone did not develop cancer. It, thus, seems likely that the initial carcinogenic stimulus in cholangiocarcinoma is multifactorial, requiring both the presence of the fluke and other precipitating factors such as dietary carcinogens.

Flavell and Lucas (1983) proposed that O. viverrini infection in the hamsters was capable of promoting dimethylnitrosamine-initiated bile duct carcinogenesis. While Thamavit et al. (1988) demonstrated the potential of infection with liver fluke parasites for promotion of liver tumour induction in both hepatocellular and cholangiocellular compartments. They also proposed that liver injury caused by parasite and non-specific compensatory regeneration might play an important role in generation of both hepatocellular carcinoma and cholangiocarcinoma in man and hamster (Thamavit et al., 1987b; Thamavit et al., 1988).

Several epidemiological studies have suggested a close geographical relationship between food contaminated with aflatoxins and the incidence of hepatocellular carcinoma (Shank et al., 1972c,d; Peers and Linsell, 1973; Shank,
1977; van Rensburg, 1985). These areas include the Northeast of Thailand where opisthorchiasis is endemic. AFB$_1$ is the most potent liver carcinogen known for the rat (Wogan and Newberne, 1967) and is toxic for a wide range of species (Newberne and Butler, 1969). The rat is extremely sensitive to the carcinogenic action of AFB$_1$ whereas the hamster is relatively resistant. The pattern of AFB$_1$ metabolism in vitro of monkey, hamster and man is similar but totally different from rat (Hsieh et al., 1977).

In the Northeast of Thailand, there are many factors that may affect the formation of liver cancer, i.e. liver fluke infestation, nitrosamine and aflatoxin ingestion, and malnutrition. These factors may act synergistically. So far only liver fluke infestation and nitrosamine have been studied (Thamavit et al., 1978; Flavell and Lucas, 1982).

In order to investigate the additive effects of liver fluke infestation and aflatoxin administration, the hamster was chosen as the experimental animal model because it was very susceptible to infection by the parasite and massive data on liver fluke pathogenicity in this species were available (Bhamaраправати et al., 1978; Flavell et al., 1980). The objectives of these experiments were to compare the pattern of urinary excretion of aflatoxin metabolites and the pathological effects of aflatoxin in hamsters with and without liver fluke infection.
4.2 MATERIAL AND METHODS

4.2.1 Animals
Golden Syrian hamsters, 8 weeks old, were obtained from the Animal Breeding Department, MRC Laboratories, Toxicology Unit, (Carshalton, Surrey). They were housed six to a cage and allowed a standard diet (from Wm. Lillico and Son, Wonham Mill Ltd., Betchworth, Surrey) and water ad libitum.

4.2.2 Chemicals
All chemicals used in these experiments were of analytical or HPLC grade.

4.2.3 Preparation of metacercariae and experimental infections
Liver fluke metacercariae were received from Thailand. The naturally infected fish were caught in a water reservoir in a small village near Khon Kaen, a Northeast province of Thailand. They were transported in ice from Khon Kaen to Bangkok. Metacercariae were obtained by the peptic digestion method described by Flavell (1982) at the Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok. The metacercariae were placed in normal saline and packed in an ice box (but not frozen) for transfer to London by air. Upon arrival, they were left at room temperature for half an hour before being infected into hamsters.
Metacercariae were administered intragastrically via a dosing needle and syringe. The successful infection was checked by counting the eggs in the stools 6 weeks after the hamsters had been infected.

4.2.4 Preparation of $^{14}$C-AFB$_1$ solution for injections

$^{14}$C-AFB$_1$, prepared by growing $A$. parasiticus in medium containing $^{14}$C-acetate, was used in the study of the effects of AFB$_1$ 10 mg/kg on hamsters with or without liver fluke infection. The solution was prepared by dissolving unlabelled AFB$_1$ (4 mg) in DMSO (400 μl), then an aliquot (400 μl) of $^{14}$C-AFB$_1$ (25 μCi in 2.5 ml methanol) was added. The concentration of the solution was 5 mg $^{14}$C-AFB$_1$/ml.

4.2.5 Histological studies

Liver tissues from hamsters were fixed in 10% buffered formalin and embedded in paraffin. Sections (5μ thick) were stained with haematoxylin and eosin.

4.3 EXPERIMENTAL PROTOCOLS

4.3.1 Histological examination of livers of hamsters with or without liver fluke infection

Male golden Syrian hamsters were divided into 2 groups of 30 animals each. One served as a control while the other was infected with liver fluke metacercariae. They were checked for the successful infection 6 weeks after metacercariae had been administered. The animals were kept
for 17 weeks before 2 animals from each group were killed. Livers were removed for histological examination.

4.3.2 Study of the effects of AFB₁ 5 mg/kg on hamsters with or without liver fluke infection

Six male golden Syrian hamsters from the previous experiment were used 17 weeks after they had been infected with metacercariae. They were injected with 5 mg AFB₁/kg intraperitoneally. AFB₁ solution was prepared at a concentration of 5 mg/ml in DMSO. Animals were placed individually in metabolism cages. Urine samples were collected over 24 h period before and after AFB₁ injection. The samples were stored at -40°C until used. They were analysed for aflatoxin levels using ELISA without any 'clean-up' procedures. It was observed that the samples required dilution before using in ELISA to obtain inhibitions in the proportional region of the competition curves. Each sample was analysed with 6 replicates using 6 wells per assay. Animals were killed 7 days after dosing. Livers were removed for histological study.

4.3.3 Study of the effects of AFB₁ 10 mg/kg on hamsters with or without liver fluke infection

Female golden Syrian hamsters were used in this set of experiments because at the time that this study was due to commence, only female hamsters were available and the study could not be postponed because the metacercariae were arriving shortly. The hamsters were divided into 2 groups of 30 animals each. One group was infected with liver
fluke metacercariae while the other one served as control. They were checked for successful infection (see Section 4.2.3) before AFB₁ was administered. The animals were kept for 15 weeks after infection. Six animals from each group were placed individually in metabolism cages 24 h before AFB₁ injection. The urines were collected to serve as untreated or control samples. In these experiments, in contrast to those described in the previous Section, the hamsters were housed in metabolism cages designed for this species which ensured maximum collection of urine samples. Four animals from each group were injected with 10 mg AFB₁/kg intraperitoneally, while the other two were injected with 10 mg ¹⁴C-AFB₁/kg (see Section 4.2.4). The animals then remained in the metabolism cages and two 24 h urine samples were collected. Animals were killed 48 h after dosing and the livers were removed for histological study. Urine samples were stored at -40°C until used.

4.3.3.1 An examination of ¹⁴C-AFB₁ metabolites excreted in hamster urine samples

Aliquots (20 μl) of 24 h urine samples from 2 hamsters with or without liver fluke infection, dosed with ¹⁴C-AFB₁ (10 mg/kg), were injected into the HPLC. The eluates were monitored by both UV and fluorescence detection. Fractions (1 min/1.2 ml) from the HPLC were collected and radioactivity was counted. Untreated (control) urine samples (20 μl) were also injected into the HPLC and chromatograms were recorded.
4.3.3.2 Aflatoxin analysis by ELISA using hamster urine samples fractionated by HPLC

Urine samples (20 μl) from untreated hamsters, and 24 and 48 h after treatment with AFB₁ (10 mg/kg), with or without liver fluke infection, were injected into HPLC using two animals from each group. Fractions from the HPLC (1 min/1.2 ml) were collected.

The HPLC fractions (1.2 ml) from untreated (control) animals of both groups were evaporated to dryness before redissolving in PBS (1 ml), and aliquots (200 μl) were analysed for apparent AFB₁ by ELISA in duplicate using 6 wells per assay.

In the case of urine samples collected 24 and 48 h after treatment with AFB₁, aliquots (200 μl) from the HPLC fractions were evaporated to dryness and redissolved in PBS (1 ml). The 24 and 48 h samples were diluted 40 and 16 times with PBS respectively, before aliquots (200 μl) were analysed for AFB₁ equivalents by ELISA in triplicate using 6 wells per assay.

4.4 RESULTS AND DISCUSSION

4.4.1 Histological comparison of hamsters with or without liver fluke infection

Livers in the liver fluke infected group were granular in appearance. The livers were enlarged and pale in colour.

Histological findings showed that in liver fluke infected animals, there was very extensive biliary
proliferation with lymphoid infiltration forming follicles. This disrupted the normal lobular pattern. In all livers, there was a pink extracellular amorphous material localized around the central veins and the area of biliary proliferation which was stained by congo red indicating the presence of amyloid (Figure 4.2).

4.4.2 Effects of AFB₁ 5 mg/kg on hamsters with or without liver fluke infection

4.4.2.1 Excretion of aflatoxins in urine samples

In collecting the urine samples, it was found in this initial experiment that the metabolism cages used in this laboratory which were designed for rats were unsuitable for use with hamsters. In many cases, little or no urine sample was collected which was due, at least in part, to the animals being able to enter and remain in the food hoppers. Only 3 samples from the non-liver fluke group and 5 samples from the liver fluke group were obtained.

The urine samples from both groups of hamsters were analysed for excretion of aflatoxins without any 'clean-up' procedures. This was because this experiment was carried out before the final procedures of aflatoxin monitoring were developed. Prior to aflatoxin analyses, different dilutions were tried and it was found that for control urines (before treating the hamsters with AFB₁), the samples had to be diluted in the range of 20 to 75 times. The concentration of apparent AFB₁ was approximately 3-12
Figure 4.2A
Liver from untreated control hamster showing normal lobular pattern. H & E stain x 120.

Figure 4.2B
Liver from hamster infected with liver fluke *Opisthorchis viverrini* for 17 weeks, showing bile duct proliferation and inflammatory infiltrate in the portal area. H & E stain x 120.
ng/ml except one animal in the non-liver fluke group which had the apparent AFB₁ content of 45 ng/ml (Table 4.1). However, there was no significant difference in the levels of apparent AFB₁ in both liver fluke infected and non-infected groups.

For the urine samples from the hamsters treated with AFB₁ at a dose of 5 mg/kg, high concentrations of aflatoxin excreted in the urine were expected. The appropriate dilutions for the ELISA lay between 1:50,000 and 1:200,000. The results (Table 4.1) were expressed as AFB₁ equivalent concentration (µg/ml). AFB₁ equivalents in hamsters treated with AFB₁ were 10⁶ times higher than the apparent AFB₁ concentrations of the urine samples obtained from the same animals prior to treatment with AFB₁. In the case of one hamster in the non-liver fluke group (animal no. C4), which had a high excretion of inhibitory substances in the urine prior to dosing with AFB₁, after dosing, showed levels of AFB₁ equivalents higher than other animals in both AFB₁ treated groups. However, at the dilutions employed, the contribution from the non-aflatoxin inhibitory substances present in the urine samples would have been negligible.

In order to examine the pathological effect of AFB₁ on hamsters infected or uninfected with liver fluke, it was necessary to dose the animals at a comparatively high level because the hamster is a species relatively resistant to AFB₁. When these animals were also used for determining aflatoxin excretion in the urine, because of the high level
<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Body weight (g)</th>
<th>Dose of AFB₁ (µg)</th>
<th>Liver fluke infection</th>
<th>AFB₁ concentration apparenta (ng/ml)</th>
<th>AFB₁ concentration equivalentb (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>85</td>
<td>425</td>
<td>-</td>
<td>2.6</td>
<td>25.7</td>
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<tr>
<td>C4</td>
<td>120</td>
<td>600</td>
<td>-</td>
<td>45.0</td>
<td>188.3</td>
</tr>
<tr>
<td>C6</td>
<td>90</td>
<td>450</td>
<td>-</td>
<td>6.3</td>
<td>89.1</td>
</tr>
<tr>
<td>LF1</td>
<td>145</td>
<td>725</td>
<td>+</td>
<td>8.4</td>
<td>89.4</td>
</tr>
<tr>
<td>LF2</td>
<td>135</td>
<td>675</td>
<td>+</td>
<td>5.7</td>
<td>92.3</td>
</tr>
<tr>
<td>LF3</td>
<td>140</td>
<td>700</td>
<td>+</td>
<td>3.8</td>
<td>39.6</td>
</tr>
<tr>
<td>LF5</td>
<td>140</td>
<td>700</td>
<td>+</td>
<td>7.9</td>
<td>80.5</td>
</tr>
<tr>
<td>LF6</td>
<td>125</td>
<td>625</td>
<td>+</td>
<td>11.8</td>
<td>61.2</td>
</tr>
</tbody>
</table>

a  Apparent AFB₁ concentration in urine before treatment with AFB₁

b  Equivalent AFB₁ concentration in urine after treatment with AFB₁
of dosing and the rate of excretion of aflatoxin in the urine, it is necessary to dilute extensively the samples before using in ELISA. This lead to a high level of variation in the results when the ELISA were corrected for the extensive dilutions employed.

Since this experiment was carried out using non-radio-labelled AFB$_1$, the pattern of metabolites excreted in the urine samples of hamsters infected with liver fluke cannot be compared with the one obtained from non-liver fluke group. Moreover, a pitfall of this experiment was the urine volumes were not measured because of the variation in collection efficiency, for reasons already referred to, so comparisons of the total aflatoxin excreted cannot be made.

4.4.2.2 Histological observations

Histological findings showed that 7 days after dosing with AFB$_1$, the livers of non-liver fluke infected hamsters dosed with 5 mg AFB$_1$/kg, showed biliary and oval cell proliferation with some residual damage to periportal hepatocytes (Figure 4.3A).

The livers of hamsters with liver fluke infection, dosed with 5 mg AFB$_1$/kg, showed more extensive biliary proliferation and lymphoid infiltration (Figure 4.3B). The extent of the biliary proliferation and lymphoid infiltration was similar in all liver fluke infected animals whether or not treated with AFB$_1$. These results indicate that the pronounced effects due to liver fluke
Figure 4.3A
Liver from hamster treated with AFB$_1$ 5 mg/kg, killed on day 7 after injection, showing slight oval cell proliferation. Periportal hepatocytes show some vesiculation and disruption of the trabecular pattern. H & E stain x 120.

Figure 4.3B
Liver from hamster infected with liver fluke, *Opisthorchis viverrini* for 17 weeks prior to injection with AFB$_1$ 5 mg/kg, killed on day 7 after injection, showing extensive biliary proliferation and inflammatory infiltrate similar to that seen in Figure 4.2B. H & E stain x 120.
infection are not enhanced by the additional treatment with 5 mg AFB₁/kg.

4.4.3 Effects of AFB₁ 10 mg/kg on hamsters with or without liver fluke infection

Animals no. 1 and 2 in the non-liver fluke infected group and no. 7 in the liver fluke group died during the period 24-48 h after injection with AFB₁, probably due to AFB₁ acute toxicity. Unfortunately, they were 3 of the 4 animals treated with radioactive AFB₁ and, therefore, no 48 h radiolabelled urine samples were obtained from these animals. The summary of the data on doses and urine volumes are shown in Table 4.2.

In this experiment, metabolism cages designed for hamsters were used. However, the urine volumes over 24 h period were still low (less than 3 ml) and, therefore, it appears probable that this is a characteristic of hamsters, and that they have a high capacity for water retention.

4.4.3.1 Histological observations

Livers from non-liver fluke infected hamsters 48 h after dosing with 10 mg AFB₁/kg showed that there was slight oval cell proliferation with moderate to extensive periportal necrosis. In one animal, the liver showed extensive haemorrhagic periportal necrosis (Figure 4.4A).

In the group that had been infected with liver fluke and dosed with 10 mg AFB₁/kg, the livers showed slight oval cell and biliary proliferation with slight to extensive
Table 4.2
Summary of data on body weight, AFB<sub>1</sub> doses and urine volumes in hamsters with or without liver fluke infection treated with 10 AFB<sub>1</sub>/kg body weight

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Liver fluke infection</th>
<th>Body weight (g)</th>
<th>AFB&lt;sub&gt;1&lt;/sub&gt; dose (mg)</th>
<th>Urine volume (μl) 24-h</th>
<th>Urine volume (μl) 48-h</th>
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<td>1*</td>
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<td>-</td>
<td>125</td>
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<td>NM&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>120</td>
<td>1.20</td>
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<tr>
<td>5</td>
<td>-</td>
<td>105</td>
<td>1.05</td>
<td>NM&lt;sup&gt;+&lt;/sup&gt;</td>
<td>NM&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>91</td>
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<td>+</td>
<td>80</td>
<td>0.80</td>
<td>550</td>
<td>-</td>
</tr>
<tr>
<td>8*</td>
<td>+</td>
<td>107</td>
<td>1.07</td>
<td>1150</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>158</td>
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<td>+</td>
<td>130</td>
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</tr>
<tr>
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<td>+</td>
<td>94</td>
<td>0.94</td>
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<td>12</td>
<td>+</td>
<td>92</td>
<td>0.92</td>
<td>2500</td>
<td>6000</td>
</tr>
</tbody>
</table>

* Animals treated with ¹⁴C-ABF<sub>1</sub>
+ NM = not measured
Figure 4.4A
Liver from hamster treated with AFB\textsubscript{1} 10 mg/kg, killed on day 2 after injection, showing biliary and oval cell proliferation, and necrosis of periportal hepatocytes. H & E stain x 120.

Figure 4.4B
Liver from hamster infected with liver fluke, Opisthorchis viverrini, for 15 weeks prior to injection with AFB\textsubscript{1} 10 mg/kg, killed on day 2 after injection, showing slight bile duct proliferation and extensive necrosis of hepatocytes involving the entire lobule. H & E stain x 120.
periportal haemorrhagic necrosis (Figure 4.4B). In two animals, there was extensive hydropic change in periportal cells and centrilobular haemorrhagic necrosis.

These results indicate that dosing hamsters in the non-liver fluke infected group with 10 mg AFB$_1$/kg resulted in more extensive damage of the livers than that observed using 5 mg AFB$_1$/kg (see Section 4.4.2.2). In addition, in the liver fluke infected group, treated with 10 mg AFB$_1$/kg, 3 out of 5 animals showed very extensive haemorrhagic periportal necrosis, suggesting that the toxicity of AFB$_1$ is accentuated by liver fluke infection.

4.4.3.2 Pattern of $^{14}$C-AFB$_1$ metabolites excreted in hamster urine samples

Hamsters excreted small volumes of concentrated urine. At the same time, the dose of AFB$_1$ given to the animals was very high (10 mg/kg), therefore, the amounts of aflatoxin metabolites present in the urine samples were high. In order to record the chromatograms from UV and fluorescence detectors of the HPLC, it was necessary to have the detectors at their lowest sensitivity settings. Even so, the high fluorescence peak went off scale.

When untreated urine samples (control) from hamsters with or without liver fluke infection were injected into the HPLC, the chromatograms from these samples showed no peaks at the detector sensitivity settings used in subsequent examination of the aflatoxin-containing urine samples. When 24 h urine samples from both groups treated
with $^{14}\text{C}}$-AFB$_1$ were used, the chromatograms (Figure 4.5) showed that there was a variety of highly fluorescent substances which could be fractionated by the HPLC and that the patterns of chromatograms for the infected and non-infected hamsters were different. The data given in Figure 4.5 is from a standard injection of 20 μl of urine samples and have not been corrected for differences in total urine volumes.

When radioactivity present in the HPLC fractions was counted, the patterns of metabolites excreted in the liver fluke (Figure 4.6A) and the non-liver fluke group (Figure 4.6B) were different, even though consistent results were obtained from the 2 animals within each group. However, in both groups there is one peak in common which appears in fraction 9. This peak corresponds with AFM$_1$ which is found in the urinary excretion shortly after exposure to AFB$_1$, as discussed in Section 3.4.2. These results are consistent with those of Hsieh et al. (1977) who demonstrated that the in vitro pattern of AFB$_1$ metabolism of monkey, hamster and man was similar.

It was, therefore, observed from UV, fluorescence and radioactive monitoring of HPLC separations of urine samples that the profile of AFB$_1$ metabolites differed between liver fluke infected and non-infected animals. Hence, it suggests that liver fluke infection has some effect on aflatoxin metabolism in hamsters. In both groups treated with AFB$_1$, there appears to be excretion of AFM$_1$ in the first 24 h after dosing but, apart from this, the overall
Figure 4.5  HPLC chromatograms of UV and fluorescence detection of aflatoxin metabolites present in the 24 h urine of hamsters treated with $^{14}$C-AFB$_1$ (10 mg/kg)

A. Non-liver fluke infected hamster
B. Liver fluke infected hamster
Figure 4.6  Radioactivity in HPLC fractions from chromatographing urine samples from hamsters treated with $^{14}$C-AFB$_1$ (10 mg/kg)

A. Liver fluke infected

B. Non-liver fluke infected

* Percentage of total radioactivity recovered in all fractions
profiles are strikingly different. In view of this result, it would be interesting to compare further the pattern of metabolism between these two groups of hamsters in more detail. However, it was outside the scope of this study.

4.4.3.3 Aflatoxin levels in hamster urine samples

When untreated urine samples from hamsters, either with or without liver fluke infection, were fractionated by HPLC, and analysed for apparent AFB\textsubscript{1} content, there was no significant difference in the pattern of apparent AFB\textsubscript{1} obtained from both groups (Figure 4.7). The levels were high in fraction 2 (approximately 3-6 ng/fraction), then they decreased to levels less than 0.5 ng apparent AFB\textsubscript{1}/fraction before, again, increasing in fraction 8 and subsequent fractions.

These results show that in urine samples from hamsters untreated with AFB\textsubscript{1}, there are high levels of interfering substances that can react with anti-AFB\textsubscript{1} serum in ELISA. It was unlikely that these animals had been fed with a diet contaminated with AFB\textsubscript{1}. It appears possible that the high levels of ELISA interfering substances present in the hamster urine are due to the concentrated nature of these excretions.

When comparing the results obtained with the hamsters before treatment with AFB\textsubscript{1} with the ones obtained using uncontaminated human samples (see Figure 2.12) and marmoset control samples (see Section 3.4.3), there was one peak in common in fraction 2 of the HPLC separations, in all three
Figure 4.7  Apparent AFB₁ by ELISA in HPLC fractions from chromatographing urine samples from non-AFB₁ injected hamsters.

- - - Liver fluke infected
▲▲▲ Non-liver fluke infected

Fraction no.
species, associated with inhibition in the ELISA. This was presumably a polar compound from its retention time on HPLC. However, a major difference between the hamster and human samples was that in the hamster samples there was a potent interfering substance which eluted in fraction 8 and subsequent fractions from the HPLC. The marmoset fractionations appeared similar to the human results and again indicated the suitability of this animal as a model for human exposure.

Dragsted et al. (1988) have carried out similar HPLC fractionations of 'uncontaminated' human urine samples and have observed that substantial amounts of ELISA interfering substances are eluted in their HPLC system with a retention time similar to that of AFB₁. However in the present study, this result was only obtained using samples from hamsters and not with the human or marmoset urines. The reason for the difference between their results and the one obtained from this study is not known at present. However, our results suggest that hamster may be a good animal model system to study UALS.

When hamster urine samples, collected 24 and 48h after injection of AFB₁ into animals of both the liver fluke and non-liver fluke groups, were fractionated by HPLC and subsequently analysed for AFB₁ equivalents by ELISA (Figure 4.8), the patterns of AFB₁ equivalents obtained from both groups at the same time point (24 or 48 h) were similar. The prominent feature of the 24 h urine samples of both liver fluke and non-liver fluke groups was the
Figure 4.8 Equivalent AFB₁ concentrations by ELISA in HPLC fractions from chromatographing urine samples from hamsters treated with AFB₁ (10 mg/kg)
major peak in fraction 9, which although still present in the 48 h samples was much reduced. The retention time of this peak was consistent with AFM$_1$. This peak was also prominent when using radioactive counting (Figure 4.6).

Radioactive monitoring of aflatoxin metabolites (Figure 4.6B) using fractions obtained from 24 h urine samples of non-liver fluke infected hamsters showed a less prominent peak in fraction 5 of the HPLC, and this peak could also be detected by ELISA (Figure 4.8A). However, a small peak in fraction 2, detected by radioactivity (Figure 4.6B) was not recognised by the antibody (Figure 4.8A). Similar discrepancies between radioactive and ELISA detection were observed using 24 h urine samples from the liver fluke group. The ELISA technique again detected the major radioactive peaks in fractions 9 and 6 but not fractions 2 and 3. These results clearly indicate that the antibody used is capable of recognising less polar metabolites of aflatoxin which may have less modification at the recognition epitope of the antibody than for the more polar metabolites which probably result from secondary metabolism, and therefore, have greater modification at the recognition epitope.

For the urine samples collected 48 h after treatment with AFB$_1$, comparisons between radioactivity and ELISA monitoring cannot be made because the animals treated with $^{14}$C-AFB$_1$ died between 24 and 48 h after dosing, therefore, no 48 h samples were obtained. But from the ELISA monitoring of unlabelled urine fractions, the qualitative
metabolite pattern was similar for the liver fluke and non-liver fluke groups (Figure 4.8B). In the absence of quantitation of aflatoxin metabolites by radioactivity, it is not possible to compare the total level of metabolites using AFB\textsubscript{1} equivalent concentrations derived from ELISA. As has been clearly demonstrated by comparing the data in Figures 4.6 and 4.8, the ELISA technique is not efficient in detecting polar metabolites and so, if the samples contain high proportion of these metabolites, the aflatoxin content would be seriously underestimated. This would be a particular drawback in the case of the 48 h samples where not only the levels of aflatoxin metabolites decreased but also it was most likely that a higher proportion of polar metabolites was present and the antibody would not detect them. It is suggested from this study, therefore, that the pattern of AFB\textsubscript{1} metabolism in liver fluke and non-liver fluke infected hamsters can best be carried out using radiolabelled AFB\textsubscript{1}, due to the difficulty in assaying polar metabolites by ELISA.

4.5 SUMMARY

The results obtained in this Chapter indicate that the pattern of aflatoxin metabolites excreted in the urine samples of liver fluke and non-liver fluke infected groups may not be the same. More animals are required to clarify this point. Concerning individual metabolites, it appeared that AFM\textsubscript{1} was a major constituent of the urine of hamsters.
treated with AFB\textsubscript{1} and this metabolite was detected sensitively by the ELISA. However, it was also found that the antibody used did not recognise efficiently the polar metabolites of AFB\textsubscript{1} excreted in the urine.

In the present study hamster urine samples were not subjected to the 'clean-up' procedures for aflatoxin monitoring developed in connection with human and marmoset samples, because the main object of the study described in this Chapter was to determine whether there was any significant differences between the aflatoxin metabolism of hamsters with or without liver fluke infection. However, from all the data available, it is possible to suggest that the level of AFB\textsubscript{1} equivalents obtained by ELISA will be underestimated as in the case of marmoset urine samples.

From histological examination, liver fluke infection can cause very extensive biliary proliferation with lymphoid infiltration forming follicles. These effects were so pronounced that even a dose of 5 mg AFB\textsubscript{1}/kg body weight of hamster did not show any significant difference from the effects of the liver fluke alone. But with a dose of 10 mg AFB\textsubscript{1}/kg, infection with liver fluke accentuated the toxicity of AFB\textsubscript{1} in the livers of the hamsters. However, in the non-liver fluke infected group, a significant difference between the dose of 5 and 10 mg AFB\textsubscript{1}/kg was clearly seen. A complicating factor in increasing the dose of AFB\textsubscript{1} to 10 mg/kg was the high mortality which resulted, but despite this drawback, it was necessary to increase the AFB\textsubscript{1} dosing to this level in
order to produce AFB$_1$-related lesion in the liver fluke infected group.

One interesting point in this study is that the hamster may be a good animal model to study the UALS. In urine samples from animals untreated with AFB$_1$ in both liver fluke and non-liver fluke infected groups, high levels of these substances were detected. This is possibly related to the small urine volumes excreted by these animals which results in the inhibitory substances being concentrated.
CHAPTER 5

Monitoring of urinary aflatoxin excretion in patients with liver disease from different areas of Thailand
There is considerable evidence indicating an association between aflatoxin ingestion and liver cancer in humans (see Section 1.6). Most of the evidence linking these two factors is based on correlation between cancer incidences and the levels of exposure to aflatoxin contamination in food. But the presence of aflatoxin-contaminated food in general food surveys does not give information concerning specific individual exposures which can only be obtained from individual monitoring (Groopman and Kensler, 1987). Although epidemiological studies may provide evidence for an association, they cannot give direct proof of a cause and effect relationship (Munoz and Linsell, 1982). In addition, despite providing unique information about the responses of humans who have been exposed to a suspect agent this is usually retrospective. In such circumstances, they can detect only relatively large increases in relative risk and huge numbers of subjects will be required to evaluate a substance having a small risk.

Some of the limitations of cancer epidemiology may be overcome by the development of methods which permit the monitoring of exposure to a suspected or known carcinogen at an individual level. Exposure monitoring provides a bridge between laboratory animal and human studies. Human risk assessment of a particular substance may be improved by correlating data obtained through the biological
monitoring of the compound in human with the results of parallel studies using laboratory animals. The incorporation of biological monitoring methods into conventional epidemiological studies can increase their power to detect carcinogenic risk earlier, and at lower exposure, as well as to estimate more accurately the magnitude of human risk. However, biological monitoring may not be straightforward and should be used in parallel with environmental monitoring, especially in the case of carcinogens, for which the latency between exposure and effects may last for decades (Vainio, 1985).

In order to monitor human exposure to any toxic substance there are two important considerations to bear in mind. Firstly, the methods used must be sensitive and specific enough to allow detection of human exposure to any substance of interest, which will often be at an extremely low level. Secondly, possible modifying and/or confounding factors, such as dietary considerations, drinking and smoking habits must be taken into account when the results of the biological monitoring are being analysed. In the present study, the assay methods for detecting aflatoxins in human urine samples using ELISA techniques have been successfully developed (see Chapters 2 and 3). It was of interest, therefore, to apply these techniques to monitor human populations in which the risk of aflatoxin exposure seems highly likely.

In Thailand, liver cancer is the most common fatal neoplasm reported in the National Cancer Registry, and is
responsible for 16.3% of all new cases in males and 5.5% in females (Srivatanakul et al., 1988). This cancer occurs in males three times more frequently than in females (Parkin et al., 1988). The overall incidence shows considerable variation in its frequency in the different regions of the country (see Section 4.1).

According to a study carried out in Thailand since 1967, there was a good correlation between the levels of aflatoxin contamination in food and incidences of liver cancer (Shank et al., 1972c,d). Another similar study was reported in 1984 (Anukarahanonta et al., 1984), the results obtained were in accordance with the previous reports. Various foodstuffs available in the local markets in Thailand have been shown to be contaminated with aflatoxins (Table 5.1). The frequency as well as the severity of aflatoxin contamination were found not to vary with the seasons of the year (Shank et al., 1972b; Anukarahanonta et al., 1984). There were two different geographical locations of great interest. The first one is Songkhla where low levels of aflatoxin contamination in food and low incidence of liver cancer were observed, and the second one, Ratburi, with high contamination of aflatoxins in food and high incidence of liver cancer. Even though the survey studies were carried out several years ago, the pattern of diet consumed and the levels of aflatoxin contamination in foodstuffs have not altered substantially in the intervening period (Anukarahanonta et al., 1984). In the present study, patients with liver
### Table 5.1

**Alfatoxin contamination in market food in Thailand**

<table>
<thead>
<tr>
<th>Commodity</th>
<th>No. of sample</th>
<th>Percent positive</th>
<th>Contamination levels* (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>test + samples</td>
</tr>
<tr>
<td>Corn</td>
<td>62</td>
<td>35</td>
<td>140 (93)</td>
</tr>
<tr>
<td>Rice</td>
<td>364</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Wheat and barley</td>
<td>44</td>
<td>11</td>
<td>7 (4)</td>
</tr>
<tr>
<td>Peanut</td>
<td>216</td>
<td>49</td>
<td>750 (426)</td>
</tr>
<tr>
<td>Sesame seeds</td>
<td>75</td>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mung beans</td>
<td>140</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Other beans</td>
<td>322</td>
<td>3</td>
<td>6 (3)</td>
</tr>
<tr>
<td>Fresh vegetables</td>
<td>140</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Onions and garlic</td>
<td>58</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Cassava starch</td>
<td>65</td>
<td>3</td>
<td>5 (2)</td>
</tr>
<tr>
<td>Peppers, chili</td>
<td>106</td>
<td>11</td>
<td>14 (9)</td>
</tr>
<tr>
<td>Fish and shrimp dried</td>
<td>139</td>
<td>5</td>
<td>8 (5)</td>
</tr>
</tbody>
</table>

* Contamination levels were expressed as average total aflatoxins

+ Numbers in parenthesis represented average level of AFB\(_1\)

\((x)\) N.A. = data not available

(Adapted from Shank *et al.*, 1972b)
disease from these two areas were monitored for aflatoxin exposure. The other area, namely Khon Kaen, was also selected due to the fact that there were high levels of aflatoxin contamination, high incidence of liver cancer and also high incidence of liver fluke infestation. This latter area was not chosen in the study of Shank and his coworkers because they did not want to complicate their study with extra aetiological factors in liver carcinogenesis (Shank et al., 1972d). However, the hamster studies (see Chapter 4) indicated alterations in aflatoxin excretion in the urine due to the presence of liver fluke infestation. Therefore, the effect of liver fluke infestation on aflatoxin disposition may also be observed by monitoring patients with liver disease in this area.

The objective of this study was to compare the levels of aflatoxin excreted in urine specimens of patients with and without liver disease in three different areas of Thailand, i.e. Ratburi, Songkhla and Khon Kaen. Since it is well established that cancer is a disease with a very long latent period, it is not to be expected that the presence of aflatoxins in the samples or the levels of aflatoxin found, would have any particular relevance in terms of the carcinogenic process in any individual patient. But giving that food consumption of Thai people has not drastically changed during the last decade (Anukarahanonta et al., 1984), the data obtained may provide additional information on human risk assessment of aflatoxin. In any case, the results obtained would
indicate the possibility of using the assay methods developed in monitoring the level of human exposure to aflatoxin which could be of value in a prospective study of liver cancer.

5.2 MATERIALS AND METHODS

5.2.1 Subjects

Patients were selected from major hospitals with good medical facilities and recording systems. The hospitals which participated in this study are located in different geographical areas with respect to differences in levels of aflatoxin contamination as being surveyed by Shank et al. (1972b, c). The hospitals are in Ratburi (area with high aflatoxin contamination and high incidence of liver cancer), Songkhla (area with low aflatoxin contamination and low incidence of liver cancer), and Khon Kaen (area with high aflatoxin contamination and high incidences of liver cancer and liver fluke infestation). All these hospitals are regional hospitals where patients from central, southern and northeastern parts of Thailand are referred to respectively.

Newly admitted patients with liver disease were recruited in the study during the period of April-December 1986. All the patients were diagnosed clinically by having the symptoms of hepatomegaly and jaundice with or without ascites. Patients without liver disease who were admitted within the same period as the patients with liver disease
were also recruited for comparison. Patients in both groups were matched with respect to their sex and age (± 5 years).

5.2.2 Sample Collections

Morning urine specimens from patients with or without liver disease were collected on the day following admission. Aliquots (15 ml) of urine samples were stored in glass bottles with screw caps and kept frozen at -20°C prior to and during transportation to Bangkok by air, except from Ratburi where the samples were sent by car (approximately one hour drive).

At the end of the field study, all urine samples were sent frozen to England by air in an ice box packed with dry ice. Upon arrival, the samples were immediately stored at -70°C until required.

5.2.3 Aflatoxin analysis

Urine samples (1 ml) were used in the assays for aflatoxin contamination. They were subjected to the 'clean-up' procedures before analysing for AFB1 equivalents by ELISA (using 4 replicates) as described in Section 2.2.4.

5.2.4 Creatinine analysis

The samples were analysed for creatinine concentrations as described in Section 2.2.3.7.

5.2.5 Statistical analysis

Because of the unequal number of the patients with and
without liver disease in each hospital, it was not possible
to carry out a combined two-way analysis of variance
(Snedecor and Cochran, 1980). Individual statistical
analyses were, therefore, performed as follows. Within
each hospital, the levels of aflatoxin per creatinine unit
in both groups of patients were compared by student t-test
using the statistical package (Minitab, Inc., University
Park, Pennsylvania). All values obtained for samples from
each individual hospital were also pooled to allow a
comparison between hospitals using one-way analysis of
variance.

5.3 RESULTS AND DISCUSSION

The age and sex of patients with or without liver
disease in the three different areas are shown in Table
5.2. In some particular cases of liver disease patients,
liver biopsies or liver scans were carried out. In Ratburi
only 15% (4 cases) of patients with liver disease was
confirmed as hepatocellular carcinoma. In Songkhla and
Khon Kaen, there were 11% (1 case) and 31% (4 cases)
respectively. The highest percentage of liver cancer among
liver disease patients was in Khon Kaen which is the area
with a high incidences of liver fluke infection and
aflatoxin contamination. The presumptive diagnosis of the
other patients with liver disease was cirrhosis. All the
patients with non-liver disease were suffering from chronic
medical diseases such as asthma, hypertension and
The age and sex of patients with or without liver disease in three different areas*

<table>
<thead>
<tr>
<th>Area</th>
<th>Disease-status</th>
<th>Numbers of subjects</th>
<th>Mean age (S.D.) (years)</th>
<th>Range of ages (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Ratburi:</td>
<td>liver disease</td>
<td>22</td>
<td>4</td>
<td>51.4 (12.3)</td>
</tr>
<tr>
<td></td>
<td>non-liver disease</td>
<td>23</td>
<td>3</td>
<td>49.3 (14.5)</td>
</tr>
<tr>
<td>Songkhla:</td>
<td>liver disease</td>
<td>7</td>
<td>2</td>
<td>54.6 (10.7)</td>
</tr>
<tr>
<td></td>
<td>non-liver disease</td>
<td>18</td>
<td>6</td>
<td>49.2 (9.1)</td>
</tr>
<tr>
<td>Khon Kaen:</td>
<td>liver disease</td>
<td>9</td>
<td>4</td>
<td>55.6 (5.9)</td>
</tr>
<tr>
<td></td>
<td>non-liver disease</td>
<td>15</td>
<td>3</td>
<td>56.2 (6.0)</td>
</tr>
</tbody>
</table>

* Ratburi : high aflatoxin, high liver cancer, no liver fluke
  Songkhla : low aflatoxin, low liver cancer, no liver fluke
  Khon Kaen : high aflatoxin, high liver cancer, high liver fluke
congestive heart failure. Since the number of patients in the groups selected were proportional to the number of people admitted with liver disease it can be seen that there were more male patients presenting with liver disease than female patients in all three areas (Table 5.2).

The mean ages of patients presenting with liver disease in the case of the males in the three different areas were in their 50's, but in the female groups, it was 46 years in Khon Kaen, whilst in the other two areas they were in their 60's. The results given in Table 5.2 are consistent with the national survey of liver cancer that males tend to have liver cancer at an earlier age than females (Srivatanakul et al., 1988). However, in the case of the female group in Khon Kaen, they presented at an earlier age than the other two female groups and also earlier than all the male groups. The reason for this is not known but it could be associated with the high incidence of cholangiocarcinoma in the case of Khon Kaen which is probably related to the high incidence of liver fluke infection.

The sizes of the three selected hospitals in this study are the same. From Table 5.2, therefore, it might appear that there was a higher incidence of liver disease in Ratburi compared with the other two regions. However, the figures may not reflect the actual incidences of liver disease in these regions during the period of study. Firstly, because some cases may not have been recruited
into the study due to the absence of responsible research assistance during the period of their admission and secondly, because the referral system in Thailand, in which patients are allowed to be treated in any hospital of their choice without having to go through their local general practitioners, often resulted in people entering hospitals other than those in their own regions. For these reasons a valid comparison between the actual incidence of liver disease in the three areas was not attempted.

In this study, the number of patients with non-liver disease is higher than the number of liver disease cases. This is due to the fact that all non-liver disease patients matching patients in liver disease group were recruited.

Morning urine specimens from both groups of patients collected on the day after their admissions were chosen as the protocol for urine sample collections because in Thailand, the average temperature is 32°C which promotes the growth of bacteria and other microorganisms. If 24 h urine samples were collected, some form of preservative would have to be added to them which could interfere with the subsequent ELISA detection system. In the case of the morning urine samples, the total daily urine volumes excreted were not recorded. Creatinine concentration in each sample was determined to serve as a reference point or internal standard for the comparison of aflatoxin levels excreted in the different urine samples. Hence, the concentrations of aflatoxin were expressed as ng AFB₁ equivalent/mg creatinine.
The AFB\textsubscript{1} equivalents in the urine samples obtained from patients with or without liver disease in the three different areas are shown in Tables 5.3-5.8 along with their sex, age and creatinine levels. When the individual levels of AFB\textsubscript{1} equivalent/mg creatinine were compared between patients with or without liver disease in any one hospital, there was no significant difference observed in the case of any of the hospitals studied. When the levels of aflatoxin excreted in the urine samples of similar disease-status patients in the three different areas were compared, no significant differences were found. When the values obtained from all patients in each individual hospital were compared with the other two hospitals, no significant differences were also observed.

Inspection of the data in Figure 5.1 shows a skewed distribution, in the case of all the groups studied, with most values clustering around 0-0.2 ng AFB\textsubscript{1}/mg creatinine with several high value outliers. In order to avoid any bias for the non-normal distribution, the above statistical tests were also carried out on the log transformation of the data, however, there was still no significant difference.

When the results for each area were analysed in more detail; in Ratburi, 4 (15\%) out of 26 patients with liver disease had liver cancer (urine sample nos. 142, 196, 197 and 199; Table 5.3). Three of them were over 50 years old while the other (sample no. 196) was only 18 years. This man had suffered from chronic viral hepatitis B infection
### Table 5.3

**AFB₁ equivalents in urine samples obtained from patients with liver disease from Ratburi+**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB₁ equivalent (ng/ml)</th>
<th>AFB₁/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>F</td>
<td>60</td>
<td>1.40</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>93</td>
<td>F</td>
<td>65</td>
<td>1.81</td>
<td>0.25</td>
<td>0.14</td>
</tr>
<tr>
<td>107</td>
<td>F</td>
<td>59</td>
<td>2.20</td>
<td>0.37</td>
<td>0.17</td>
</tr>
<tr>
<td>164</td>
<td>F</td>
<td>62</td>
<td>4.02</td>
<td>0.20</td>
<td>0.05</td>
</tr>
<tr>
<td>76</td>
<td>M</td>
<td>47</td>
<td>5.54</td>
<td>0.31</td>
<td>0.06</td>
</tr>
<tr>
<td>78</td>
<td>M</td>
<td>48</td>
<td>1.36</td>
<td>0.23</td>
<td>0.17</td>
</tr>
<tr>
<td>79</td>
<td>M</td>
<td>42</td>
<td>1.07</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td>84</td>
<td>M</td>
<td>62</td>
<td>2.05</td>
<td>0.37</td>
<td>0.18</td>
</tr>
<tr>
<td>113</td>
<td>M</td>
<td>58</td>
<td>4.60</td>
<td>0.48</td>
<td>0.10</td>
</tr>
<tr>
<td>127</td>
<td>M</td>
<td>51</td>
<td>4.29</td>
<td>0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>135</td>
<td>M</td>
<td>54</td>
<td>1.60</td>
<td>1.35</td>
<td>0.84</td>
</tr>
<tr>
<td>142*</td>
<td>M</td>
<td>52</td>
<td>2.08</td>
<td>0.25</td>
<td>0.12</td>
</tr>
<tr>
<td>144</td>
<td>M</td>
<td>46</td>
<td>1.52</td>
<td>0.44</td>
<td>0.29</td>
</tr>
<tr>
<td>146</td>
<td>M</td>
<td>31</td>
<td>2.18</td>
<td>0.19</td>
<td>0.09</td>
</tr>
<tr>
<td>149</td>
<td>M</td>
<td>63</td>
<td>2.40</td>
<td>0.21</td>
<td>0.09</td>
</tr>
<tr>
<td>155</td>
<td>M</td>
<td>65</td>
<td>3.30</td>
<td>0.30</td>
<td>0.09</td>
</tr>
<tr>
<td>165</td>
<td>M</td>
<td>57</td>
<td>1.43</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td>167</td>
<td>M</td>
<td>68</td>
<td>0.63</td>
<td>0.38</td>
<td>0.61</td>
</tr>
<tr>
<td>172</td>
<td>M</td>
<td>39</td>
<td>7.33</td>
<td>0.76</td>
<td>0.10</td>
</tr>
<tr>
<td>184</td>
<td>M</td>
<td>46</td>
<td>7.21</td>
<td>0.40</td>
<td>0.06</td>
</tr>
<tr>
<td>186</td>
<td>M</td>
<td>45</td>
<td>0.27</td>
<td>0.14</td>
<td>0.52</td>
</tr>
<tr>
<td>196*</td>
<td>M</td>
<td>18</td>
<td>2.87</td>
<td>0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>197*</td>
<td>M</td>
<td>60</td>
<td>8.43</td>
<td>0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>199*</td>
<td>M</td>
<td>50</td>
<td>6.40</td>
<td>0.65</td>
<td>0.10</td>
</tr>
<tr>
<td>201</td>
<td>M</td>
<td>44</td>
<td>0.40</td>
<td>0.08</td>
<td>0.21</td>
</tr>
<tr>
<td>206</td>
<td>M</td>
<td>55</td>
<td>2.04</td>
<td>0.23</td>
<td>0.11</td>
</tr>
</tbody>
</table>

| Mean       | 53  | 3.00 | 0.33 | 0.18 |
| S.D.       | 12  | 2.27 | 0.26 | 0.19 |

+ Area with high aflatoxin contamination and high incidence of liver cancer  
* Patients with liver cancer
Table 5.4

AFB₁ equivalents in urine samples obtained from patients with non-liver disease from Ratburi+

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB₁ equivalent (ng/ml)</th>
<th>AFB₁/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>83</td>
<td>F</td>
<td>60</td>
<td>1.33</td>
<td>0.15</td>
<td>0.11</td>
</tr>
<tr>
<td>137</td>
<td>F</td>
<td>65</td>
<td>4.79</td>
<td>0.16</td>
<td>0.03</td>
</tr>
<tr>
<td>145</td>
<td>F</td>
<td>63</td>
<td>0.93</td>
<td>0.33</td>
<td>0.36</td>
</tr>
<tr>
<td>86</td>
<td>M</td>
<td>39</td>
<td>1.74</td>
<td>0.18</td>
<td>0.10</td>
</tr>
<tr>
<td>91</td>
<td>M</td>
<td>59</td>
<td>1.40</td>
<td>0.18</td>
<td>0.13</td>
</tr>
<tr>
<td>99</td>
<td>M</td>
<td>20</td>
<td>3.23</td>
<td>0.32</td>
<td>0.10</td>
</tr>
<tr>
<td>105</td>
<td>M</td>
<td>20</td>
<td>3.13</td>
<td>0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>106</td>
<td>M</td>
<td>60</td>
<td>2.10</td>
<td>2.69</td>
<td>1.28</td>
</tr>
<tr>
<td>114</td>
<td>M</td>
<td>61</td>
<td>0.29</td>
<td>0.11</td>
<td>0.39</td>
</tr>
<tr>
<td>121</td>
<td>M</td>
<td>72</td>
<td>2.28</td>
<td>0.35</td>
<td>0.16</td>
</tr>
<tr>
<td>123</td>
<td>M</td>
<td>44</td>
<td>0.97</td>
<td>0.29</td>
<td>0.30</td>
</tr>
<tr>
<td>129</td>
<td>M</td>
<td>56</td>
<td>1.79</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>133</td>
<td>M</td>
<td>30</td>
<td>0.81</td>
<td>0.17</td>
<td>0.21</td>
</tr>
<tr>
<td>139</td>
<td>M</td>
<td>69</td>
<td>2.23</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>140</td>
<td>M</td>
<td>50</td>
<td>0.06</td>
<td>0.07</td>
<td>1.17</td>
</tr>
<tr>
<td>143</td>
<td>M</td>
<td>49</td>
<td>0.20</td>
<td>0.06</td>
<td>0.31</td>
</tr>
<tr>
<td>148</td>
<td>M</td>
<td>54</td>
<td>1.97</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>168</td>
<td>M</td>
<td>57</td>
<td>1.39</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>169</td>
<td>M</td>
<td>72</td>
<td>1.44</td>
<td>0.26</td>
<td>0.18</td>
</tr>
<tr>
<td>182</td>
<td>M</td>
<td>48</td>
<td>2.50</td>
<td>0.53</td>
<td>0.21</td>
</tr>
<tr>
<td>198</td>
<td>M</td>
<td>31</td>
<td>1.43</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>202</td>
<td>M</td>
<td>58</td>
<td>1.73</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>203</td>
<td>M</td>
<td>52</td>
<td>0.79</td>
<td>0.25</td>
<td>0.31</td>
</tr>
<tr>
<td>204</td>
<td>M</td>
<td>41</td>
<td>0.79</td>
<td>0.53</td>
<td>0.68</td>
</tr>
<tr>
<td>205</td>
<td>M</td>
<td>47</td>
<td>0.63</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>207</td>
<td>M</td>
<td>45</td>
<td>1.72</td>
<td>0.18</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Mean 51 1.60 0.30 0.26
S.D. 14 1.04 0.50 0.33

+ Area with high aflatoxin contamination and high incidence of liver cancer
since the age of 10. In these liver cancer patients, their levels of AFB₁ equivalents, expressed per unit creatinine, were all in the range of 0.02-0.12 ng/mg, compared with the range of 0.05-0.84 ng/mg creatinine in other patients with liver disease. In the patients with non-liver disease, there were two cases (sample nos. 106 and 140; Table 5.4) which had high levels of AFB₁ equivalents of 1.28 and 1.17 ng/mg creatinine respectively. These patients were both suffering from asthmatic attack. In one case (sample no. 140), the urine sample was extremely dilute, with a creatinine level of only 0.06 mg/ml, and even though the patient had a low level of AFB₁ equivalents (0.07 ng/ml) in his urine, when the aflatoxin excretion was corrected for the creatinine level, the aflatoxin excretion was high. In contrast, sample no. 106 had a creatinine level in the normal range but a very high level of AFB₁ equivalents, resulting in a high level of AFB₁ equivalent per unit creatinine. The ranges of AFB₁ equivalent/mg creatinine in patients with or without liver disease in this area were 0.02-0.84 and 0.02-1.28 ng/mg respectively.

In Songkhla, there were 9 liver disease cases (Table 5.5), of which 1 case (11%) had liver cancer (sample no. 183). He was 36 years old with the level of AFB₁ equivalents of 0.07 ng/mg creatinine. This level was low when compared with the levels obtained from other patients with liver disease in the same hospital, in which the range was 0.03-0.41 ng/mg creatinine. In the patients with non-liver disease (Table 5.6), the range of AFB₁
Table 5.5
AFB₁ equivalents in urine samples obtained from patients with liver disease from Songkhla+

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB₁ equivalent (ng/ml)</th>
<th>AFB₁/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>F</td>
<td>54</td>
<td>1.38</td>
<td>0.19</td>
<td>0.14</td>
</tr>
<tr>
<td>175</td>
<td>F</td>
<td>67</td>
<td>1.84</td>
<td>0.26</td>
<td>0.14</td>
</tr>
<tr>
<td>103</td>
<td>M</td>
<td>49</td>
<td>2.20</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>173</td>
<td>M</td>
<td>64</td>
<td>12.26</td>
<td>1.14</td>
<td>0.09</td>
</tr>
<tr>
<td>178</td>
<td>M</td>
<td>48</td>
<td>0.39</td>
<td>0.16</td>
<td>0.41</td>
</tr>
<tr>
<td>180</td>
<td>M</td>
<td>66</td>
<td>3.80</td>
<td>0.22</td>
<td>0.06</td>
</tr>
<tr>
<td>183*</td>
<td>M</td>
<td>36</td>
<td>4.46</td>
<td>0.32</td>
<td>0.07</td>
</tr>
<tr>
<td>187</td>
<td>M</td>
<td>58</td>
<td>8.81</td>
<td>0.28</td>
<td>0.03</td>
</tr>
<tr>
<td>189</td>
<td>M</td>
<td>61</td>
<td>1.76</td>
<td>0.33</td>
<td>0.18</td>
</tr>
<tr>
<td>Mean</td>
<td>56</td>
<td>4.1</td>
<td>0.35</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>10</td>
<td>3.9</td>
<td>0.30</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

+ Area with low aflatoxin contamination and low incidence of liver cancer
* Patient with liver cancer
Table 5.6
AFB₁ equivalents in urine samples obtained from patients with non-liver disease from Songkhla+

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB₁ equivalent (ng/ml)</th>
<th>AFB₁/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>118</td>
<td>F</td>
<td>50</td>
<td>1.65</td>
<td>0.18</td>
<td>0.11</td>
</tr>
<tr>
<td>157</td>
<td>F</td>
<td>58</td>
<td>0.77</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>174</td>
<td>F</td>
<td>53</td>
<td>3.16</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>181</td>
<td>F</td>
<td>60</td>
<td>1.57</td>
<td>0.58</td>
<td>0.37</td>
</tr>
<tr>
<td>188</td>
<td>F</td>
<td>55</td>
<td>5.73</td>
<td>0.37</td>
<td>0.07</td>
</tr>
<tr>
<td>196</td>
<td>F</td>
<td>68</td>
<td>1.99</td>
<td>0.20</td>
<td>0.10</td>
</tr>
<tr>
<td>92</td>
<td>M</td>
<td>36</td>
<td>3.03</td>
<td>0.62</td>
<td>0.20</td>
</tr>
<tr>
<td>94</td>
<td>M</td>
<td>46</td>
<td>2.16</td>
<td>0.33</td>
<td>0.15</td>
</tr>
<tr>
<td>95</td>
<td>M</td>
<td>41</td>
<td>1.86</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>96</td>
<td>M</td>
<td>46</td>
<td>2.33</td>
<td>0.20</td>
<td>0.09</td>
</tr>
<tr>
<td>98</td>
<td>M</td>
<td>51</td>
<td>1.09</td>
<td>0.29</td>
<td>0.26</td>
</tr>
<tr>
<td>100</td>
<td>M</td>
<td>56</td>
<td>2.43</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>115</td>
<td>M</td>
<td>54</td>
<td>1.55</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>119</td>
<td>M</td>
<td>52</td>
<td>6.55</td>
<td>0.71</td>
<td>0.11</td>
</tr>
<tr>
<td>131</td>
<td>M</td>
<td>52</td>
<td>2.03</td>
<td>0.26</td>
<td>0.13</td>
</tr>
<tr>
<td>151</td>
<td>M</td>
<td>26</td>
<td>2.44</td>
<td>0.16</td>
<td>0.07</td>
</tr>
<tr>
<td>163</td>
<td>M</td>
<td>52</td>
<td>4.58</td>
<td>0.29</td>
<td>0.06</td>
</tr>
<tr>
<td>176</td>
<td>M</td>
<td>60</td>
<td>2.16</td>
<td>0.22</td>
<td>0.10</td>
</tr>
<tr>
<td>177</td>
<td>M</td>
<td>57</td>
<td>1.38</td>
<td>1.35</td>
<td>0.98</td>
</tr>
<tr>
<td>179</td>
<td>M</td>
<td>60</td>
<td>3.27</td>
<td>0.26</td>
<td>0.08</td>
</tr>
<tr>
<td>190</td>
<td>M</td>
<td>61</td>
<td>1.76</td>
<td>0.33</td>
<td>0.18</td>
</tr>
<tr>
<td>191</td>
<td>M</td>
<td>51</td>
<td>7.53</td>
<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>192</td>
<td>M</td>
<td>43</td>
<td>1.57</td>
<td>0.13</td>
<td>0.08</td>
</tr>
<tr>
<td>193</td>
<td>M</td>
<td>42</td>
<td>0.69</td>
<td>0.12</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Mean 51 2.64 0.31 0.15
S.D. 9 1.77 0.28 0.19

+ Area with low aflatoxin contamination and low incidence of liver cancer
equivalents was 0.02-0.98 ng/mg creatinine. The highest level of AFB1 equivalents in this group of patients (0.98 ng/mg) was found in a 57 years old man (sample no. 177) who suffered from urinary tract infection and had a urinary creatinine content in the normal range.

In Khon Kaen, there were 13 cases of liver disease (Table 5.7), of which 4 cases (31%) had liver cancer (sample nos. 152, 162, 111 and 209). These were two women who were in their 30's and two men who were over 50 years. The range of AFB1 equivalents for these cancer patients was 0.06-0.17 ng/mg creatinine which was nearly the same as the range obtained for the other liver disease (non-cancer) patients (0.04-0.23 ng/mg). In the patients with non-liver disease (Table 5.8), the range of AFB1 equivalents was 0.03-1.69 ng/mg creatinine. The highest level (1.69 ng/mg) was observed in sample no. 81 which was obtained from a 54 year old man, suffering from congestive heart failure. This high result was due to the fact that his urine sample was extremely dilute with a creatinine concentration of only 0.09 mg/ml.

In Khon Kaen hospital, all the patients were screened for liver fluke ova in the stools. They were positive in 4 (31%) and 3 (17%) cases of patients with and without liver disease respectively. There was one case (sample no. 161) who had both liver fluke infection and liver cancer. The levels of AFB1 equivalents in the patients with liver fluke infection were higher in the non-liver disease group than in the liver disease patients. There is no clear
Table 5.7

AFB₁ equivalents in urine samples obtained from patients with liver disease from Khon Kaen+

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB₁ equivalent (ng/ml)</th>
<th>AFB₁/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>88</td>
<td>F</td>
<td>74</td>
<td>1.26</td>
<td>0.25</td>
<td>0.20</td>
</tr>
<tr>
<td>152*</td>
<td>F</td>
<td>39</td>
<td>2.76</td>
<td>0.35</td>
<td>0.13</td>
</tr>
<tr>
<td>158</td>
<td>F</td>
<td>35</td>
<td>3.48</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>161*x</td>
<td>F</td>
<td>37</td>
<td>1.59</td>
<td>0.22</td>
<td>0.14</td>
</tr>
<tr>
<td>77</td>
<td>M</td>
<td>52</td>
<td>0.58</td>
<td>0.13</td>
<td>0.23</td>
</tr>
<tr>
<td>104</td>
<td>M</td>
<td>58</td>
<td>2.79</td>
<td>0.33</td>
<td>0.12</td>
</tr>
<tr>
<td>108*x</td>
<td>M</td>
<td>54</td>
<td>4.53</td>
<td>0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>109*x</td>
<td>M</td>
<td>63</td>
<td>3.08</td>
<td>0.35</td>
<td>0.11</td>
</tr>
<tr>
<td>111*</td>
<td>M</td>
<td>65</td>
<td>0.63</td>
<td>0.11</td>
<td>0.17</td>
</tr>
<tr>
<td>138</td>
<td>M</td>
<td>46</td>
<td>2.62</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>147*x</td>
<td>M</td>
<td>57</td>
<td>2.52</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>170</td>
<td>M</td>
<td>52</td>
<td>3.67</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>209*</td>
<td>M</td>
<td>53</td>
<td>2.53</td>
<td>1.62</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Mean   53  2.46  0.32  0.11
S.D.  11  1.17  0.40  0.06

+ Area with high aflatoxin, high incidence of liver cancer and high incidence of liver fluke infestation
* Patients with liver cancer
x Patients with liver fluke infection
Table 5.8
AFB$_1$ equivalents in urine samples obtained from patients with non-liver disease from Khon Kaen$^+$

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Sex</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB$_1$ equivalent (ng/ml)</th>
<th>AFB$_1$/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>F</td>
<td>40</td>
<td>0.87</td>
<td>0.33</td>
<td>0.38</td>
</tr>
<tr>
<td>125</td>
<td>F</td>
<td>36</td>
<td>4.48</td>
<td>0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>141</td>
<td>F</td>
<td>63</td>
<td>0.66</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>81$^x$</td>
<td>M</td>
<td>54</td>
<td>0.09</td>
<td>0.15</td>
<td>1.69</td>
</tr>
<tr>
<td>82</td>
<td>M</td>
<td>55</td>
<td>2.78</td>
<td>0.22</td>
<td>0.08</td>
</tr>
<tr>
<td>85</td>
<td>M</td>
<td>65</td>
<td>2.23</td>
<td>0.28</td>
<td>0.13</td>
</tr>
<tr>
<td>89</td>
<td>M</td>
<td>63</td>
<td>2.72</td>
<td>0.67</td>
<td>0.25</td>
</tr>
<tr>
<td>112</td>
<td>M</td>
<td>54</td>
<td>2.48</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td>124</td>
<td>M</td>
<td>63</td>
<td>0.45</td>
<td>0.21</td>
<td>0.45</td>
</tr>
<tr>
<td>130$^x$</td>
<td>M</td>
<td>54</td>
<td>0.53</td>
<td>0.18</td>
<td>0.34</td>
</tr>
<tr>
<td>136</td>
<td>M</td>
<td>51</td>
<td>1.40</td>
<td>0.22</td>
<td>0.16</td>
</tr>
<tr>
<td>153</td>
<td>M</td>
<td>61</td>
<td>1.47</td>
<td>0.91</td>
<td>0.13</td>
</tr>
<tr>
<td>154</td>
<td>M</td>
<td>63</td>
<td>0.22</td>
<td>0.15</td>
<td>0.66</td>
</tr>
<tr>
<td>156$^x$</td>
<td>M</td>
<td>45</td>
<td>3.67</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>159</td>
<td>M</td>
<td>60</td>
<td>5.88</td>
<td>0.58</td>
<td>0.10</td>
</tr>
<tr>
<td>160</td>
<td>M</td>
<td>54</td>
<td>9.89</td>
<td>0.25</td>
<td>0.03</td>
</tr>
<tr>
<td>162</td>
<td>M</td>
<td>48</td>
<td>4.49</td>
<td>0.24</td>
<td>0.05</td>
</tr>
<tr>
<td>171</td>
<td>M</td>
<td>53</td>
<td>1.72</td>
<td>0.11</td>
<td>0.07</td>
</tr>
</tbody>
</table>

| Mean       | 55  | 2.56 | 0.29 | 0.27 |
| S.D.       | 8   | 2.47 | 0.22 | 0.39 |

$^+$ Area with high aflatoxin contamination, high incidence of liver cancer and high incidence of liver fluke infestation

$^x$ Patients with liver fluke infection
explanation for this difference but one point that is worth noting is that the urine samples obtained from 2 out of the 3 liver fluke infected patients without liver disease were dilute with creatinine concentrations less than 0.6 mg/ml.

From these data, it appeared that correcting for creatinine concentration in the case of several dilute urine samples resulted in apparent significantly high levels of aflatoxin content where the actual aflatoxin assays themselves gave results in the normal range. Therefore, the validity of correcting the samples on the basis of the creatinine content warrants further investigation.

When the results from the three areas are combined (Figure 5.1), there appears to be a trend towards a wider distribution in the levels of AFB₁ equivalents in samples obtained from the non-liver disease patients. When the 'background' level of 0.2 ng AFB₁ equivalent/mg creatinine, obtained from presumably uncontaminated Western urine samples, was applied as the lower limit of detection of significant aflatoxin contamination (see Section 2.4.7.2), there were 5 (8%) and 10 (38%) cases of the patients with and without liver disease respectively in Ratburi who had levels of AFB₁ equivalents higher than 0.2 ng/mg creatinine. While in Songkhla and Khon Kaen, there were 1 case (11%) and 1 case (8%) of liver disease and 4 cases (17%) and 6 cases (33%) of the non-liver disease group respectively exhibiting significant levels of aflatoxin in their urines. These higher levels in patients with
Figure 5.1  AFB$_1$ equivalents in urine samples from patients with or without liver disease from different areas of Thailand

- Liver disease patients
- Non-liver disease patients
non-liver disease may be due to the fact that the patients with liver disease came to the hospitals when they were very sick. From medical records, most of them were suffering from their symptoms at least one week before admission to the hospitals, therefore, their dietary patterns might have completely changed during this period, and the levels of aflatoxin excreted in the urine might not represent the normal level of exposure. In contrast, the patients with non-liver disease came to the hospitals because of their acute symptoms such as asthmatic attack. In these cases, the nature of their ailments were probably such as not to have had a significant effect on their dietary habits. Thus, there was a possibility that they had consumed their normal diet which contained aflatoxin and, therefore, their urine samples would be more likely to reflect the aflatoxin contamination of this food.

When the levels of aflatoxin excreted in urine samples of patients with non-liver disease from the three different areas were compared, the levels of AFB\textsubscript{1} equivalents, higher than 0.2 ng/mg creatinine in Ratburi, Songkhla and Khon Kaen were 38, 11 and 33\% of cases respectively. If these numbers represent the levels of exposure, the results are consistent with the survey of aflatoxin contamination in food (Shank \textit{et al.}, 1972b), i.e. there is a high level of aflatoxin contamination in Ratburi and Khon Kaen and a relatively low contamination in Songkhla.

Even though the numbers of patients participating in this study were too small to make any valid statistical
comparisons, nevertheless a trend in the levels of aflatoxin excreted in human urine samples is indicated. These levels are in agreement with the levels of aflatoxin-contaminated food for each area. This study also shows that the methods developed to monitor aflatoxin excretion in human urine samples are able to detect variations in the levels of contamination. It also provides useful information for designing further epidemiological studies.

5.4 SUMMARY

The methods for monitoring aflatoxin excretion in human urine samples were applied to samples obtained from patients with or without liver disease from three different areas of Thailand. These areas differ in terms of the levels of aflatoxin contamination in food and incidences of liver cancer and liver fluke infestation. The results obtained show that the methods can detect variations in the levels of aflatoxin in urine samples. However, there is no statistically significant difference between the aflatoxin content of samples obtained from patients with or without liver disease in the same areas, or between the levels found in samples obtained from the different areas. This may be due to the small number of samples analysed. This study also suggests that patients with liver disease may not be the group that should be monitored for urinary aflatoxin levels, because their dietary patterns may have
changed as a result of their illness prior to their admission to hospitals. However, when samples obtained from patients with non-liver disease were compared, a trend in the levels of aflatoxin found in the urine samples was observed which was consistent with the known levels of food contamination in each area.
CHAPTER 6

Monitoring of urinary aflatoxin excretion

in Thai vegetarians
Studies of the geographical distribution of cancer have demonstrated a variation in the incidences of specific cancers (Parkin et al., 1988). It is estimated that 80% of human cancers are environmentally determined and only a small part can be attributed to genetic factors (Doll, 1986). The wide variation in incidence of cancers of various sites in the gastrointestinal tract and the liver may be due to carcinogenic factors in the diet. In some parts of Thailand, human foodstuffs have been found to be contaminated with small amounts of aflatoxins and this is believed to contribute to a high incidence of hepatocellular carcinoma in some areas (Shank et al., 1972d). Aflatoxins have been reported to contaminate mainly vegetarian products (see Table 5.1) such as peanuts, rice and maize (Shank et al., 1972b,c; Anukarahanonta et al., 1984). Since the vegetarians consume more of these products than the meat-eaters, it is likely that the former have a higher risk of exposure to aflatoxins than the latter. Starting from the early 1970's, there has been a surge of interest in vegetarianism as an alternative food pattern (Guthrie, 1975). In Thailand, as in other parts of the world, increasing numbers of people are turning to vegetarianism for a variety of reasons. To some it represents a form of religion or spiritual release through which they hope to purify their bodies. For others, vegetarian diets are an alternative food to relatively
costly animal proteins. There are two major types of vegetarianism, namely the true vegetarian and ovolacto-vegetarian (Guthrie, 1975; Davidson et al., 1977). The former relies exclusively on vegetable proteins whereas the latter has milk, eggs and other dairy products in addition to vegetables. Thai vegetarians usually consume rice, peanuts, beans and maize daily.

Evidence has been advanced suggesting a significant difference in drug metabolism between vegetarians and meat-eaters. Asian vegetarians were shown to metabolize antipyrine more slowly than Asians or Europeans who ate meat regularly (Fraser et al., 1978; Mucklow et al., 1979; Wilmana et al., 1979). Half-lives of antipyrine and paracetamol were slightly longer in the vegetarians than non-vegetarians (Dollery et al., 1979; Brodie et al., 1980). The low protein content of the Asian vegetarian diet may be responsible for the differences in drug handling between Asian and European vegetarians. However, a study of antipyrine metabolism has suggested that a significant difference in antipyrine clearance between lactovegetarians and meat-eaters appears to be related to differences in consumption of animal fat rather than protein (Fraser et al., 1978).

There is little doubt that dietary factors influence the development of cancer in humans (Rogers, 1978) and animals (Newberne, 1976). The complexities of dietary interactions with chemical carcinogens, especially aflatoxin, in animal models, are evident from many studies.
(Boyd et al., 1982; Emerole et al., 1984; Llewellyn et al., 1985; Chang and Bjeldanes, 1978; Baldwin and Parker, 1987; Bailey et al., 1987), and the situation very probably is no less complicated in the case of human cancer. In some parts of the world, especially in the developing countries, primary liver cancer is a major problem, particularly in males (Parkin et al., 1988; Srivatanakul et al., 1988). In most populations, the livers of hepatic cancer patients are usually fatty and often cirrhotic, although the populations generally do not consume excessive levels of fat. It should be noted, however, that the fat that they do consume is often unsaturated (fish or vegetable) and that their diets frequently are contaminated with mycotoxins, nitrosamines or other hepatic carcinogens. They also suffer from a high incidence of viral hepatitis and, in some cases, liver parasitism. Any or all of these factors may interact with dietary nutrients to enhance liver cancer (Newberne et al., 1979).

In view of the above evidence, vegetarians would appear to be a high risk group of the population for aflatoxin carcinogenicity, although there is, at present, no epidemiological evidence to support this hypothesis. It is also possible that the handling of aflatoxins in the body may be different between the vegetarians and non-vegetarians.

The objective of this study was to compare the levels of aflatoxins excreted in urine of these two groups of the population. A well-established vegetarian community in
Bangkok was selected for this study. In this community, they follow the same doctrine of Buddhism that believes in a very modest style of living. They tend to follow the same activities and patterns of eating throughout the year. They usually consume rice, peanuts, beans and maize which are transported from areas where high levels of contamination with aflatoxins have been reported.

6.2 MATERIALS AND METHODS

6.2.1 Subjects
The subjects in this study consisted of vegetarians and non-vegetarians who live in Bangkok. The non-vegetarians were third-year medical students at Mahidol University while the vegetarians were from one vegetarian community, Santi Asoke. Forty-five people from each group participated in the study. They were told about the objective of the study. The vegetarians had been on a strict vegetarian diet for more than 3 months. None of the subject in either group was a regular smoker or drinker.

6.2.2 Sample collections
Single voided urine samples were collected from these two groups of people in August 1987. For the non-vegetarian group, the urine samples were collected in 50 ml plastic containers at Mahidol University. The data on their age and sex were also collected. The samples
(15 ml) were transferred into glass bottles with screw caps and stored at -20°C before sending to England.

For the vegetarian group, the urine samples were collected at their community in 50 ml plastic containers. Then they were placed in ice boxes and brought back to Mahidol University. The samples (15 ml) were also transferred into glass bottles and stored at -20°C. All the pertinent data were collected.

All the samples were sent frozen to England in a box with dry ice. Upon arrival, they were stored at -70°C until required.

6.2.3 **Aflatoxin analysis**

Urine samples (1 ml) were used in the assay for aflatoxin contamination. They were subjected to the 'clean-up' procedures before analysing for AFB₁ equivalents by ELISA (using 4 replicates) as described in Section 2.2.4.

6.2.4 **Creatinine analysis**

The samples were analysed for creatinine concentrations as described in Section 2.2.3.7.

6.2.5 **Statistical analysis**

Results were analysed comparing the levels of aflatoxin per creatinine unit in urine samples from the various groups by student t-test.
The age and sex of the subjects participating in this study are summarized in Table 6.1. There was a wide variation between the means of the ages of the vegetarians and the non-vegetarians. The mean age of the non-vegetarians was 20 while that of the vegetarians was around 40 years. This was due to the fact that the non-vegetarians were medical students who were in their 20's, while in the case of the vegetarian group, they were a mixed population of all ages. Comparison between the sexes within each group were possible because the mean ages in both sexes of each group were similar.

Single voided urine samples were chosen as the method of collection for the reasons stated in Section 5.3, and creatinine levels were expressed as mg/ml. For the purpose of comparison, aflatoxin levels, analysed by ELISA, were expressed as ng AFB₁ equivalent/mg creatinine. In the vegetarian group, the levels of aflatoxin in the male and female ranged from 0.04-0.39 and 0.04-3.22 ng AFB₁ equivalent/mg creatinine respectively, with the mean values of 0.14 and 0.31 ng/mg (Tables 6.2-6.3). The levels in the female samples were higher but these values did not reach statistical significance. In the non-vegetarians, the ranges were 0.02-1.82 and 0.04-1.85 ng AFB₁ equivalent/mg creatinine for male and female, with mean values of 0.20 and 0.24 ng/mg respectively (Tables 6.4-6.5). When the data obtained from the male and female
Table 6.1

Age and sex distribution in vegetarian and non-vegetarian populations

<table>
<thead>
<tr>
<th></th>
<th>MALE</th>
<th></th>
<th>FEMALE</th>
<th></th>
</tr>
</thead>
<tbody>
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<td>non-vegetarian</td>
<td>vegetarian</td>
<td>non-vegetarian</td>
<td>vegetarian</td>
</tr>
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<td>number</td>
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<td>19</td>
<td>20</td>
<td>26</td>
</tr>
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<td>mean age (year)</td>
<td>20.12</td>
<td>38.47</td>
<td>20.25</td>
<td>39.42</td>
</tr>
<tr>
<td>standard deviation</td>
<td>1.01</td>
<td>13.46</td>
<td>1.21</td>
<td>16.46</td>
</tr>
<tr>
<td>range of ages (year)</td>
<td>18-23</td>
<td>26-64</td>
<td>18-24</td>
<td>20-78</td>
</tr>
</tbody>
</table>
### Table 6.2

**Urinary aflatoxin levels in male vegetarians**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB$_1$ equivalent (ng/ml)</th>
<th>AFB$_1$/Creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>304</td>
<td>53</td>
<td>2.34</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>307</td>
<td>40</td>
<td>2.34</td>
<td>0.62</td>
<td>0.26</td>
</tr>
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<td>311</td>
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<td>1.16</td>
<td>0.19</td>
<td>0.16</td>
</tr>
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<td>313</td>
<td>53</td>
<td>1.93</td>
<td>0.36</td>
<td>0.19</td>
</tr>
<tr>
<td>314</td>
<td>58</td>
<td>3.32</td>
<td>0.52</td>
<td>0.16</td>
</tr>
<tr>
<td>319</td>
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<td>0.99</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
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<td>3.17</td>
<td>0.27</td>
<td>0.09</td>
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<td>2.91</td>
<td>0.17</td>
<td>0.06</td>
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<td>324</td>
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<td>0.26</td>
<td>0.07</td>
<td>0.27</td>
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<td>2.34</td>
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<td>0.12</td>
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<td>330</td>
<td>27</td>
<td>4.14</td>
<td>0.54</td>
<td>0.13</td>
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<td>2.74</td>
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<td>0.09</td>
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<td>0.15</td>
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<tr>
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<td>0.18</td>
<td>0.13</td>
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<tr>
<td>344</td>
<td>64</td>
<td>2.79</td>
<td>0.11</td>
<td>0.04</td>
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</table>

|            | mean | 38.47 | 2.14 | 0.25 | 0.14 |
|            | S.D.  | 13.46 | 1.02 | 0.16 | 0.09 |
Table 6.3

Urinary aflatoxin levels in female vegetarians

<table>
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<th>Sample No.</th>
<th>Age</th>
<th>Creatinine (mg/ml)</th>
<th>AFB1 equivalent (ng/ml)</th>
<th>AFB1/Creatinine (ng/mg)</th>
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<td>300</td>
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<td>0.38</td>
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<td>0.10</td>
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<td>0.15</td>
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<td>0.24</td>
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<td>0.28</td>
<td>0.05</td>
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<td>48</td>
<td>2.37</td>
<td>0.29</td>
<td>0.12</td>
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<td>310</td>
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<td>0.30</td>
<td>0.43</td>
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<td>0.41</td>
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<td>0.10</td>
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<td>0.25</td>
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<td>0.82</td>
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<td>3.22</td>
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<td>0.09</td>
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</table>

Mean 39.42  2.25  0.42  0.31
S.D. 16.46  1.33  0.56  0.62
<table>
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<th>Sample No.</th>
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<th>AFB₁ equivalent (ng/ml)</th>
<th>AFB₁/Creatinine (ng/mg)</th>
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</thead>
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<td>0.23</td>
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<td>0.24</td>
<td>0.22</td>
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<tr>
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<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
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<td>0.10</td>
<td>0.05</td>
</tr>
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<td>0.07</td>
<td>0.05</td>
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<td>0.96</td>
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<td>1.63</td>
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<td>0.07</td>
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<td>0.09</td>
<td>0.04</td>
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<td>AFB₁/Creatinine (ng/mg)</td>
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216
Table 6.5

Urinary aflatoxin levels in female non-vegetarians

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<th>AFB\textsubscript{1} equivalent (ng/ml)</th>
<th>AFB\textsubscript{1}/Creatinine (ng/mg)</th>
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<td>0.13</td>
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<td>0.09</td>
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</tr>
<tr>
<td>279</td>
<td>24</td>
<td>1.38</td>
<td>0.19</td>
<td>0.14</td>
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Mean 20.25 1.68 0.23 0.24
S.D. 1.21 1.21 0.21 0.40
urine samples were analysed statistically, no significant differences were found either within or between vegetarians and non-vegetarians. This might be due to the small number of samples being analysed. If the number of subjects was increased, a statistically significant difference might possibly be observed.

However, the trend that vegetarians appear to excrete higher level of AFB₁ equivalents than the non-vegetarians is further illustrated when the data are plotted as in Figure 6.1. In addition, the levels obtained in the female samples of both vegetarians and non-vegetarians appear to be higher than those found in the males of comparable groups. These data raise the possibility that the metabolism of aflatoxin in males and females is different, and that females seem to excrete higher levels of aflatoxin. Higher excretion in females could also result from higher intake. However, it would appear that females excrete more of a given dose than males based on the results of the marmoset experiments. In the marmoset, the level of aflatoxin in the female urine sample (as a percentage of dose) was higher than that observed in the male sample (see Section 3.4.4.4). However, the effect of sex difference on aflatoxin metabolism, especially in humans, needs to be further investigated.

From the study using samples from non-exposed European people, the lower limit of significant detection of aflatoxin in the urine was set at 0.2 ng/mg creatinine (see Section 2.4.7.2). When this limit was applied to the data
Figure 6.1 AFB₁ equivalents in urine samples from Thai vegetarians

- Vegetarian
- Non-vegetarian

Male Female Male & Female
obtained in this study, 13 samples (29%) of the vegetarians were positive compared with 11 samples (24%) in the case of the non-vegetarians. However, within the levels detected below the limit of significance of 0.2 ng/mg creatinine, there was a difference between the vegetarian and non-vegetarian samples. In the case of the vegetarians, 6 samples (13%) had values less than 0.05 ng/mg creatinine whereas in the non-vegetarians, there were 14 samples (31%). This may indicate that the level of significant detection deduced from the European samples may not be appropriate to use with samples from Thai people, possibly because the dietary patterns of the Thais and the Western people are different. It has been suggested that the amounts of UALS present in the urine samples, which are used to set a level of significance of aflatoxins detection, depend on the dietary intake of the subjects (Dragsted et al., 1988). However, this limit cannot easily be set using samples from Thai people, because it is usually unknown if they have consumed aflatoxin-contaminated food. In other words, on the evidence of the results from ELISA detection, the relative contribution from UALS and actual aflatoxins is difficult to assess. When the results of the Thai samples were compared with those obtained using presumed uncontaminated urine samples from Western people, the levels observed in the Thai samples were actually much lower than the values obtained using the Western samples even though the Thai samples were obtained from an area where high levels of aflatoxin-
contaminated food were found. This discrepancy cannot be explained on the basis of the level of aflatoxin contamination, but it appears possible that the limit of significance of 0.2 ng/mg creatinine set on the basis of the Western samples may not be appropriate to the Thai samples and that dietary patterns may, at least in part, play an important role.

In the case of the vegetarians, there is one point that is worth mentioning. Since their sources of diet are entirely vegetables and fruit, if they do not choose their diet carefully, they may experience some degree of malnutrition. It has been shown that vegetarians may not have protein deficiency if they eat a correct diet but they will probably have some kind of fat deficiency due to the difficulty in substituting for animal fats (Frazer et al., 1978). This could result in an alteration in metabolism. Such a difference has been shown for the metabolism of antipyrine between vegetarian and non-vegetarian groups (Fraser et al., 1978; Dollery et al., 1979; Brodie et al., 1980). Therefore, it is possible that the metabolic pathways of many other xenobiotics may differ between vegetarians and non-vegetarians. If this is the case, the aflatoxin metabolites excreted by these two groups of people may be different. Because the same antibody was used to detect the aflatoxin in the urine of the two groups, differences in the nature of metabolites excreted by them could have a considerable effect on the results obtained. This could lead to a non-significant difference
in the levels of aflatoxins excreted, because the antibody used may not be able to detect them. However, this subject needs further investigation which was beyond the scope of this thesis.

6.4 SUMMARY

From these results, it is confirmed that the methods developed for monitoring aflatoxin excretion can be used in the case of actual human samples. It can detect wide variations in the levels of aflatoxins in the urine. In this study, vegetarian people were selected as the study population because they were a high risk group who were more likely to consume aflatoxin-contaminated food. But when the results were compared with a non-vegetarian group, the statistical analyses failed to show any significant difference. However, it is possible to suggest that vegetarians may excrete different forms of metabolites from non-vegetarians. This factor needs to be investigated thoroughly.
CHAPTER 7

General Discussion
The aim of this study was to develop a method for monitoring individual exposure to aflatoxin. The method should be able to be used in large scale epidemiological studies without requiring any expensive equipment. It should be sensitive enough to detect a very low level of aflatoxin exposure with reliable results. The system should not be too complicated to perform and ideally it should be able to be used in field studies; i.e. in the areas where there is a high level of contamination with aflatoxins. In order to monitor aflatoxin exposure at an individual level, urine is the easiest material to collect and the technique is non-invasive. The method for monitoring urinary aflatoxin excretion has been developed with a 'clean-up' procedure to be applied to the samples prior to the aflatoxin analysis by ELISA. The method was validated in the conventional way by using uncontaminated urine samples from European people spiked with radiolabelled AFB₁. In addition, the marmoset monkey, an animal model which has similar pathways of aflatoxin metabolism to human (Hsieh et al., 1977; Yourtee et al., 1987), was also used to validate the method because it is probable that their urine, after treating the animals with AFB₁, would contain a spectrum of aflatoxin metabolites similar to that expected to be present in urine samples of exposed humans. The details of the development of the method will be discussed in Section 7.2.
After the method had been successfully developed, it was used to examine human exposure in samples collected from different areas of Thailand where there is considerable evidence of aflatoxin contamination in foodstuffs (Shank et al., 1972b; Anukarahanonta et al., 1984). Two groups of people were monitored. In the first group, the objective was to compare the urinary aflatoxin excretion in patients with or without liver disease in the areas where there are high and low aflatoxin contamination of aflatoxins in food which correlate with high and low incidence of liver cancer respectively.

In one of the areas being studied, in addition to high incidences of aflatoxin contamination of foodstuffs and liver cancer, there is also a prevalence of liver infection with Opisthorchis viverrini, which presents a serious health problem and which is believed to be involved in the development of cholangiocarcinoma (Harinasuta and Harinasuta, 1984; Thamavit et al., 1988). It was not known whether or not infection with liver fluke would result in any change in the metabolic pathway of aflatoxin. In order to examine this point, the hamster was used as an animal model system to study this interaction because it is a species that is susceptible to infection with the human liver fluke, O. viverrini. The second group of people monitored were vegetarians because they would have a very high probability of consuming aflatoxin-contaminated food. The details of the human monitoring will be discussed in Section 7.4.
ELISA techniques are considered to be suitable methods for measuring the levels of aflatoxins in human body fluids especially in epidemiological studies (Garner et al., 1985). When the techniques were applied to human urine samples, it was found that some substances were present in control, presumed uncontaminated, urine samples which precluded their direct use in ELISA (see Section 2.4.4.). These results are in agreement with other studies (Martin et al., 1984; Groopman et al., 1984). A 'clean-up' procedure for the urine samples was introduced by Groopman et al. (1984) which included the use of a Sep-Pak C\textsubscript{18} cartridge and an immunoaffinity column. In the present study a method for analysing aflatoxins in human urine samples using ELISA techniques with some prior 'clean-up' of the samples was successfully developed (see Chapters 2 and 3). The individual steps in the method developed will be discussed in detail.

7.2.1 **The antibody**

The crucial factor in the development of the method used was the antibody. In this study, it was a rabbit anti-AFB\textsubscript{1} serum which was a polyclonal antibody raised against an AFB\textsubscript{1}-antigen. The antibody had as its major epitope for antibody recognition, in the coumarin and cyclopentenone rings of the aflatoxin molecule (see Section 2.4.3). The anti-serum showed cross-reactivity with
various aflatoxin metabolites but with differing degrees of sensitivity (Table 2.1). A polyclonal antibody was used in order to obtain an overall estimation of exposure to aflatoxin since a range of metabolites would be involved. However when the polyclonal antibody used is compared with a monoclonal antibody raised against AFB₁, for example, that raised by Groopman et al. (1984), it is seen that both antibodies detect a range of AFB₁ metabolites, such as AFM₁, AFQ₁, AFB₁-Gua and AFB₁-FAPy, with a similar order of sensitivities. It is apparent that a monoclonal antibody does not necessarily possess a higher degree of specificity than a polyclonal antibody, and since it is more difficult to raise, the polyclonal antibody used in this study is suitable for screening urine samples for aflatoxin contamination.

However, the use of the polyclonal antibody, raised against AFB₁, in the present study also has several drawbacks. Firstly, the sensitivity for detecting AFQ₁ and its conjugates, which are the most likely metabolites excreted in human urine, is low. These metabolites have been shown to account for more than 30% of the total metabolites in in vitro system using human liver microsomes (Moss and Neal, 1985). This means that the level of aflatoxin detected by this antibody would be underestimated. On the other hand, the antibody can detect AFM₁ with a high sensitivity (Table 2.1). AFM₁ has been found in human urine samples by many investigators (Campbell et al., 1970; Sun et al., 1983; Zhu et al.,
immediately after exposure to aflatoxin-contaminated food. AFM$_1$ excreted in human urine samples represents 1-4% of the dose received. Despite the low level of conversion of AFB$_1$ consumed to AFM$_1$ excreted, the sensitivity with which this metabolite is detected by the antibody used gives a sensitive indication of actual exposure to aflatoxins. Secondly, when urine samples from marmoset monkeys, treated with $^{14}$C-AFB$_1$, were used in validating the capacity of the antibody in detecting the actual metabolites excreted, the antibody had a very low capacity for most of the polar metabolites (see Section 3.4.4.4), even though it was capable of detecting AFB$_1$-NAcCys with high sensitivity (Table 2.1). This demonstrates that it is not the polarity of the compounds which determine the sensitivity of the antibody, but it is the modification at some specific parts of the molecule, especially in the coumarin and cyclopentenone rings, which plays an important role.

In order to increase the sensitivity of the method, further investigations particularly regarding the polar metabolites of aflatoxin need to be carried out in detail. It is most likely that, most if not all of the polar metabolites are in the form of conjugates. One way to increase the sensitivity of the ELISA in detecting conjugated materials in urine samples would, theoretically, be to pretreat them with hydrolytic enzymes in order to cleave the conjugates. This would appear to offer the possibility of increasing the sensitivity of the aflatoxin
ELISA analysis using urine samples. However, in some preliminary experiments (results not given), it appeared that even if the polar aflatoxin metabolites were glucuronide or sulphate conjugates, they were not capable of easy cleavage with β-glucuronidase or sulphatase. Treatment of these polar metabolites with these enzymes did not significantly increase the sensitivity of the ELISA or result in the release of chloroform-soluble metabolites. Some of the polar metabolites might be of considerable significance in relation to long-term exposure to aflatoxins. It is possible that in long-term exposure, aflatoxin which has been metabolized and bound to protein or other macromolecules could subsequently be released by repair systems or turnover of macromolecules, followed by entry into the bloodstream and excretion in the urine.

In this study, six rabbits were used for antibody production. The ELISA and immunoaffinity gel both depend on an antigen-antibody reaction and hence, an antibody which has the capacity to bind an AFB₁ antigen in the ELISA should be usable in the preparation of immunoaffinity gels. In some cases in the present study, the antibody detected aflatoxin in ELISA with a high degree of sensitivity, but did not form active immunoaffinity gels. No explanation is readily apparent for this phenomenon. Presumably there was some factor which affected the ability of the IgG fractions to bind to the gel. This point requires further investigation. Therefore, in this study the antibody used in the ELISA and in preparing the
immunoaffinity gel came from different rabbits but both antibodies were tested in ELISA and were shown to have similar capacities and sensitivities for AFB1 and its metabolites.

7.2.2 The 'clean-up' procedures

The 'clean-up' procedures used in this study involved the use of Sep-Pak C18 cartridges and immunoaffinity columns which are now standard procedures used by many investigators (Groopman et al., 1986; Zhu et al., 1987; C.P. Wild, personal communication). The advantages and disadvantages of these procedures are discussed step by step.

7.2.2.1 Sep-Pak C18 cartridge

Sep-Pak C18 cartridges have been used for collecting and concentrating urine samples for aflatoxin analysis since 1983 (Autrup et al., 1983). Apart from being used as the preliminary 'clean-up' procedure, the cartridge have also been used to transfer the samples from Third World countries where most of the field studies were carried out (Autrup et al., 1983; Groopman et al., 1985; Dragsted et al., 1986). In these studies, not all the samples were collected as 24 h total urines, so it was not possible to obtain the total volumes of urine excreted. To overcome this problem, creatinine concentrations were determined in samples collected at single time points to correct for individual variation in volume voided and allow the
comparison of aflatoxin excreted from each individual (Garner et al., 1985). If the cartridges are used as the means of sample transportation, creatinine is not retained and comparisons between each individual cannot be performed. This problem may be solved either by sending untreated urine samples or by analysing them for their creatinine content before absorbing them onto the Sep-Pak cartridges.

From the results of the experiments performed in the present study using marmoset urine samples, absorption onto Sep-Pak C₁₈ cartridges provided a suitable system for removing some of the substances which interfere with the ELISA, whilst at the same time retaining not only the non-polar but also the polar metabolites of aflatoxin (see Section 3.4.4.1). More than 95% of the radiolabelled metabolites, excreted in marmoset urine, were retained by the cartridge and subsequently could be eluted with methanol. This demonstrated that this step in the 'clean-up' procedures did not cause any significant loss of aflatoxin metabolites and also that, some of the interfering substances could be removed, suggesting that they were polar in nature.

7.2.2.2 Immunoaffinity column

The objective of using the immunoaffinity column is to concentrate and purify the samples based on the antigen-antibody reaction. Substances which are not recognized by the antibody will not be retained, regardless of their
polarity, only substances of interest will be retained in the concentrated samples. The affinity characteristics of the antibodies used in the preparation of the affinity gels determine the range of metabolites which will be retained. Groopman et al. (1984) was the first group who suggested the use of such columns in the assay of aflatoxin, and later reported that the columns were reusable (Groopman et al., 1985, 1986). When the immunoaffinity column was adapted for the present study, apart from the problem encountered in the preparation of immunoaffinity gels with some samples of immune serum as discussed in Section 7.2.1, the columns were found to be reusable only under certain circumstances. When \( \text{AFB}_1 \) was applied to the column in PBS, the column could be reused at least 4 times without losing any capacity. In contrast, when \( \text{AFB}_1 \) was added in uncontaminated human urine samples, no loss of efficiency was observed using the column for two sample assays, but when subsequent column loadings were attempted quantitative binding of \( \text{AFB}_1 \) onto the column did not take place and so the effective use of the column was limited to two preparations. The interfering substances which limited the use of the columns could be partly removed by passage through the Sep-Pak \( \text{C}_{18} \) cartridges. These results are similar to those reported in other studies, for example Groopman et al. (1984) had reported that 60\% of the radiolabelled \( \text{AFB}_1 \) in spiked urine samples applied directly to the affinity column failed to bind, but when the samples were pre-purified by absorption onto Sep-Pak
cartridges, quantitative absorption of AFB₁ subsequently took place on the affinity column. The results from the present study indicated that the volume of urine loaded onto the affinity column should be kept as low as possible consistent with the level of contamination with aflatoxin which determined the probability of obtaining significant results in the subsequent ELISA. It was decided that routinely only 1 ml of urine samples would be used and the column would not be used more than twice.

The capacity of a column, having a 1 ml bed volume, to retain AFB₁ was found to be 20 ng/ml PBS or 5 ng/ml urine. This capacity was quite low when compared with 1-1.3 μg AFB₁ from 10 ml PBS (100-130 ng AFB₁/ml PBS), also using a 1 ml column bed volume as reported by Groopman et al. (1984). The reason for this may be that when a monoclonal antibody, such as that used by Groopman et al. (1984), is used in the preparation of affinity gel, there is a greater concentration of specific binding sites attached to the gel than in the case of polyclonal antibody, even if a purified IgG is used. Furthermore in the present case whole serum was used to bind to the gel. If necessary, the capacity of the gel could be increased by purifying the IgG fraction from the serum to remove serum protein not involved in the antigenic response. This purification step was not attempted in this study because the columns were designed as disposable after two sample loadings and the capacity of the column appeared adequate for the requirement of the study.
When the binding characteristics of the column were examined, using urine samples from marmoset monkeys treated with $^{14}$C-AFB$_1$, only 50% of the radiolabelled metabolites was retained on the column and most of the polar metabolites were removed in the washing fraction. This is due to the lack of sensitivity of the antibody for the polar metabolites present in the urine as discussed in Section 7.2.1. This study appears to be the first one reported examining the retention on affinity gels of those metabolites most likely to be found in human urine. It provides an assurance that the system being developed is suitable for detecting actual aflatoxin metabolites in urine rather than just AFB$_1$.

7.2.2.3 Evaporation process

Since methanol is used at almost every step of the procedures and it had been found that it affected the analysis of aflatoxin in ELISA (see Section 2.4.5), it was necessary to examine the efficiency of methods for its removal. Various evaporation processes were tried and the results indicated that the Speedvac Concentrator was the most efficient and reliable method which gave more than 90% recovery (based on radiochemical recovery) after evaporation at 40°C. Recoveries using some of the other evaporation techniques examined were found to be as low as 20-50% for as yet unknown reasons. The limitation of the chosen method is that it takes a long time to process samples with large volumes, for example, 5 h to concentrate.

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10 ml samples, containing 85% methanol, to 0.5 ml at 40°C, and only 8 samples can be run at a time. When large volumes of methanol-containing samples needed to be evaporated, rotary evaporation was used, but it required great care to avoid any loss of the samples, and much more careful attention than the Speedvac Concentrator.

When samples were fractionated by HPLC, different fractions contained different concentrations of methanol due to the gradient elution used. In order to remove these differences in methanol concentration, the samples were evaporated to dryness in the Speedvac Concentrator at 40°C. The practice of evaporation to complete dryness whilst suitable for the quantitative HPLC analyses performed, was not advisable when samples from the Sep-Pak and affinity column were used in subsequent ELISA analysis because of very variable recoveries. But in the case of the HPLC fractionating it was used since absolute quantitation was not the object of these experiments.

7.2.3 ELISA

The ELISA technique proved to be sensitive and reliable in analysing aflatoxin in human urine samples and the results were reproducible. It detected a level of AFB₁ as low as 10 pg/ml in PBS. However, when the actual urine samples were used after passing through all the 'clean-up' procedures, they were diluted with PBS in order to achieve a content of AFB₁ equivalents in the range of 0.03-1.00 ng/ml which was in the proportionate part of the
standard ELISA inhibition curves. The actual dilution required depended on the level of aflatoxin contamination of the individual urine samples. At the outset, this level, of course, was not known and so the dilution required was determined as the results of the preliminary experiments using a range of dilutions. The samples were always analysed in triplicate using 6 wells per assay and the results obtained were reproducible (within ± one standard deviation). When the levels of aflatoxin in individual samples were compared, $\text{AFB}_1$ equivalents were expressed per creatinine unit as has been discussed (see Section 5.3). However, all of these aflatoxin values were underestimates, due to the lack of sensitivity of the antibody in recognizing some of the aflatoxin metabolites. But as far as the monitoring system is concerned, the absolute level of contamination may not be crucial. The most important objective is to correlate the relative levels of contamination with the relative incidences of liver cancer in the different regions. It will be valuable in future to gather more information on the level of individual exposure in many parts of the world and relate them to food contamination and liver cancer incidence.

7.3 **URINARY AFLATOXIN-LIKE SUBSTANCES (UALS)**

There is considerable evidence that urine samples cannot be used directly in the immunoassays due to the presence of UALS (Martin et al., 1984; Groopman et al., 1984).
1984; Dragsted et al., 1988). In the present study using 10 presumed uncontaminated urine samples from West Germany, the levels of UALS detected varied in the range of 1.63-10.69 ng apparent AFB\textsubscript{1}/mg creatinine. These levels could be reduced by 97% by passing the samples through Sep-Pak C\textsubscript{18} cartridges and immunoaffinity columns, when the levels were substantially reduced to 0.04-0.24 ng apparent AFB\textsubscript{1}/mg creatinine (Table 2.16). These results indicate that there are some substances in the urine that can react with antibody and interfere with ELISA. However, even after the 'clean-up' procedures, although the levels are reduced, the materials remaining in the samples react in the ELISA giving measurable blank values. These findings are essentially in agreement with the study of Martin et al. (1984) who found that human urine samples collected in France were positive for aflatoxins in an ELISA using a polyclonal antibody against AFB\textsubscript{1}, and with the study of Groopman et al. (1984, 1986) who noted that materials present in human urine samples collected in the USA, inhibited the binding of AFB\textsubscript{1} to an affinity column which was prepared using a monoclonal antibody against AFB\textsubscript{1}. The interference due to these substances could be abolished by extraction or dilution (Martin et al., 1984), or effectively removed by concentration of the urine samples on Sep-Pak C\textsubscript{18} cartridges (Groopman et al., 1986). However, in a more recent study using Danish urine samples, Dragsted et al. (1988), who found levels of 0.0-6.5 ng AFB\textsubscript{1} equivalent/mg creatinine analysed by ELISA using a
monoclonal antibody against AFB₁, reported that a non-specific inhibitor from some urine samples could be removed in the purification step using a Sep-Pak C₁₈ cartridge, but however, a competitive component for the antibody still remained. This finding is consistent with the results from the present study that UALS can be partially, but not completely, removed by the 'clean-up' procedures using a Sep-Pak and an affinity column.

An attempt has been made to isolate and characterize the structure of UALS (Dragsted et al., 1988). They found that excretion of UALS showed inter- and intra-individual variations and indicated that dietary components, especially beer, dairy products and meat, were responsible for the high level of UALS present in the Danish samples. They also suggested that different aflatoxin-like compounds may be present in the different dietary sources. It is obvious that dietary patterns vary considerably in different parts of the world and since they appear to be highly correlated with the excretion of UALS, therefore, this subject requires thorough investigation in different populations. However, this may not be practical in areas with high contamination with aflatoxins because of difficulties in differentiating between the relative contributions of UALS and actual contamination with aflatoxins in the ELISA. To solve this problem, monitoring of UALS levels and aflatoxin contamination in food have to be carried out in parallel, and the levels of UALS could be established using the samples obtained from people known to
consume aflatoxin-free food. However, a great number of samples would have to be analysed.

In order to infer the possible chemical structures of UALS, Dragsted et al. (1988) used a map of epitopes, recognized by the antibody, to conduct an on-line computer search and came up with strong evidence that UALS contain an aflatoxin-like structure, possibly with modification at the 8,9-position. The UALS can be resolved into several peaks by HPLC, indicating that they represent a group of compounds with retention times similar to or slightly longer than that of AFB1. By comparison with the results of Dragsted et al. (1988) it appears that the hamster may be a good animal model system to study the UALS because in HPLC separations of control urine samples before treating with AFB1 (Figure 4.7), the apparent AFB1 was present in fractions having retention time corresponding to the retention time of AFB1 itself. Since hamsters produce relatively concentrated urine, no preliminary evaporation processes are required and so they should provide a suitable source of UALS for characterization.

7.4 STRATEGY FOR MONITORING AFLATOXIN EXPOSURE USING HUMAN URINE

The purpose of aflatoxin monitoring in human body fluids is to provide useful information on the risk assessment of this compound with regard to the development of liver cancer. In this study urine samples from patients with or without liver disease from different parts of
Thailand were monitored. It was found that the use of hospitalized people as the study group, especially patients with liver disease, was not ideal for monitoring aflatoxin exposure. Although it is relatively easy to collect biological fluids from these people, it is possible that they may be too sick to have their normal dietary patterns. By changing from their normal diet even for a short period of time, aflatoxin will be readily removed from their bodies. In humans, similar to the marmoset monkey, most of the aflatoxin metabolites will be excreted within the first 48 h of exposure (see Section 3.4.5). Therefore, monitoring the levels of aflatoxin excretion after admission to hospital is unlikely to be representative of their previous exposure because the majority of the aflatoxin detected represents immediate pre-exposure only. From this viewpoint, the target population for urinary aflatoxin monitoring should be normal healthy people in the high risk areas since they are likely to have their normal dietary patterns. By monitoring this population, more useful information regarding exposure should be obtained.

When patient populations are monitored for aflatoxin excretion, one point which should be considered is the status of their drug metabolizing systems. Any condition that has an affect on aflatoxin metabolism may lead to alterations in the metabolites excreted. It was clearly shown in this study, using hamsters infected with liver fluke, *Opisthorchis viverrini*, that the pattern of
aflatoxin metabolites excreted in the urine was different from the non-infected animals. If this is also the case in humans, comparison of aflatoxin excretion by ELISA monitoring of urine samples between people with or without liver fluke infection, may be invalid because the results of the monitoring method are dependent on the relative antibody recognition of specific aflatoxin metabolites. Alteration of the pattern of metabolites excreted to compounds that are not recognized by the antibody or which are more poorly recognized will lead to a further underestimation of exposure levels. It is quite clear therefore that any condition which may affect or alter the pathways of aflatoxin metabolism should be investigated in detail. This could be carried out by examining the changed pattern of metabolites excreted and, in addition, by using a variety of antibodies with differing specificities toward aflatoxin metabolites. This would provide a better understanding of the aflatoxin excretion pattern.

However, it was found from this study that there was some evidence of a correlation between the level of aflatoxin excreted in urine samples by patients with non-liver disease and the level of aflatoxin-contaminated food in those areas of study, although the correlation did not achieve statistical significance. These patients, even though they do not have liver disease, may not be an ideal population for aflatoxin monitoring but, at least, they indicate that using the methods developed, it is possible to obtain valid results using actual urine samples obtained
from areas of differing aflatoxin contamination. In a future study, it would be preferable to compare groups of normal healthy people living in those areas.

In the study on vegetarians, there is a trend that they may excrete higher levels of aflatoxin in their urine (Figure 6.1), but more samples need to be analysed. In addition, it was also found in this study that females tend to excrete higher levels of aflatoxin than males. The higher levels of aflatoxin found in urine samples may indicate that they are exposed to higher levels of aflatoxin in food, or alternatively that they may excrete more aflatoxin from a comparable level of exposure. From the marmoset monkey study which is consistent with other animal studies, the female excretes a higher percentage of the aflatoxin dosed and is less susceptible to the toxic effects of AFB\textsubscript{1} than the male (Busby and Wogan, 1984). High initial excretion of aflatoxin metabolites in urine may indicate that AFB\textsubscript{1} is metabolized to less toxic compounds which are then more readily excreted, without binding to any biologically active macromolecule. However, monitoring of food intake is necessary before any firm conclusion could be drawn.

In order to monitor aflatoxins in the community more precisely, surveys of exposure in the general population need to be carried out. However, this will pose some problems. The first problem is concerned with the logistics as it is not an easy task to monitor a large group of population living in a widespread community and it
would require a large number of staff to collect the appropriate urine specimens for aflatoxin analysis. The other important scientific consideration is the well known fact that the latent period of cancer is very long and it may take several years before cancers developed after exposure to the causative carcinogenic agent. Therefore, the level of aflatoxin exposure observed at any one time may not reflect the actual situation at the time of the carcinogenic induction. Providing that the life style, eating habits and levels of contamination are relatively constant, as appears to be the case in Thailand (Anukarahanonta et al., 1984), the monitoring of aflatoxin exposure will be appropriate. However, it is still quite difficult to establish the link between aflatoxin contamination in food and level of excretion if only a single time point sample is collected. This is because the levels of aflatoxin found in the urine samples will represent only the immediately previous exposure. Therefore, pilot studies may be needed using a stable community in which patterns of diet are constant. Food and urine samples should be collected for at least 7 consecutive days to determine whether there is a variation within a week. This study would need to be repeated to determine seasonal variation. If the variations within a week and also between the seasons are low, samples collected at any single time point would be appropriate, otherwise the study design has to take all of these variations into account. In addition, the number of
samples collected should be large enough to allow statistical analysis to be performed, especially when there are multiple factors involved in the study.

7.5 CONSIDERATIONS IN THE APPLICATION OF THE MONITORING SYSTEM TO AFLATOXIN EXPOSURE IN HUMAN

The monitoring of urinary aflatoxin excretion should be applied to prospective studies in order to prevent liver cancer. However, there are some points that need to be borne in mind.

1. There is strong evidence supporting the hypothesis of multifactorial aetiology of human liver cancer in which aflatoxin and HBV both play important roles (see Section 1.5). In order to study the effect of aflatoxins in the development of liver cancer, confounding factors should be taken into consideration, especially those which may alter the pattern of metabolites excreted in urine. The results of the present study indicate such a role for infection with liver fluke in the hamster animal model system. Clearly a similar situation could exist in the case of infection with HBV and this aspect should be considered in any aetiological studies.

2. The UALS present in samples from different parts of the world may differ because of the patterns of diet consumed. The UALS levels may differ between children and adults, and also between males and females, aspects which require further investigation.
3. Concerning the metabolic aspect of aflatoxin action, it is quite obvious that with any alteration in liver status, e.g. as a result of liver parasitism, the patterns of aflatoxin metabolites could be changed as indicated in the hamster study. Thus liver fluke infection and other conditions that may affect the drug metabolism systems should be borne in mind when carrying out monitoring of aflatoxin in urine. The results of the study using urine samples from the Philippine children show a wide range of AFBI equivalents (0.19-5.50 ng/mg creatinine) (D.W. Denning, personal communication). This may be due to differences in aflatoxin metabolism between adults and children. It would be very interesting to study the levels of aflatoxin excretion in children of various ages because, from experimental studies, young animals appear to be more susceptible to the carcinogenic and/or toxic effects of many substances (Vesselinovitch and Mihailovich, 1968). In addition, for the purpose of prospective monitoring, children would be the appropriate group to start with.

4. The results of urinary aflatoxin monitoring very much depend on the antibody used, and therefore it would be desirable to have a range of antibodies which could recognize the appropriate metabolites present in urine samples from different conditions such as infection with liver fluke, vegetarians as well as 'control' people.
5. Many studies monitoring urinary aflatoxin levels are concerned with the presence of primary metabolites formed principally in the liver or other organs. These aflatoxin metabolites only reflect acute exposure. Other metabolites present in the urine or perhaps blood which are more indicative of chronic exposure, such as aflatoxin-protein or DNA adducts should be considered in future studies. In this connection, the average life-time in human of e.g. albumin (20 days) and haemoglobin (120 days) render these molecules better candidates for monitoring chronic exposure than e.g. AFM₁ which is excreted within 48 h of exposure to aflatoxin. The influence of adduct formation in proteins on the life-time of these compounds is also largely unknown. The possibility of shorter or extended life-time should be investigated. In addition, the pattern of metabolites excreted by people exposed to chronic low doses of aflatoxin may not be the same as those resulting from acute high doses. In this study, the urine samples from marmoset monkeys used in the validation of the methods developed, represent only acute high dosing in which the antibody used could not detect most of the polar metabolites excreted. In the chronic situation, it is most likely that more polar metabolites would be excreted. This is indicated by the results of the marmoset study in which 48 h post-dosing, samples contained a higher percentage of polar metabolites.
than the 24 h samples (see Section 3.4.2). Therefore, it is necessary to develop antibodies capable of detecting these polar metabolites to achieve higher sensitivities in monitoring for chronic exposure.

7.6 SUMMARY

The immunological methods necessary for monitoring aflatoxin excretion in human urine samples were successfully developed using ELISA techniques with prepurification of the samples with Sep-Pak C_{18} cartridges and immunoaffinity columns. The crucial part of these procedures is the antibody used in ELISA and in the preparation of the immunoaffinity gel. The antibody used has as its major epitope, the coumarin and cyclopentenone rings of the aflatoxin molecule. The overall recoveries of the procedures developed, validated using urine samples from aflatoxin-treated marmoset monkeys were approximately 50%. This low recovery was apparently due to the inability of the antibody to recognize most of the polar metabolites excreted. These methods have been used to monitor the levels of aflatoxin excretion in urine samples from various groups of people from Thailand. The results indicate that the methods developed are capable of detecting a wide range of aflatoxin excretion and, therefore, are suitable for use in epidemiological studies provided that certain modifying factors, e.g. differences in patterns of metabolite excretion, are borne in mind.
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