Corrections

Page 10	1.1	compound
Page 14	6.2, 6.3	cumulative
Page 21	line 5	O-methyl guanine more readily pairs
Page 40	line 10	that dose orally had no effect!
Page 56	line 11	(200mg)
Page 64	line 6	anhydrous $MgClO_4$ (1:1 by weight)
Page 105	line 3 from bottom - conversion	
Page 157	line 10	HCl
Page 162	line 4 from bottom - unaffected	
Page 165	x axis	δppm
Page 174	line 6 from bottom - conversion	
Page 227	line 9	at

THE METABOLISM AND DISPOSITION OF *trans*-ANETHOLE AND *p*-PROPYLANISOLE IN RODENT SPECIES AND MAN

by

SUSAN ANN SANGSTER

a thesis submitted
for the degree of
Doctor of Philosophy
in the University of London

September, 1983.

Department of Pharmacology,
St. Mary's Hospital Medical School,
London, W2 1PG.

'Where there is much desire to learn,
there of necessity will be much arguing,
much writing, many opinions; for opinion
in good men is but knowledge in the
making.'

John Milton (1608-1674),
Areopagitica 1644.

ABSTRACT

1. The metabolism of two food flavours, [^{14}C-methoxy]-*trans* anethole and [^{14}C-methoxy]-*p*-propylanisole were investigated in rodents. Both compounds underwent extensive oxidative O-demethylation, shown by $^{14}CO_2$ elimination. Urinary metabolites were isolated by solvent extraction and chromatography and identified by their chromatographic characteristics, mass spectra and nuclear magnetic resonance spectra. These comprised products of side chain oxidation and cleavage. Ten ^{14}C-metabolites were identified after [^{14}C]-*trans*-anethole administration, the major being two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol and 4-methoxyhippuric acid, and four after [^{14}C]-*p*-propylanisole administration, the major being 1-(4'-methoxyphenyl)propan-1- and 2-ols, and 4-methoxyhippuric acid.

2. An investigation into the conversion of 1-(4'-methoxyphenyl)-propan-3-ol to 4-methoxyhippuric acid indicated that the latter metabolite of *p*-propylanisole may arise in this instance from ω-hydroxylation of the side chain.

3. The effect of dose size on the metabolism in rodents of [^{14}C-methoxy]-*trans*-anethole and [^{14}C-methoxy]-*p*-propylanisole, was studied over the range 0.05-1500mg/kg. Oxidative O-demethylation was found to decrease with increasing dose from 64% and 78% of the dose respectively at 0.05mg/kg to 33% and 48% respectively at 1500mg/kg. Over this dose range the elimination of ^{14}C urinary metabolites increased significantly after both compounds.

4. The rate of oxidative O-demethylation of p-propylanisole was shown to be independent of dose size in mice until 1.5h post dosing after which the rate decreased in high dose animals.

5. Investigation of the disposition of [^{14}C-methoxy]-trans-anethole and [^{14}C-methoxy]-p-propylanisole in two human volunteers at doses close to the normal dietary exposure showed that both compounds were rapidly absorbed and eliminated (within 24 hours) and completely metabolised by similar routes, but to different extents, as the rodent species.

6. In view of the great discrepancy between human dietary exposure to these compounds (15-65µg/day) and the doses used in their toxicological evaluation in animals, these results emphasize the importance of considering the possibility of dose-dependent metabolism when interpreting toxicity data from high level rodent experiments with respect to potential human risk.

ACKNOWLEDGEMENTS

I am very grateful to Prof. Bob Smith for his continuous advice and encouragement and for allowing me the opportunity to work in his department, and also to Dr John Caldwell under whose guidance and help this research was undertaken. I would like to thank all my colleagues in the department, past and present who have helped to make this studentship rewarding both intellectually and socially.

Special thanks are due particularly to Dr Andy Hutt and to Philippa Chilvers for her patience in typing this thesis. I also much appreciated the advice that Dr Tim Sloan gave with the statistical analysis of the data.

Outside the department I am grateful to Dr Peter Farmer for the facilities of mass spectrometry that he made available to us.

Lastly and by no means least I should like to thank my family, most especially Tom for help, patience and not understanding a word during this studentship.

This work was supported by a grant from the Flavor and Extract Manufacturers' Association, Washington, D.C., U.S.A.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

General Introduction

1.1. INTRODUCTION

An ultimate aim of much research in the life sciences is to achieve an improvement in the quality of life. The past 120 years have brought with them tremendous advances in medicine. There is now the capability to prevent, treat and in most cases cure infectious diseases. In the middle of the last century 80% of all mortalities were as a result of some type of infectious disease (McKeown, 1979). By 1931 the corresponding figure had fallen to 30% (Offices of Health Economics, 1980) and by 1981 less than 1% of all deaths were as a result of infectious disease (Central Statistical Office, Annual Abstract of Statistics, 1983). This dramatic decrease can be attributed to the prophylactic use of immunisation (first introduced for smallpox prior to 1848, McKeown, 1979) and the introduction of chemotherapy, arsenicals (Erhlich, 1913), sulphonamides (Domagk, 1935), natural penicillins (Fleming, 1929; Chain et al., 1940; Florey and Florey, 1943) and semi-synthetic penicillins (1959).

The major causes of mortality in the 1980's are the diseases of the circulatory system (40% of all mortalities) and neoplasia (20% of all mortalities) (see table 1.1.). Cardiovascular diseases have been described as 'diseases of civilisation' and many epidemiological studies have suggested that such diseases are associated with certain 'risk factors', such as cigarette smoking, intake of a highly animal fat laden diet, obesity and stress, which could predispose a person to cardiovascular disorders (for references see Inglis, 1981). Neoplasia*, the second major cause of death, can take many forms attacking any organ or tissue of the body.

*Most neoplasias are cancers

Table 1.1. Death analysed by cause in England and Wales, 1981

Cause	Number of deaths	% of total deaths
Infectious/parasitic disease	2,107	0.3
Digestive disease	16,455	2.5
Respiratory disease	83,514	12.6
Accidents and violence	19,880	3.0
Circulatory disease	286,326	43.3
Neoplasia	131,758	19.9
Other disease	121,479	18.4

taken from the Central Statistical Office, Annual Abstract of Statistics, 1983.

Cancer is a disorder of cellular growth, in which the cells are no longer subject to the restraining influences normally controlling their behaviour. The fault causing this disorder is a result of interference with the DNA (and/or RNA and proteins) in the cell, and once established this fault is irreversible and transmitted to succeeding generations of cells in which the disorders first developed.

Many hypotheses have been suggested to account for this postulated alteration (see section on possible mechanisms of carcinogenesis), and table 1.2. lists some known exposures, diseases and predispositions and their associated cancers.

Table 1.2. Exposures, diseases and pre-dispositions, and associated associated cancers

Causal influence	Type of cancer
heredity	retinoblastoma, familial polyposis
ethnic origin	nasopharyngeal cancer (Southern Chinese)
viral infection - Epstein-Barr	Burkitt's lymphoma
radiation - X-ray	Skin cancer
- atomic bomb	leukaemia (Hiroshima)
injury, inflammation - sunlight	skin cancer
- ulcerative colitis	cancer of large bowel after approx. 20 years
parasites - Schistosoma haematobium	chronic bladder inflammation leading to cancer
environmental, dietary chemicals	see table 1.3.

taken from Bodley-Scott, 1981.

With the exception of some specific and readily identifiable causes, e.g. smoking and lung cancer (Smoking and Health, 1964), chewing betel nuts and cancer of the mouth (Muir and Kirk, 1960), schistosomiasis and bladder cancer and certain industrial chemicals (Tomatis et al., 1978) the cause can seldom be identified or the type is extremely rare.

Over the past two decades several authors have estimated that 60-90% of all human cancer cases can be attributed to environmental causes (Miller, 1978; Tomatis, 1979 and references therein). These percentages were based upon two main considerations : a) a certain number of environmental factors have been identified in the aetiology of human cancer (table 1.3.) and b) significant variations in the incidence of

cancer in different population groups in different countries suggest that environmental factors play an important role (Miller, 1978).

The first reported observations that environmental exposure to chemicals is associated with cancer dates back to the sixteenth century when it was noted that pitch-blende miners in Saxony and Bohemia frequently suffered from lung cancer, and to 1775 when Percival Potts reported that chimney sweeps were particularly prone to a rare cancer of the scrotum. Since that date many types of exposure have been implicated in cancer initiation, Table 1.3. lists a few examples where laboratory animals administered the compound by the same route as human exposure acquire cancer in the same target organ as man (Tomatis et al., 1978). There are also many examples of environmental hazards resulting in human cancer where animal exposure does not result in tumours in the same target organ(s) (Tomatis et al., 1978).

1.2. Mechanisms of Carcinogenesis

There is at present no single theory to explain the induction of cancer in either man or animals. Cancer as a result of exposure to chemicals is thought to implicate many processes, the compound interacting with host receptors (RNA, DNA or protein) in competition with the natural defence mechanisms (DNA repair and immune response), which attempt to control the onset of cancer. It is of interest to note that children who suffer from the rare disease of immune insufficiency (agammaglobulinaemia, Wiskott-Aldrich syndrome) have increased incidence of neoplasm (Crampton and Charlesworth, 1975).

Table 1.3. Some chemicals or industrial processes associated with cancer induction in humans with the same target organs when administered to animals by the same route.

Chemical or industrial process	humans			animal species
	type of exposure	target organ	main route of exposure	
Aflatoxins	diet	liver	oral inhalation	rat, fish, duck, marmoset, monkey, tree shrew
Asbestos	occupational	lung, pleura, gastrointestinal tract	inhalation oral	mouse, rat, hamster, rabbit
Diethylstilboestrol	therapeutic	uterus, vagina	oral	mouse (local administration)
Soot, tars and oils	occupational environmental	lung, skin (scrotum)	inhalation skin	mouse, rabbit
Vinyl chloride	occupational	lung, liver brain	inhalation	mouse, rat

data from Tomatis et al., 1978.

There are many research areas within the field of chemical carcinogenesis and several theories have been suggested to explain these findings and other associated exposures that may lead to cancer.

Investigations by E.C. and J.A. Miller and collaborators have indicated that some chemical carcinogens e.g. aflatoxins, acetylaminofluorene, estragole, safrole and pyrolizidine alkaloids are metabolised in vivo to electrophilic intermediates that are able to interact with cellular macromolecules (Miller and Miller, 1981). The reactive electrophilic metabolites can interact with nucleophilic centres in proteins and nucleic acids. Interactions in DNA are at nitrogen and oxygen atoms in purine and pyrimidine bases and at oxygen atoms in the phosphate moiety, and form a wide variety of adducts (Farber, 1981 and references therein).

DNA damaged by chemicals may be repaired by normal cellular mechanisms. However if repair does not occur prior to replication then the repairable lesion is converted at replication to a non-repairable, inheritable change to the base sequence of the daughter strand.

Chemicals can cause several types of mutation of the DNA strand. Base pair substitutions are particularly though not exclusively associated with alkylation of DNA by ethyl- or methyl-groups. Guanine normally pairs at replication with cytosine, however O-methylguanine more readily pair with thymine, therefore DNA containing O-methylguanine can cause G-C to A-T transition (ECETOC, 1982). Another type of mutation is frame shift. Large bulky molecules such as aflatoxin B, and acetylaminofluorene form DNA adducts which are excised, leaving apurinic sites in DNA. This type of mutation is known as frame shift

because the ordered translation of codons, triplet by triplet is profoundly disturbed.

The Miller's theory is supported by the good correlation of known carcinogens and their ability to induce mutations in bacteria (Purchase et al., 1976), when the carcinogens were metabolically activated to electrophilic intermediates by a liver activating system (Ames et al., 1973). The natural carcinogens, the aflatoxins, estragole and safrole and their metabolites 1'-hydroxy-estragole and 1'-hydroxysafrole have all been found to be carcinogenic in rats and mice, and the 1'-acetoxymetabolites of estragole and safrole have been shown to be mutagenic in bacteria (Butler and Barnes, 1963; Borchert et al., 1973a,b; Drinkwater et al., 1976; Miller et al., 1983).

The strong electrophilic nature of ultimate carcinogens is consistent with both genetic and epigenetic mechanisms of carcinogenesis. In the former, the electrophiles binding to DNA, and in the latter to proteins.

The epigenetic theory of carcinogenesis is based upon the fact that complex organisms develop from a fertilised ova. If it is accepted that normal differentiations are a consequence of epigenetic phenomena, then similarly epigenetic modifications of cellular transcription or translation or both may also be involved in the conversion of apparently normal cells to tumour cells with relatively stable phenotypes. Data from a variety of experiments indicate that malignant cells and non malignant cells may possess the same genomes, although detailed analyses at the molecular level have not yet been feasible.

Gurdon (1974) has transplanted nuclei from frog renal carcinomas into enucleated fertilised frog eggs with the subsequent development of apparently normal swimming tadpoles. These studies showed that the nuclei from the tumours retained in expressible form at least the major share of the information that was present in the fertilised ova (McKinnell et al., 1969; Gurdon, 1974).

Chimeric mice have been obtained by implantation of single cells from embryoid bodies of mouse teratocarcinoma cells into mouse blastulas. The chimeric mice contained a wide variety of apparently normal somatic tissues that developed from the progeny of the teratocarcinoma cells.

The initiation-promotion theory of tumour growth developed to explain the observation that following a small initiating dose of a carcinogen, the prolonged or repeated treatment with a non-carcinogenic promoter caused a progression of changes leading to tumour development (Berenblum, 1974). The relatively slow and reversible nature of promotion is in accord with an epigenetic concept.

Promoters cause increased synthesis of phospholipids, RNA, protein and DNA, and increase the mitotic rate (Boutwell, 1974). One of the earliest changes seems to be increased ornithine carboxylase activity (Boutwell, 1978), and inhibition of the promotion stage of epidermal carcinogenesis by protease inhibitors suggest that specific proteases may be of critical importance in promotion (Matsushima et al., 1976; Troll, 1976).

The genotoxic theories of carcinogenesis assume that the change from a normal to a tumour cell is dependent on genomic alterations. This

latter point of view receives primary support from the fact that the potential of a cell is determined by the information coded in the genome and from the abilities of carcinogenic agents to alter cellular genomic content. Quite compelling evidence for tumour development depending on an alteration of genomic information is available from u.v.-induced carcinogenesis. Xeroderma pigmentosa patients are very susceptible to exposure to sunlight. It has been shown that cells from these patients have greatly impaired capacity for error-prone repair of DNA that contains u.v.-induced or chemical induced lesions (Miller, 1978).

1.3. Dietary Hazards

Identification of many human carcinogens (Tomatis, 1979) has increased interest in the possibility of food-mediated cancer. A number of natural carcinogens have been identified in human diets including one of the most toxic compounds known to man, aflatoxin B, (Table 1.4.; Crampton and Charlesworth, 1975).

Man's diet consists of many thousands of chemical compounds, many of which, with the advent of more powerful analytical techniques it has been possible to identify. However, only a few are of nutritional significance. Macromolecules such as carbohydrates, fats and proteins are essential for fuel and growth, and micromolecules and elements such as vitamins and minerals are necessary to maintian biochemical processes. A diet made up exclusively of macro- and micro-molecules would support life, but the quality and palatability of food is the result of the so-called anutrients in

Table 1.4. Carcinogens in food as identified by animal experiments

Compound	Food	Origin	Reference
Luteoskyrin	rice	microbiological contaminant	See Crampton and Charlesworth (1975) for references
Cyclochlorotine	rice	"	
Aflatoxins	ground nuts and cereals	"	
Ergot	rye	"	
Cycasin	cyad nut	anutrient	
Selenium	crops	anutrient	
Pyrrolizidine alkaloids	cereals	anutrient	
Polycyclic hydrocarbons	many plant foods	anutrient, processing	
Estragole	herbs and spices	anutrient	Drinkwater et al., (1976) Miller et al., (1983)
Safrole	herbs and spices	anutrient	Homburger et al., (1961) Long et al., (1964)
Amino acid pyrolysates	cooked foods	processing	Sugimura et al., (1980)

food. Even the humble potato contains over 250 different anutrient compounds (Crampton and Charlesworth, 1975).

In an address to the Institute of Food Technologists (1971), the President of F.E.M.A. listed the potential hazards in the diet in 6 main categories, microbiological, nutritional, environmental contamination, natural toxicants, pesticide residue and food additives (Wendt, 1971). Food additives are perhaps the most controversial, which is reflected in the large number of guidelines and regulations that control their usage in human foodstuffs. Flavours come under the category of natural contaminants, but the use of natural and synthetic flavour compounds in processed foods are also subject to regulations and guidelines.

1.4. Toxicity testing of food additives

The regulatory authorities in the U.K. (Ministry of Agriculture, Fisheries and Foods, M.A.F.F.) and in the U.S.A. (Food and Drug Administration, F.D.A.) require that potential food additives are screened for potential toxicity or carcinogenicity by performing experiments on laboratory animals. There are no standard protocols for testing in the U.K., and no regulations govern the toxicity testing of food flavours. However, M.A.F.F. does have guidelines for animal testing, outlining the type of tests required for other food additives (M.A.F.F. 1965 and ammendments). In the U.S.A. a formal protocol is laid down which must be satisfied for all proposed food additives (F.D.A., 1983). The guidelines are dependent upon which 'concern level' the compound is placed

(F.D.A., 1983). This is determined based upon the possible level of human exposure, and the chemical structure of the compound, the latter using a "decision tree" (Cramer et al., 1978).

Table 1.5. lists the type of tests that may be required for the highest level of concern (Level III). The information and testing required is not very different for the U.K. and U.S.A.

1.5. Assessment of toxicity data - risk versus benefit

It is impossible to provide an assurance that any substance is absolutely safe in all circumstances for man or animal. The regulatory authorities must decide whether the benefit derived from the compound is greater than the possible risk involved in its consumption. The benefit is difficult to define in the case of a flavour or a food colour. It is far more obvious in the case of a drug or even a food preservative or antioxidant where food is protected from pathogenic organisms or chemical toxins. Even once passed as a permitted additive, further data may become available that persuades the authorities that a compound is no longer 'safe', for example saccharin, now a controlled additive in the U.S.A. but permitted in the U.K. It has been shown to cause bladder cancer in rodents, but there are obvious benefits from use of such a sweetener in decreasing the incidence of obesity and subsequent heart disease resulting from use of sugar only.

Table 1.5. Animal toxicity testing requirements for food additives

Test	Species	dose levels	mode of administration	duration	comments
Acute toxicity	rodents	-	stomach tube	-	determination of LD_{50}, lowest fatal and highest non-fatal dose
Short term toxicity	rodents	control low dose - no toxicity, intermediate high dose-adverse toxicity	stomach tube	7 days - 28 days	range finding studies usually more than 3 dose levels
Subchronic toxicity	rodents	as above	stomach tube or in diet or water	90 days - 12 months	determination of no adverse effect level
Chronic toxicity	rodents and non rodent	as above	as above	at least 12 months	daily observations, gross pathology on death or at termination of study
Carcinogenicity tests	rodents (2 species)	control + 3 doses low dose should not interfere with growth. High dose should elicit toxicity	in diet	major part of life span	An additional dose may be used if the pharmacokinetics or metabolic profile from dose 1 to 3 are different. The level should yield pharmacokinetics similar to lower doses daily observations and gross pathology

continued over

Table 1.5. In continuation

Test	Species	dose levels	mode of administration	duration	comments
Reproductive studies	rodents	control and 3 levels as before	diet/stomach tube	two generations	If toxicity in $F_2 > F_1$ then the study should include F_3 generation
Teratogenicity studies	rodents	as above	stomach tube	during gestation	observed daily foetal examination
Absorption, distribution metabolism and elimination studies	rodents	more than 1 may be required	stomach tube	-	desirable to aid in the evaluation of test results from toxicity tests for extrapolation to man

The material used in the tests must be of the same composition and purity as for human consumption.

based upon F.D.A. (1983) and M.A.F.F. (1965)

1.6. Exposure levels used in toxicity and carcinogenicity tests and their relevance to human exposure

The doses used in toxicity tests are in some cases millions of times the estimated per capita daily intake of food additives in the diet.

The intention of using such large doses of test compound is to counteract the poor sensitivity of the test method and allow statistical evaluation of the toxic/tumour incidences found in the treated and control animals (ECETOC, 1982). The administration of lower doses would necessitate the use of very large numbers of animals, which in many cases would be economically prohibitive.

An assumption is made that in these tests the toxic/carcinogenic response to a chemical is dose related. Thus high doses would therefore increase the incidence of toxicity/tumours and shorten the latency period of the latter. However the pharmacokinetics of compounds may be different at high doses to that at low doses. Many metabolic pathways are saturable (see introduction to Chapter 3) and so linear first order pharmacokinetics can only be applied to a defined concentration range. For higher doses an expression involving Michaelis-Menten kinetics must be derived (Wagner, 1974).

If a metabolic pathway of a test compound is saturable, then administration of a high dose will not expose the laboratory animal to the same relative amounts of metabolites and unchanged compound as administration of a low dose would produce. There are three possible consequences of this phenomenon: a) if it is the

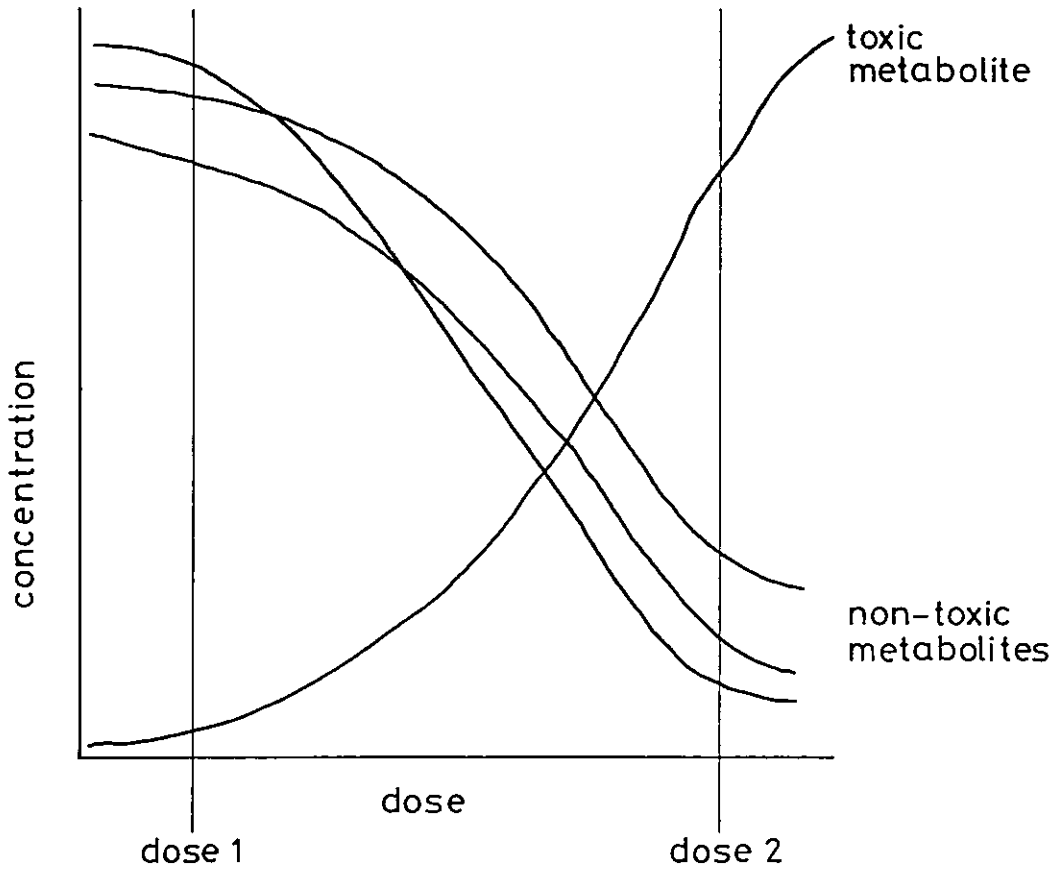
activating/toxic pathway that is saturated, then a higher dose than that resulting in maximal effect will not increase the rate of transformation to toxic metabolites. b) If a de-activating pathway is overwhelmed this could mean that the tumour/toxicity risk would increase disproportionately with dose above the point of saturation. c) Alternative pathways could show dissimilar saturation characteristics, and there would be variation in the ratio of various excreted metabolites as a function of the dose applied.

Figure 1.1. illustrates the situation with a hypothetical compound which at a low dose 1 is metabolised to three non-toxic metabolites and a minor amount of a toxic metabolite. However at a high dose 2, the majority of the compound is metabolised to a toxic metabolite, due to the saturation of a detoxication pathway and little to the non-toxic metabolites. If the toxicity tests of this compound were performed at dose 2 when the expected human exposure was dose 1 it is obvious that the tests would not model the human exposure because the relative levels of metabolites and unchanged compound would be different.

1.7. Metabolism of Safrole and Estragole

Both estragole and safrole are presumed to be carcinogenic by metabolic activation to proximate carcinogens. They are both derived from the volatile oils of plants and are flavouring agents.

Figure 1.1. Hypothetical metabolite profile of a compound at various doses



Safrole has been the subject of many metabolism studies both *in vivo* and *in vitro* (Borchert *et al.*, 1973b; Janiaud *et al.*, 1977; Stillwell *et al.*, 1974) and has been shown to be metabolised by two major routes, oxidation of the allylic side chain and oxidation of the methylenedioxy moiety with subsequent cleavage to catechol. One of the side chain oxidations leads to the excretion of 1'-hydroxysafrole glucuronide. 1'-Hydroxysafrole was more hepatotoxic and hepatocarcinogenic to animals than the parent compound when administered in the diet (Borchert *et al.*, 1973a,b; Wislocki *et al.*, 1976; Wislocki *et al.*, 1977), and more mutagenic than safrole (Swanson *et al.*, 1979). Its mutagenicity was increased by an activating system, suggesting that 1'-hydroxysafrole is metabolised to more potent mutagen(s).

Esterification of 1'-hydroxysafrole to 1'-acetoxysafrole *in vivo* (Wislocki *et al.*, 1976) was demonstrated and this compound has recently been shown to be more hepatocarcinogenic than 1'-hydroxysafrole (Miller *et al.*, 1983).

Estragole like safrole is metabolised by two major routes, side chain oxidation and oxidative O-demethylation (Solheim and Scheline, 1973; Zangouras *et al.*, 1981). 1'-Hydroxysafrole was shown to be excreted as a glucuronide in the urine of mice after estragole administration (Drinkwater *et al.*, 1976). This metabolite is more hepatocarcinogenic and mutagenic than the parent compound (Drinkwater *et al.*, 1976; Miller *et al.*, 1983).

Zangouras et al (1981) demonstrated that the extent of oxidative O-demethylation of estragole in rats and mice was dependent on the dose administered. At 0.05mg/kg 34% of a dose of estragole was metabolised by O-demethylation but at 1000mg/kg only 20% of the dose was metabolised by this route. The percentage of the dose eliminated as the presumed proximate carcinogen 1'-hydroxyestragole rose from 1% of the dose at 0.05mg/kg to 12% of the dose at 1000mg/kg.

The information confirms the fears of the Food Safety Council of the U.S.A. (1978) and ECETOC (1982) when they advised that care should be taken in choosing appropriate doses for long term toxicity tests for carcinogens whose action is the result of metabolic activation.

1.8. Usage of Food Flavours

The aromatic herbs and spices from which today's chemical food flavours are derived have been used as food flavours for many centuries. As today, their usage was economically very important, one of the earliest records of the value of spices is in the Old Testament (1 Kings). Much of the wealth of the Phoenician, Roman and Arab conquerors were derived from spice trade. It is thought that the stories of 'Sinbad the Sailor' are based upon the legends of various spice trading sailors. The cities of Venice and Genoa thrived as spice markets until the Portugese explorer Vasco de Gama in 1498 discovered the sea route to India thus

winning the spice trade from the Venetian merchants. The centre of the trade then passed to the Dutch and finally to London and the British East India Company who for more than two centuries were the greatest spice market in the world.

Trade is now world wide and more synthetic compounds have been introduced into usage along with the more traditional flavours.

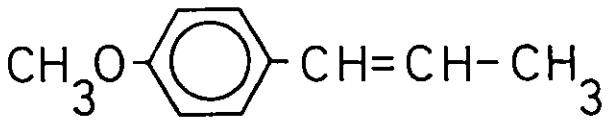
The substituted anisole food flavours trans-anethole, derived from the ancient spice - anise and the synthetic p-propylanisole are of particular toxicological concern because of their similarity in structure to the known carcinogens safrole (Homburger et al., 1961) and estragole (Drinkwater et al., 1976; Miller et al., 1983) as is illustrated in figure 1.2.

1.9. Origins and Usage of anethole and p-propylanisole

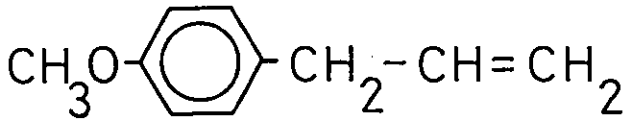
Anethole is to be found in the volatile oils of some twenty different species and varieties of plants (Le Bourhis, 1973a). It is found in particularly large amounts in Anise, Chinese Star Anise and Fennel (table 1.6.). Anise has been used as a food flavour for many centuries, and Chinese medicine has used Star Anise as an aromatic carminative, stimulant and expectorant for 1,300 years (Leung, 1980).

p-Propylanisole (4-methoxypropylbenzene) is a synthetic flavour, described as being a 'sweet, herbaceous, quite powerful odour' (F.E.M.A., 1978). It was first reported in flavour usage in 1948

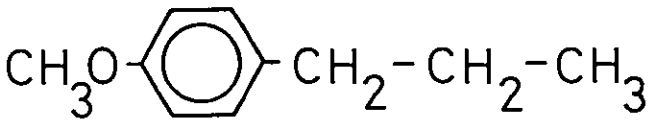
Figure 1.2. Structures of trans-anethole, estragole, p-propylanisole and safrole



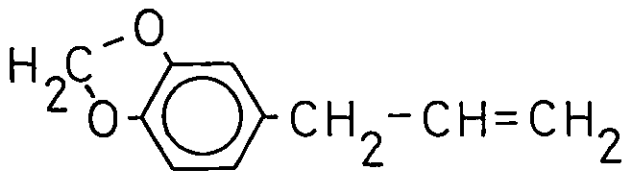
trans-anethole



estragole



p-propylanisole



safrole

Table 1.6. Natural sources of anethole

Species of plant	Common name	Part of plant used	%volatile oil	%anethole in volatile oil
Pimpinella anisum Linné Umbelliferae	Anise or Aniseed	dried fruit	1 - 4	75 - 90
Illicium verum Hooker filius Magnoliaceae	Chinese Star Anise	dried fruit	5	80 - 90
Foeniculum vulgare Miller Umbelliferae	Fennel, finocchio	dried fruit	9 - 28	50 - 90

taken from Leung, 1980.

and was in general use in 1952 (F.E.M.A., 1970).

Both trans-anethole and p-propylanisole are widely used in the food industry. In a survey of usage made for 1970 in the U.S.A., it was estimated that 70 tons of anethole was consumed, which represented an estimated average daily intake of 65µg per capita (2-65 years range) (F.E.M.A., 1978). Data based upon the French consumption of anethole estimates the average annual world usage as 400 tons of anethole, and the French consumption as 200 tons per annum (Le Bourhis, 1973a).

The annual usage of p-propylanisole in the U.S.A. is only about 3,200 lbs (F.E.M.A., 1978) and this represents an estimated average daily intake of 15µg per capita (F.E.M.A., 1978).

For both flavours the major food category of use was in baked goods (table 1.7.) and beverages. A large amount of the annual usage of anethole in beverages is in anisette type liquors. The large annual consumption of anethole in France reflects the larger intake of such liquors in that country. The other major food category where anethole and p-propylanisole are used are sweets, this is significant because the greatest consumers of sweets are children.

1.10. Toxicity of anethole

The long usage of anise has led to a relatively long history of animal toxicity tests involving anise oil. The first reports of

Table 1.7. Usage of trans-anethole and p-propylanisole in various categories of prepared foods

Food category	trans-anethole		p-propylanisole	
	% total intake	intake (μg) per capita per day	% total intake	intake (μg) per capita per day
baked goods	55.6	32.6	78.8	11.5
frozen dairy products	1.7	1.1	2.1	0.3
meat products	0.6	0.4	0.7	0.1
relish, condiments and gravies	0.03	0.02	1.4	0.2
soft and hard sweets, chewing gum	3.4	2.2	4.1	0.6
puddings and sauces	1.8	1.2	2.7	0.4
beverages (including alcoholic)	36.8	24.0	10.3	1.5
all categories		61.52		14.6

animal toxicity tests involving anise oil date back to the eighteenth century. The major observations from these studies were that very large doses of anise oil or absinth oil (now prohibited) could produce convulsions or depression in various animal species; guinea pig, rabbit, rat and dog. Much later, as a result of the large consumption of anise liquors in France, Vignoli et al (1949) compared the effects of various liquors in guinea pigs. He reported that 10ml/kg liquor intravenously produced trembling but double that dose had no effect! and he concluded that as Tanon (cited in Le Bourhis, 1973a), "L'essense d'anis n'est pas toxique".

The propenyl double bond of anethole exhibits cis-trans isomerism. However, the two isomers of anethole were not isolated until 1958 (Naves, 1958a,b; Naves et al., 1958). The natural oils from plants contain much larger amounts of the trans isomer than the cis isomer. However the percentage of cis-anethole in natural oils varies from species to species and locality to locality within the same species (Leung, 1980). Estimates of cis-anethole content in oils vary from 0.045% (Colombo and Manitto, 1971) to 0.8% (Toth, 1967).

Several groups have compared the acute toxicites of the two isomers after oral and intraperitoneal administration.

Table 1.8. LD_{50} data for *cis* and *trans*-anethole after oral and intraperitoneal administration to rats and mice

Species	Isomer	LD_{50} after i.p. injection	LD_{50} after p.o. administration
rat	*trans*	0.8g/kg	3.2 g/kg
	cis	0.1g/kg	-
mouse	*trans*	1.0g/kg	5.0g/kg
	cis	0.1g/kg	0.2g/kg

taken from Caujolle and Meynier, 1958 a,b; Le Bourhis, 1973b.

These studies indicated that i.p. administration caused greater toxicity (lower LD_{50}) than oral administration, and that mice were less susceptible than rats after administration by either route. However *cis*-anethole was by both routes of administration approximately 10 times more toxic than *trans*-anethole. Other studies (Jenner *et al.*, 1964) using anethole from natural sources (>99% *trans*) have reported LD_{50} values in the range 2-5g/kg body weight in rodents. All long term toxicity tests have been confined to *trans*-anethole because it is the more abundant isomer and also *cis*-anethole is chemically unstable and is difficult to obtain in large amounts (*trans* configuration is more stable).

However the greater toxicity of the *cis*-isomer prompted one study into the possibility of *cis*-anethole formation from *trans*-anethole during normal storage of food products. Beaud and Ramaz (1975) showed that it requires the exposure of an anethole containing beverage in a clear glass bottle to direct sunlight for 150 days for the percentage

of anethole as the cis-isomer assayed according to Martin and Berner (1972) to rise from 0.3% to 13.8%. Normal storage in a coloured bottle in the semi-darkness had no effect.

Acute pharmacological studies using trans-anethole have been reported, administration of up to 300mg/kg to mice caused psycholeptic, hypnotic, analgesic and anticonvulsant activity (Le Bourhis and Soene, 1973) and Boissier et al., (1967) have reported reduced motor activity to 60% of control values in mice after 800mg/kg trans-anethole. Prolonged administration of trans-anethole in the diet of rodents at levels up to 500mg/kg and above caused toxic symptoms, namely hepatic lesions, which were particularly prevalent in the male of the species (Taylor et al., 1964; Shelanski, 1958; Hagan et al., 1967) and growth retardation or reduced weight gain (Shelanski, 1958; Le Bourhis, 1973b; Miller et al., 1983) (table 1.9.). However Vignoli et al (1965) reported no toxic effects after administration of 11.4mg/kg to rabbits in the drinking water for up to 90 days (table 1.9.).

trans-Anethole was described as having equivocal carcinogenic risk in mice (F.D.A., 1982). Since that date, data from Miller et al (1983) indicates that trans-anethole was not shown to have any carcinogenic activity at doses in preweanling and weanling mice higher than those required for hepatocarcinogenicity of estragole and safrole.

Anethole was granted GRAS status in 1964 by F.E.M.A. and is an approved additive for food use by the F.D.A. (Opdyke, 1973). The Council of Europe listed trans-anethole (1970) giving an ADI

Table 1.9. Toxicity data for trans-anethole

Reference	dose(oral) g/kg/day	duration of study	species	toxic symptoms
Shelanski, (1958)	0.05 0.15 0.5 1.5	90 days	rat	growth retardation; hepatic cell oedema; changes in hepatic cell degeneration and regeneration, all but lowest dose.
Taylor et al., (1964)	1.8 (1.3-2.4)	4 hours - 4 days LD_{50}	rat	depression at low doses; comatose at high doses
	0.695	4 days	rat	minor hepatic lesions
	0.125	1 year	rat	no effect
	0.5	15 weeks	rat	slight hydropic changes in hepatic cells, males only
Vignoli et al., (1965)	0.0114	2 months 90 days 90 days	rat rat rabbit	no toxic effects
Hagan et al., (1967)	0.5	16 weeks	rat	slight hydropic changes of liver cells, males only
	0.125	1 year	rat	no effect
Le Bourhis, (1973)	1.00 0.1	6 weeks	rat	35% reduction in growth
	0.25 0.5 1.0	12-22 months	rat	1.0g/kg/day growth retardation 0.5g/kg/day growth retardation males only
Miller et al., (1983)	0.23	18 months	mice CD-1	weight loss, and low weight gain no carcinogenic activity

(acceptable daily intake) of 1.5mg/kg and the Joint FAO/WHO Expert Committee on Food Additives (1967) published an ADI of 0-1.25mg/kg for man.

1.11. Toxicity of p-Propylanisole

There is little toxicity data available on p-propylanisole, reflecting its short life as a food flavour, and its low usage. That which is available is summarised in table 1.10.

p-Propylanisole has a high LD_{50} in rodents, 4.4g/kg in rats (Taylor et al., 1964) and 7.3g/kg in mice (Jenner et al., 1964). Administration of 1.47g/kg/day (approximately one third of the LD_{50} value) for 4 days produced mild hepatic lesions but no mortality (Taylor et al., 1964). In a 32 day study (Hagan et al., 1967) where rats were administered 2g/kg/day rising to 5g/kg/day, 16 out of 20 animals survived to receive the highest dose of p-propylanisole and seven survived the entire 32 day study. Substantial toxicity was reported, including stomach ulceration and hyperkeratosis and moderate to severe osteoporosis. In a larger study where doses of 50-500mg/kg/day were administered in the diet, skeletal osteoporosis was reported which increased with severity with dose size.

Table 1.10. Toxicity data for p-propylanisole

Reference	dose oral g/kg/day	duration of study	species	toxic symptoms
Caujolle and Meynier, (1958a)	0.75-4 i.p.	acute	mouse	
Taylor et al., (1964)	1.9(0.6-5.6) 1.475	4 hours - 3 days 4 days	rat	depression, porphyrin like deposit around eyes and nose, wet posterior, mid hepatic lesions
Jenner et al., (1964)	7.30 LD_{50}	acute	mouse	as Taylor et al., 1964 acute data in rats
Hagan et al., (1967)	0.05 0.125 0.5	19 weeks	rat	slight to moderate skeletal osteoporosis increasing in severity with dose
	2-5	32 days	rat	flaky white coalescent areas of forestomach, minute ulcers. Moderate skeletal osteoporosis. Moderately severe hyperkeratosis of the forestomach squamous epithelium.

1.12. Aims of the present research

trans-Anethole and p-propylanisole are important and widely used food flavours. There has been much toxicity data published on these compounds at high doses, however the relevance of these results comes into question because the metabolism of the related food flavour estragole has been shown to be dependent upon the size of the dose administered. This brings forward the suspicion that any toxicity reported for substituted anisoles may not be as a result of the parent compound but a metabolite whose production is only significant at high doses as with estragole and its metabolite 1'hydroxyestragole.

Therefore the objectives of this present study are as follows :-

a) To establish the metabolism of [^{14}C-methoxy]-trans-anethole and [^{14}C-methoxy]-p-propylanisole in the rat and mouse, by isolation and identification of urinary metabolites and measurement of the extent of $^{14}CO_2$ elimination in the expired air.

b) To investigate the influence of dose size administered on the extent of oxidative O-demethylation and urinary elimination of [^{14}C-methoxy]-trans-anethole and p-propylanisole, and on their metabolism by quantification of the urinary metabolites that have been identified.

c) To investigate the disposition of trans-anethole and p-propylanisole in man, at doses close to the normal levels of exposure from the diet.

It is anticipated that this information will help in the interpretation of high dose animal toxicity data, with respect to the risk from these substances to man from the diet, taking particular account of the extrapolation of data from high doses to low doses and from one species to another.

It is hoped that the conclusions will aid in the prediction of the metabolism and toxicity of chemicals with similar structures and low human exposure levels.

CHAPTER 2

Metabolism of *trans*-anethole in the rat and mouse

2.1. INTRODUCTION

Anethole (4-methoxypropenylbenzene) is the compound responsible for the well known aniseed flavour. It is found naturally occurring in the plant kingdom, and herbs and spices containing anethole have been used as flavours for many centuries. However, it was not until 1832 that Dumas (cited in Le Bourhis, 1973a) isolated the flavour component from anise oil, and not until 1958 (Naves, 1958a; Naves et al., 1958) that the two isomers of anethole, cis- and trans- anethole were separated and identified.

The trans-isomer is the most abundant in natural oils (Colombo and Manitto, 1971; Toth, 1967) accounting for greater than 99% of anethole.

Metabolism studies

Metabolic studies on anethole have thus far been restricted to the trans isomer, of which there have been two independent studies : i) Le Bourhis (1970) and Strolin-Benedetti and Le Bourhis (1972) administered [^{14}C]-trans-anethole to rodents and used tlc for metabolite identification, and ii) Solheim and Scheline (1973) administered unlabelled trans-anethole to rats and separated and quantified metabolites by gc and identified them by ms. (Neither study was comprehensive and not all metabolites of trans-anethole were quantified).

trans-[^{14}C]-Anethole, administered orally, is readily absorbed from the gastrointestinal tract as indicated by the high recovery of radioactivity in the urine (Strolin-Benedetti and Le Bourhis, 1972), although previously Le Bourhis (1968) had reported poor absorption of trans-anethole. Fritsch et al., (1975) confirmed the extensive gastric and intestinal absorption of trans-anethole using an in situ perfusion technique.

Solheim and Scheline (1973) reported that the metabolic fate of trans-anethole was independent of the route of administration if given by intraperitoneal injection or orally. They showed that the major route of metabolism was via O-demethylation (52% of dose) with 4-hydroxypropenylbenzene and 4-hydroxycinnamic acid as the major excretion products in the urine (table 2.1.). Le Bourhis (1970) reported that only 5% of the dose was metabolised by O-demethylation as measured by 4-hydroxybenzoic acid content in the urine. This low figure may have been the result of loss of urinary metabolites during acid hydrolysis of urine in work up.

Both studies showed that approximately 40% of dosed trans-anethole was excreted in the urine as 4-methoxyhippuric acid.

Figure 2.1. illustrates the major routes of metabolism of trans-anethole discovered thus far, based upon the more extensive study by Solheim and Scheline (1973). In this same study the metabolism of estragole (4-methoxyallylbenzene) was also investigated. Comparison of the metabolism of the two compounds trans-anethole and estragole

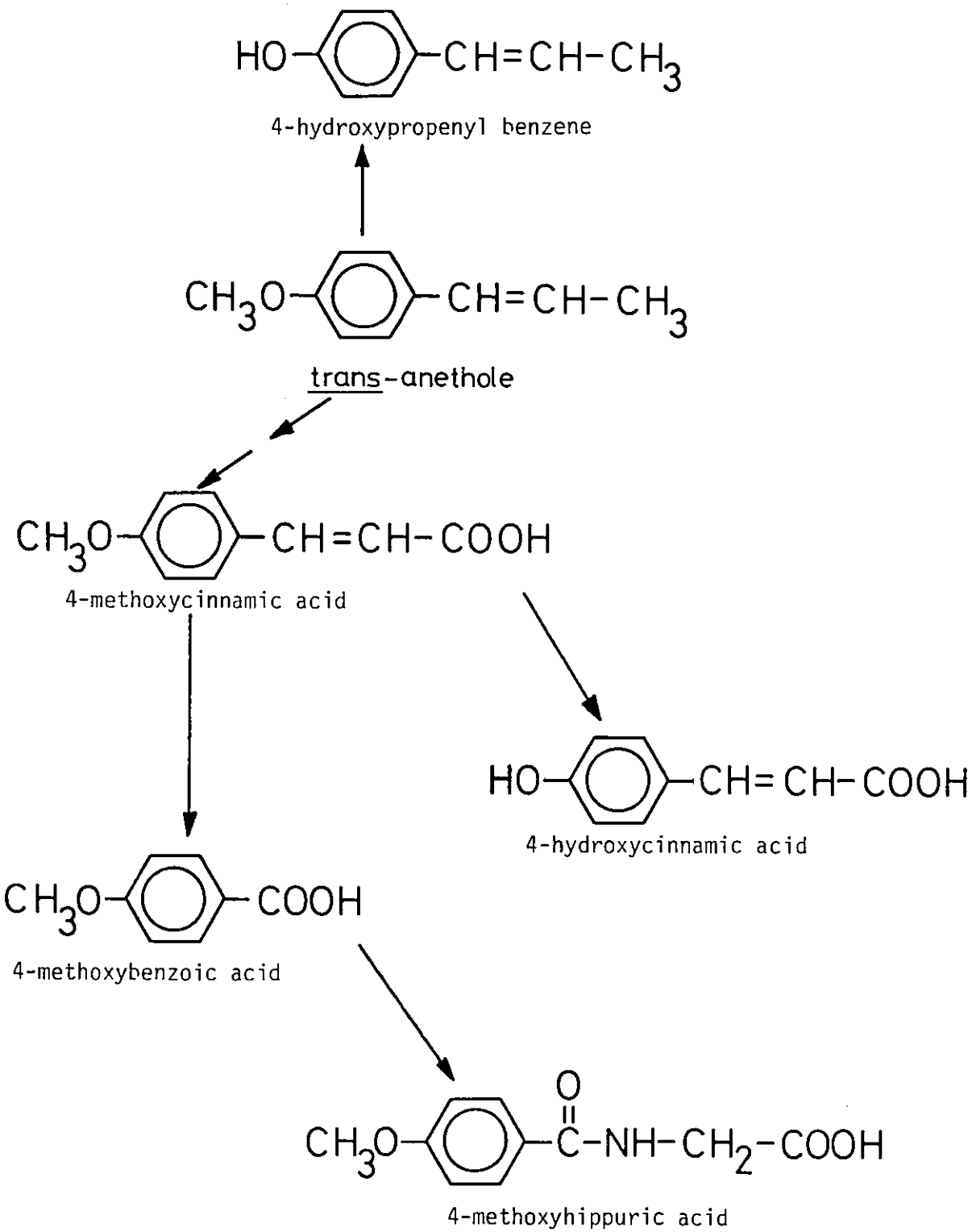
Table 2.1. Major urinary metabolites of 4-methoxypropenylbenzene in the rat

(taken from Solheim and Scheline, 1973)

route of administration	treatment of urine	METABOLITES 4-hydroxy-propenyl-benzene	4-hydroxy-cinnamic acid	4-methoxy-benzoic acid	4-methoxy-hippuric acid	recovery of major urinary metabolites
oral	none	1	7(5-9)	3 (2-3)	49(44-53)	60
oral	hydrolysed	32 (20-37)	15 (9-21)	4 (3-4.5)	43 (36-63)	94
intra-peritoneal	none	1	9 (6-15)	3 (1-5)	41 (35-50)	54
intra-peritoneal	hydrolysed	31 (20-45)	21(14-33)	3 (1-5)	40 (34-49)	95

Figures are % of dose with ranges in parentheses.

Figure 2.1. Major routes of metabolism of trans-anethole in the rat



taken from Scheline (1978)

(Solheim and Scheline, 1973) indicates that metabolism via the cinnamyl derivatives, 4-methoxycinnamyl alcohol and 4-methoxycinnamic acid is more extensive with trans-anethole than with estragole of which only 6-12% of the dose is excreted as 4-methoxyhippuric acid. With trans-anethole, as much as 75% of the dose is excreted as the oxidation products 4-methoxybenzoic acid and its glycine conjugate, 4-methoxyhippuric acid, and 4-methoxycinnamic acid. It is perhaps not unexpected as in trans-anethole the double bond is in the 1',2' position and does not require migration, while in estragole it is in the 2',3' position, and to proceed by metabolism along this pathway the double bond would need to migrate.

This chapter reports the results of a study of the metabolism of [^{14}C-methoxy]-trans-anethole in the rat and mouse, at a dose level of 50mg/kg. In view of the position of ^{14}C-labelling the extent of O-demethylation has been determined by the estimation of $^{14}CO_2$ in the expired air. Urinary metabolites have been separated by chromatography and solvent extraction, characterised by comparative chromatography with authentic standards and ms, and quantified from tlc plates and by radio-hplc.

2.2. MATERIALS AND METHODS

Compounds

[^{14}C]-Estragole (4'-[^{14}C-methoxy]-allylbenzene) specific activity 9.1 mCi/mmol purity >99% by radio-hplc was a custom synthesis by Amersham International, Amersham, U.K.

General laboratory chemicals were purchased from usual U.K. commercial sources. Chemicals used in syntheses and as reference standards are listed along with their sources in appendix 2.1.

[^{14}C-methoxy]-*trans*-Anethole

Estragole (51.5mg) was added to ether (50ml) containing [^{14}C-methoxy]-estragole (4mg, 252µCi). This was dried (anhyd. Na_2SO_4) and reduced to dryness under an air stream in a 1 ml screw-capped vial. Powdered anhydrous KOH was added to fill the vial which was sealed and heated at 130°C for 16 hours. The vial was allowed to cool, then opened under water (50ml) covered with hexane (50ml). The phases were shaken together, allowed to separate and the organic phase removed. The aqueous residue was re-extracted with hexane (50ml), and the hexane extracts were combined and dried (anhyd. Na_2SO_4). A sample of the combined hexane extracts were examined by radio-hplc (system 1). The ms of anethole made by this method is shown in appendix 2.2. It is identical to that of a sample of authentic *trans*-anethole, as was the gc retention time. Gc of the hexane extract using a method known to separate the two isomers of anethole (Martin and Berner, 1972) gave only one

peak at the same relative retention time as authentic trans-anethole compared to estragole.

Trioctanoin (4ml) was added to the dried hexane extract, and the hexane removed in vacuo, leaving a solution of [^{14}C-methoxy]-trans-anethole in trioctanoin (26.3μCi/ml; sp. act. 4.53μCi/mg).

4-Methoxyhippuric acid (4-methoxybenzoylglycine)

4-methoxybenzoyl chloride (5g) was added dropwise over 30 minutes to glycine (2.5g) dissolved in M NaOH (50ml) with stirring. The reaction mixture was stirred at room temperature for 4 hours, and acidified to pH 1.0 (conc. HCl) giving a precipitate which was filtered. The filtrate was washed thoroughly with water and recrystallised three times from hot water, giving the title compound as white crystals, yield 4.3g (70% of theoretical) mp 168-171 °C (Solheim and Scheline (1973) give 168-170 °C). The compound gave a ms consistent with the proposed structure by direct probe insertion as such and by gc-ms of the methyl ester (appendix 2.3. a and b).

4-Methoxymandelic acid

4-hydroxymandelic acid (275.4mg; 0.0016mol) in 0.1M NaOH (28ml) was refluxed with iodomethane (200mg) in acetone (30ml) for 3 hours. The reaction mixture was allowed to cool, and the acetone removed in vacuo. The residue was adjusted to pH1.0 (6M HCl) and extracted with ether (100ml).

The ether extract was dried (anhyd. $NaSO_4$) and evaporated *in vacuo*. The residue was taken up in methanol and subjected to tlc in system B. The band at R_f 0.62 seen under u.v. light was scraped off, and extracted with methanol which was evaporated to give the title compound yield 14.5mg (50% of theoretical) and gave one peak by hplc (system 3, R_t 8.4 min). The compound gave ms consistent with the proposed structure by direct probe insertion ms as such and by gc-ms of the TMS derivative (appendix 2.4. a and b).

4-Methoxyphenyllactic acid

4-hydroxyphenyllactic acid (205mg, 0.0011mol) in 0.1 M NaOH (25ml) was refluxed for 3 hours with iodomethane (200µg) in acetone (30ml). The same procedure and purification was followed as for the 4-methoxymandelic acid synthesis. The off-white product (mp 160°C) purified by tlc (system B, R_f 0.5) yield 94mg (44% of theoretical) gave a ms consistent with the proposed structure by direct probe insertion ms as such (appendix 2.5.).

4-Methoxyphenylpyruvic acid

4-hydroxyphenylpyruvic acid (294mg, 0.0016mol) in 0.1 M NaOH (28ml) and iodomethane (200mg) in acetone (30ml) were refluxed together for 3 hours. The procedure and purification was followed as for 4-methoxymandelic acid. The product was purified by tlc (system B, R_f 0.67) as a brown oil, yield 40mg (8% of theoretical). The product gave a ms consistent with the proposed structure by direct probe insertion ms as such (appendix 2.6.).

Thin layer chromatography (tlc)

Tlc was performed on aluminium backed pre-coated silica-gel plates (cat. no. 5554, E. Merck, Darmstadt, W. Germany) with solvents listed below.

System	Solvents
A	Hexane:ether (1:1 by vol.)
B	Benzene:acetone:glacial acetic acid (6:2:1 by vol.)
C	Benzene:dioxane:glacial acetic acid (90:25:4 by vol.)
D	Benzene:methanol (9:1 by vol.)
E	Chloroform:acetone:glacial acetic acid:water (4:2:2:1 by vol.)
F	Hexane:chloroform:glacial acetic acid (6:3:1 by vol.)

Plates were developed by the ascending technique to 15cm from the origin, and compounds were detected on the plates by inspection under u.v. light (254nm), where aromatic compounds showed as dark quenching spots against the fluorescent background. In addition the following spray reagents were used.

Naphtharesorcinol: 5:1 mixture of 0.2% $^{W}/_{v}$ naphtharesorcinol in ethanol - 85% orthophosphoric acid was sprayed on the plate, and after heating for 10min. at 100°C, glucuronic acid derivatives gave a blue spot on a pink background.

p-Dimethylaminobenzaldehyde (p-DMAB): 4% $^{W}/_{v}$ p-DMAB in acetic anhydride with a few crystals of sodium acetate added before spraying; glycine conjugates give a yellow/orange spot after heating at 100°C for 5 mins.

Chloroplatinic acid reagent: (Toennis and Kolb, 1951) 0.05% chloroplatinic acid with a small amount of potassium iodide (1M) added just prior to spraying. The plates are allowed to dry and divalent sulphur gives a pink reaction.

Potassium chromate/silver nitrate: (Knight and Young, 1958). Firstly the plate was sprayed with 0.1M $K_2Cr_2O_7$ in 50% acetic acid, then after 5 min. sprayed with 0.1 M $AgNO_3$ (aqueous). Divalent sulphur groups give an orange reaction on a brown background.

The R_f values of trans-anethole and related compounds are listed in table 2.2.

High pressure liquid chromatography (hplc)

Hplc was carried out using a Rheodyne 7205 valve loop injector, a Waters Associates M6000A pump and model 441 absorbance detector, a stainless steel column (100 x 5 mm) and the systems listed in table 2.3.

The retention times of trans-anethole and related compounds are listed in table 2.2.

Table 2.2. Chromatographic characteristics of trans-anethole and related compounds by tlc and hplc

Compound	R_t (min) in hplc systems			R_f values in solvent systems					
	1	2	3	A	B	C	D	E	F
Anethole	17.6	>2h	-	0.86	0.95	-	-	-	-
Estragole	16.0	>2h	-	0.86	0.95	-	-	-	-
Chavicol	4.1	35.6	-	0.52	0.70	-	-	-	-
4-methoxyacetophenone	-	12.0	24.8	-	0.72	-	-	-	-
1-(4'-methoxyphenyl)-propan-1-ol	-	17.4	-	0.58	0.64	0.77	0.59	0.95	0.54
1-(4'-methoxyphenyl)-propan-2-ol	-	8.4	-	-	-	-	-	-	-
1-(4'-methoxyphenyl)-propan-3-ol	-	14.4	-	-	-	-	-	-	-
1-(4'methoxyphenyl-propane-1,2-diol	-	5.0	-	0.10	0.70	-	-	-	-
4-methoxybenzaldehyde	-	8.2	-	-	-	-	-	-	-
4-methoxybenzoic acid	-	9.0	21.6	0.25	0.91	0.78	0.40	0.95	0.67
4-methoxycinnamic acid	-	24.0	42.8	-	0.85	-	-	-	-
4-methoxycinnamyl alcohol	-	15.0	-	0.20	0.80	-	-	-	-
4-methoxycinnamoyl glycine	-	3.0	-	0	0.30	-	-	-	-
4-methoxyhippuric acid	-	3.1	6.2	0	0.38	0.16	0.07	0.63	0.02
4-methoxymandelic acid	-	3.2	8.4	-	0.62	-	-	-	-
4-methoxyphenylacetic acid	-	7.1	14.4	0.16	0.79	0.75	0.36	0.95	0.54
4-methoxyphenyllactic acid	-	6.5	-	-	0.50	-	-	-	-
4-methoxyphenylpropionic acid	-	13.8	27.6	0.27	0.80	0.81	0.41	0.95	0.60
4-methoxyphenylpyruvic acid	-	7.0	-	-	0.67	-	-	-	-
4-methoxyphenylaceturic acid	-	3.0	-	0	0.40	-	-	-	-

Table 2.3. Hplc systems used in the study of *trans*-anethole metabolism

System	Column packing*	mobile phase	flow rate	detector wavelength
1	MOS Hypersil 5μ (C_8 reverse phase)	33% aqueous CH_3CN	2ml/min	254nm
2	ODS Hypersil	30% aqueous methanol containing 1% glacial acetic acid	1ml/min	254nm
3	ODS Hypersil 5μ (C_{18} reverse phase)	25% aqueous methanol containing 1% glacial acetic acid	1ml/min	254nm

* all packings from Shandon Southern Products, Runcorn, Cheshire, U.K.

Mass spectrometry (ms) and gas chromatography-mass spectrometry (gc-ms)

Direct probe insertion mass spectra were obtained in the electron impact mode with the VG-micromass ZAB-IF instrument of the University of London Intercollegiate Mass Spectrometry Service. The ionisation energy was 70eV and the probe temperature 160°C.

Mass spectrometric analyses were also made using a VG 70-70 Analytical Mass Spectrometer linked with a VG 2035 data system of the MRC Toxicology Unit, Carshalton, Surrey, U.K.

Electron impact mass spectra were obtained at an ionisation energy of 70eV and a source temperature of 180-200°C. Chemical ionisation using isobutane as reagent gas was used for some metabolites. Samples were admitted to the source either on a direct insertion probe or by a gc-ms interface, and spectra were obtained at 1sec/decade.

Gas-chromatographic separations were made on a fused silica capillary column (20m x 0.3mm), coated with SE52 or OV1 701 stationary phase and housed in a Pye Unicam Series 204 gas chromatograph. Samples were injected using either a falling needle solid injector or an on-column injector. The temperature programme for the SE52 column was 100°C for 20 sec, followed by a 32°/min temperature rise to 250°C and for the OV1 701 column 100°C for 3 min rising by 30°/min to 250°C.

Trimethylsilyl derivatives (TMS) were made by treatment with N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (50µl) in pyridine (50µl) at 70°C for 30 min. Samples were methylated by dissolving them in a saturated ethereal solution of diazomethane. The reaction time was ten minutes except in the analysis of the phenolic metabolite, when a longer time increased the yield of product.

Radiochemical techniques

Radioactivity in excreta and solutions was assayed by liquid scintillation spectrometry with a Packard TriCarb Model 3385 instrument. Counting efficiency was determined by reference to an external standard, using a regularly determined quench correction curve. For urine and water-miscible solutions, a Triton-toluene based Scintillation fluid was used (5.5g PPO, 0.1g POPOP per litre toluene)Triton X 100; 2:1 by vol), and for ethanolamine-2-methoxyethanol mixtures a scintillant of 5.5 PPO per litre toluene/2-methoxyethanol (2:1 by vol) was used (Jeffrey and Alvarez, 1961).

^{14}C on tlc plates was located with a Packard radiochromatogram scanner model 7201, and quantified by cutting plates into 0.5cm wide sections placing these in vials with 5ml Triton/toluene scintillant and counting for ^{14}C.

Radio-hplc was performed by collecting fractions of eluant at 30 sec or 1 min intervals with a LKB RediRac fraction collector and counting these for ^{14}C.

^{14}C in faeces was assayed after homogenisation according to Caldwell *et al* (1972). Radioactivity remaining in the carcass after administration of [^{14}C]-*trans*-anethole was assayed by dissolving the body in concentrated ethanolic KOH (20%^{W}/v) and counting aliquots of the neutralised (6M HCl) digest for ^{14}C.

Animals and Dosing

Female albino Wistar rats (body weight 200-250g) (Anglia Laboratory Animals, Alconbury, Cambs., U.K.) and male CD-1 mice (body weight 15-20g) (Charles River U.K. Ltd., Margate, Kent, U.K.) were used in this study. They were maintained on Labsure 41B pellets, and allowed food and water *ad libitum* throughout the experiment.

The rodents were administered [^{14}C-methoxy]-*trans*-anethole (50mg/kg, 100μCi/kg) in trioctanoin by gavage to rats and by intraperitoneal injection to mice.

The animals were housed in all-glass metabolism cages equipped for the separate collection of urine, faeces and expired air

(Metabowls for rats and Minimetabowls for mice, Jencons Ltd., Hemel Hempstead, Herts., U.K.). Air was drawn into the cages through a sodalime trap (Durasorb, M.I.E. Ltd., Sowton, Devon, U.K.) to absorb CO_2. The effluent gas from the cages passed through a drying bottle containing anhydrous $CaCl_2$ and anhydrous (1:1 by weight) then a 'cold finger' containing methanol at -20°C to trap volatile compounds in the expired air, and through two traps (200ml each for mice and 350ml each for rats) containing ethanolamine-2-methoxyethanol (1:2 by vol) to trap expired $^{14}CO_2$ as described by Jeffray and Alvarez (1961). Drying agents and trapping solutions were changed at 24 hour intervals.

Urine and faeces were assayed for ^{14}C immediately after collection and stored at -20°C without preservative until analysed. Trapping solutions were counted for ^{14}C immediately after changing.

Identification of urinary metabolites

Neat urine from rodents was subjected to tlc (system B) and strips of the plate were sprayed with p-DMAB reagent and naphtharesorcinol reagent.

The urinary metabolites were separated and isolated as follows.

Urine (5ml from rats and total 0-24 hour urine from mice) from animals dosed with [^{14}C]-*trans*-anethole was mixed with equal volumes of 0.2M pH5.0 acetate buffer, and extracted with ether (2 x 5 vol). The ether extracts were combined and retained. β-Glucuronidase (Glucurase, from bovine liver, 5000 units/ml in pH5.0 acetate buffer (Sigma Chemical Company) was added to the aqueous residue in equal volume, and the whole was incubated at 37°C for 16 hours. In some tubes, the specific inhibitor of β-glucuronidase, saccharo-1,4-lactone (Sigma) (20mg) was added to the β-glucuronidase prior to incubation, and in other tubes buffer only was added. As a positive control a tube was incubated containing phenolphthalein diglucuronide (1mg) dissolved in rat urine (1ml). After incubation, the appearance of a purple colour on the addition of alkali confirmed enzyme activity.

After incubation the samples were allowed to cool and were extracted with ether (2 x 5 vol). The aqueous residue was adjusted to pH1.0 (5 M HCl) and again extracted with ether (2 x 5 vol). The volume and ^{14}C content of each organic and aqueous layer was measured, to allow calculation of the extraction of radioactivity at each step.

The ether extracts were dried (anhyd. Na_2SO_4) evaporated to dryness *in vacuo* and the residues taken up in small volumes of methanol for further analysis.

The metabolites extracted at pH5.0 were separated by hplc (system 2) and isolated by collection of the appropriate ^{14}C peaks as they were eluted from the column. The radioactivity in the fractions of eluant was extracted into ether (2 vols) which was dried and evaporated as before. The identities of the metabolites were deduced in part by comparison of retention times and R_f values with known authentic compounds in hplc system 2 and tlc system B. The identities were confirmed by direct probe insertion ms as such and gc-ms as their TMS and methylated derivatives.

The metabolites extracted at pH1.0 were separated and isolated initially by tlc (system B). The radioactive bands were located on the plates by radiochromatogram scanning, and scraped from the plates and eluted with methanol, which was removed *in vacuo*. The metabolites were further purified by hplc (system 3) and extracted from the eluant as described above. The metabolites were identified by comparison of retention times with authentic known compounds by hplc (system 3) and tlc (systems B-F), and their structures confirmed by direct probe insertion ms as such, and gc-ms as their TMS and methylated derivatives.

A portion of the pH1.0 extract was hydrolysed at 100°C for 6 hours with 6M HCl (1ml), then extracted into ether (5 vol). The residue after evaporation to dryness *in vacuo* was analysed by hplc (system 3) and tlc (system B).

Treatment of aqueous residue from rat urine

The aqueous residue was subjected to tlc (system B) and the plates sprayed for divalent sulphur groups with the chloroplatinic acid and potassium chromate/silver nitrate reagents.

A sample of the aqueous residue (2ml) was adjusted to pH7.4 with 0.1 M phosphate buffer and incubated with 5000 units of Jack Bean urease (Sigma) for 3 hours at 37°C. ^{14}C in the residue before and after incubation was assayed to establish if there was any loss of radioactivity as a result of the treatment.

The remaining aqueous residue was freeze-dried then dissolved in water (5ml). This was refluxed together with activated Raney nickel catalyst (1g) in ethanol (100ml) for 2 hours. After cooling the supernatant was separated from the nickel by centrifugation. The ethanol in the supernatant was removed *in vacuo*, and the aqueous remaining adjusted to pH5.0 with 0.2M acetate buffer and extracted into ether (5 vol.). The extract was treated as before, and examined by hplc (system 2).

2.3. RESULTS

Excretion

Table 2.4. presents the excretion of radioactivity by rats and mice after a single 50mg/kg dose of [^{14}C-methoxy]-*trans*-anethole.

In both species the principal route of elimination of ^{14}C was via the expired air as $^{14}CO_2$. In the case of rats this represents some 42% of the dose and in mice 47%. Less than 0.1% of the dose was trapped in the 'cold finger' in either species indicating that no volatile radioactive metabolites nor *trans*-anethole were eliminated in the expired air.

Rats excreted 41% of the dose in the urine and in mice the corresponding figure was 37%. In both species the ^{14}C recovery in the faeces was less than 2% of the dose.

Urinary metabolites of [^{14}C-methoxy]-*trans*-anethole in the rat

Tlc of neat rat urine in system B revealed three radioactive bands, (R_f 0.1, R_f 0.29 and R_f 0.38). That at R_f 0.1 was positive (blue) to naphtharesorcinol indicating the presence of glucuronides, and that at R_f 0.38 was positive to *p*DMAB spray (orange) indicating the presence of glycine conjugate(s) (figure 2.2.).

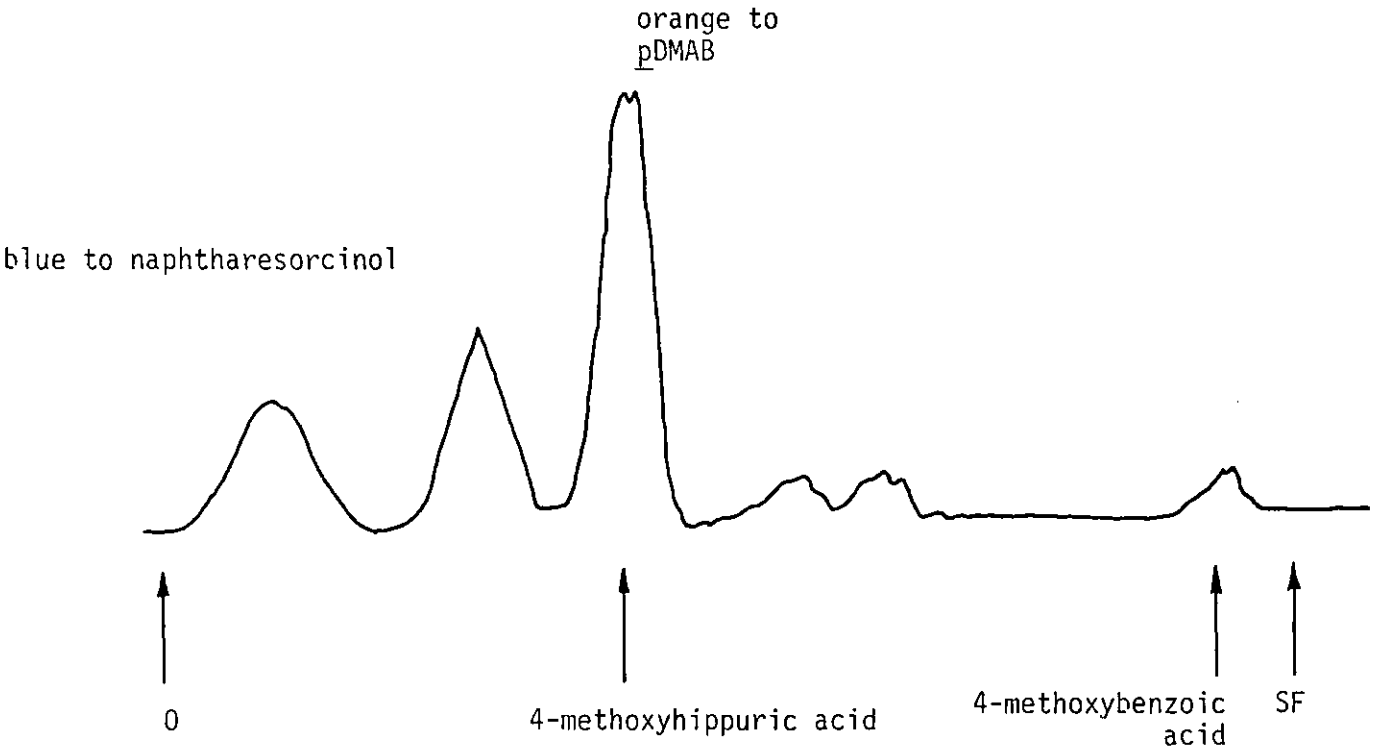
The methods described in the previous section revealed 6 radioactive metabolites in the pH5.0 extract prior to β-glucuronidase treatment (figure 2.3.) and, in addition to these, 2 extra radioactive and one

Table 2.4. Elimination of radioactivity from rats and mice after a single 50mg/kg dose of [^{14}C-methoxy]-*trans*-anethole

Species	route of administration	% of dosed ^{14}C* urine	expired air	faeces	total recovery
Mouse (CD-1,M)	intraperitoneal	36.5(35.6-38.6)	47.3(45.3-48.5)	1.0(0.9-1.1)	84.8(82.2-88.1)
Rat (Wistar,F)	oral	41.2(38.7-43.5)	41.8(37.3-51.0)	1.7(1.4-2.1)	84.7(81.5-91.0)

* means of 4 animals with ranges in parentheses

Figure 2.2. Separation of radioactive metabolites in the urine of rats after a single oral dose of [^{14}C-methoxy]-*trans*-anethole by tlc (system B)



0 = origin

SF = solvent front

Figure 2.3. Separation of the metabolites in the pH5.0 extract of rat urine after 50mg/kg [^{14}C]-trans-anethole by hplc (system 2) showing u.v. trace and radio-histogram

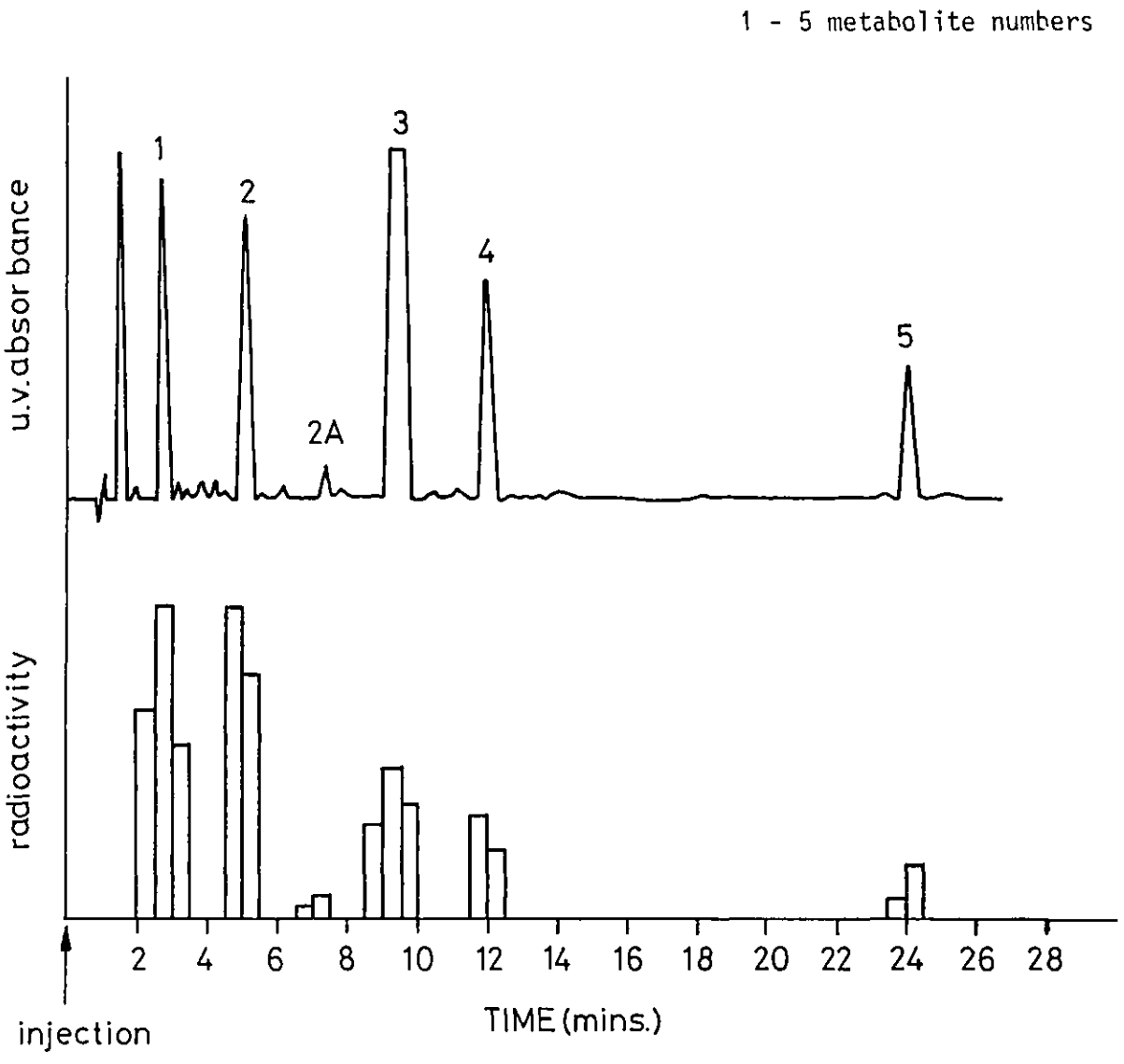
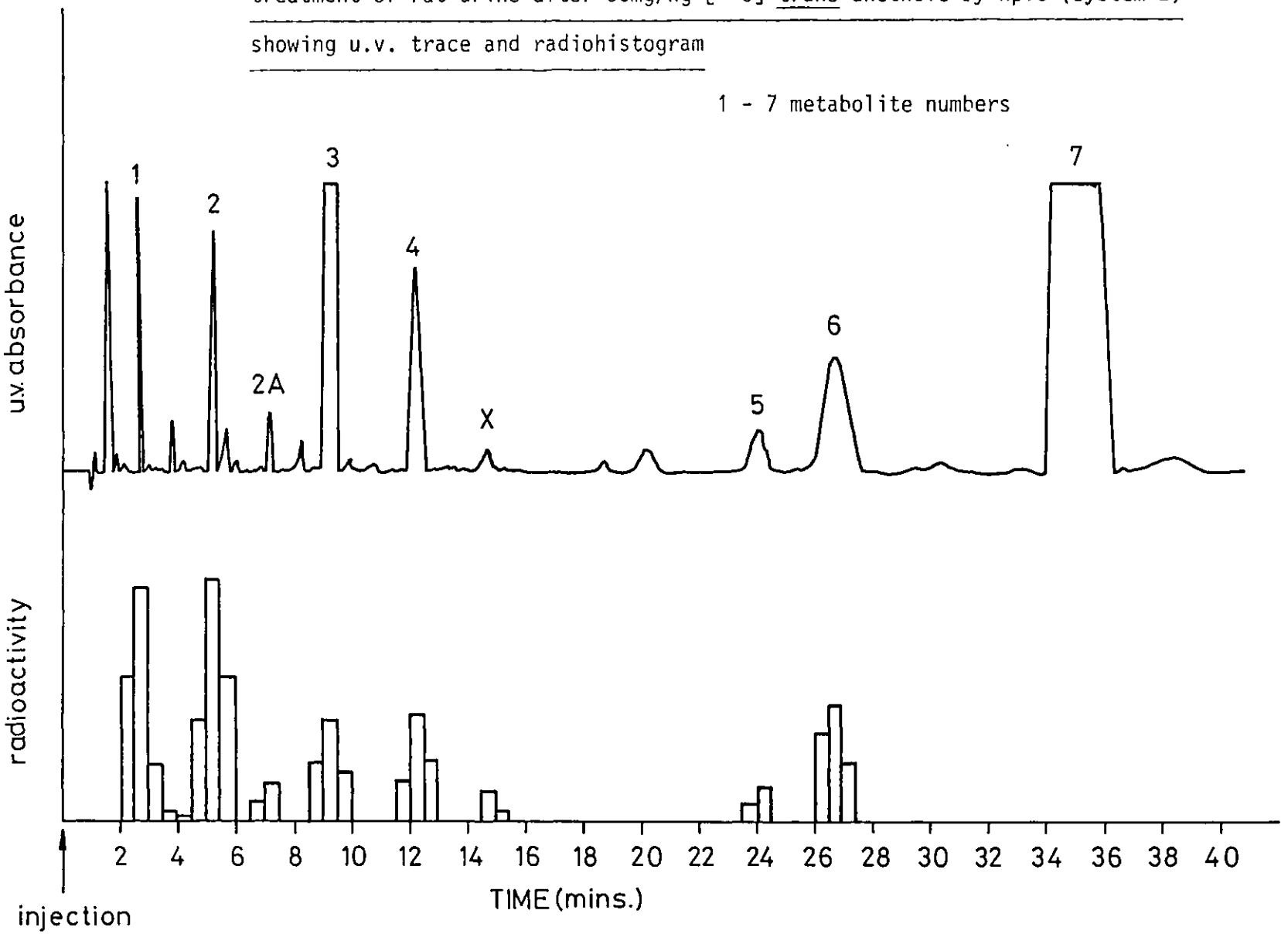


Figure 2.4. Separation of the metabolites in the pH5.0 extract after β-glucuronidase treatment of rat urine after 50mg/kg [^{14}C]-*trans*-anethole by hplc (system 2) showing u.v. trace and radiohistogram



non-radioactive metabolite in the pH5.0 extract after β-glucuronidase treatment (figure 2.4.). The metabolites were provisionally numbered for convenience in increasing order of hplc retention times.

Inhibition of β-glucuronidase with saccharo-1,4-lactone or incubation with buffer alone prevented further extraction of radioactivity at pH5.0 into ether.

The radioactivity extracted at pH1.0 was separated by tlc into 2 major and 4 minor radioactive metabolites (figure 2.5.). The 4 minor metabolites were shown after further chromatographic and ms analysis to be compounds labelled metabolites 1-4 that are extracted in greater amounts at pH5.0.

Metabolites 1 and 2

Metabolite 1 (R_t 3.0 min, hplc system 2), metabolite 2 (R_t 5.0 min, hplc system 2) and authentic 1 -(4'-methoxyphenyl)-propane-1,2-diol (R_t 5.0min. hplc system 2) all had similar mass spectra by direct insertion (appendix 2.7.). Further examination showed that metabolite 2 had the same retention time by gc as the authentic diol, and the mass spectra of metabolite 2 obtained by gc-ms as such, and as its TMS derivative (figure 2.6. a and b) were identical with those for authentic 1-(4'-methoxyphenyl)propane-1,2-diol (appendix 2.8a and b).

The mass spectrum of metabolite 1 obtained by gc-ms as such, (figure 2.7a.) was also identical with that for 1-(4'-methoxyphenyl) propane-1,2-diol but the metabolite had a different retention time

Figure 2.5. Radiochromatogram scan of a tlc of the pH1.0 extract of β-glucuronidase treated urine from rats given a single dose of [^{14}C-methoxy]-*trans*-anethole (50mg/kg) (system B)

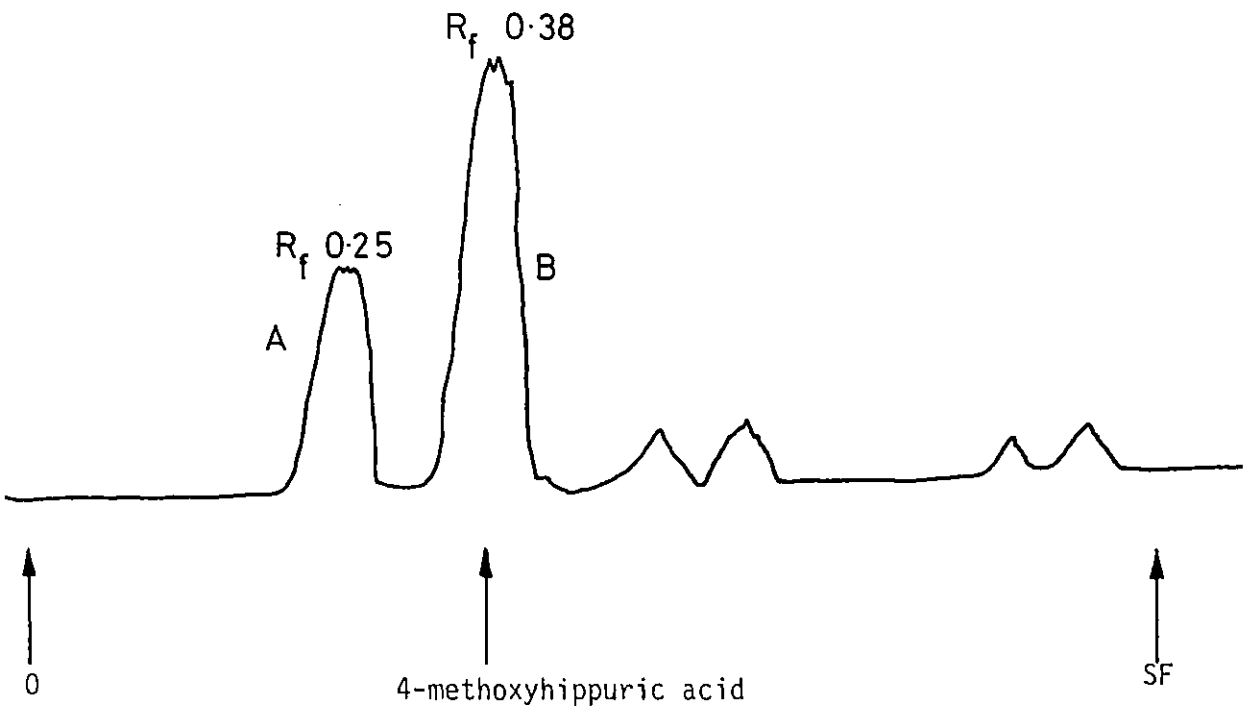


Figure 2.6. Mass spectrum of 1-(4'-methoxyphenyl)propan-1,2-diol, diastereoisomer 2, extracted from the urine of rats administered [^{14}C]-*trans*-anethole a) as such and b) as its TMS derivative

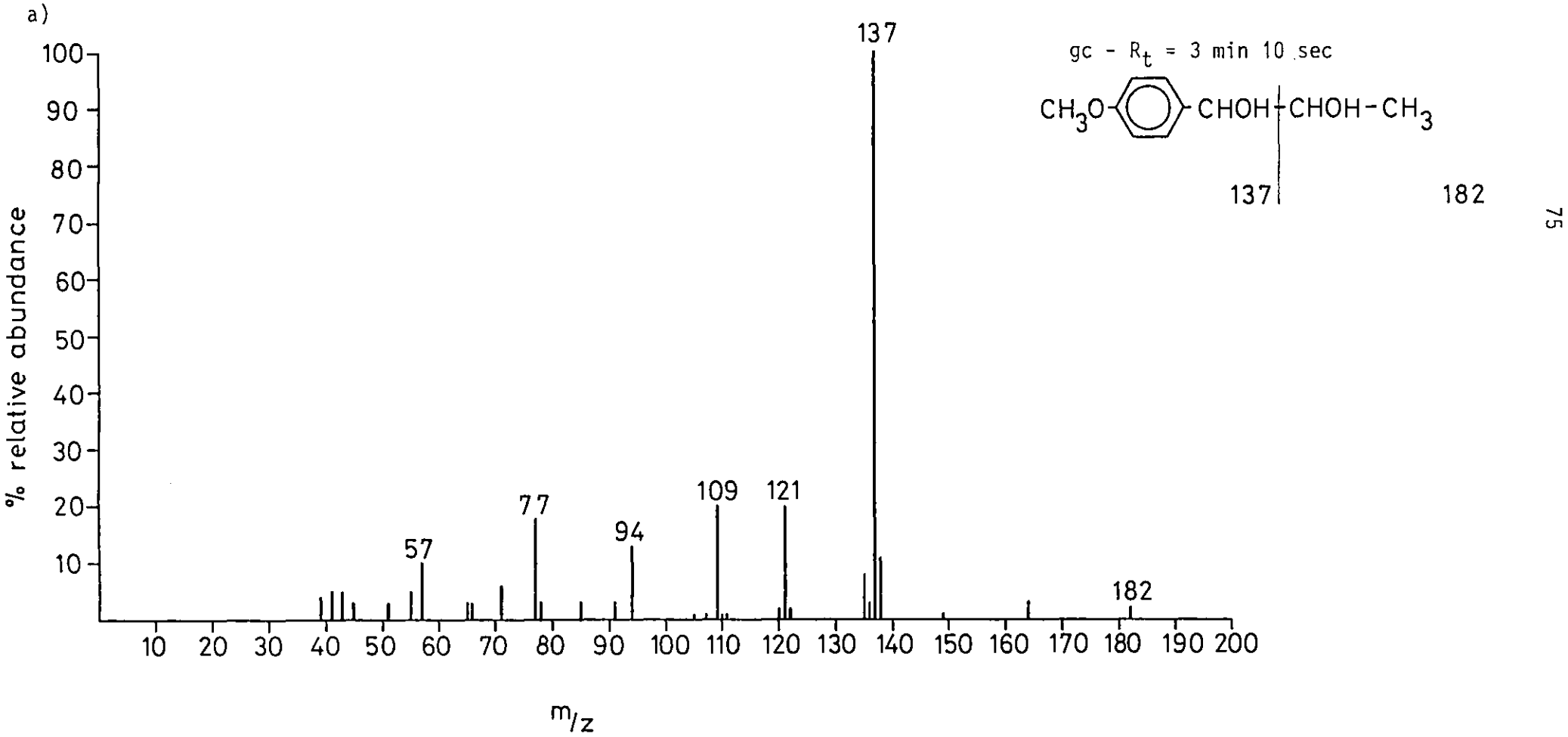
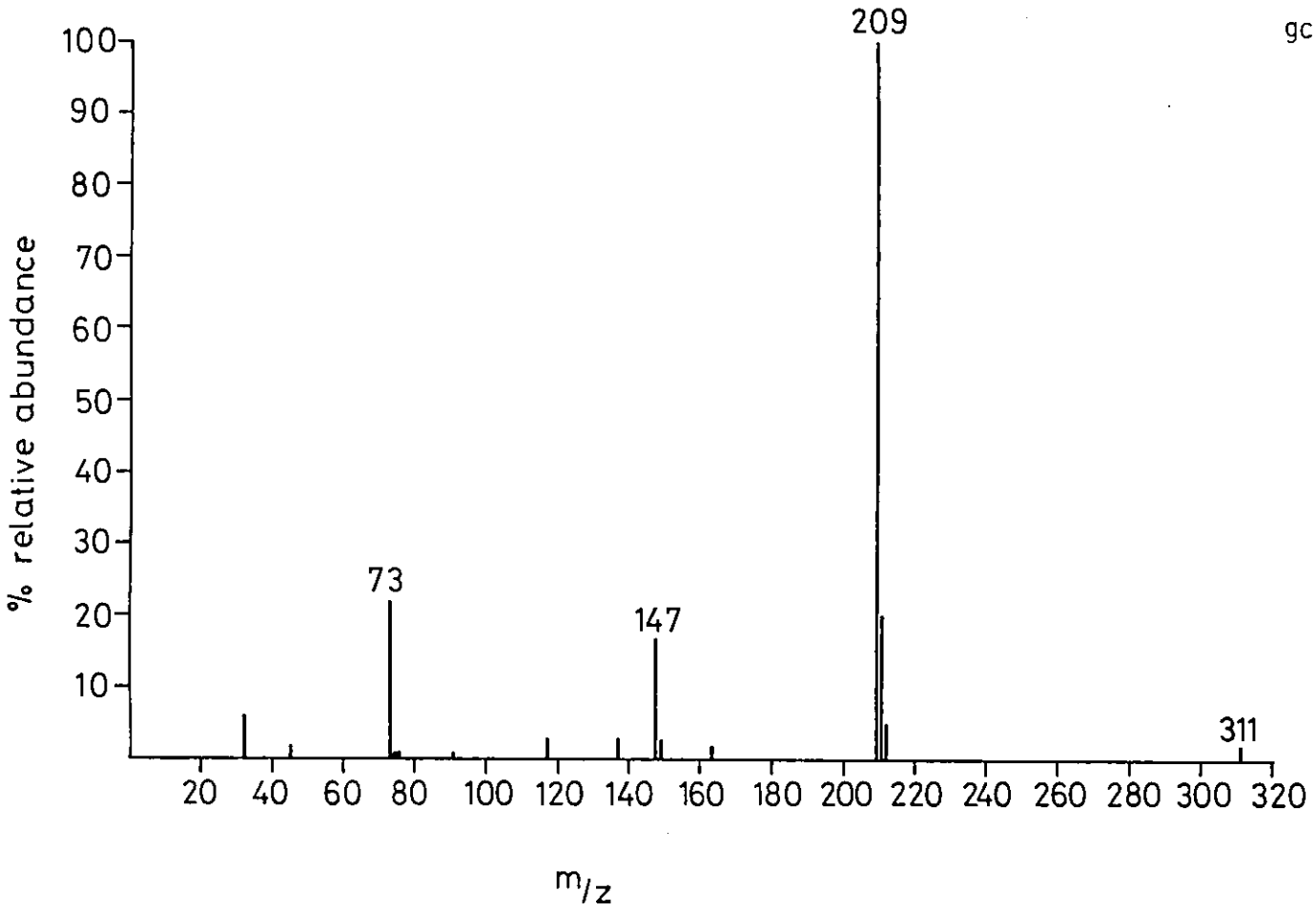
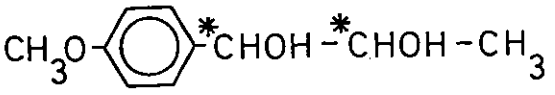


Figure 2.6b)


209
gc - R_t = 3 min 20 sec
100
90
80
70
60
50
40
30
20
10
% relative abundance
73
147
311
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320
m/z

on gc to the authentic diol. The retention time of the TMS derivative of metabolite 1 was also different to that of the authentic 1-(4'-methoxy-phenyl)propane-1,2-diol TMS derivative, but again the mass spectra obtained by gc-ms of the two TMS-derivatives showed the same fragmentation (figure 2.7b. and appendix 2.8b.). The only slight differences were in the percentage relative abundances of some fragments, for example $^{m}/_{z}$ 73 for metabolite 1 - TMS was 71.3% while for metabolite 2 - TMS and the authentic diol, the corresponding figures were 28.8% and 21.4% respectively.

From the above it was concluded that metabolite 1 and metabolite 2 were diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol.



Metabolites 1 and 2

The stereochemistry of this diol is such that there are four possible isomers, the techniques used in this study are likely to have separated them into two pairs of diastereoisomers.

Figure 2.7. Mass spectrum of 1-(4'-methoxyphenyl)propane-1,2-diol, diastereoisomer 1, extracted from urine of rats administered [^{14}C]-*trans*-anethole a) as such and b) as its TMS derivative

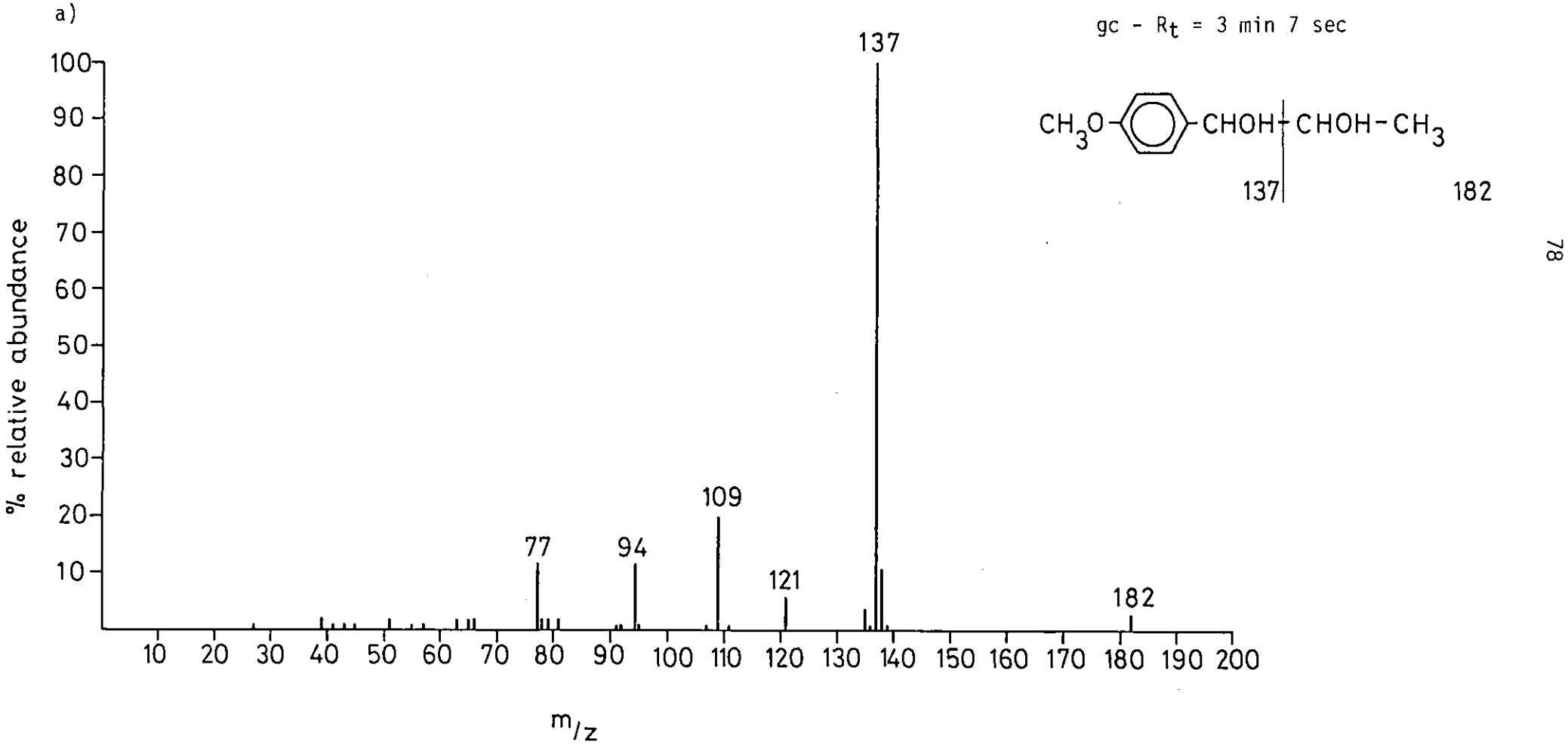
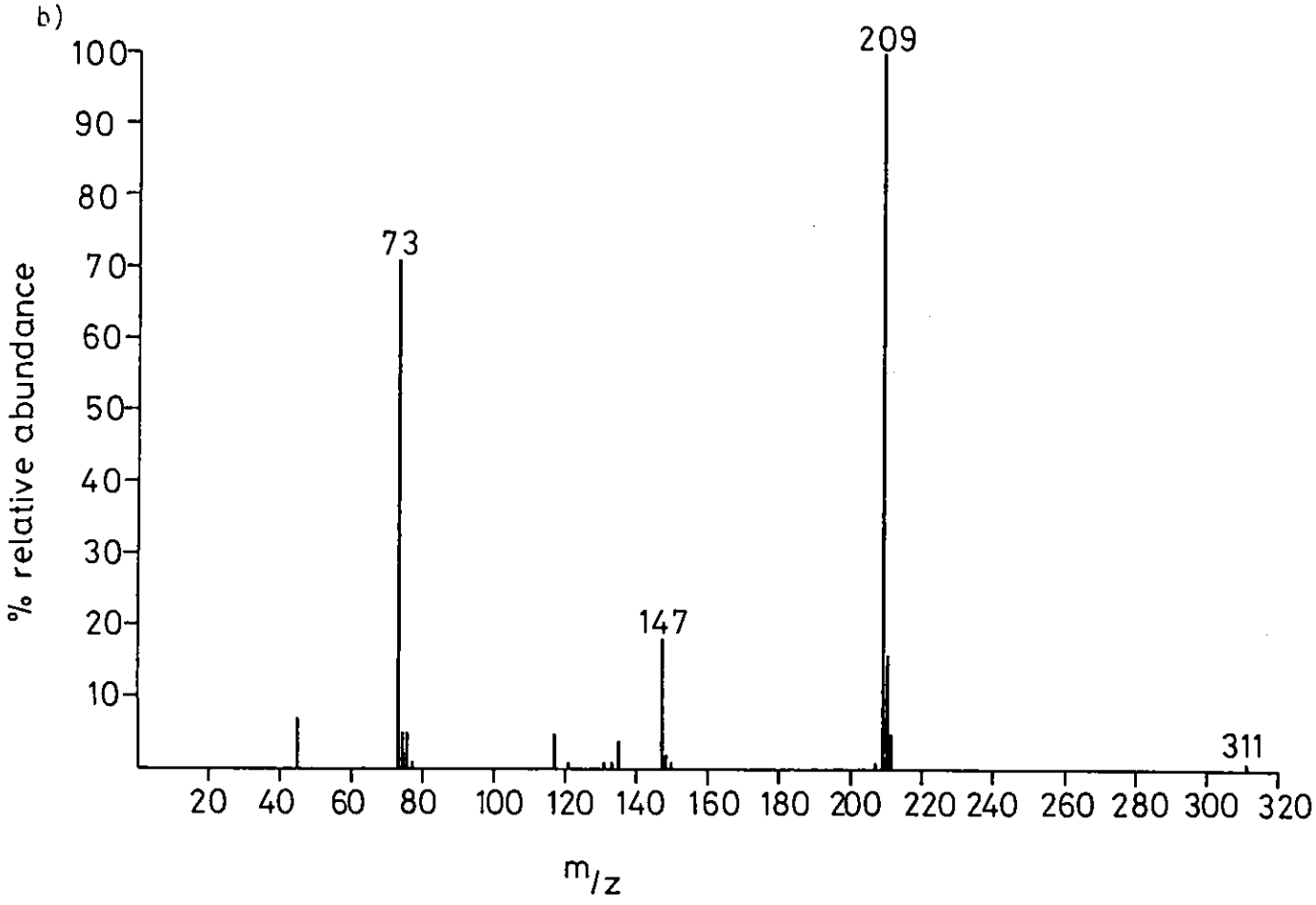


Figure 2.7b

gc - R_t = 3 min 15 sec



Both metabolites 1 and 2 were excreted free (diastereoisomer 1 3.0% of the dose, diastereoisomer 2 2.6% of the dose) and conjugated with glucuronic acid (diastereoisomer 1 2.8% of the dose, diastereoisomer 2 2.4% of the dose). A total of 10.6% of the dosed radioactivity was excreted as these two metabolites.

Metabolite 3

Metabolite 3 was excreted free (1.1% of the dose) and conjugated with glucuronic acid (1.7% of the dose). It had the same retention time by hplc (systems 2 and 3) and R_f values by tlc (systems B-F) as 4-methoxybenzoic acid. The mass spectrum of metabolite 3 obtained by gc-ms was identical with that for authentic 4-methoxybenzoic acid (figure 2.8. and appendix 2.9a.).

Metabolite 4

Metabolite 4 (1.5% of the dose) had the same retention time by hplc (system 2) as 4 -methoxyacetophenone, and the mass spectrum of this metabolite obtained by gc-ms (figure 2.9.) was identical to that of 4-methoxyacetophenone (appendix 2.9b.).

4-methoxycinnamyl alcohol (X)

A small percentage of the administered radioactivity (<0.5%) had the same retention time as 4-methoxycinnamyl alcohol (hplc system 2). The metabolite was excreted conjugated with glucuronic acid and the mass spectrum obtained by gc-ms (figure 2.10.) was identical with that of authentic 4-methoxycinnamyl alcohol (appendix 2.9c.).

Figure 2.8. Mass spectrum of 4-methoxybenzoic acid, extracted from the urine of rats administered [^{14}C]-*trans*-anethole

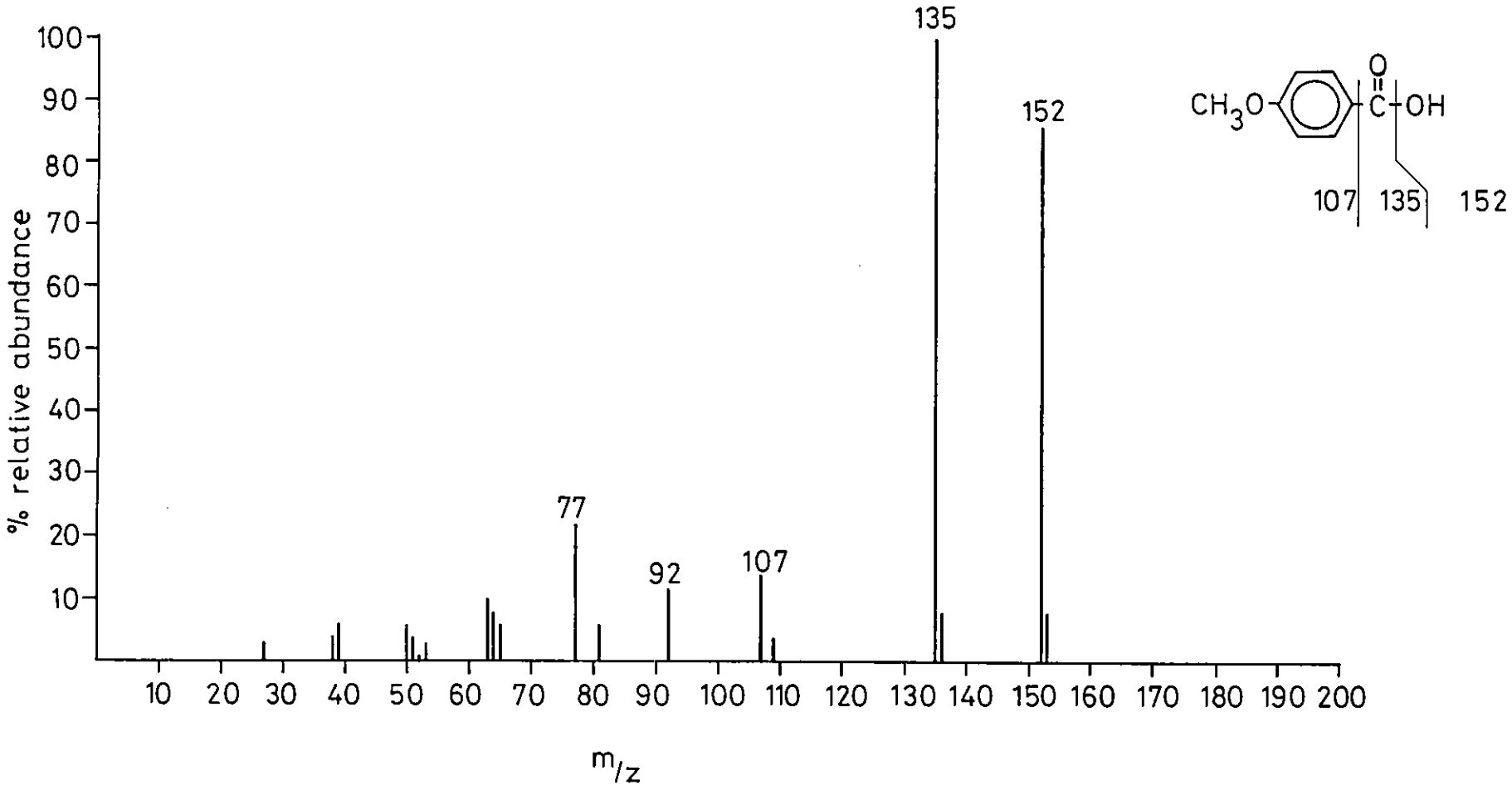


Figure 2.9. Mass spectrum of 4-methoxyacetophenone, extracted from the urine of rats administered [^{14}C]-*trans*-anethole

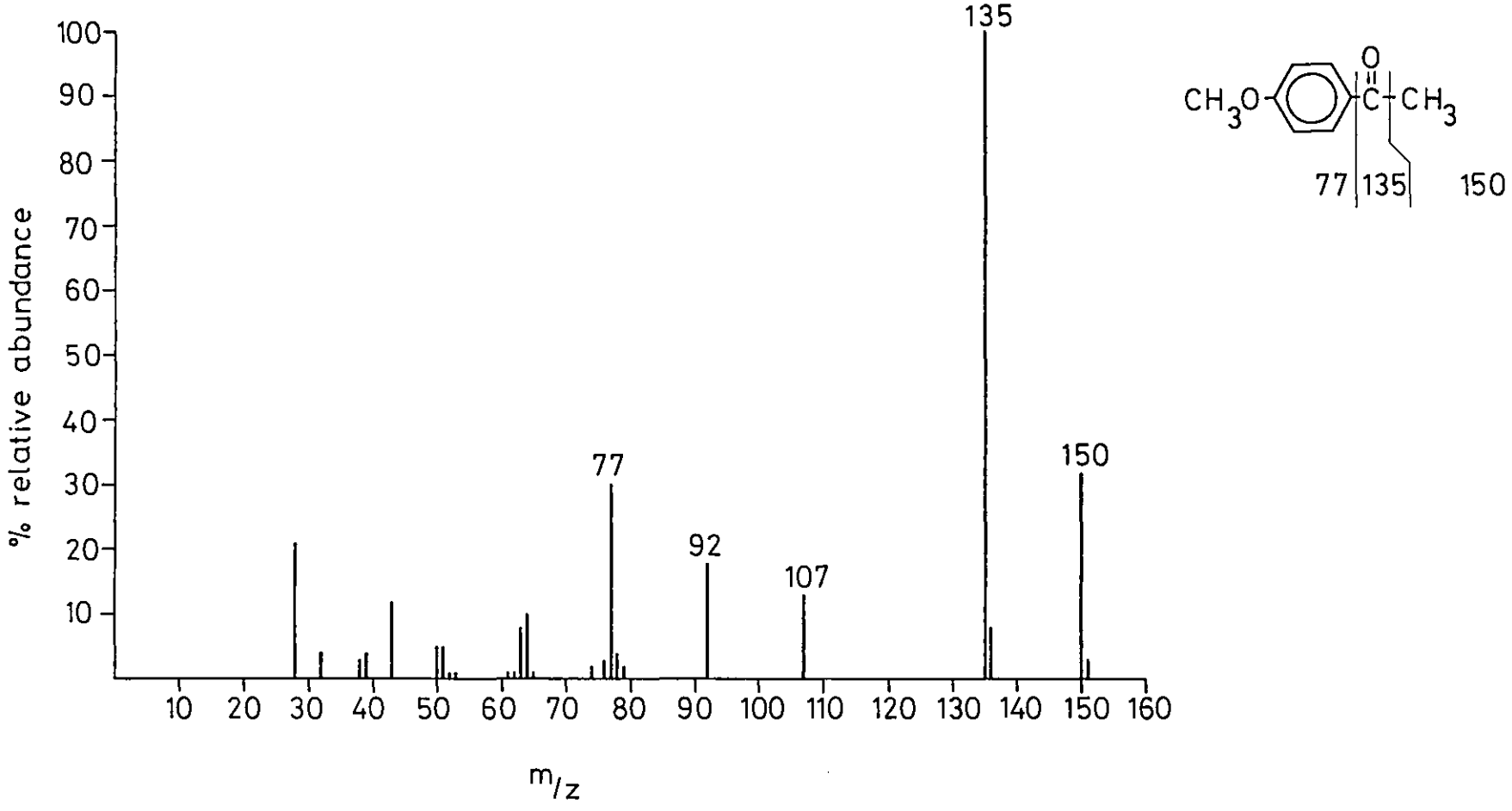
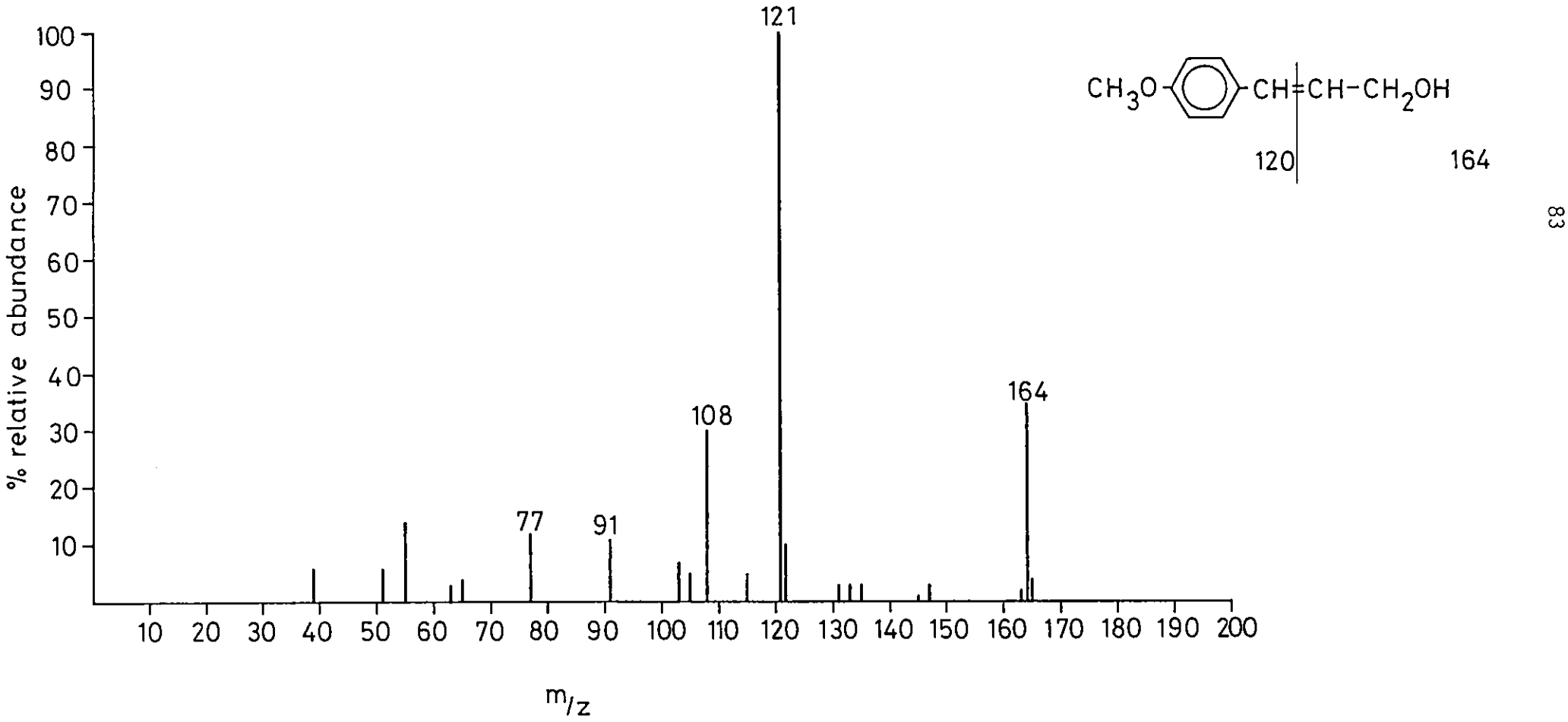


Figure 2.10. Mass spectrum of 4-methoxycinnamyl alcohol, extracted from the urine of rats administered [^{14}C]-*trans*-anethole



Metabolite 5

Metabolite 5 had the same retention time by hplc (system 2) and R_f value by tlc (system B) as trans-4-methoxycinnamic acid. The direct probe mass spectrum of metabolite 5 and the mass spectrum obtained by gc-ms of its TMS derivative were identical to those for authentic trans-4-methoxycinnamic acid (figure 2.11b and appendix 2.10a). However, methylation of the metabolite yielded two products, which could be separated by gc, one having the same retention time as methylated authentic trans-4-methoxycinnamic acid, but both having identical mass spectra by gc-ms as the authentic methylated compound, (figure 2.11a. and appendix 2.10b.).

This data suggests that metabolite 5 contains both cis and trans isomers of 4-methoxycinnamic acid, excreted free and conjugated with glucuronic acid (1.5% of the dose).

Metabolites 2A and 6

Metabolite 2A was found to be resolved into two compounds by gc. The chemical ionisation mass spectrum of the first peak obtained by gc-ms is shown in figure 2.12. The fragment at $^{m}/z$ 181 (MH^+) demands the addition of two oxygen atoms to trans-anethole, and the base peak at $^{m}/z$ 135 indicates an oxygen atom on the benzylic carbon atom. The spectrum is consistent with the fragmentation expected for 1-(4'-methoxyphenyl)-1-oxo-propan-2-ol, drawn below.

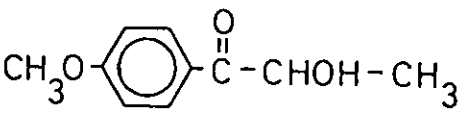


Figure 2.11. Mass spectrum of derivatives of 4-methoxycinnamic acid extracted from the urine of rats administered [^{14}C]-trans-anethole, a) its methyl ester and b) its TMS derivative.

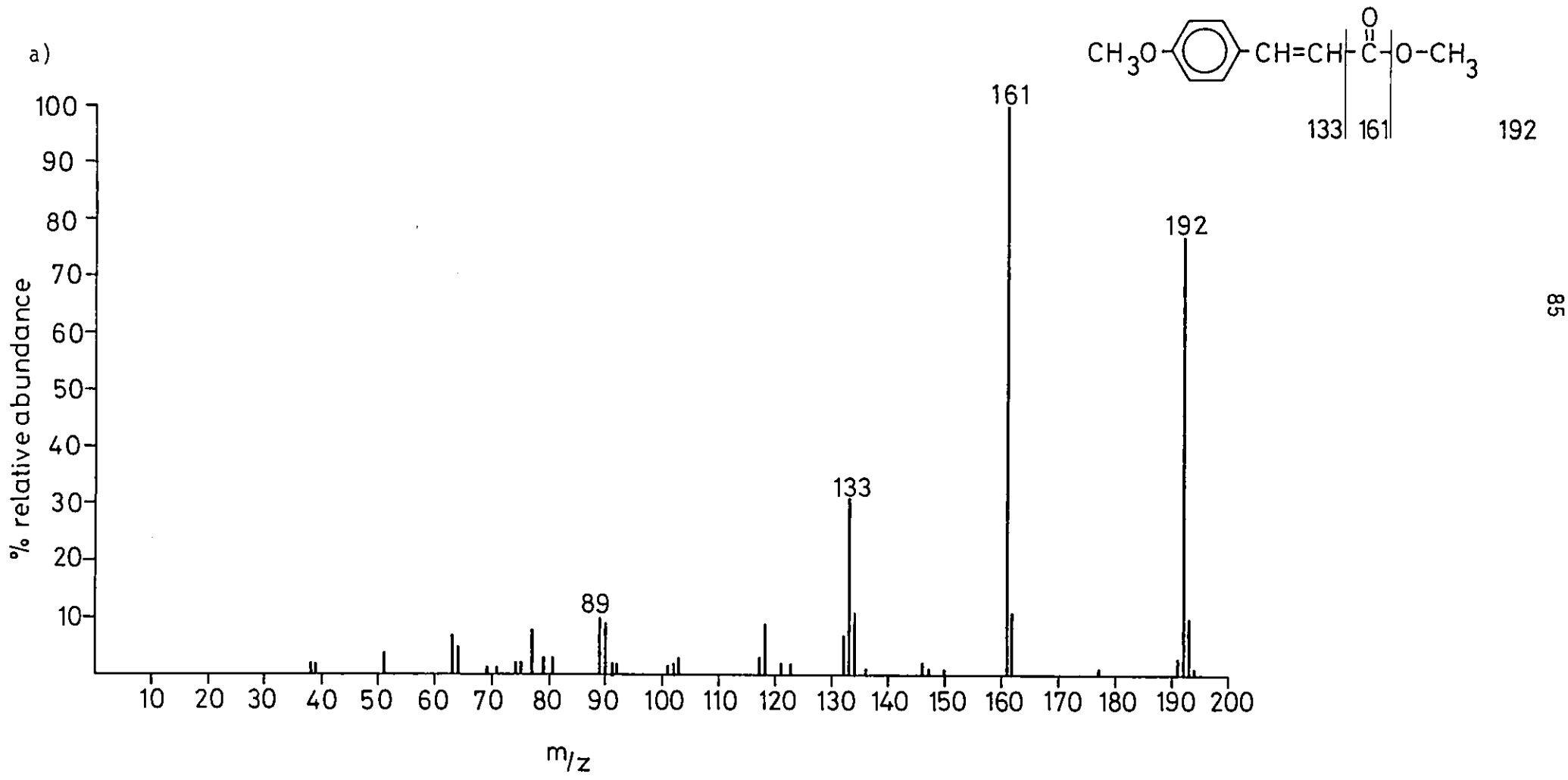
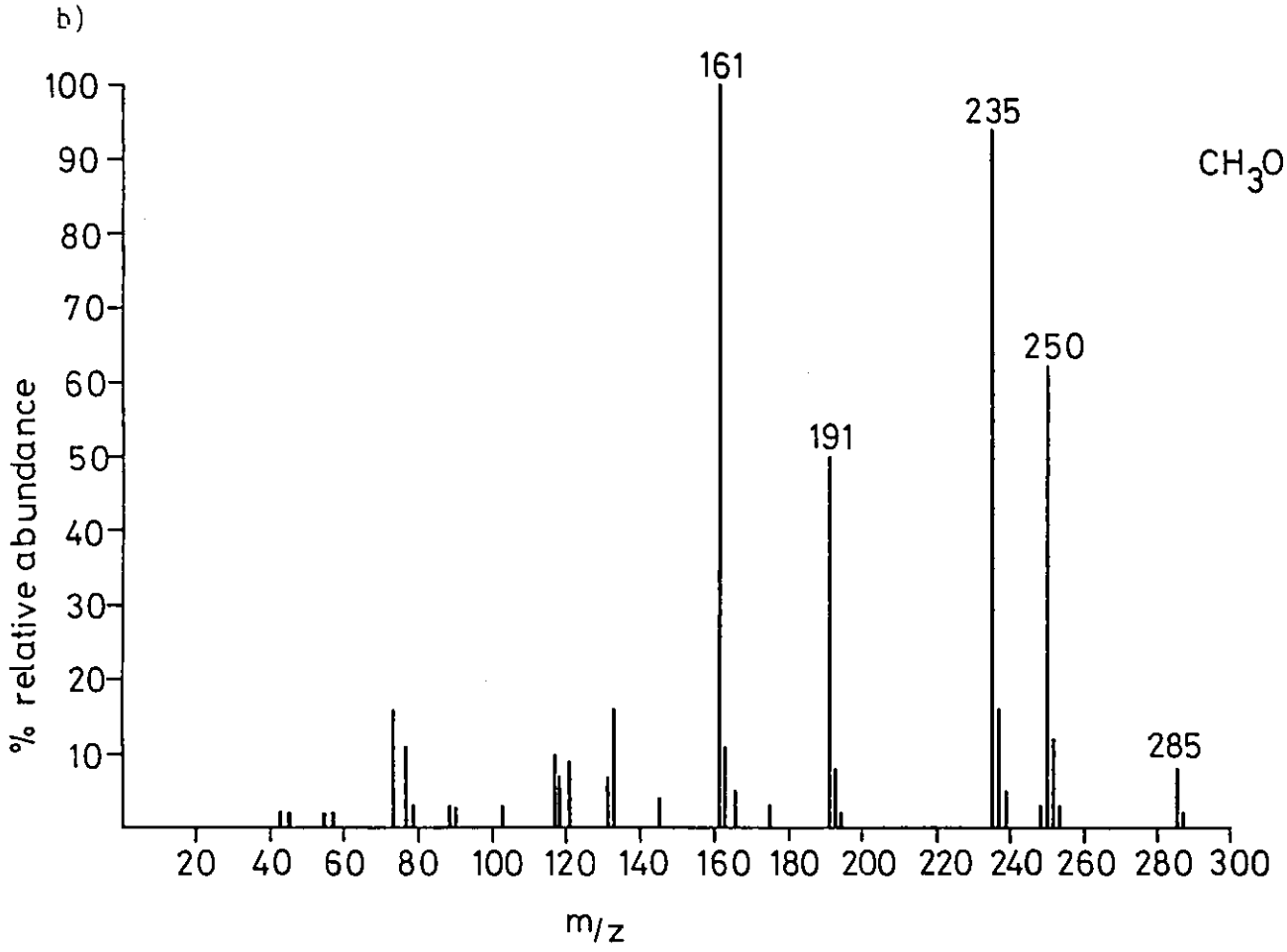


Figure 2.11b.


b)
% relative abundance
m/z
161
235
250
191
285
100
90
80
70
60
50
40
30
20
10
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300

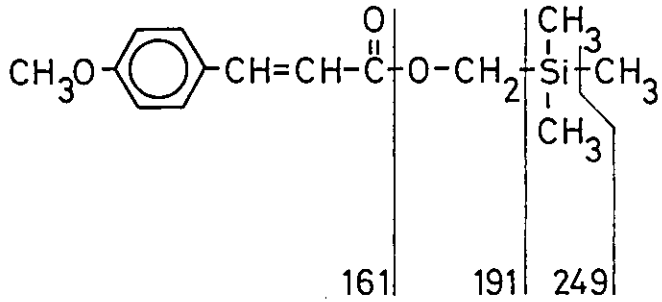
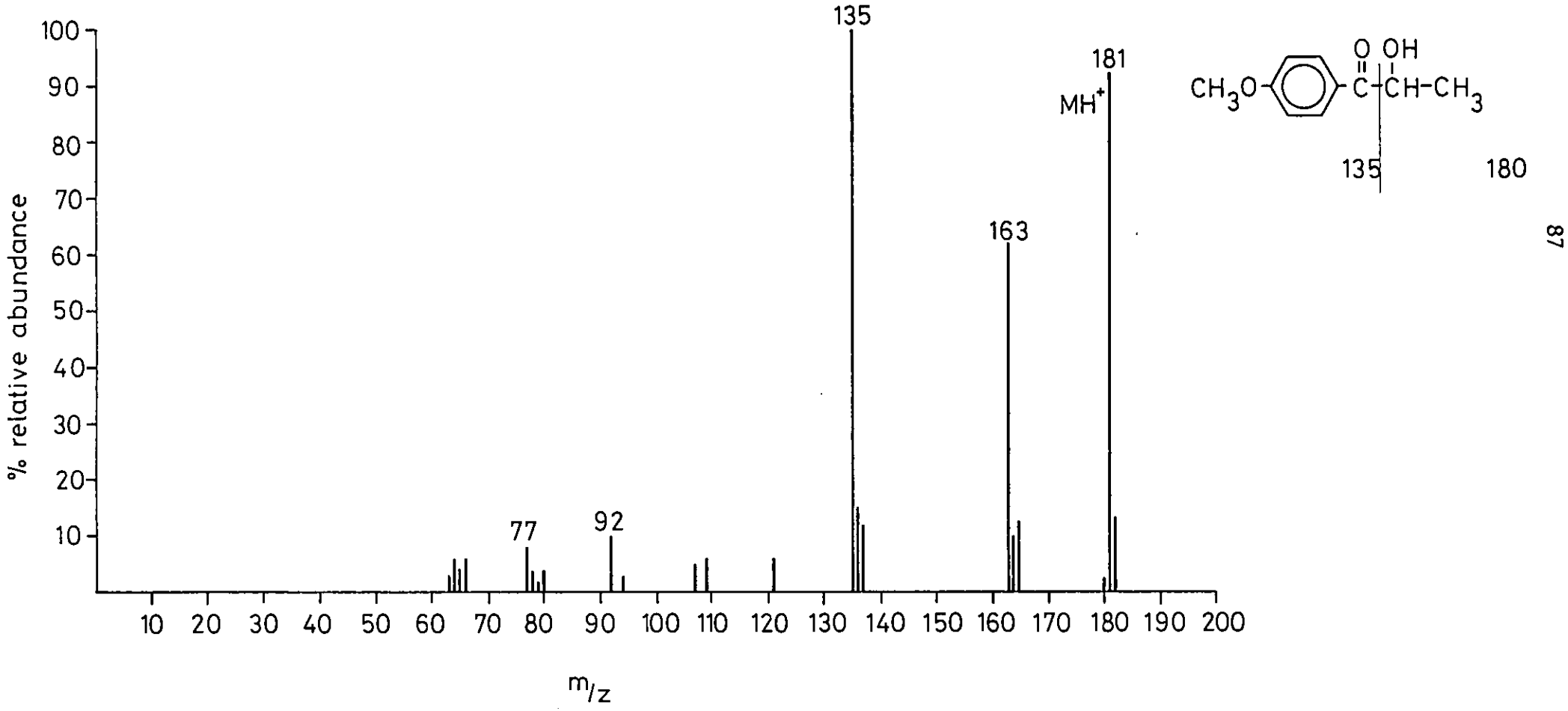
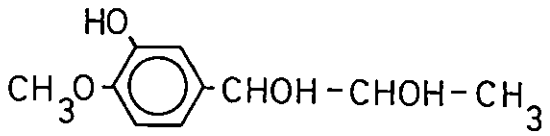

CH3O
CH=CH-C
O
O-CH2
Si
CH3
CH3
CH3
161
191
249

Figure 2.12. Chemical ionisation mass spectrum of 1-(4'-methoxyphenyl)-1-oxo-propan-2-ol extracted from the urine of rats administered [^{14}C]-trans-anethole



The electron impact mass spectrum of the second peak obtained by gc-ms is shown in figure 2.13. The molecular ion at $^{m}/z$ 198 demands the addition of 3 oxygen atoms to trans-anethole. Methylation of this peak gave a product with a mass spectrum by gc-ms identical with that for metabolite 6 (figure 2.14.). The molecular ion of this compound was $^{m}/z$ 212, and the base peak $^{m}/z$ 167. The latter fragment is consistent with oxygen groups on positions 1 and 2 in the three carbon side chain and the other fragments of the spectrum indicate that the third oxygen atom is in the benzene ring, most likely in position 3. The lack of ions at $^{m}/z$ 135 or 137 and the presence of $^{m}/z$ 121 precludes the metabolite from being an aliphatic triol, and additionally such a triol would not be methylated. Therefore the proposed structure for the second gc peak of metabolite 2A is thus: 1-(3'-hydroxy-4'-methoxy)propane-1,2-diol.



Metabolite 2 A/2

Metabolite 6 is suspected to be an artefact produced by methylation of metabolite 2A (peak 2) to 1-(3',4'-dimethoxyphenyl)-propane-1,2-diol, as a result of the workup of the extracts during analysis of the urine.

Figure 2.13. Mass spectrum of 1-(3'-hydroxy-4'-methoxyphenyl)propane-1,2-diol, extracted from the urine of rats administered [^{14}C]-*trans*-anethole

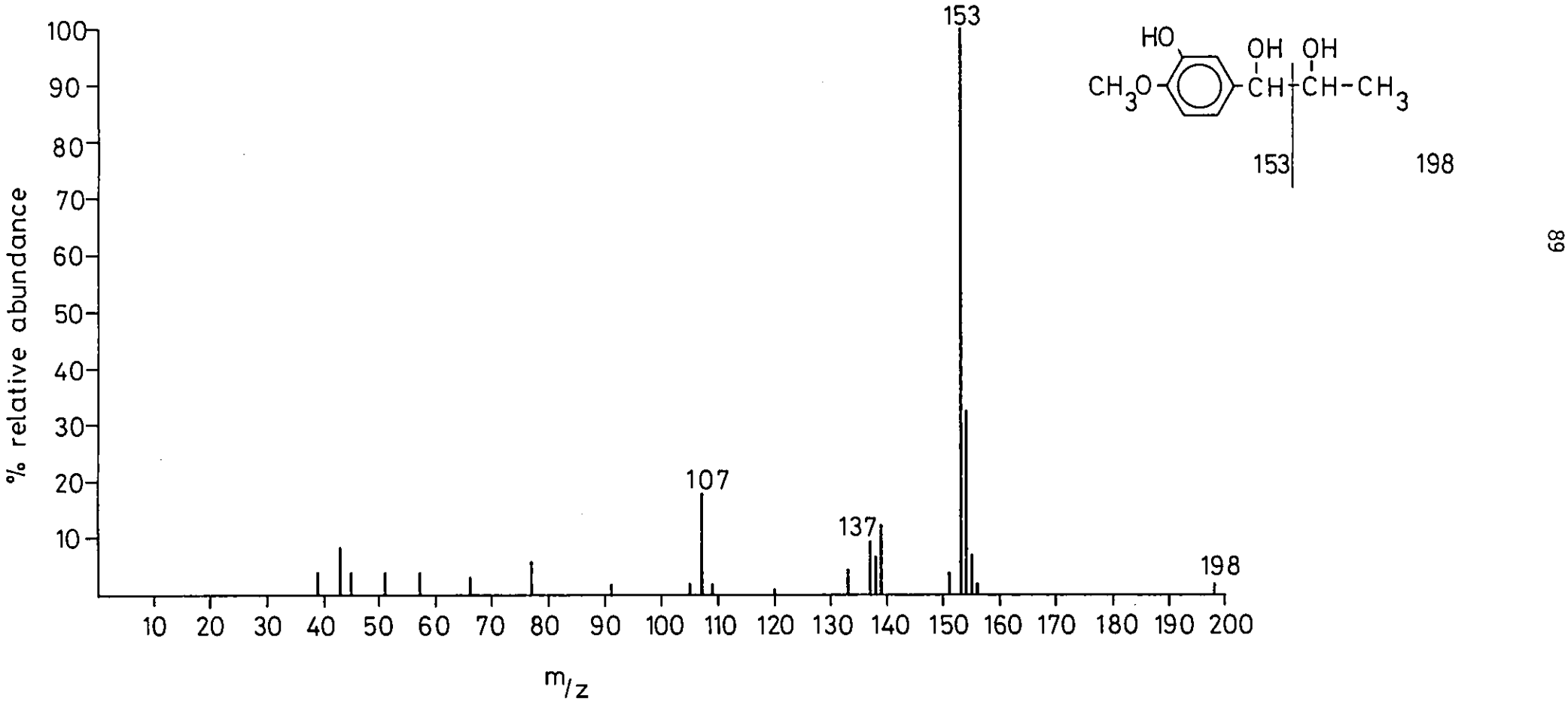
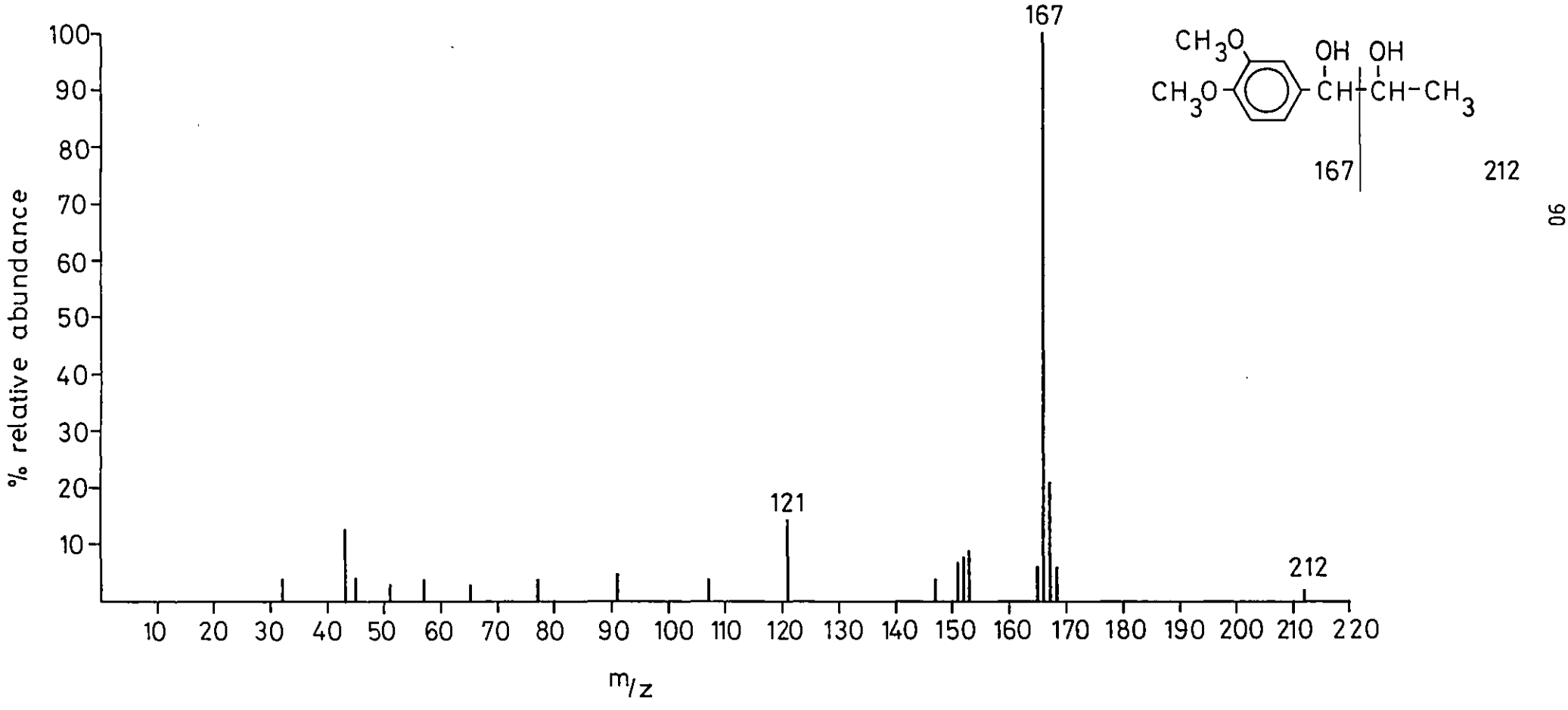


Figure 2.14. Mass spectrum of 1-(3',4'-dimethoxyphenyl)propane-1,2-diol extracted from the urine of rats administered [^{14}C]-*trans*-anethole, and also obtained by methylation of 1-(3'-hydroxy-4'-methoxyphenyl)propane-1,2-diol.



Metabolite 7

After β-glucuronidase treatment a very large u.v. absorbing peak was observed in extracts from rat urine at pH5.0. This metabolite was not radioactive which indicated that the compound was O-demethylated. The mass spectrum of metabolite 7 (figure 2.15.) was similar to that of chavicol (4-hydroxyphenyl-allylbenzene, appendix 2.11.). Methylation of the metabolite gave a product with the same retention time by gc and an identical mass spectrum by gc-ms as authentic trans-anethole (figure 2.16. and appendix 2.2.).

It was deduced that metabolite 7 was anol (4-hydroxypropenylbenzene) excreted in the urine conjugated with glucuronic acid.

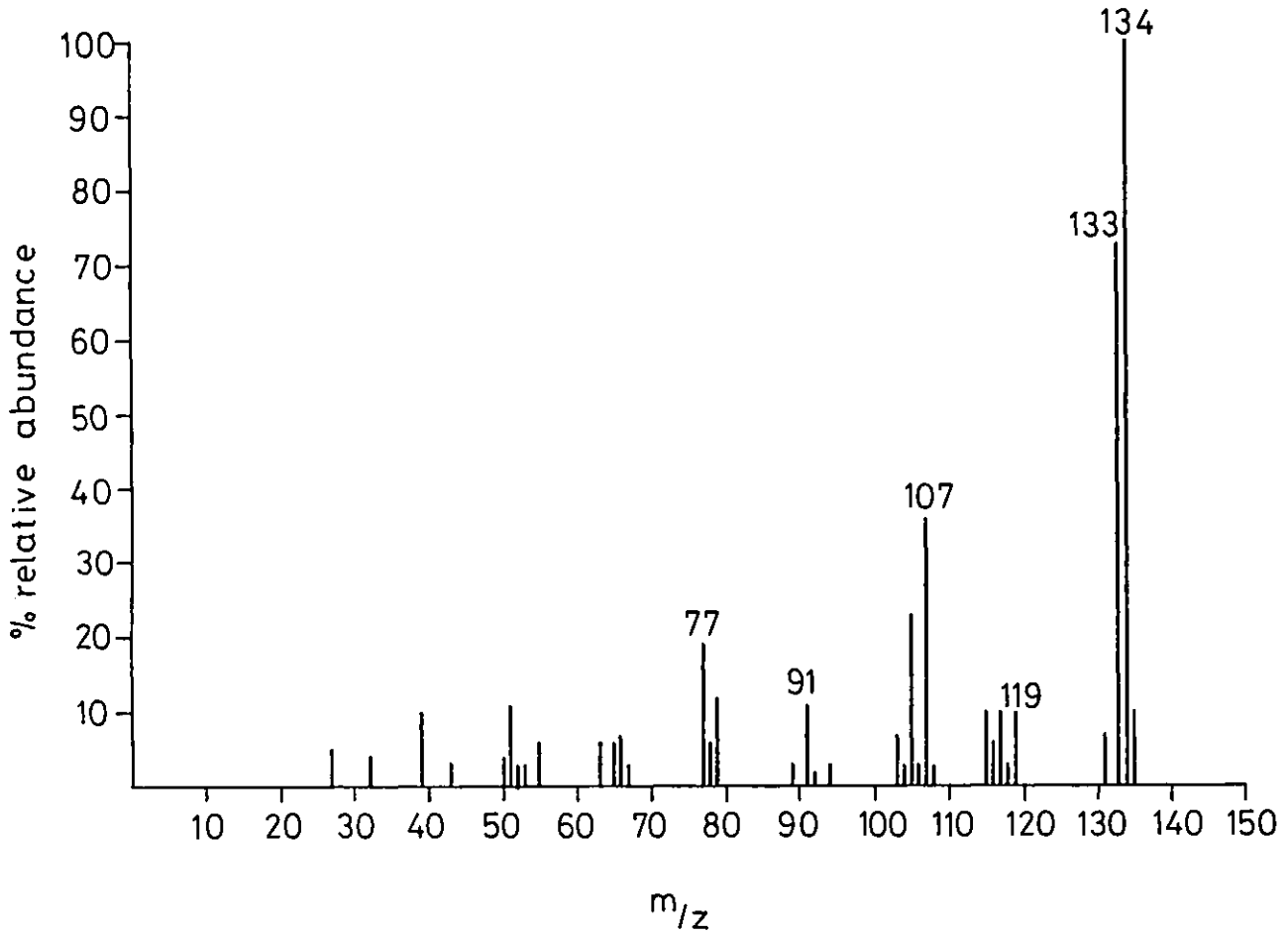
Metabolite A

Metabolite A was not extractable at pH1.0 prior to β-glucuronidase treatment of urine, and acid hydrolysis degraded it to 4-methoxy-benzoic acid as shown by hplc (system 3). This suggests that it may be a conjugate or a chemical precursor of 4-methoxybenzoic acid. It accounted for 5.8% of the dose. No interpretable mass spectra were obtained and it remains so far unidentified.

Metabolite B

Metabolite B was indicated to be a glycine conjugate by a positive reaction with p DMAB spray and it had the same retention time by hplc (system 3) and R_f by tlc (system B,C and E) as 4-methoxyhippuric acid. Methylation of the metabolite gave a product with a mass spectrum obtained by gc-ms identical with

Figure 2.15. Mass spectrum of 4-hydroxypropenylbenzene (anol), extracted from the urine of rats administered [^{14}C]-trans-anethole



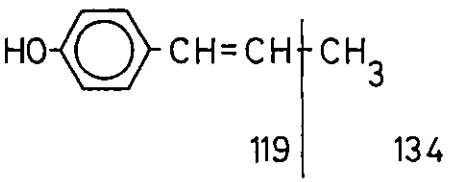
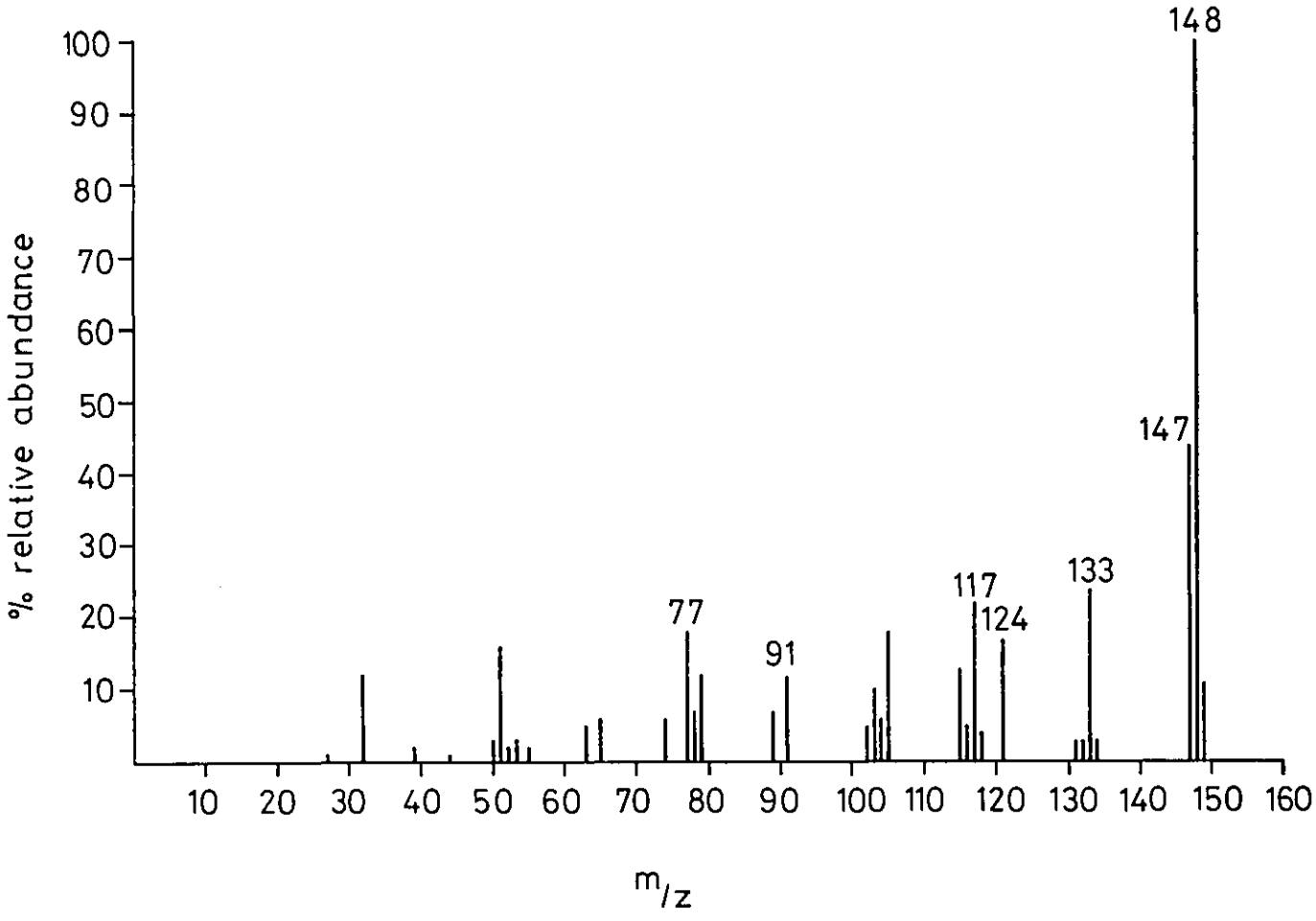
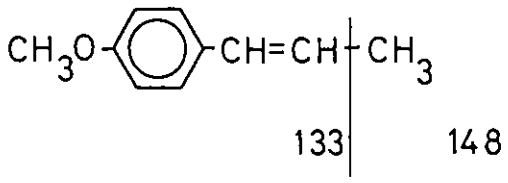


Figure 2.16. Mass spectrum of methylated 4-hydroxypropenylbenzene (anol; figure 2.15.)
(cf. mass spectrum of trans-anethole appendix 2.2.)





that of the methyl ester of 4-methoxyhippuric acid (figure 2.17. and appendix 3). This major metabolite accounted for 6.8% of the dose.

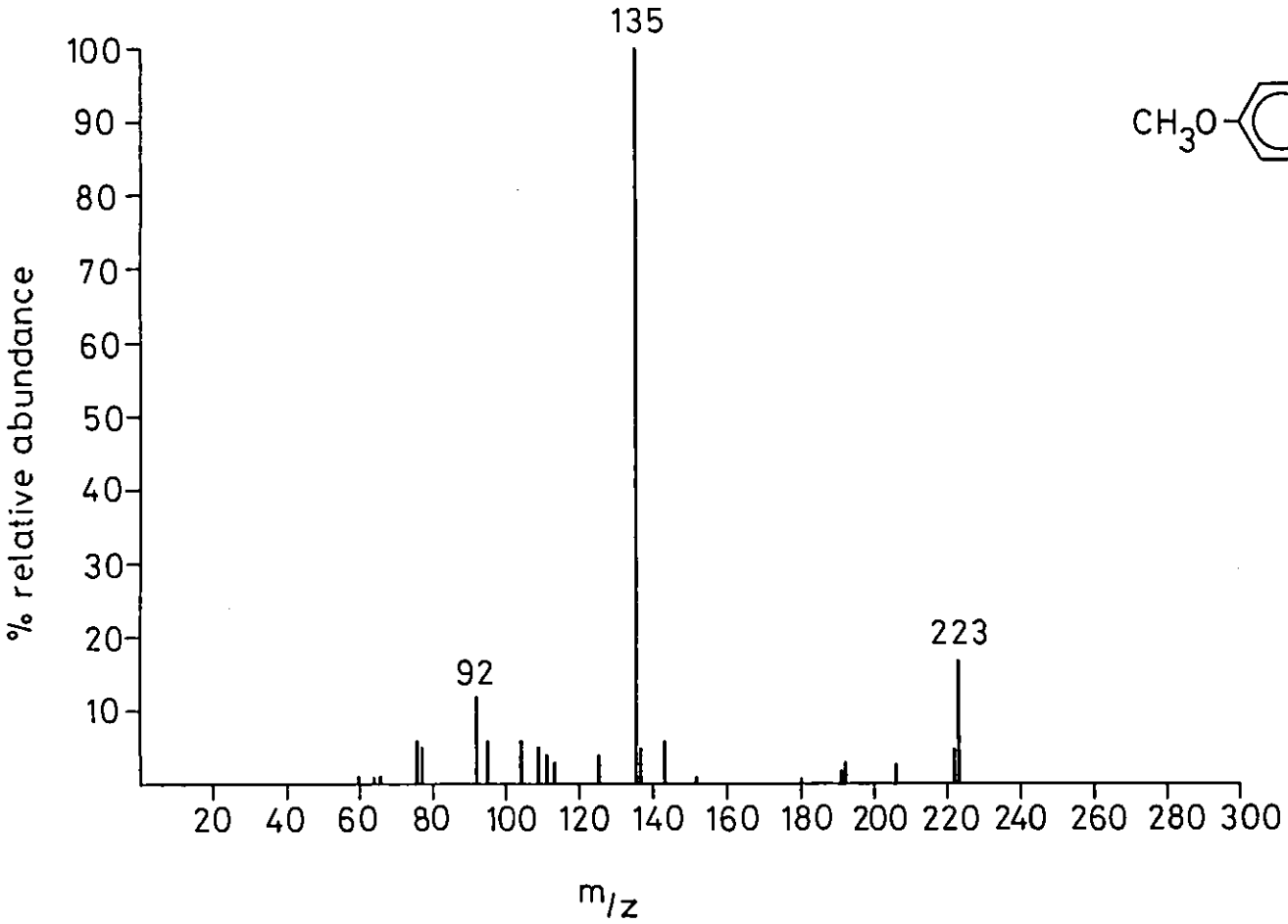
Radioactivity remaining in the aqueous residue

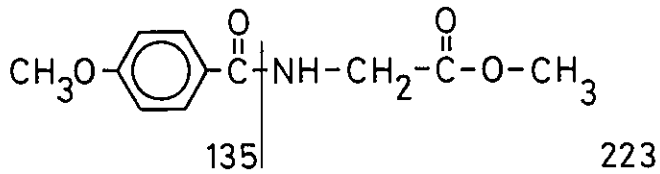
Tlc of the aqueous residue in system B did not show any distinct radioactive bands, but a broad smear of radioactivity from R_f 0.00-0.25. This broad band gave positive reactions to chloroplatinic acid reagent (pink) and $K_2CrO_7/AgNO_3$ reagent (orange) both indicating divalent sulphur groups.

Treatment of the aqueous residue with Jack Bean urease resulted in a small reduction in the radioactivity in the residue (5% of the total; 0.5% of the dose) indicating that some 0.5% of the [^{14}C]-trans-anethole was excreted as ^{14}C-urea.

After Raney nickel treatment of the aqueous residue, 70% of the remaining activity (6.5% of the dose) was extractable into ether. Analysis of the extract by hplc (system 2) revealed three major radioactive bands. Two had the same retention times as the two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol (system 2) and the third had the same retention time as 1-(4'-methoxyphenyl)-propan-1-ol.

Figure 2.17. Mass spectrum of methylated 4-methoxyhippuric acid, extracted from the urine of rats administered [^{14}C]-*trans* anethole





Metabolism of [^{14}C]-*trans*-anethole in the mouse

The metabolism of *trans*-anethole in the mouse is qualitatively the same as in the rat with one exception, in that the mouse does not excrete any metabolite A, extractable from rat urine at pH1.0 after β-glucuronidase treatment. Other differences between the species are quantitative (table 2.5.).

The mouse excretes less of all but one of the ^{14}C-urinary metabolites identified in rat urine after [^{14}C]-*trans*-anethole; this deficit is made up by a much greater excretion of 4-methoxyhippuric acid (16.9% of the dose as compared to 6.7% of the dose in the rat).

As in the rat, 4-hydroxypropenylbenzene glucuronide was found in the urine of mice, and all the ^{14}C metabolites were excreted free and conjugated as discussed for the rat.

The aqueous residue of mouse urine contained 0.5% of the dose as ^{14}C-urea, but no other analyses were performed.

Table 2.5. Metabolites of [^{14}C-methoxy]-*trans*-anethole in the rat and mouse after a single dose of 50mg/kg***

Metabolite	% of the dose rat*	mouse**
Anethole	n.d.	n.d.
4-methoxycinnamylalcohol	<0.5	
1-(4'-methoxyphenyl)propane-1,2-diol diastereoisomer 1	5.3(4.1-6.3)	2.2(2.0-2.5)
1-(4'-methoxyphenyl)propane-1,2-diol diastereoisomer 2	5.3(5.2-5.4)	2.2(2.1-2.3)
1-(4'-methoxyphenyl)-1-oxo-propan-2-ol 1-(3'-hydroxy-4'-methoxyphenyl)-propane-1,2-diol	0.5(0.3-0.6)	1.1(0.9-1.2)
1-(3',4'-dimethoxyphenyl)propane-1,2 diol	3.2(2.9-3.7)	0.7(0.6-0.8)
4-methoxyacetophenone	1.5(1.1-1.9)	0.2(0.1-0.1)
4-methoxybenzoic acid	2.8(2.1-3.3)	1.4(1.3-1.5)
4-methoxycinnamic acid (*cis* and *trans*)	1.3(1.0-1.6)	0.2(0.1-0.3)
4-methoxyhippuric acid	6.7(5.7-8.0)	16.0(15.9-16.2)
Unknown metabolite	5.8(5.1-6.8)	n.d.
^{14}C urea	0.5	0.5
$^{14}CO_2$	41.8(37.3-51.0)	47.3(45.3-48.5)
Aqueous residue	8.8(7.7-9.6)	12.0(11.0-13.7)
Total	84.0(81.5-91.0)	84.8(82.2-88.1)

n.d. none detected

* oral

** intraperitoneal

*** mean of 4 animals with ranges in parentheses

2.4. DISCUSSION

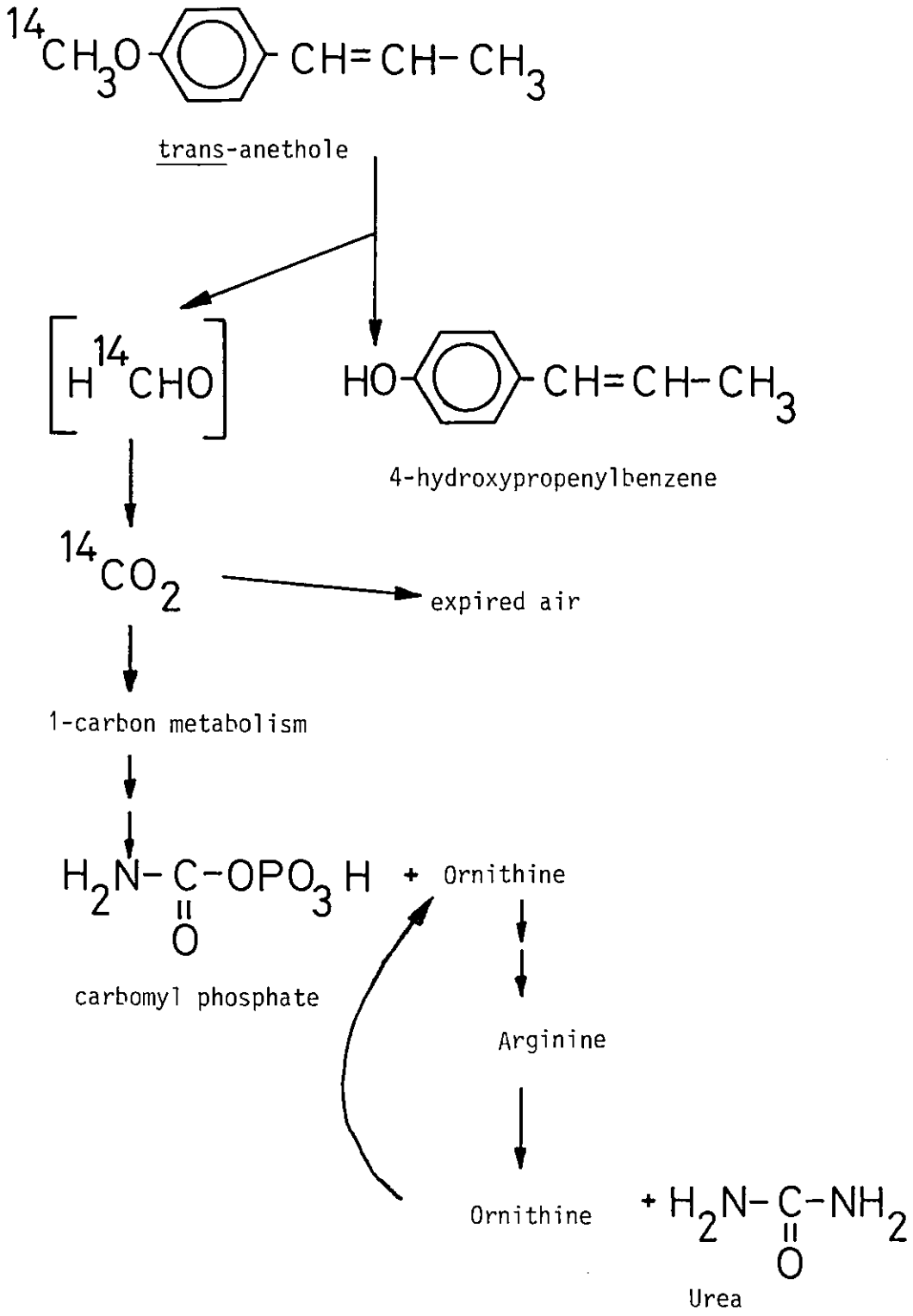
The overall recovery of radioactivity in the urine, faeces and expired air of rats and mice after the administration of a single dose of 50mg/mg [^{14}C-methoxy]-*trans*-anethole was some 85% of the dose, with less than 0.1% of the dose remaining in the body after 72 hours. The recovery was acceptable as more than half of the radioactivity was detected as $^{14}CO_2$, which has previously been shown to result in low total recoveries due to incomplete trapping of $^{14}CO_2$ (Strolin-Benedetti and Le Bourhis, 1972). The *trans*-anethole in this study was well absorbed following oral administration as indicated by the low faecal radioactivity.

trans-Anethole was totally metabolised by rodents with no detectable unchanged compound in the urine, and less than 0.1% of the dose trapped as volatile radioactivity from the expired air where one might expect to find some unchanged *trans*-anethole.

The major route of metabolism of [^{14}C]*trans*-anethole was oxidative O-demethylation, as indicated by the amount of $^{14}CO_2$ in the expired air. This route accounted for more than 40% of the dose and was in agreement with previously reported data (Solheim and Scheline, 1973).

The $^{14}CO_2$ in the expired air probably arose from [^{14}C]-formaldehyde, (figure 2.18.), the initial product of O-demethylation of [^{14}C-methoxy]-*trans*-anethole, and the ^{14}C urea in the urine from the incorporation

Figure 2.18. Fate of the [^{14}C]-methoxy group after O-demethylation of [^{14}C-methoxy]-trans-anethole

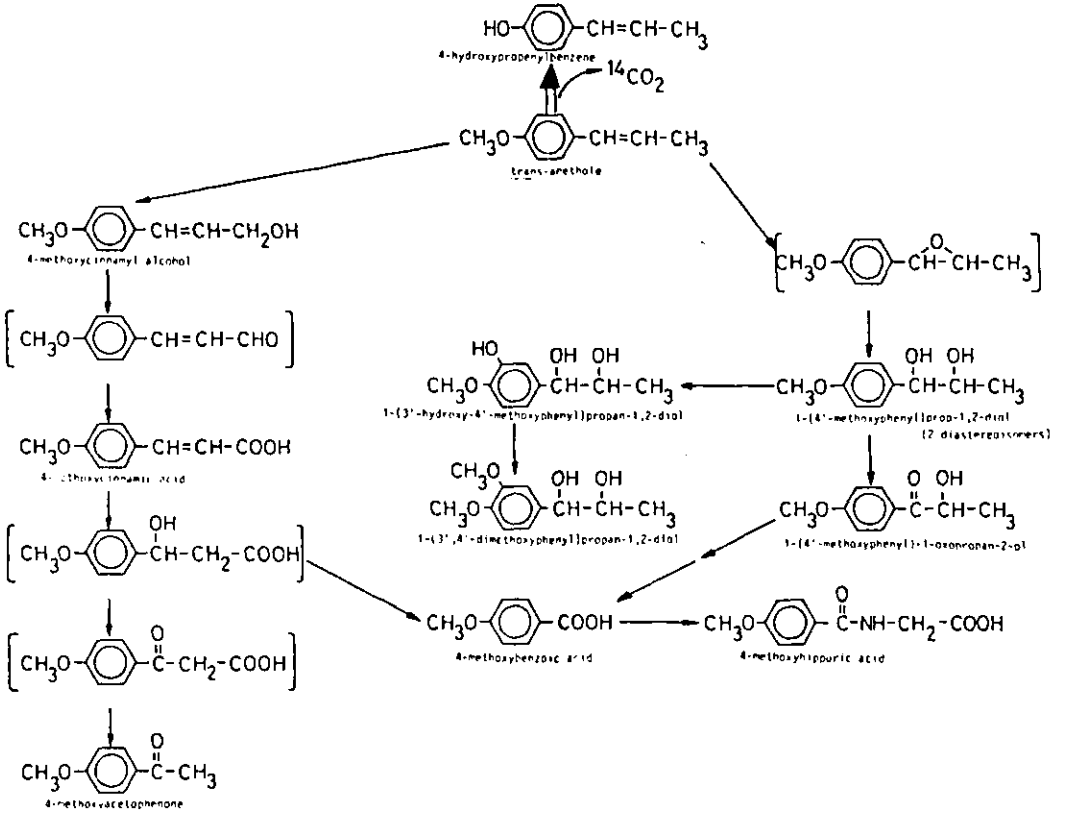


of the [^{14}C]-formaldehyde into pathways of 1-carbon fragment intermediary metabolism. By transfer of [^{14}C]-carbamyl phosphate into the urea cycle, ^{14}C can be incorporated into arginine, then into ^{14}C-urea when the amino acid is converted to urea and ornithine (White, Handler and Smith, 1973). This incorporation of ^{14}C in urea was also demonstrated after decarboxylation of phenylcarbamic acid,a metabolite of phenylthiourea (Smith, 1960).

The urinary metabolites of the two rodent species indicate that both rats and mice metabolise the propenyl side chain of trans-anethole via similar routes, which are consistent with previously published data (Solheim and Scheline, 1973). Where variations exist between the two rodent species they are mainly in the percentages of each metabolite excreted and indicate some species preference for one route rather than another. Figure 2.19. illustrates the proposed routes of metabolism of [^{14}C-methoxy]-trans-anethole in rodents based upon the present study and earlier work (Solheim and Scheline, 1973).

One of the major metabolites in the rat urine and the most important metabolite in mouse urine was 4 -methoxybenzoic acid, excreted largely as its glycine conjugate but also free and as a glucuronide. This acid could arise either via ω-hydroxylation or by α,β hydroxylation. The former route is consistent with identities of other urinary metabolites, which are possible intermediates in β-oxidation of the propenyl side chain. In normal intermediary metabolism, fatty acids

Figure 2.19. Proposed routes of metabolism of trans-anethole in rodents



are oxidised in the mitochondria by a sequence of reactions in which the fatty acyl chain is shortened by 2-carbon atoms at a time. The β-oxidation of acids was first proposed by Knoop (1905) and extended to investigations of many acids by Dakin (1909) and Quick (1928).

The identification of 4-methoxycinnamyl alcohol and 4-methoxycinnamic acid in rodent urine supports this theory, although β-hydroxy-3-(4'-methoxyphenyl)propionic acid which would be an expected metabolite was not identified in this study. Solheim and Scheline (1973) did detect this metabolite in the urine of rats given 100mg/kg trans-anethole. This β-hydroxy-acid is an obligate intermediate for the metabolism of the propenyl side chain to 4 -methoxybenzoic acid via this route. Other studies of the metabolism of compounds with the propenyl side chain have been reported that show cleavage to the benzoic acid derivative. Peele and Oswald (1977) report the metabolism of propenylbenzene in rats to hippuric acid (benzoylglycine) via cinnamyl alcohol and cinnamic acid, and Quick (1928) showed the excretion of benzoyl glucuronide and hippuric acid in the urine of dogs given cinnamic acid

The identification of two isomers of 4-methoxycinnamic acid in rodent urine leads one to suspect that part of this metabolite may have arisen by chemical breakdown of another metabolite during analysis, which 'opened' to both the cis and the trans isomers of 4 -methoxycinnamic acid. This deduction was based upon the improbability of the trans-4-methoxycinnamic acid having isomerised in vivo, as this phenomena would have undoubtedly have also taken

place with the metabolites anol and 4-methoxycinnamyl alcohol, and isomerisation would also have been observed during analysis by gc-ms of these compounds, nor the possibility of the isomerisation occurring in gc, because the same procedure was followed for gc-ms of authentic trans 4-methoxycinnamic acid and no isomerisation occurred.

Further confirmation for this pathway of metabolism in rodents comes from the identification of 4-methoxyacetophenone in the urine of rats and mice given trans-anethole. Solheim and Scheline attributed the identification of this metabolite in their study to the decarboxylation of the unstable 3-keto-3-(4'-methoxyphenyl)propionic acid. They supported this theory by showing that authentic 3-keto-3-(4'-methoxyphenyl)-propionic acid could be readily extracted into ether and undergo facile decarboxylation to 4-methoxyacetophenone. Marsh et al (1982) reported that acetophenone in an extract of horse urine was also attributable to the spontaneous decarboxylation of 3-keto-3-phenylpropionic acid during workup.

The data from this study and evidence from previous investigations supports the pathway illustrated in figure 2.19. to 4-methoxy-hippuric acid via the cinnamyl compounds.

The remaining extractable urinary radioactivity was accounted for by the two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol 1-(4'-methoxyphenyl)-1-oxo-propan-2-ol and 1-(3'-hydroxy-4'-methoxy-phenyl)propane-1,2-diol,(and the methylated derivative of the latter, presumably arising by methylation during workup).

1-(4'-methoxyphenyl)-1-oxo-propan-2-ol is assumed to have arisen by oxidation of an hydroxyl group in the diol. Ohtsuji and Ikeda (1971) reported the elimination of phenylglyoxylic acid from rats administered styrene oxide, and it is possible that such a keto intermediate may occur in the pathway of elimination of styrene or styrene oxide as hippuric acid (El Masri et al., 1958). This possibility suggests that oxidation of the diol may provide another pathway for the elimination of trans-anethole as 4-methoxyhippuric acid (figure 2.19.).

Ring hydroxylation of 1-(4'-methoxyphenyl)propane-1,2-diol in the ortho position to the 3'-hydroxy-metabolite accounted for about 3% of the dose to rats. Workup unfortunately methylated this metabolite to the 3'-4'-dimethoxy-derivative. Ortho ring hydroxylation is a well documented route of metabolism for aromatic compounds. As long ago as 1942, gentisic acid was reported as being excreted in the urine of volunteers given salicylic acid (Kapp and Coburn, 1942) and later gentisic acid was demonstrated to be excreted in the urine of rodents (Bray et al., 1948; Quilley and Smith, 1952).

Analyses indicated that the unextractable aqueous residue of rodent urine contained metabolites with divalent sulphur groups, which were broken down by treatment with Raney nickel to extractable hydroxylated compouds. These sulphur-containing metabolites could be thioethers, most likely mercapturic acids (S-substituted-N-acetylcysteines).

These two groups of compounds, the diols and their subsequent metabolites and the thioethers have been shown in other related compounds to arise via an epoxide intermediate. In 1968 it was shown independently by two groups that epoxides were obligatory intermediates in the metabolism of olefins to glycols (Leibman and Ortiz, 1968 and 1970; Watabe and Maynert, 1968). However prior to this it was known that styrene oxide was metabolised to the same mercapturic acids as were excreted after styrene administration (James and White, 1967). As early as 1958 styrene was shown to be excreted as the monoglucuronide of phenylethylene glycol (El Masri et al., 1958).

The evidence from styrene and styrene oxide metabolism suggests that based on the metabolites identified in the rodent urine after trans-anethole administration, metabolism via anethole epoxide is logically to be expected, and accounts in total for more than 20% of the dose. Solheim and Scheline, however report epoxidation to be a minor route of anethole metabolism (Solheim and Scheline, 1973) and no other studies discussed the pathway.

The unsaturated propenyl side chain of trans-anethole has the structural capability to undergo oxidative metabolism by the microsomal monooxygenase system, which is present in the liver and other extrahepatic tissues, to give rise to an epoxide (Burke and Orrenius, 1979). The epoxide could then become a substrate for enzymic and non-enzymic reactions. Convertion to the diols could occur chemically or by the action of an enzyme, epoxide hydrolase (EC3.3.2.3) found in the endoplasmic

reticulum (Oesch et al., 1970), the nucleus and the cytosol (Ota and Hammock, 1980). Anethole epoxide is more likely to be a substrate for the cytosolic enzyme. Ota and Hammock (1980) showed that the structurally similar β-methyl styrene epoxide was hydrolysed extensively by cytosolic epoxide hydrolase, whereas no detectable hydrolysis occurred with microsomal epoxide hydrolase and styrene oxide was exclusively hydrolysed by the microsomal enzyme.

The anethole epoxide may also be converted to glutathione conjugates by a family of cytosolic glutathione transferases (EC.2.5.1.18) (Boyland and Williams, 1965; James and White, 1967). These glutathione conjugates would be further metabolised to mercapturic acids.

The stereochemistry of trans-anethole which results in the excretion of two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol is very complex. The epoxides of trans-anethole and styrene have chiral centres and can exist as various optical isomers (Delbressine et al., 1981). From chiral epoxides, in turn chiral diols and glutathione conjugates may be expected to be produced. The metabolism of styrene and of styrene epoxide and its enantiomers has recently been the subject of much attention. There is very little information available on the orientation of the introduction of the glutathione sulphhydryl group into xenobiotic epoxides. With respect to the oxirane fused to a ring system, the enzymic conjugation reaction has been shown to occur regiospecifically with dihydronaphthalene (Booth et al., 1960; Boyland and Sims, 1960),

naphthalene oxide (Jeffray and Jerina, 1975; Jerina et al., (1970) and cholesterol α-epoxide (Watabe et al., 1979) and regioselectively with benzo(a)pyrene-4,5-oxide (Hernandez et al., 1980). Styrene oxide was reported to conjugate specifically with glutathione at the benzylic carbon under enzymic and non-enzymic conditions to yield a single conjugate (Ryan and Bend, 1977). Under similar conditions Pachecka et al. (1979) reported the conjugation of glutathione with racemic styrene oxide at both carbon atoms. However, the conclusion was based upon the treatment of the inseparable conjugate mixture with Raney nickel; they yield phenethyl alcohol and methylphenylcarbinol as products. Recently Watabe et al.(1981; 1983) have shown that the Raney nickel desulphuration method is inadequate for the determination of sulphur bearing atoms in the styrene oxide glutathione conjugate since it affords styrene oxide as the major desulphuration product from conjugates at either carbon atom. The epoxide in the catalytic medium is then hydrogenated to either phenethyl-alcohol or methylphenylcarbinol.

This study of styrene metabolism may explain the identification of the identification of the two diastereoisomers of 1-(4'-methoxy-phenyl)propane-1,2-diol and 1-(4'-methoxyphenyl)propan-1-ol as desulphuration products from the aqueous residue of urine from trans-anethole treated rodents. Due to the highly reactive nature of anethole epoxide (Miller et al., 1979) any anethole epoxide formed in the catalytic medium during Raney nickel treatment would not be detected because it would immediately hydrolyse to the diols or alcohols. Based upon this information the identification of the regioisomers was not possible.

The metabolic information on trans-anethole in rodents together with the high reactivity of anethole epoxide suggest that the simplest explanation of this data is that trans-anethole is oxidised to a single epoxide which is opened chemically to two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol and that some glutathione conjugation, possibly at the benzylic carbon, takes place.

CHAPTER 3

Dose-dependent variations in the metabolism of *trans*-anethole in the rat and mouse

3.1. INTRODUCTION

Human lifetime exposure to all food additives, including the food flavouring trans-anethole is generally continuous and at a low level (anethole; 65µg/day, F.E.M.A., 1978), assuming a maintained dietary pattern. However, toxicity tests are carried out at high doses (up to 1g /day, Hagan et al., 1967) often at the maximum tolerated dose for up to 1 year (Jones, 1981). Since large doses may overwhelm detoxication mechanisms, caution must be observed when making comparisons between the effects of very large doses and very small ones.

The pattern of metabolism of a compound can be altered by administering different doses, in so far as a large dose may saturate the mechanisms by which a small dose is metabolised. The compound may then accumulate temporarily, may be excreted partly unchanged or may undergo different metabolic reactions. Alternative pathways may be followed when certain conjugating agents are limited in supply, but these pathways are not necessarily associated with increased toxicity. In the following sections there are examples of the consequences of administering large doses of compounds whose metabolism and/or elimination is known to be dose dependent.

Saturation of metabolism

Once the dose administered is sufficient to saturate one of the pathways involved in the drugs' disposition, its elimination can no longer be described by first order kinetics and an expression

involving Michaelis-Menten kinetics must be derived (Rowland, 1977). The non-linearity so introduced has important consequences, which have been summarised by Wagner (1974) and are listed in table 3.1.

Table 3.1. Consequences of saturable metabolic pathways

1. Elimination kinetics non-exponential
2. Elimination $t_{\frac{1}{2}}$ increase with dose
3. Area under plasma level versus time curve (AUC) after oral dosing not proportional to percentage of dose absorbed
4. Pattern of urinary metabolites varies with dose
5. Competitive interaction with other drugs also metabolised by the saturable pathway(s) is frequent

(taken from Caldwell, 1980)

The problem of saturation of metabolic pathways is far more frequent in conjugation reactions than phase I reactions when the dosage used is within the therapeutic range. However, toxicity tests are often carried out at doses far above the therapeutic range, and therefore examples of saturation of phase I reactions have been reported.

Saturation of phase I metabolism

Estragole (4'-methoxyallybenzene) exhibits dose-dependent metabolism in both rats and mice (Zangouras et al., 1981). The proportion of the dose undergoing oxidative O-demethylation declines as the dose is increased, from 36% of the dose at 0.05mg/kg to 21% of the dose at 1000mg/kg, and this is accompanied by an increase in the proportion of the dose that undergoes urinary elimination. The authors concluded that at high doses the enzymes responsible for O-demethylation became saturated, therefore relatively greater amounts of the substrate were available for alternative metabolic reactions. The carcinogenicity of estragole is thought to be mediated via the proximate carcinogen 1'-hydroxyestragole (Drinkwater et al., 1976) and the formation of this metabolite was found to increase as the dose was increased, from 1% to 9% of the dose over the range 0.05-1000µg/kg. Therefore it may be deduced that tumour induction may prove to be dose-dependent.

Ethylene glycol, a component of antifreeze, appears to be more toxic to man than to laboratory animals (minimum reported lethal dose in man about 1.6g/kg; LD_{50} in cat 2ml/kg; LD_{50} in mouse 13ml/kg). The metabolic conversion of ethylene glycol to oxalate has been postulated as the reason for the toxic effects (Gessner et al., 1961).

In rodents ethylene glycol is metabolised via glyoxylic acid to CO_2 and oxalic acid. At lower doses (0.1-1g/kg) the pathway to oxalic acid is a minor route (about 0.05% of the dose excreted as oxalate) and most glyoxylic acid is decarboxylated and excreted

as CO_2. However,at higher doses (up to 5g /kg) larger amounts of oxalate are excreted (0.5% of dose) with an accompanying decline in decarboxylation and CO_2 elimination.

When 1,4-dioxane was administered to rats the percentage of the dose excreted as the only urinary metabolite hydroethoxyacetic acid decreased from 92% to 60% of the dose as the dose was increased from 10 to 1000mg/kg (Young et al., 1976). At the same time the percentage of unchanged 1,4-dioxane eliminated in the urine rose from 5% to 38% of the dose. Pharmacokinetic studies showed increased plasma clearance of 0.25 to 2.88ml/min over the dose range revealing that the observations were the result of saturation of metabolism rather than saturation of excretory pathways. Toxicological studies supported the conclusion that toxicity of dioxane occurs only when doses are given sufficient to saturate the metabolic pathway for its detoxication.

Table 3.2. lists some drugs whose phase I metabolism is saturable within the normal dosage range in man, together with the saturable pathway.

Table 3.2. Drugs exhibiting phase I dose-dependent metabolism and kinetics in humans as a consequence of saturation of metabolism

Drug	Pathway	Reference
phenytoin	4-hydroxylation	Gerber & Wagner (1972)
Theophylline	N-1-demethylation	Caldwell et al., (1977)
Ethanol	oxidation	Lundquist & Wolthers (1958)

Saturation of phase II metabolism

Sulphate conjugation represents for phenols an alternative to glucuronidation and the relative extent of these two reactions has been shown, in many studies, to be dependent on the size of the dose administered. At low doses (Capel et al., 1972), sulphation predominates and at larger doses this pathway seems to be overloaded (Levi and Matsuzama, 1967). This has been demonstrated for salicylamide in man over the dose range 150-1000mg, the percentage of the dose excreted as the sulphate conjugate being significantly reduced at the higher doses. When L-cysteine was administered concomitantly as a source of sulphate, the amount of salicylamide conjugated with sulphate increased (Levi and Matsuzawa, 1967; Levy and Procknal, 1971).

Paracetamol can undergo detoxication by glutathione, glucuronic acid and sulphate conjugation (Jollow et al., 1973). Conjugation with glutathione is quantitatively the least important pathway (Mitchell et al., 1974). However it is suspected that paracetamol hepatotoxicity is related to the metabolic activation of the drug to an arylating agent which binds covalently to liver macromolecules (Jollow et al., 1973). Binding of the reactive metabolite to macromolecules and liver necrosis only occurs when liver glutathione levels have been depleted, suggesting that the reactive metabolites are normally detoxified by conjugation with glutathione (Mitchell et al., 1973). Necrosis and binding can be increased by pretreatment of the animals with diethyl malonate, an agent causing glutathione depletion (Mitchell, et al., 1973; Potter et al., 1974).

These and other examples of saturable conjugation reactions are listed in table 3.3.

Table 3.3. Drugs exhibiting dose-dependent phase II metabolism and kinetics in humans and animals as a consequence of saturation of metabolism

drug	pathway(s)	reference
paracetamol	glutathione, glucuronic acid and sulphate conjugations	Jollow et al., 1973
salicylate	glycine conjugation, ester glucuronidation	Levy et al., 1972
salicylamide	sulphate & glucuronic acid conjugations	Levy & Yamada, 1971 Levy & Procknal, 1971
Isoniazid	N-acetylation	Kakemi et al., 1963
para-aminobenzoic	N-acetylation	Drucker et al., 1964
benzoic acid	glycine conjugation	Caldwell et al., 1970

Influence of dose size on excretion

It was found that when low dose of safrole (0.6mg/kg) an allylbenzene related to trans-anethole and estragole, was administered to rats it was rapidly absorbed, metabolised and excreted in the urine within 24 hours (90% of dose) (Strolin-Benedetti et al., 1977). However, when the dose was increased to 750mg/kg a marked decrease in the rate of elimination occurred. Only 25% of the dose was recovered in the urine in 24 hours and only 70% in 48 hours. The tissue levels of safrole also remained elevated for 48 hours and the ratio of tissue to plasma radioactivity was much greater than observed at the low dose, indicating a marked accumulation

of compound in the tissues. Furthermore, tissue and plasma levels of unchanged safrole are proportionally higher than at the low dose, suggesting that the metabolism of the compound was altered at the higher dose.

Piper et al. (1973) showed that the pharmacokinetics of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was dose-dependent in the rat. The plasma half-life of 2,4,5-T in the rat increased after single doses (p.o.) of 5-200mg/kg from 4.7 to 25.2 hours. The elimination half-life from the body also increased with increasing dose. It was concluded that at 200mg/kg the dose may have exceeded the excretion capacity of the body. At the highest dose an unidentified minor metabolite was detected, otherwise the compound was not metabolised. In this study it was also demonstrated that the plasma half-life of 2,4,5-T in the dog was 770 hours at 5.0mg/kg (cf. 4.7 hours in the rat), which may explain in part why this herbicide is more toxic in the dog than in the rat.

All these observations are consistent with the view that the pattern of metabolism of a test compound in a high dose toxicity trial can be quantitatively and/or qualitatively different from the metabolic profile seen at 'normal' levels of exposure, due to the possibility of metabolic overload leading to alternative pathways of metabolism.

This chapter described the results of a study of the metabolism of [^{14}C-methoxy]-trans-anethole in rats and mice over the

dose range 0.05-1500mg/kg. The findings are discussed in relation to the known metabolic behaviour of certain chemically related structures, namely safrole and estragole. In this way it is hoped to gain an insight into the predictability of the disposition of other chemically related substances under conditions of low-level exposure consistent with that of the real dietary exposure situation that occurs for man.

3.2. MATERIALS AND METHODS

The materials and methods used in this study were as described in chapter 2. Any variations are indicated below.

Animals and dosing

Rats and mice (as described in chapter 2) were administered [^{14}C-methoxy]-*trans*-anethole appropriately diluted with unlabelled *trans*-anethole in trioctanoin (5ml/kg). Rats were administered doses of 0.05, 5, 50 and 1500 mg/kg by oral intubation and mice 0.05, 5, 50, 250 and 1500mg/kg by intra-peritoneal injection. Four animals of each species were dosed at each level. Every animal received at least 2μCi of radioactivity except those administered the low doses where the following amounts of radioactivity were dosed; rats at 0.05mg/kg; 0.05μCi, mice at 0.05mg/kg; 0.005μCi and at 5mg/kg; 0.5μCi. The animals were maintained and housed as previously described for up to 72 hours. Excreta and trapping solutions were handled and stored as before.

Quantitation of [^{14}C] labelled urinary metabolites of *trans*-anethole in the rat and mouse.

Urine samples were extracted and analysed as described in chapter 2. 0-24 hour urine samples were analysed except from the 1500mg/kg dosed animals where 0-48 hour samples were used.

The metabolites extracted at pH5.0 were quantified in duplicate by radio-hplc (system 2), and pH1.0 extractable metabolites by tlc (system B) and radio-hplc (system 3). ^{14}C-urea in the aqueous residues was analysed and quantified as described in chapter 2.

3.3. RESULTS

Elimination of [^{14}C-methoxy]-*trans*-anethole in rodents over the dose range 0.05-1500mg/kg

In both rats and mice greater than 85% of a single dose of [^{14}C-methoxy]-*trans*-anethole was recovered after all levels of exposure. The excretion data is summarised in figures 3.1. and 3.2.

At all doses the majority of the radioactivity was recovered in the expired air as $^{14}CO_2$ and in the urine, with only 1% of the dose being excreted in the faeces.

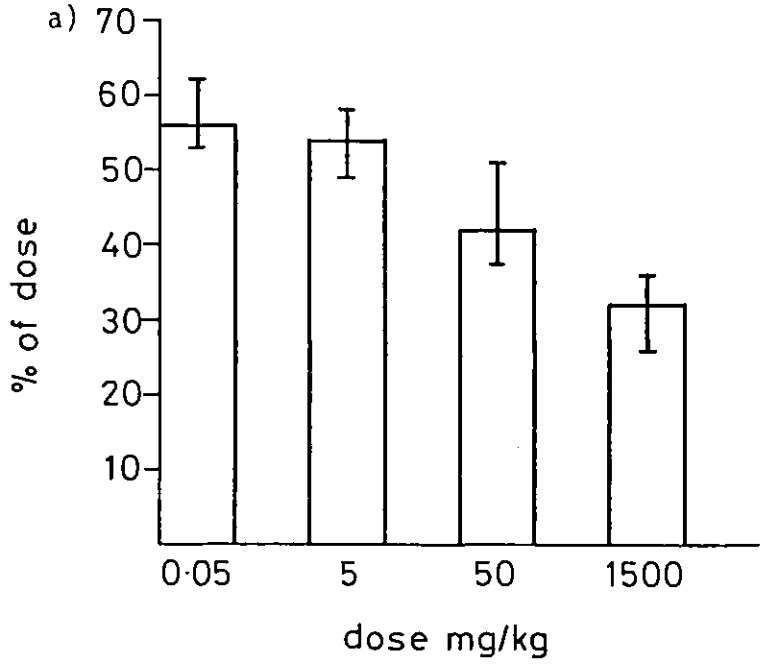
The expired air was the major route of elimination of radioactivity at the lowest dose investigated, accounting for 56% of the dose at 0.05mg/kg in rats and 72% of the dose in mice. However, as the dose was increased the percentage of the dosed radioactivity eliminated as $^{14}CO_2$ fell to 32% in rats at 1500mg/kg and 35% in mice at 1500mg/kg.

Paralleling this variation, the percentage of the dose excreted in the urine rose as the dose was increased in both species from 28% of the dose in the rat urine at 0.05mg/kg to 57% of the dose at 1500mg/kg, and for mice this change was from 20% of the dose at 0.05mg/kg to 67% of the dose at 1500mg/kg. These changes were all highly significant by the Spearman rank correlation (figures 3.1. and 3.2.), and it can be seen that over this dose range the percentage

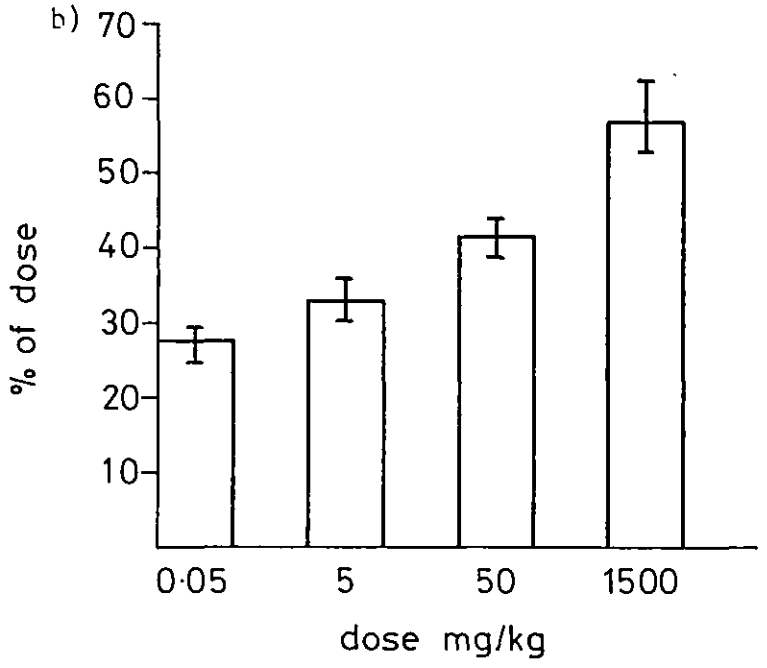
Figure 3.1. Excretion of a single dose of [^{14}C-methoxy]-trans-anethole in rats in the range 0.05-1500mg/kg

a) in the expired air as $^{14}CO_2$ and

b) in the urine

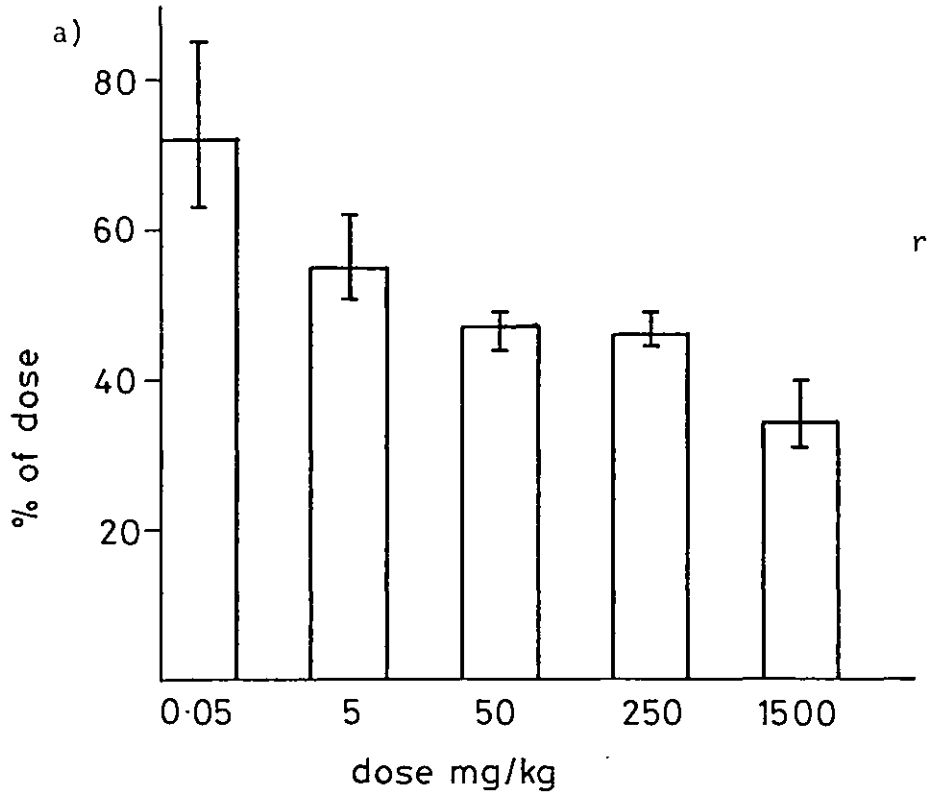


$r_{(s)} = 0.950$; $p < 0.002$

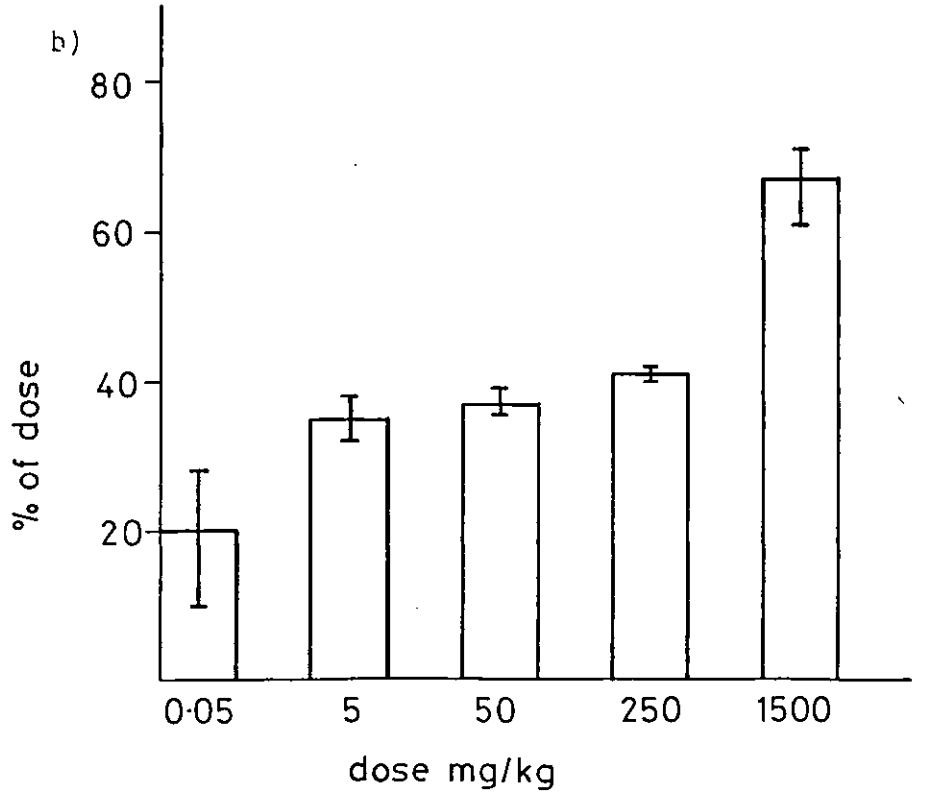


$r_{(s)} = 0.960$; $p < 0.001$

Figure 3.2. Excretion of a single dose of [^{14}C-methoxy]-*trans*-anethole in mice in the range 0.05-1500mg/kg a) in the expired air as $^{14}CO_2$ and b) in the urine



$r_{(s)} = 0.960$; $p < 0.001$



$r_{(s)} = 0.853$; $p < 0.002$

of the dosed ^{14}C in the urine of rats nearly doubled, while that in the mouse it more than doubled.

After administration of 1500mg/kg trans-anethole to rats the volume of urine excreted was very large, up to 40ml/24 hours, as compared to the normal volume of 15ml/24 hours. It was not possible to confirm this observation with mice, as the volume of urine excreted in 24 hours in this species is very small. At the highest dose in both species the rate of elimination of urinary radioactivity was slower, taking up to 72 hours, whereas at lower doses elimination was complete within 24 hours.

Variation in the urinary elimination of metabolites of [^{14}C-methoxy]-trans-anethole in the rat at various doses over the range 0.05-1500mg/kg.

Over the dose range 0.05-1500mg/kg there was no change in the number of metabolites isolated, although the quantities of 4-methoxycinnamyl alcohol present at 0.05 and 5mg/kg were only just above the limit of detection. Twelve metabolites were isolated and including ^{14}C-urea which accounted for about 0.5% of the dose at all levels of exposure. The percentages of all metabolites isolated at all the doses investigated are given in table 3.4.

Table 3.4. Percentage of administered [^{14}C]-*trans*-anethole excreted as each metabolite in the rat at various doses

(mean of at least 4 animals)

Metabolite	0.05mg/kg	5mg/kg	50mg/kg	1500mg/kg	Spearman Rank correlation coefficient $r_{(s)}$	2p
$^{14}CO_2$	56.3(52.5-62.1)	53.9(49.2-58.4)	41.8(37.3-51.0)	31.8(25.9-35.8)	0.950	0.002
4-methoxyhippuric acid	1.6(1.1-2.0)	4.8(4.0-5.6)	6.7(5.7-8.0)	12.1(11.2-12.9)	0.971	0.0003
Unknown A	8.2(7.1-8.9)	8.6(5.3-11.2)	5.8(5.1-6.8)	5.2(4.7-5.7)	0.780	0.005
4-methoxybenzoic acid	1.1(0.8-1.2)	2.5(1.9-2.7)	2.8(2.1-3.3)	5.4(3.5-8.0)	0.850	0.002
4-methoxycinnamic acid	0.3(0.2-0.3)	1.0(0.7-1.0)	1.3(1.0-1.6)	2.2(1.5-2.6)	0.954	0.0004
4-methoxyacetophenene	0.3(0.1-0.1)	0.5(0.4-0.7)	1.5(1.1-1.9)	1.9(0.7-4.1)	0.973	0.0003
1-(4'methoxyphenyl)-1-oxo-propan-2-ol 1-(3-hydroxy-4'-methoxyphenyl)-propane-1,2-diol	0.1	0.4(0.1-0.5)	0.5(0.3-0.7)	0.6(0.5-0.6)	-*	-
1-(3',4'-dimethoxyphenyl)-propane-1,2-diol	0.6(0.3-0.7)	0.8(0.5-1.1)	3.1(2.9-3.7)	3.2(2.5-3.7)	0.861	0.002
1-(4'-methoxyphenyl)propane-1,2-diol diastereoisomer 1	1.1(0.8-1.4)	1.9(1.5-2.0)	5.4(5.2-5.4)	8.7(6.7-10.4)	0.972	0.0003
1-(4'-methoxyphenyl)propane-1,2-diol diastereoisomer 2	1.1(0.8-1.4)	2.3(1.9-2.3)	5.3(4.1-6.3)	6.2(3.5-7.1)	0.949	0.0004

Continued over page

Table 3.4. In continuation

Metabolite	0.05mg/kg	5mg/kg	50mg/kg	1500mg/kg	Spearman Rank correlation coefficient $r_{(s)}$	2p
4-methoxycinnamyl alcohol	< 0.1	< 0.1	0.5(0.3-0.5)	0.8(0.5-1.0)	-	-
Aqueous residue faecal radioactivity	11.9(9.3-14.8)	9.7(8.4-10.8)	9.3(8.2-10.1)	11.1(9.8-12.3)	-	-
Total	82.7	86.5	84.1	89.2		

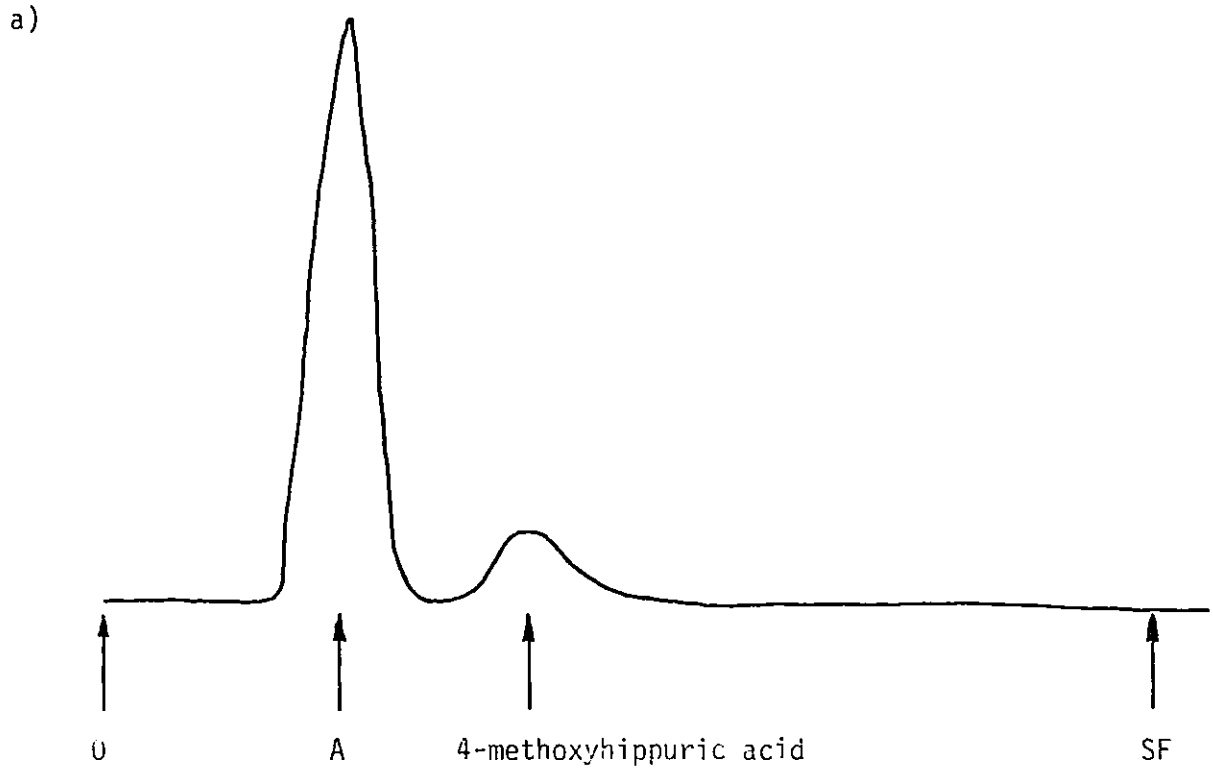
*insufficient values for statistical calculations

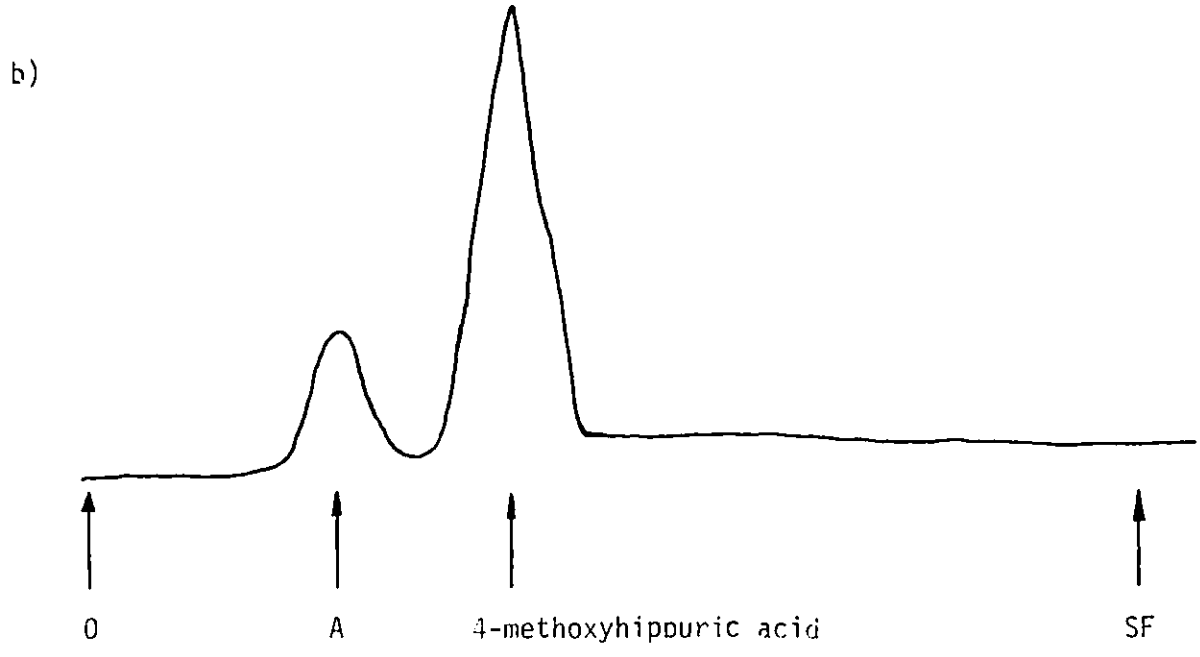
Acidic metabolites

4-methoxyhippuric acid and unknown metabolite A were the major metabolites isolated from the pH1.0 extracts of rat urine. At 0.05mg/kg metabolite A was the major metabolite in this extract (figure 3.3a.), but at 1500mg/kg, 4-methoxyhippuric acid was the major metabolite (figure 3.3b.). Over this dose range 4-methoxyhippuric acid excretion significantly increased as a percentage of the dose from 2% at 0.05mg/kg to 12% at 1500mg/kg, a six fold increase (figure 3.4a.)($r_{(s)} = 0.971$, $p < 0.001$). Metabolite A excretion decreased significantly from 8% of the dose at 0.05mg/kg to 5% of the dose at 1500mg/kg (figure 4b.)($r_{(s)} = 0.780$, $p < 0.01$). The ratio of the percentage of the dose excreted as metabolite A to the percentage of the dose excreted as 4-methoxyhippuric acid also decreased significantly with dose (figure 3.4c.)($r_{(s)} = 0.971$, $p < 0.001$).

The variations in the percentage of the dose excreted as 4-methoxybenzoic acid and 4-methoxycinnamic acid are illustrated in figure 3. 5a and b respectively. Both metabolites show significant increases in elimination over the dose range, 4-methoxybenzoic acid excretion increasing from 1% to 5% of the dose ($r_{(s)} = 0.850$, $p < 0.002$) and 4-methoxycinnamic acid excretion increasing from 0.3% to 2% of the dose ($r_{(s)} = 0.954$, $p < 0.001$).

Figure 3.3. Radioscans of the chromatography of the pH1.0 extracts of β-glucuronidase treated rat urine after administration of a) 0.05mg/kg and b) 1500mg/kg [^{14}C]-*trans*-anethole, developed in tlc system B.





O

O = origin; SF = solvent front

Figure 3.4. Variation in the percentage of the dose of [^{14}C]-trans-anethole excreted as a) 4-methoxyhippuric acid b) metabolite A and c) the ratio of metabolite A to 4-methoxyhippuric acid elimination in the rat, over the dose range 0.05-1500mg/kg.

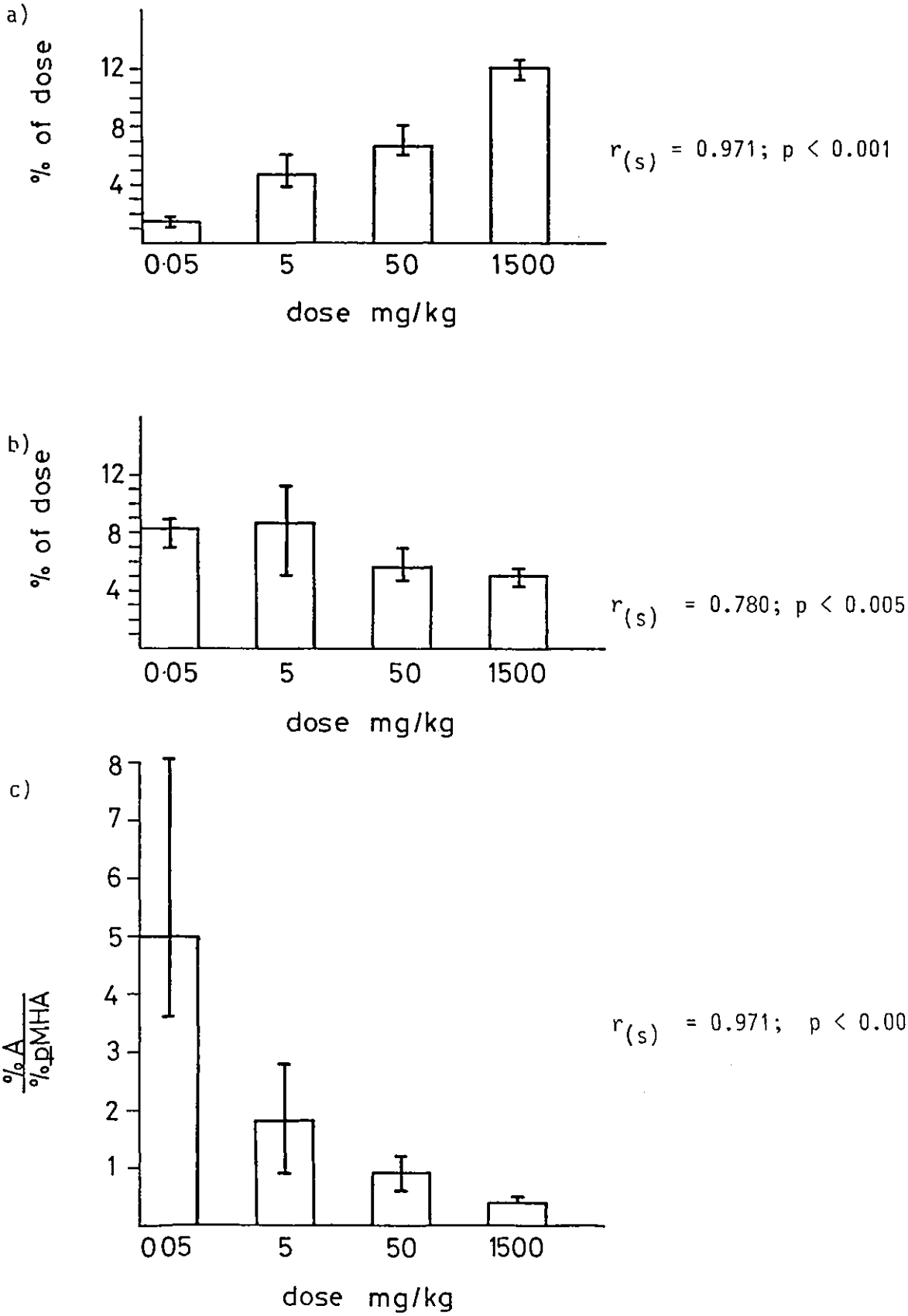
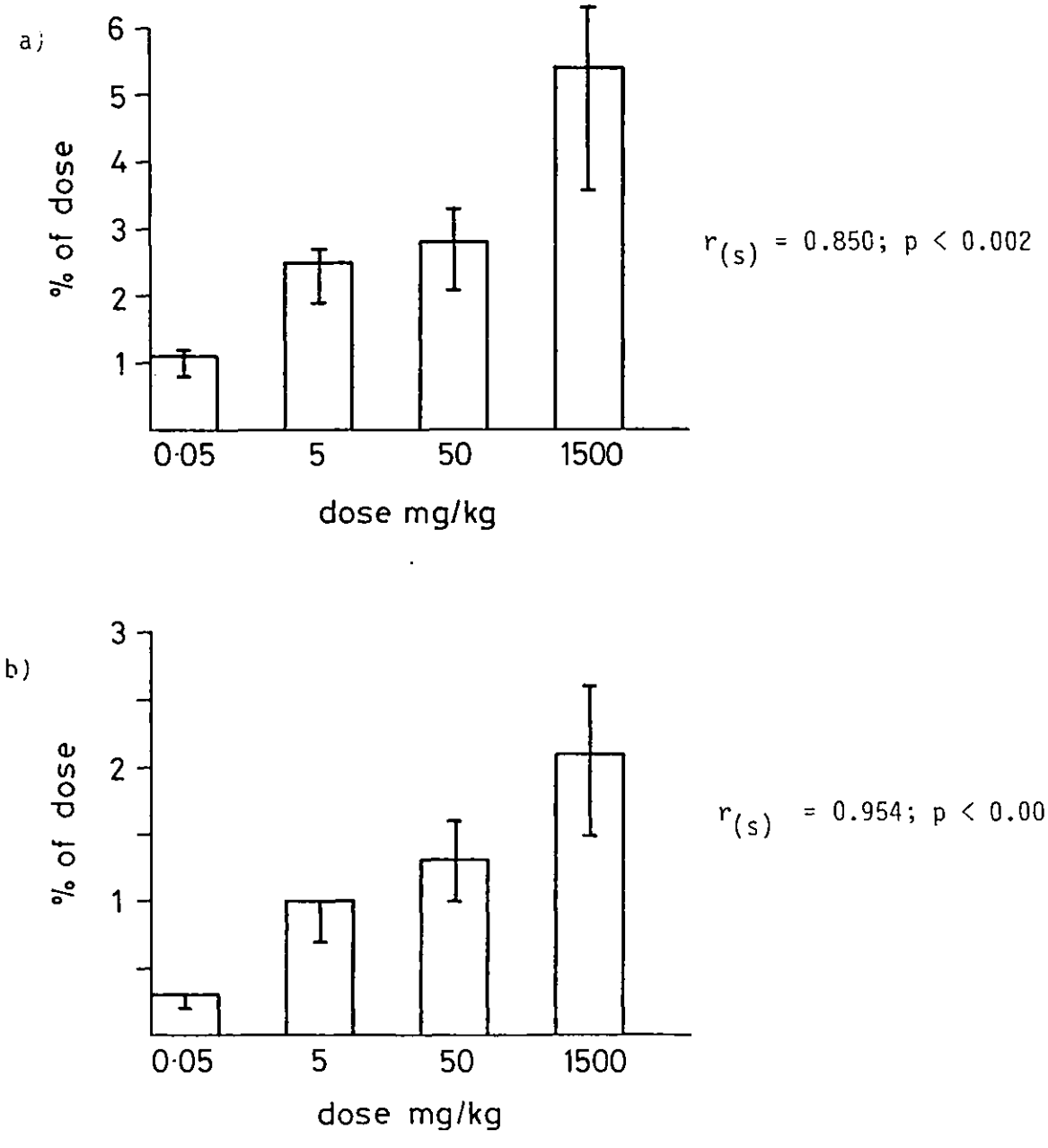


Figure 3.5. Variation in the percentage of the dose of [^{14}C]-*trans*-anethole excreted as a) 4-methoxybenzoic acid, and b) 4-methoxycinnamic acid (*cis* and *trans*) in the rat over the dose range 0.05-1500mg/kg.



4-methoxyacetophenene excretion also showed a significant increase ($r_{(s)}$ = 0.954, $p < 0.001$) over the dose range, but this metabolite only accounted for less than 2% of the dose at 1500mg/kg (table 3.4.).

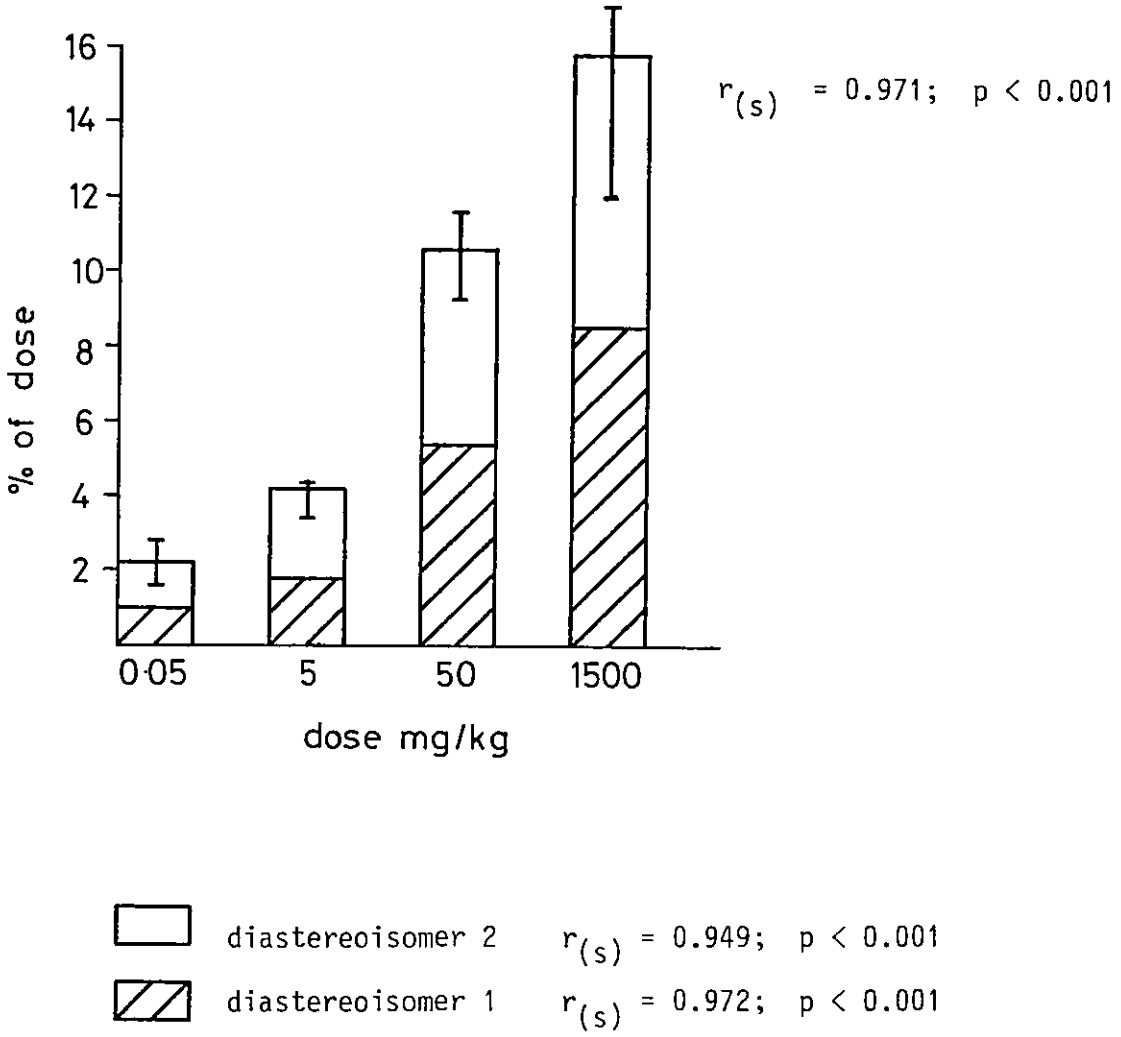
Hydroxylated metabolites

The major hydroxylated metabolites of trans- anethole at all doses were the two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol. These metabolites accounted for a significantly larger percentage of the dose as the dose was increased. At 0.05 mg/kg 2% of the dose was excreted as these diols (1% as each diastereoisomer) which increased 7-fold to 15% of the dose (9% of diastereoisomer 1, 6% of diastereoisomer 2) after a 1500mg/kg dose ($r_{(s)}$ = 0.971, $p < 0.001$). This data is illustrated in figure 3.6. A portion of both diastereoisomers was excreted free and a portion conjugated with glucuronic acid at all dose levels.

4-methoxycinnamyl alcohol, 4-(4'-methoxyphenyl)-1-oxo-propan-2-ol and 1-(3'-hydroxy-4'-methoxyphenyl)propane-1,2-diol (see below) each accounted for less than 1% of the dose at 1500mg/kg, although they all showed increased elimination at high doses compared to low doses.

1-(3',4'-dimethoxyphenyl)propane-1,2-diol is thought to be an artefact produced during analytical workup due to the methylation of 1-(3'-hydroxy-4'-methoxyphenyl)propane-1,2-diol. The methylated metabolite accounts for less than 1% of the dose at 0.05mg/kg but at 1500mg/kg,

Figure 3.6. Variation in the percentage of the dose of [^{14}C]-*trans*-anethole excreted as the two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol in the rat over the dose range 0.05 - 1500mg/kg



3% of the dose is eliminated as this metabolite (figure 3.7.) ($r_{(s)}$ = 0.861, $p < 0.002$). This suggests that the O-demethylation compound (the probable 'real' metabolite) shows increased elimination with dose.

Variation in the urinary elimination of [^{14}C]-metabolites of [^{14}C-methoxy]-*trans*-anethole in mice over the dose range 0.05-1500mg/kg

Over the dose range 0.05-1500mg/kg there was no change in the number of metabolites isolated. At the lowest dose the metabolites that were quantified after pH5.0 extraction were assayed for all animals in a pooled sample, because the small amounts of metabolites excreted at this dose are only just above the limits of detection of the techniques used. The excretion data for the mouse is summarised in table 3.5.

Acidic metabolites

4-methoxyhippuric acid was the major metabolite at all doses (figure 3.8.). The excretion of the free acid of this conjugate (4-methoxybenzoic acid) accounted for less than 2% of the dose, however at a dose of 1500mg/kg, 17% of the dose was eliminated as 4-methoxybenzoic acid. 4-methoxyhippuric acid accounted for 10% of the dose at 0.05mg/kg but this rose to 25% of the dose at 1500mg/kg (r_s = 0.948, $p < 0.001$). The data for the elimination of

Figure 3.7. Variation in the percentage of the dose of [^{14}C]-*trans*-anethole excreted as 1-(3',4'-dimethoxyphenyl)propane-1,2-diol in the rat over the dose range 0.05-1500mg/kg.

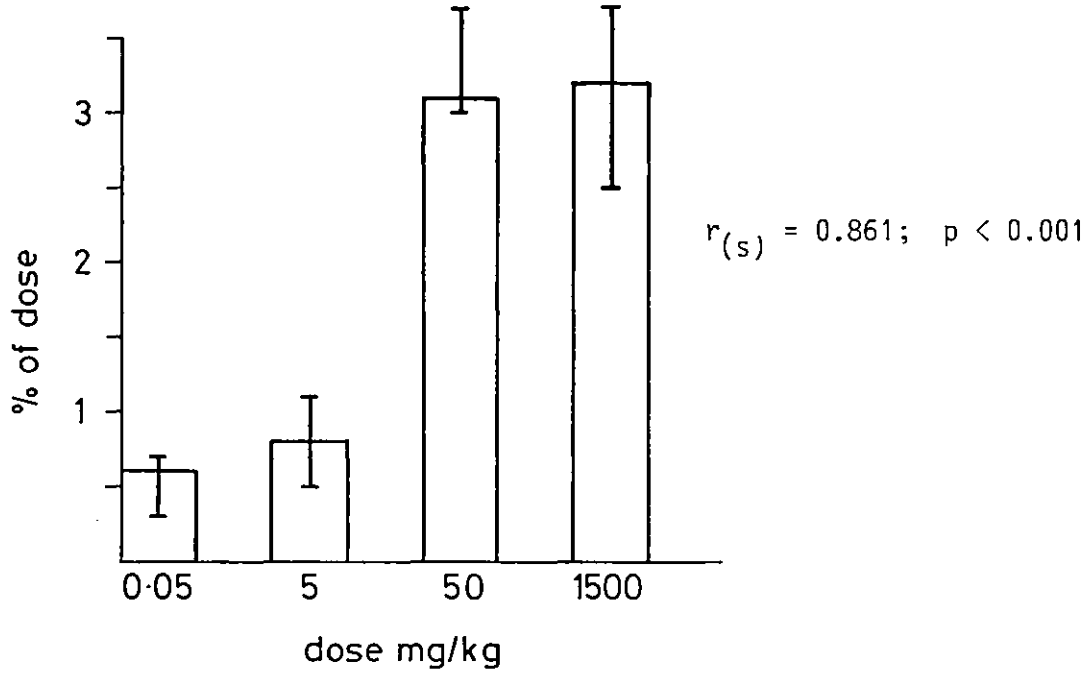


Table 3.5. Percentage of administered [^{14}C] *trans*-anethole excreted as each metabolite in the mouse at various doses

(mean of at least 4 animals)

Metabolite	0.05mg/kg	5mg/kg	50mg/kg	1500mg/kg	Spearman Rank correlation coefficient $r_{(s)}$	2p
$^{14}CO_2$	71.8(63.0-85.0)**	55.4(50.4-61.8)	47.3(45.3-48.5)	34.6(31.2-40.1)	0.960	0.0001
4-methoxyhippuric acid	10.0(5.0-13.2)	13.2(11.3-14.4)	16.0(15.9-16.2)	25.0(23.5-26.9)	0.948	0.0004
4-methoxybenzoic acid	<0.1	1.8(0.8-2.6)	1.4(0.9-2.0)	16.6(14.7-17.0)	0.701	0.01
4-methoxycinnamic acid	<0.1	0.1	0.2(0.1-0.3)	0.3(0.2-0.3)	* -	
4-methoxyacetophenene	<0.1	0.1	0.2(0.1-0.2)	1.3(0.7-2.1)	-	
1-(4'-methoxyphenyl)-1-oxo-propan-2-ol 1-(3'-hydroxy-4'-methoxy-phenyl)propane-1,2-diol	<0.5	1.1(0.7-1.2)	1.1(0.9-1.2)	1.4(1.0-1.5)	-	
1-(3',4'-dimethoxyphenyl)-propane-1,2-diol	<0.1	0.1	0.7(0.6-0.8)	2.9(2.3-3.5)	-	
1-(4'-methoxyphenyl)propane-1,2-diol diastereoisomer 1	<0.5	1.4(1.2-1.6)	2.2(2.0-2.5)	2.0(1.3-2.9)	0.944	0.0004
1-(4'-methoxyphenyl)propane-1,2-diol diastereoisomer 2	<0.5	1.5(1.2-1.7)	2.2(2.1-2.3)	2.5(1.7-3.5)	0.967	0.0004

Continued over page

Table 3.5. In continuation

Metabolite	0.05mg/kg	5mg/kg	50mg/kg	1500mg/kg	Spearman Rank correlation coefficient $r_{(s)}$ 2p
4-methoxycinnamyl alcohol	<0.1	<0.1	0.5(0.4-0.5)	0.6(0.4-0.7)	-
Aqueous residue faecal radioactivity	9.3(4.5-11.5)	14.7(13.4-16.3)	12.5(11.5-14.2)	18.7(15.0-21.7)	-
Total	92.6	89.5	84.3	103.9	

*insufficient data for statistical calculation

**figures are means with ranges in parentheses

4-methoxyhippuric acid and 4-methoxybenzoic acid is illustrated together in figure 3.8. for the dose range 0.05-1500mg/kg.

4-methoxycinnamic acid was a minor metabolite of trans-anethole in the mouse, accounting for less than 0.5% of the dose at 1500mg/kg (table 3.5.).

Hydroxylated metabolites

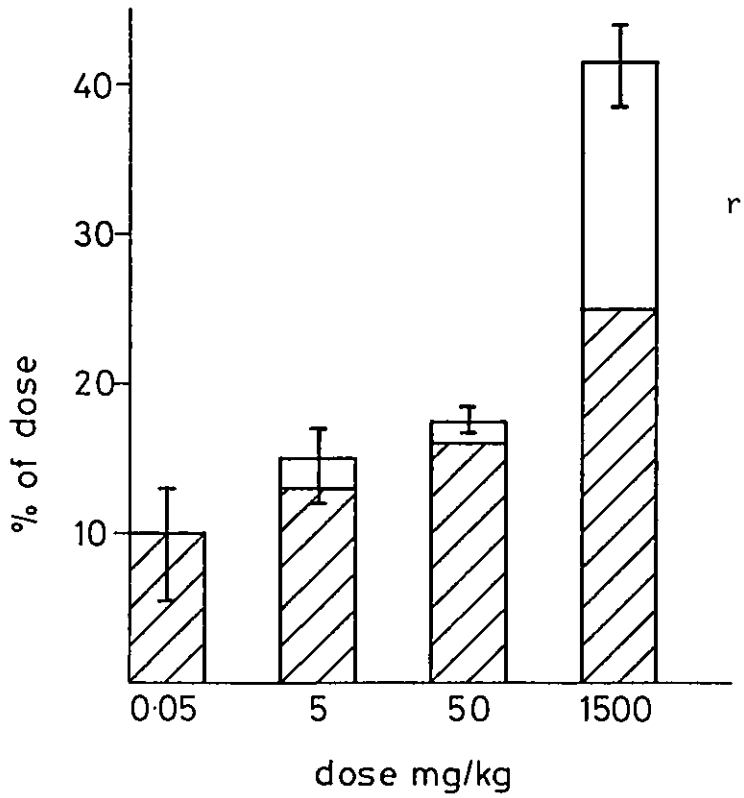
The diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol were less important metabolites of trans-anethole in the mouse than in the rat at all doses. Together they accounted for 1% of the dose at 0.05mg/kg which increased significantly to 5% of the dose at 1500mg/kg (figure 3.9) ($r_{(s)}$ = 0.930, $p < 0.002$).

4-methoxyacetophenene, 4-methoxycinnamyl alcohol and 1-(4-methoxyphenyl)-1-oxo-propan-2-ol all showed increased excretion as the dose was increased but at the highest dose accounted for 1% of the dose or less.

1-(3',4'-dimethoxyphenyl)propane-1,2-diol accounted for 3% of the dose at 1500mg/kg having risen from less than 0.1% of the dose at 0.05mg/kg. However statistical calculations were not possible due to the low amounts excreted at the lowest doses.

No dose-dependent variation in the percentage of the dose remaining unextractable in the rat or mouse urine was observed.

Figure 3.8. Variation in the percentage of the dose of [^{14}C]-*trans*-anethole excreted as 4-methoxybenzoic acid in the mouse over the dose range 0.05-1500mg/kg

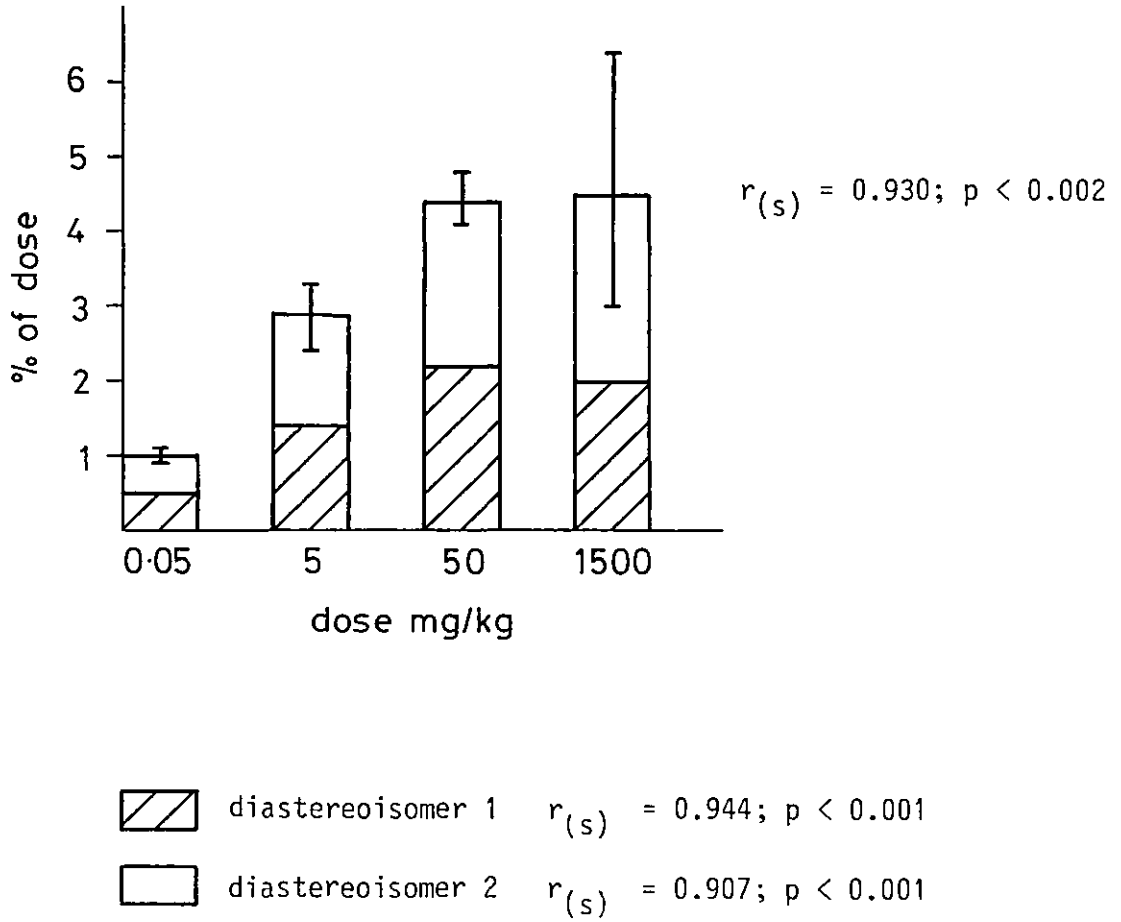


$r_{(s)}$ = 0.967; $p < 0.001$.

% of the dose excreted as 4-methoxyhippuric acid (4-methoxybenzoyl glycine)

$r_{(s)}$ = 0.948; $p < 0.001$.

Figure 3.9. Variation in the percentage of the dose of [^{14}C]-trans-anethole excreted as the two diastereoisomers of 1 -(4'-methoxyphenyl)propane-1,2-diol in the mouse over the dose range 0.05-1500mg/kg



3.4. DISCUSSION

Administration of [^{14}C-methoxy]-*trans*-anethole to rats and mice in the dose range 0.05-1500mg/kg resulted in a dose-dependent change in the route of elimination of radioactivity. At low doses elimination was mainly via the expired air as $^{14}CO_2$ and at high doses mainly in the urine.

The decreased elimination of $^{14}CO_2$ indicates that oxidative O-demethylation of *trans*-anethole falls with increased dose. This suggests that the enzymes responsible for this reaction were either inhibited or overloaded as the dose was increased. Investigation of the rate of elimination of $^{14}CO_2$ after administration of different doses of *trans*-anethole could help to clarify this question. This type of investigation was performed with the saturated analogue, *p*-propylanisole (chapter 5) and this point is discussed further in that chapter. A reduction in oxidative O-demethylation with increasing dose was also observed with the related food flavour estragole in rodents over a similar dose range (Zangouras *et al.*, 1981).

The decreased oxidative O-demethylation of *trans*-anethole at high results in the availability of larger amounts of unchanged compound for metabolism by enzyme systems capable of oxidising the propenyl side chain to ^{14}C products excreted in the urine. As a result of this there is a two-fold increase in the urinary elimination of radioactivity at 1500mg/kg compared with that of 0.05mg/kg, which

completely compensates for the reduced elimination of $^{14}CO_2$ in the expired air recorded over this dose range.

The total recovery of radioactivity was greater than 85% of the dose administered, at all levels. No unchanged trans-anethole was detected in the expired air or urine, even at the highest dose. These facts together indicated that trans-anethole was extensively absorbed, metabolised and eliminated at all doses up to 1500mg/kg in rodents and that there was no irreversible tissue binding by the compound or its radioactive metabolites. Strolin-Benedetti and Le Bourhis (1972) have also reported the complete elimination of a 100mg/kg dose of [^{14}C]-trans-anethole from rats within 24 hours.

However in this present study the rate of elimination of a single dose of 1500mg/kg in rats and mice was slower than at doses of 50mg/kg or lower, taking up to 72 hours. This slow elimination at high doses has similarities to data reported for the elimination of safrole (Strolin-Benedetti et al., 1977). In this paper the authors proposed that at high doses of safrole (750mg/kg), the metabolism was impaired due to saturation of metabolic enzymes, which led to the accumulation of unchanged safrole in the tissues. It is possible that the same situation could occur for trans-anethole at high doses, because both have similar chemical structures and are lipophilic oils which are not excreted unchanged by the kidney.

The increased diuresis observed in rats after 1500mg/kg [^{14}C]-*trans*-anethole may also be related to the slow elimination of the dose which may help to explain the weight loss, or reduced weight gain reported in toxicity tests when *trans*-anethole was administered in the diet at levels of 0.46% or higher (Le Bourhis, 1973b, Miller *et al.*, 1983).

All the ^{14}C urinary metabolites of [^{14}C-methoxy]-*trans*-anethole in rats and mice showed dose-dependent variation in elimination For only one metabolite, metabolite A detected in rat urine, did urinary excretion decrease with increasing dose. Suggestions as to the identity of this so far unidentified metabolite were discussed in chapter 2.

Observation of the excretion data for the other major urinary metabolites of [^{14}C]-*trans*-anethole, 4 -methoxyhippuric acid, 4 -methoxybenzoic acid, the two diastereoisomers of 1 -(4'-methoxyphenyl)-propane-1,2-diol and 1-(3',4'-dimethoxyphenyl)-propane-1,2-diol, indicate clear species differences in the preference for one metabolic route, either via epoxidation (rat), or via the cinnamyl compounds to 4 -methoxybenzoic acid by β-oxidation (mouse). In both species however, there is a dose-dependent increase in the elimination of the major and all the minor metabolites.

4 -methoxyhippuric acid was the major urinary ^{14}C metabolite in the mouse at 1500mg/kg, but such a large percentage of the dose was metabolised to 4 -methoxybenzoic acid in the mouse that 17% of the dose was excreted unconjugated as free 4-methoxybenzoic acid in the

urine in addition to the 25% of the dose excreted as its glycine conjugate, 4-methoxyhippuric acid. This evidence suggests that in the mouse at 1500mg/kg trans-anethole the capacity of the mechanism of glycine conjugation is reached by the large extent of metabolism to 4-methoxybenzoic acid. The limited capacity of glycine conjugation was first reported by Quick (1931) who showed that the maximum rate of synthesis of hippuric acid in man was 7.3-9.3mmol/hour, and later by Bray et al.,(1951) who showed a similar limited capacity to convert benzoic acid to hippuric acid in rabbits. However the reason for the limited capacity of the glycine conjugation mechanism is not entirely clear. Early evidence suggested that it may be due to a limitation in the amount of free glycine available. Attempts to alter the conjugation mechanism by replenishing the glycine pool through exogenous administration of the amino acid have produced variable results. Administration of glycine in the diet of dogs has been shown to enhance the rate of conversion of benzoic and p-aminobenzoic acids to their respective glycine conjugates (Quick et al., 1931). However co-administration of glycine to dogs does not apparently affect the conversion of salicylic acid to salicyluric acid (Quick, 1933).

In addition to the possibility that limited glycine availability may be an important phenomena in limiting the capacity of the mechanism of conjugation of 4 -methoxybenzoic acid in mice,

enzyme binding sites may be the rate limiting step, either by substrate or product inhibition. Hippuric acid was found to be inhibitory to glycine N-acyltransferase at a concentration of 6×10^{-4}M (Schachter & Taggart, 1954).

In the rat only 12% of the dose at 1500mg/kg was excreted as 4-methoxyhippuric acid and no limit in glycine capacity was observed.

In the rat the preferred route of metabolism of trans-anethole was via epoxidation to the diols. At 1500mg/kg, 15% of the dose was eliminated as diols and 3% of the dose as substituted diols (cf. mouse 4%) showing that this route is not saturated at the highest dose. This data differs slightly from that obtained with estragole at 100mg/kg (Zangouras, 1982) where metabolites thought to arise via estragole epoxide were 'slightly less important' at this dose than at lower doses.

Anethole epoxide most likely also undergoes glutathione conjugation as deduced from the possible presence of thioethers in rodent urine after administration of [^{14}C]trans-anethole. The percentage of the dose eliminated in the urinary fraction containing these metabolites does not show any dose-dependency, suggesting that high doses of trans-anethole do not exhaust glutathione stores contrary to what has been reported after acetaminophen treatment (Mitchell et al., 1973; Jollow et al., 1974).

Epoxides, by virtue of their electrophilic nature are associated with fears of carcinogenicity, mutagenicity and teratogenicity (Daly et al., 1972). However it is clear that the chemical properties of epoxides are an important determinant of their toxicity. Anethole epoxide provides an efficient route of elimination for trans-anethole. The epoxide was not isolated from rodent urine, which was undoubtedly due to the high reactivity of this compound as previously reported by Miller et al (1979). Anethole epoxide was probably rapidly hydrated to the two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol as has been reported for the hydration of estragole epoxide to 1-(4'-methoxyphenyl)-propane-2,3-diol (Phillips et al., 1981).

This study has confirmed the species difference in the metabolism of trans-anethole in the rat and mouse observed at 50mg/kg, (chapter 2) for other doses.

Both species extensively oxidatively O-demethylate trans-anethole but once this pathway is overloaded there is a clear preference in the CD-1 mouse strain for the proposed β-oxidation route of metabolism of the propenyl side chain to 4-methoxybenzoic acid. However, both species are able to metabolise high doses of trans-anethole, with different patterns of metabolites to those at lower doses.

The information gained from this metabolism study of [^{14}C-methoxy]-trans-anethole in rats and mice at various doses supports the theory suggested in the introduction, that use of very high doses of trans-anethole in animal toxicity trials causes a metabolic overload, thereby distorting the pharmacokinetics of the test compound. This is particularly relevant when assessing the risk accruing to man from the use of trans-anethole at low levels in human foodstuffs. The possible uses of such data in risk assessment are described in later chapters.

CHAPTER 4

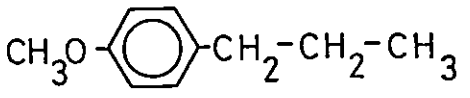
Metabolism of p-Propylanisole in the rat and mouse

4.1. INTRODUCTION

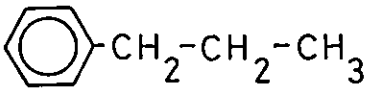
p-Propylanisole is the saturated analogue of trans-anethole and estragole. It has a similar flavour to these two compounds, but is not found naturally. Only comparatively recently has it been introduced into flavour usage in the food industry, its first reported usage being in 1948, although it was not in general use until 1952 (F.E.M.A., 1970).

Of the three analogues, p-propylanisole is generally least toxic although mild toxicity and marked osteoporosis have been reported following the oral administration of large doses chronically to rats (Jenner et al., 1964; Taylor et al., 1964; Hagan et al., 1967).

The metabolism of p-propylanisole does not appear to have been reported in the literature. However, that of the related compound n-propylbenzene has been studied in the rabbit (Smith et al., 1954; El Masri et al., 1956).



p-propylanisole



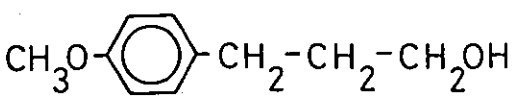
propylbenzene

The three carbon side chain of propylbenzene undergoes α- and ω-1-hydroxylation to yield two alcohols which are excreted as glucuronides, and side chain oxidation to benzoic acid, excreted

as the glycine conjugate, hippuric acid. The authors of the more recent study proposed that benzoic acid arose principally by oxidation of the 2 -hydroxymetabolite even though this carbinol is not itself extensively converted to benzoic acid when administered.

Benzoic acid may also arise from ω-hydroxylation of the three carbon side chain. The ω-alcohol would rapidly be oxidised to phenylpropionic acid, which has long been known to be converted by β-oxidation to benzoic acid (Dakin, 1909). Although it is known that both propylbenzene and phenylpropionic acid are metabolised to hippuric acid (Dakin, 1909; El Masri et al., 1956), phenylpropan-3-ol has not been investigated.

The purpose of the study of p-propylanisole metabolism in the rat and mouse described in this chapter was to elucidate the metabolism of this compound with particular interest in the metabolic pathways leading to 4-methoxybenzoic acid formation. To help accomplish this, 1-(4'-methoxyphenyl)propan-3-ol was also administered to rats and the 4-methoxyhippuric acid produced quantified.



1-(4'-methoxyphenyl)propan-3-ol

[^{14}C-methoxy]-p-propylanisole was administered to rats and mice at 50mg/kg, $^{14}CO_2$ in the expired air was measured and urinary ^{14}C metabolites isolated and quantified by solvent extraction and chromatography, and identified by mass spectrometry and nuclear magnetic resonance spectroscopy.

4.2. MATERIALS AND METHODS

Chemicals

Chemicals and standard laboratory reagents were purchased from usual laboratory sources as described in appendix 2.1.

Radiochemicals

See Chapter 2.

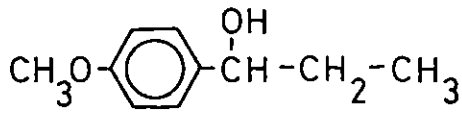
Synthesis of [^{14}C-methoxy]-p-propylanisole

[^{14}C-methoxy]-Estragole (2mCi, Amersham International) was carefully washed out of the packaging ampoule with ethanol. This was transferred to a pear-shaped flask containing activated Raney nickel catalyst (100mg). The reaction mixture of [^{14}C]-estragole, Raney nickel and ethanol (10ml) was refluxed together for 2 hours. This was allowed to cool and then centrifuged in glass vials. The supernatant was removed, and the nickel pellet washed with ethanol and re-centrifuged. The supernatant was again removed and combined with the first one. A portion of the combined supernatants was removed for ^{14}C assay to check the recovery of radioactivity, and to assay the product for purity by tlc and hplc.

The ethanol supernatants were evaporated *in vacuo*, but radioactivity carried over with the ethanol on the rotary evaporator. This problem was solved by mixing the ethanol supernatant with

trioctanoin (dosing vehicle, 2ml), and the ethanol again removed *in vacuo*, this time leaving the radioactive product dissolved in the trioctanoin. Tlc of the reaction product (system A) and hplc (system 4) showed that it co-chromatographed with authentic *p*-propylanisole. The yield was 52.4% (1.047mCi; specific activity 61.7μCi/mg), and the purity greater than 99% by radio-hplc. Its ir spectrum was consistent with the loss of the double bond of the allylic side chain, thus the absorbancies at 910 and 990 cm^{-1} were absent. The direct probe mass spectrum was also identical to authentic *p*-propylanisole (appendix 4.1.).

1-(4'-methoxyphenyl)propan-1-ol



4-methoxypropiophenone (10g; 0.0016mol) dissolved in sodium dried ether (50ml) was added slowly over 30 minutes to lithium aluminium hydride (0.57g; 0.015mol) in dry ether. The mixture was refluxed for 2 hours, and water (20ml) was added until no further hydrogen was evolved. Further water was added (80ml), and the ether was separated and dried (anhyd. Na_2SO_4). The ether was removed *in vacuo* to yield 8.1g of a colourless oil. The oil was purified by tlc (system A) on preparative plates. The purified

oil gave one peak by hplc (system 5; R_t 11.8 min) and had a mass spectrum and nmr spectrum consistent with the structure of the title compound (appendix 4.2a and 4.3).

1-(4'-methoxyphenyl)propan-2-ol.

This compound was synthesised in the department (Zangouras, 1982) from 4-methoxybenzaldehyde with nitroethane according to the method of Blackburn and Burghard (1965) to yield 2'-nitro-1-methoxy-4-(prop-1-enylbenzene) which was converted by the method of Pearl and Beyer (1951) to 4-methoxyphenylacetone. This was then reduced to 1-(4'-methoxyphenyl)propan-2-ol with $LiAlH_4$.

The oily product was purified as for 1-(4'-methoxyphenyl)propan-1-ol, and gave one peak by hplc (system 5; R_t 9.4 min.). The mass spectrum and nmr spectrum were consistent for the structure of the title compound (appendix 4.2b and 4.3).

Thin layer chromatography (tlc)

This technique is described in chapter 2. The solvent systems used in this study are listed below, and the R_f values of p-propylanisole and related compounds are given in table 4.1.

System	Solvents
A	Hexane:ether (1:1 by vol)
B	Benzene:acetone:glacial acetic acid (6:2:1 by vol)
C	Benzene:dioxane:glacial acetic acid (90:25:4 by vol)

Table 4.1. Chromatographic characteristics of p-propylanisole and related compounds in various hplc and tlc systems.

Compound	R_t min hplc systems				R_f values tlc systems						
	3	4	5	6	A	B	C	D	F	G	H
p-propylanisole	-	31.8	-	-	0.87	0.95	0.79	0.90	0.95	0	0.90
estragole	-	19.2	-	-	-	-	-	-	-	-	-
4-hydroxypropylbenzene	-	-	20.1	-	0.63	0.90	0.91	0.41	0.60	0.92	0.83
1-(4'-methoxyphenyl)propan-1-ol	-	-	11.8	13.0	0.58	0.64	0.77	0.59	0.54	-	0.87
1-(4'-methoxyphenyl)propan-2-ol	-	-	9.4	10.8	0.36	0.60	0.71	0.54	0.52	-	0.86
1-(4'-methoxyphenyl)propan-3-ol	-	-	10.0	11.7	0.35	0.57	0.67	0.47	0.41	-	0.82
1-(4'-methoxyphenyl)propane-1,2-diol	-	-	2.0	-	0.10	0.70	-	-	-	-	-
4-methoxyhippuric acid	5.4	-	2.2	2.0	0	0.38	0.16	0.07	0.02	0.58	0.01
hippuric acid	3.2	-	-	-	-	-	-	-	-	-	-
4-methoxyphenylacetylglycine	-	-	2.2	2.0	0	0.40	0.17	0.06	-	0.60	-
4-methoxybenzoic acid	-	-	7.0	8.0	0.25	0.91	0.78	0.40	0.67	0.79	0.03
4-methoxyphenylacetic acid	-	-	5.2	6.0	0.16	0.69	0.75	0.36	0.54	0.79	0.04
4-methoxyphenylpropionic acid	-	-	10.4	11.8	0.27	0.80	0.81	0.41	0.60	0.79	0.05
4-methoxycinnamic acid	-	-	-	12.4	-	0.85	-	-	-	-	-

System	Solvents
D	Benzene:methanol (9:1 by vol)
F	Hexane:chloroform:glacial acetic acid (6:3:1 by vol)
G	Benzene:acetone:glacial acetic acid (2:2:1 by vol)
H	Chloroform:methanol:ammonia (90:10:1 by vol)

High pressure liquid chromatography (hplc)

This was performed as described in chapter 2. The solvent systems used in this study are listed in table 4.2. below and the retention times of p-propylanisole and related compounds are given in table 4.1.

Table 4.2. Hplc systems used in the p-propylanisole study

System	column packing*	mobile phase	flow rate	u.v. detector wavelength
3	ODS Hypersil 5μ (C_{18} reverse phase)	30% methanol 1% glacial acetic acid	1ml/min	254nm
4	MOS Hypersil 5μ (C_8 reverse phase)	33% CH_3CN (aqueous)	2ml/min	254nm
5	ODS Hypersil 5μ (C_{18} reverse phase)	30% methanol (aqueous)	2ml/min	280nm
6	ODS Hypersil 5μ	30% methanol pH3.0 (HCl)	2ml/min	280nm

*purchased from Shandon

Infra-red spectroscopy (Ir)

Ir spectra was obtained using a Perkin Elmer 157G grating spectrophotometer. Spectra of oils were recorded using the pure compound.

Mass spectrometry (ms)

Details of ms are given in chapter 2. For this study the direct probe insertion technique was used. Accurate masses were measured by peak matching with PFK as reference.

Nuclear magnetic resonance spectroscopy (nmr)

Nmr spectra were recorded at 250MHz with the Bruker WM250 instrument of the University of London Intercollegiate NMR Service.
The internal standard was TMS.

Radiochemical techniques

See Chapter 2.

Animals and dosing

Rats and mice were used and maintained as described in Chapter 2. [^{14}C-methoxy]-p-propylanisole dissolved in trioctanoin was diluted to the appropriate concentration with unlabelled p-propylanisole. Rats and mice were administered [^{14}C]-p-propylanisole 50mg/kg; (100µCi/kg and 5.0ml/kg); rats by gavage and mice by intraperitoneal injection. Sample collection and storage was as in Chapter 2.

Analysis and sequential extraction of urinary metabolites

Neat urine was subjected to tlc (system B) and the chromatograms sprayed with p-DMAB and naphtharesorcinol reagents to detect glycine

and glucuronic acid conjugates. Aliquots of rat urine (5ml) and total 0-24 hour mouse urine from animals dosed with p-propylanisole were mixed with equal volumes of 0.2M pH5.0 acetate buffer and then extracted with ether (2 x 5 vol). β-glucuronidase (Glucurase (Sigma), 5000 units/ml in acetate buffer) in equal volume was added to the aqueous phase and the whole incubated at 37°C for 16 hours. Positive controls to test the enzyme, and controls using enzyme and inhibitor saccharo-1,4-lactone and buffer alone were incubated at the same time (chapter 2).

After incubation the cooled incubates were extracted into ether (2 x 5vol). The aqueous phase was adjusted to pH1.0 with 5M HCl and again extracted with ether (10vol) in a liquid-liquid extractor for 6 hours.

After each extraction the excess ether in the aqueous phase was removed by a cool air stream. The volumes of the aqueous phases and their ^{14}C content were measured before and after each extraction, to facilitate the following of the extraction of the radioactivity.

The ethereal extracts were dried (anhyd. Na_2SO_4) and evaporated *in vacuo*. The residues were stored at -20°C until analysed.

Metabolites extractable at pH5.0 were separated by tlc (system A) using preparative plates, the radioactive metabolites were located by radio-scanning, scraped from the plates and eluted into methanol which was reduced *in vacuo*. The metabolites were purified by hplc

(system 5). Purified metabolites were subjected to tlc with authentic standards (systems A-F and H) and analysed by ms and nmr spectroscopy to confirm their identifies.

Radioactivity extractable at pH1.0 revealed the presence of a single radioactive component on tlc (system B) which was eluted from preparative plates, and purified by hplc (system 6). The purified band was subjected to tlc against authentic standards (systems B,C and G) and analysed by ms.

A portion of the pH1.0 extract was treated with 6M Hcl (1ml) at 100°C for 6 hours. The incubate was extracted into ether under acidic conditions, and the extract was concentrated as previously described then subjected to hplc (system 6) and tlc (systems A-G).

Analysis of the aqueous residue

Aliquots (1ml) of the aqueous residue remaining after solvent extraction of rat urine were subjected to a series of treatments listed below.

1. Sulphatase (Sigma) 5000 units incubated at 37°C for 24 hours.
2. as 1 but in addition saccharo-1,4-lactone (20mg).
3. 6M HCl (1ml) incubated at 100°C for 2 hours.
4. 3M NaOH (1ml) incubated at 100°C for 2 hours.
5. 0.1M HCl (1ml) incubated at 100°C for 2 hours.

These treatments were followed by extraction in ether under acid (pH1.0) and alkaline (pH10.0) conditions. The aqueous residues were also extracted into ethyl acetate at pH1.0 and pH5.0 and n-butanol at pH1.0 without prior hydrolysis.

Aliquots of the aqueous residue were applied to an XAD-2 column (7cm long). The column was washed with 2 volumes of water and 3 volumes of methanol.

The extracts and residues of all treatments were assayed for ^{14}C.

A portion of the aqueous residue was also treated with Jack Bean urease as described in chapter 2. The aqueous residue was subjected to tlc (system G) before and after urease treatment.

Administration of 1-(4'-methoxyphenyl)propan-3-ol to rats

1-(4'-methoxy)propan-3-ol (Aldrich) was administered to female Wistar rats at 50mg/kg (10mg/ml dissolved in trioctanoin) by gavage. The animals were housed and maintained as described in chapter 2, without traps for expired air. The urine was stored at -20°C without preservatives.

Extraction and analysis of urine from animals administered 1-(4'-methoxyphenyl)propan-3-ol, for 4-methoxyhippuric acid

Samples of 0-24 hour urine (2ml) were diluted with equal volumes of 0.2M pH5.0 buffer, then extracted with hexane (2 x 5vol.). Hippuric acid was added to the aqueous residue (10µl, 50mg/ml solution). Aliquots of the aqueous residue were analysed by hplc (system 3) and the peak height ratios (peak height 4-methoxyhippuric acid/peak height hippuric acid) compared to a standard curve (made using blank rat urine) to calculate the concentration of 4-methoxyhippuric acid in the urine.

4.3. RESULTS

Elimination of radioactivity from rats and mice administered [^{14}C-methoxy]-p-propylanisole at 50mg/kg

The elimination of radioactivity from the two rodent species is summarised in table 4.3. The total recoveries were 84% of the dose for rats and 87% of the dose for mice, and the major elimination routes were in the urine and expired air. Greater than 55% of the dose was detected in the expired air as $^{14}CO_2$. A small percentage of the dosed ^{14}C was detected in the faeces, 1% in mice and 2% in rats, this was not further investigated, nor was the small amount of ^{14}C (0.1% of the dose) trapped in the 'cold finger' from the expired air.

Identification of urinary metabolites in the rat and mouse after [^{14}C-methoxy]-p-propylanisole

Tlc of neat urine from both species (system B) followed by radio-scanning and ^{14}C-quantification revealed the presence of two radioactive bands, R_f 0.00 (68% of urinary radioactivity) and R_f 0.38 (31% of urinary radioactivity). The band at the origin (R_f 0.00) gave a positive blue reaction to naphtharesorcinol reagent indicating glucuronides and the band at R_f 0.38 was positive (orange/red) to p-DMAB spray for glcyine conjugates.

Prior to treatment with β-glucuronidase less than 2% of the urinary radioactivity was extractable at pH5.0. However after enzyme

Table 4.3. Elimination of radioactivity from rats and mice administered [^{14}C-methoxy]-p-propylanisole (50mg/kg)*

	% of the dose			
Species	urine	expired air	faeces	%total recovery**
Rat	26.0(25.2-27.1)	56.2(55.5-57.8)	2.0(1.1-3.0)	84.1(82.1-85.9)
Mouse	27.6(26.2-30.0)	59.0(50.8-69.1)	0.5(0.1-0.8)	87.1(81.6-98.9)

* means of 4 animals with ranges in parentheses

** recovery in 48 hours

treatment this figure rose to 56% in the rat and 50% in the mouse. Tlc (system A) and hplc (system 5) revealed two major radioactive peaks (figure 4.1.) with the same retention times as 1-(4'-methoxyphenyl)propan-1-ol and 1-(4'-methoxyphenyl)propan-2-ol respectively. The identities of these metabolites were confirmed by ms (figures 4.2a, 4.3a.) and additionally by nmr spectroscopy (figures 4.2b, 4.3b), which were all identical to those of the authentic compounds (appendices 4.2a,b. and 4.3). The nmr spectrometric analyses were required to distinguish the 2'-hydroxylated metabolite from 1-(4'-methoxyphenyl)propan-3-ol, as these two compounds have similar ms (appendices 4.2b and 4.2c.) but characteristic nmr spectra (appendix 4.3).

An unlabelled metabolite was clearly visible under uv light on a chromatographic plate of the pH5.0 extract of β-glucuronidase-treated urine. This compound had the same R_f values as 4-hydroxypropylbenzene by tlc (systems A-D and F-H). Comparison of the mass spectrum of authentic 4-hydroxypropylbenzene with that of the metabolite (figure 4.4. and appendix 4.5.) confirmed its identity as this compound.

At pH1.0 a further 30% of the urinary radioactivity was extractable into ether. This figure was uneffected by prior treatment of the urine with β-glucuronidase. Tlc of this extract revealed only one radioactive band (system B; R_f 0.38) which was positive to p-DMAB reagent (orange/red), and had the same R_f values as

Figure 4.1. Separation of ^{14}C-metabolites, extractable at pH5.0 from β-glucuronidase treated rodent urine after 50mg/kg [^{14}C-methoxy]-p-propylanisole, showing the uv trace and the radio-histogram

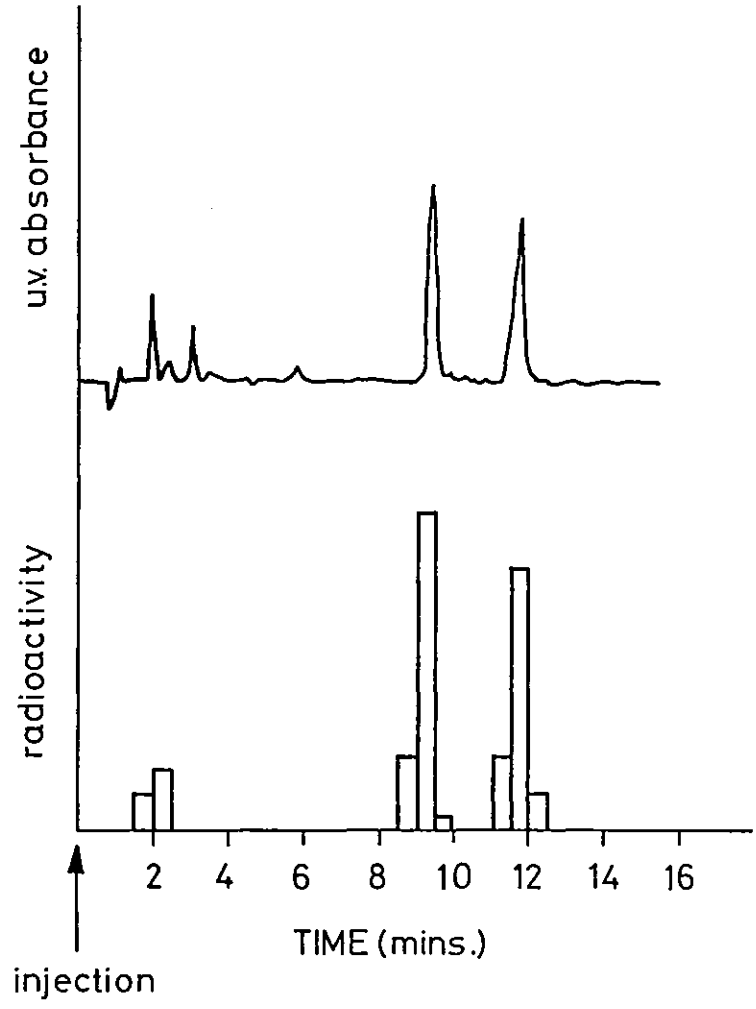


Figure 4.2. a) Mass spectrum and b) nmr spectrum of 1-(4'-methoxyphenyl)propan-1-ol extract of the urine of rats administered [^{14}C]-p-propylanisole

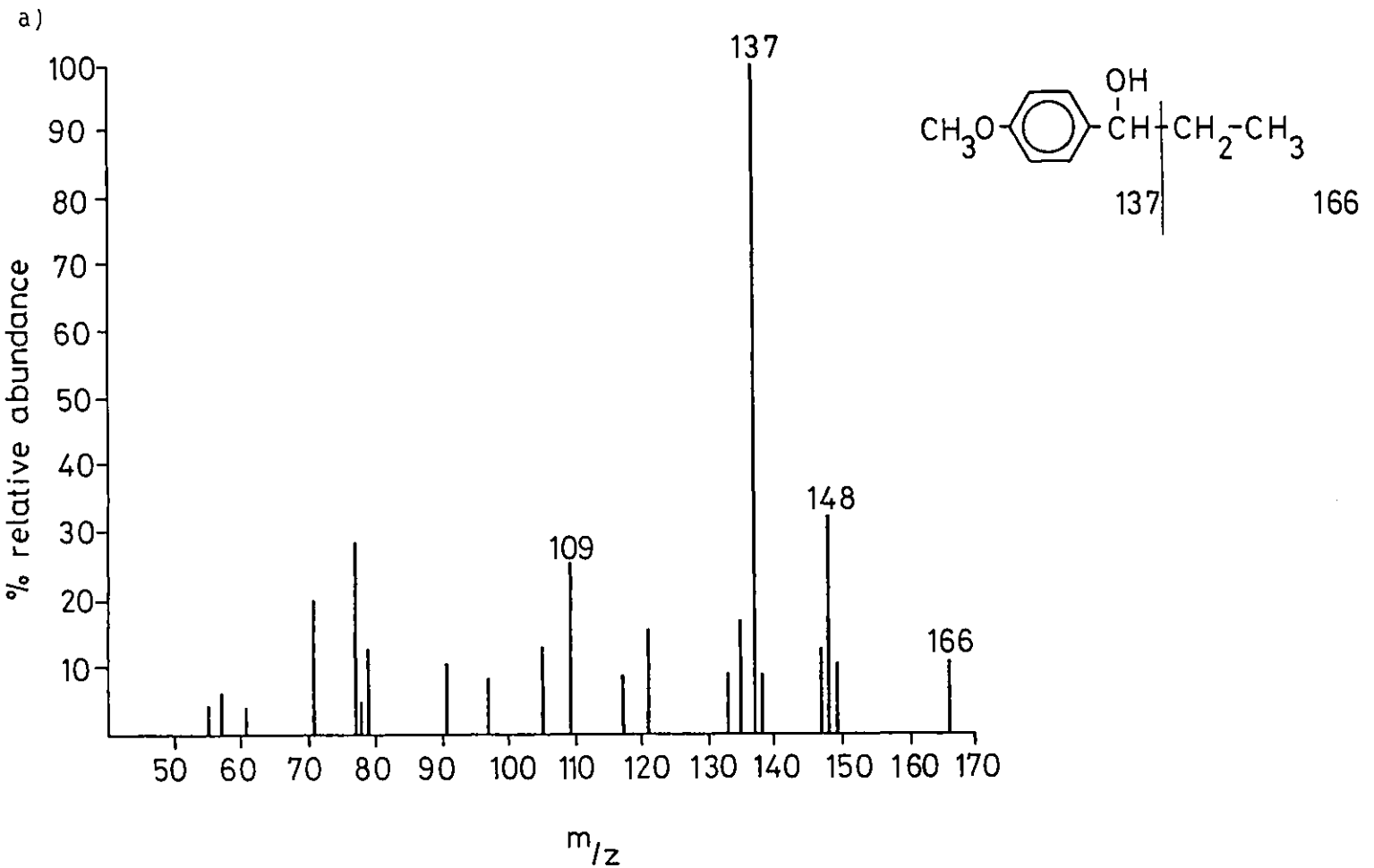


Figure 4.2.b

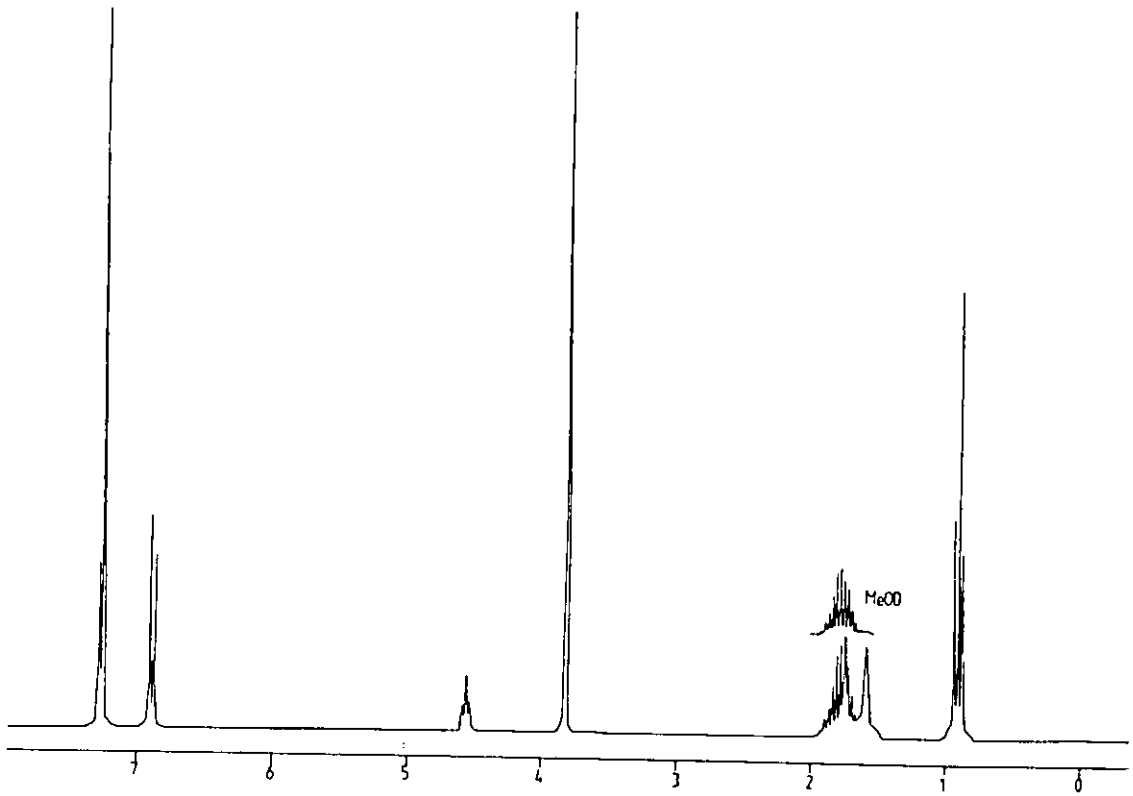


Figure 4.3. a) mass spectrum and b) nmr spectrum of 1-(4'-methoxyphenylpropan-2-ol, extracted from the urine of rats administered [^{14}C]-p-propylanisole

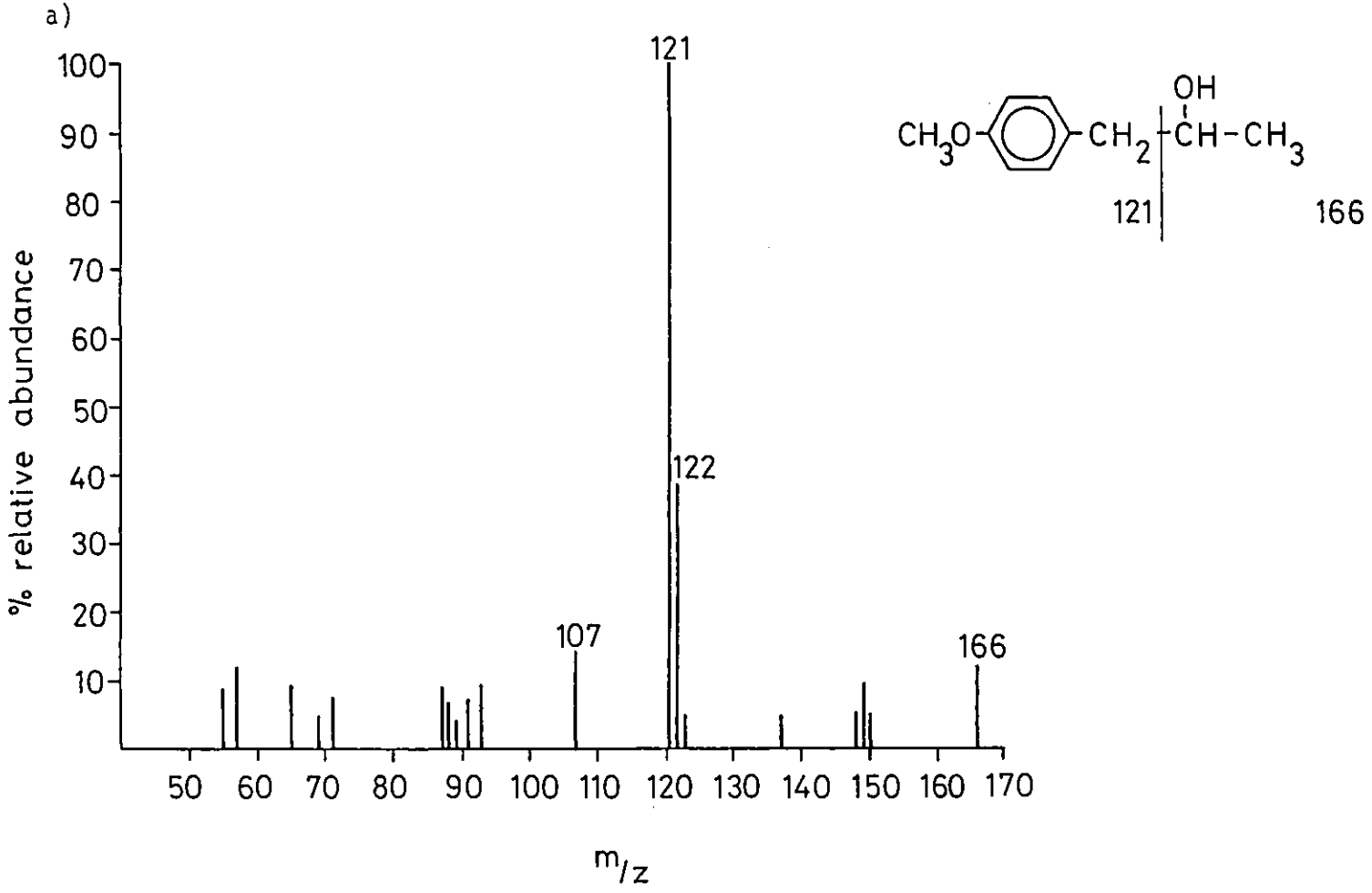


Figure 4.3b.

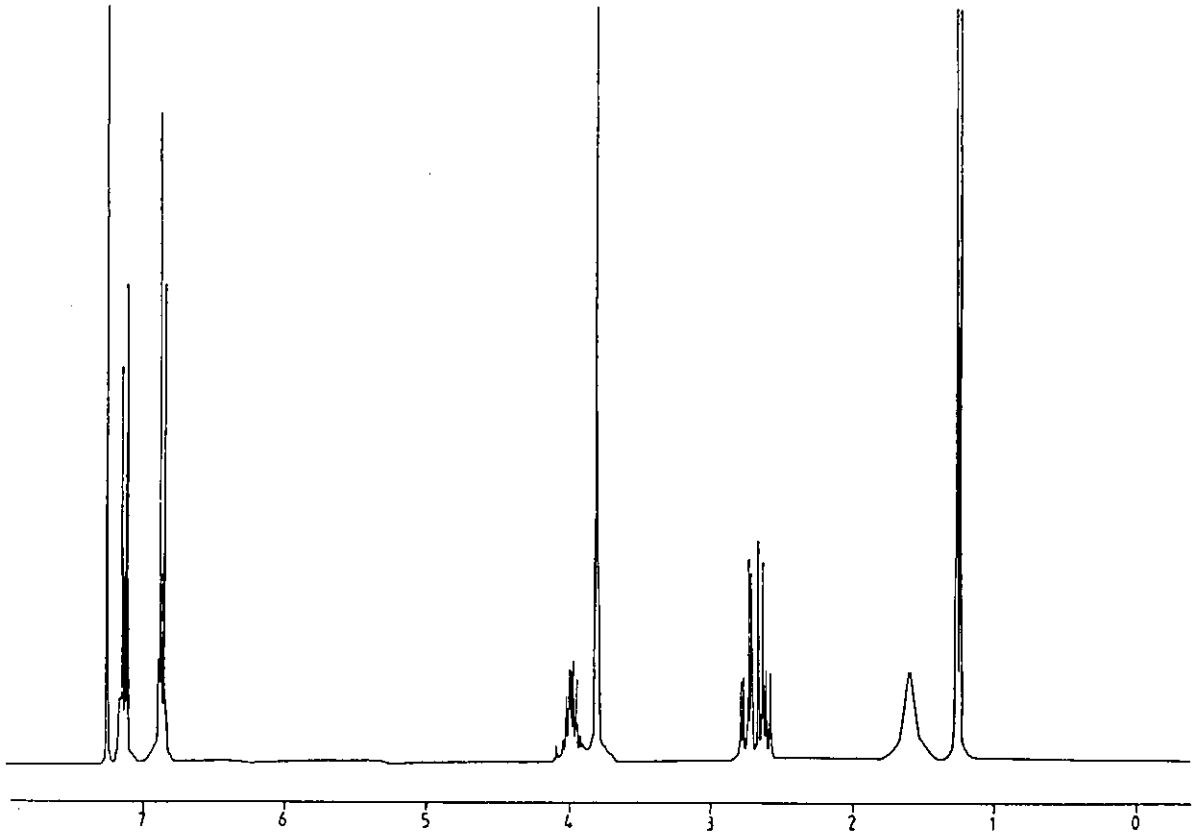
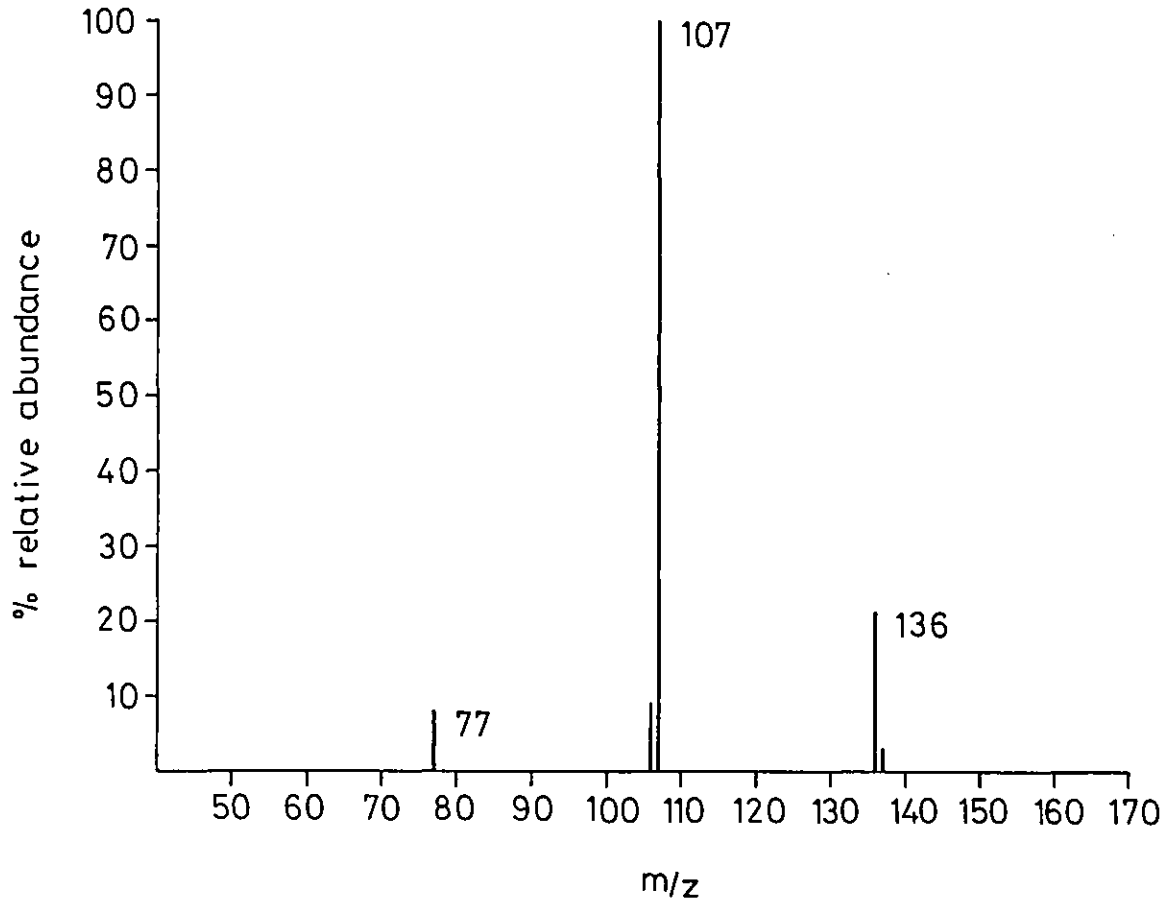


Figure 4.4. Mass spectrum of 4-hydroxypropylbenzene extracted from β-glucuronidase treated rodent urine after administration of [^{14}C]-p-propylanisole

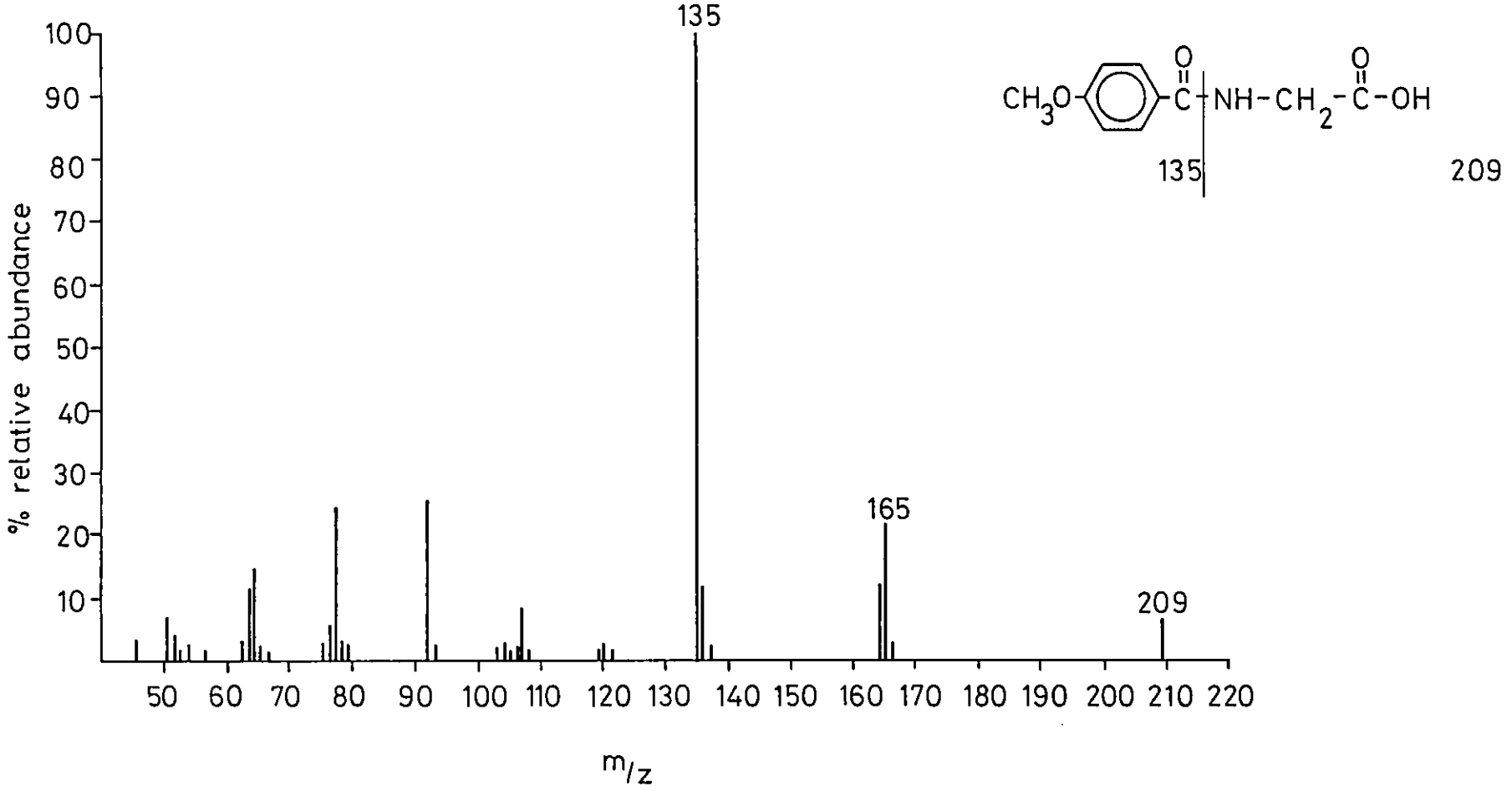


4-methoxyhippuric acid in this solvent system and systems C,D and G. However these systems could not resolve 4-methoxyhippuric acid and 4-methoxyphenylacetylglycine. Therefore the metabolite was acid hydrolysed to release the acid(s). This treatment revealed one acid, which had the same R_f values as 4-methoxybenzoic acid by tlc (systems A-D and F-H) and retention time by hplc (system 5). The direct probe mass spectrum of the unhydrolysed metabolite was identical (figure 4.5.) with that of authentic 4-methoxyhippuric acid (appendix 2.3a).

Radioactivity remaining in the aqueous residue

Some 15% of the urinary radioactivity (in the rat) was unextractable at pH5.0 or pH1.0 into ether, ethyl acetate or n-butanol. The latter formed an emulsion which could not be separated. A very small amount of radioactivity was extractable into ethyl acetate at pH1.0 but this represented less than 0.05% of the dose. Neither sulphatase treatment nor acid or alkaline hydrolysis released any extractable radioactivity, nor was XAD-2 separation useful. Tlc of aqueous residue (system G) revealed two radioactive bands (R_f 0.50 and 0.90), after urease treatment the ^{14}C in the aqueous residue had reduced by 27% (1% of the dose) and the radioactive band at R_f 0.50 was no longer present. The remaining band gave a mass spectrum which could not be interpreted.

Figure 4.5. Mass spectrum of 4-methoxyhippuric acid, extracted from the urine of rats administered [^{14}C]-p-propylanisole



4-Methoxyhippuric acid excretion after administration of 1-(4'-methoxyphenyl)propan-3-ol to rats at 50mg/kg

Greater than 50% of the dose of 1-(4'-methoxyphenyl)propan-3-ol was excreted as 4-methoxyhippuric acid (47.8-65.4%). In all animals the volume of urine excreted from 0-24 hours was about 40ml.

4.4. DISCUSSION

Almost 60% of the dose of [^{14}C-methoxy]-*p*-propylanisole was excreted as O-demethylated metabolites as measured by the amount of $^{14}CO_2$ in the expired air (55% of the dose in the rat, and 59% of the dose in the mouse) and the amount of ^{14}C-urea in the urine (1% of the dose in the rat urine, 2% of the dose in the mouse urine) (table 4.4.). The ^{14}C-urea arose as with [^{14}C]-*trans*-anethole administration from incorporation of small amounts of [^{14}C]-formaldehyde the initial labelled product of O-demethylation, into the pathways of 1-carbon fragment metabolism, and the bulk of the [^{14}C]-formaldehyde being excreted as $^{14}CO_2$. The larger amount of ^{14}C-urea seen in the urine after the administration of [^{14}C]-*p*-propylanisole than after [^{14}C]-*trans*-anethole reflecting the greater proportion of the dose that undergoes O-demethylation after administration of the saturated compound.

The three routes of side chain metabolism expected from the work previously reported on the metabolism of propylbenzene (Smith *et al.*, 1954; El Masri *et al.*, 1956) were shown to occur with *p*-propylanisole, namely α- and ω-1-hydroxylation, to yield 1-(4'-methoxyphenyl)propan-1-ol (6% of the dose) and 1-(4'-methoxyphenyl)propan-2-ol (7% of the dose), excreted in the urine as glucuronides, and side chain degradation yielding 4-methoxybenzoic acid conjugated with glycine. 4 -methoxyhippuric acid accounted for 8% of the dose (table 4.4.).

Table 4.4. Disposition of [^{14}C-methoxy]-p-propylanisole in rat and mouse at 50mg/kg *

Compound	Species	
	rat	mouse
$^{14}CO_2$	56.2(53.9-57.8)	39.0(50.8-69.1)
p-propylanisole	0 **	0
1-(4'-methoxyphenyl)-propan-1-ol	5.6(4.1-7.1)	3.8(3.6-4.0)
1-(4'-methoxyphenyl)-propan-2-ol	6.6(6.0-7.7)	8.2(7.0-9.3)
4-methoxyhippuric acid	7.8(7.0-8.7)	8.1(6.8-9.5)
^{14}C-urea	1.0(0.9-1.4)	2.0(1.8-2.4)
Unknown in aqueous residue	2.7(2.0-3.0)	3.2(2.7-4.3)
Unknown in pH5.0 extract	2.0(0.9-2.8)	1.0(0.7-1.4)
faecal radioactivity	2.0(1.1-3.0)	0.5(0.1-0.8)
Total	83.9	85.6

* percentage of dose, means of at least 4 animals with ranges in parentheses.

** none detected

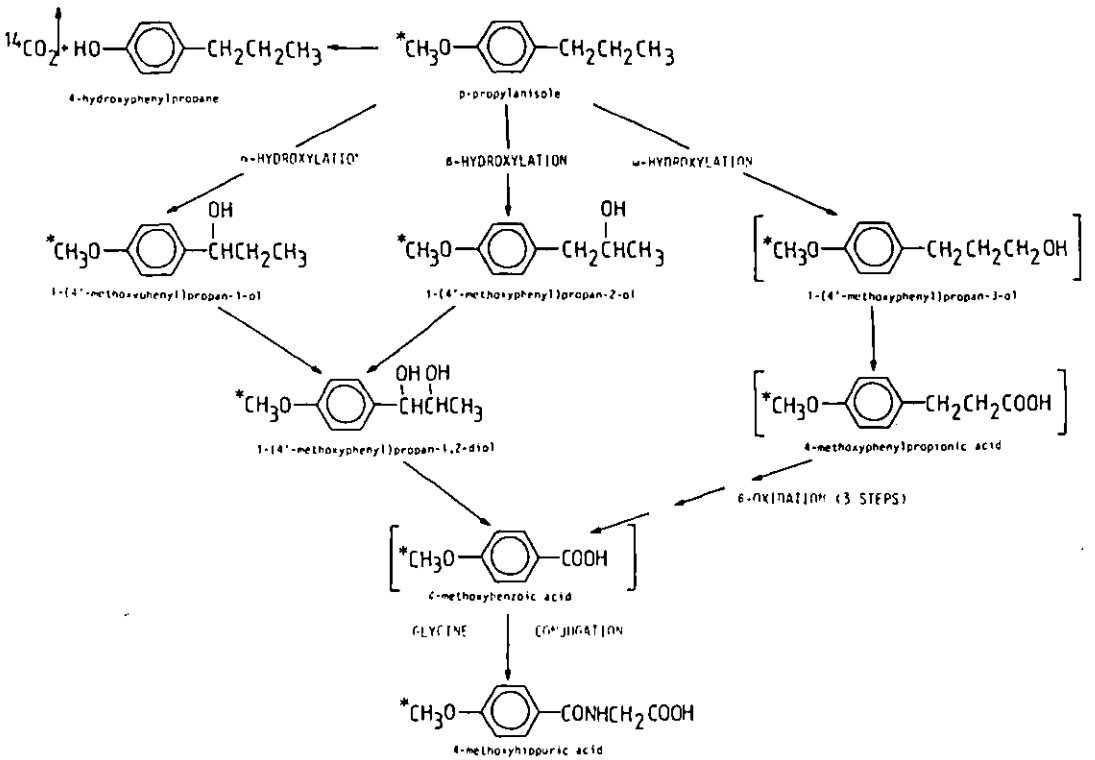
The unequivocal identification of the metabolite assigned the structure 1-(4'-methoxyphenyl)propan-2-ol, demanded the analysis of the metabolite by nmr spectroscopy (figure 4.3b.). This was required because the mass spectra of authentic samples of 1-(4'-methoxyphenyl)propan-2-ol (appendix 4.2b.) and 1-(4'-methoxyphenyl)propan-3-ol (appendix 4.2c.) were very similar. However, nmr spectroscopy of these compounds (appendices 4.3b. and 4.3c.) show that the positional isomers are distinct by this method of analysis.

Figure 4.6. shows the proposed possible routes of metabolism of p-propylanisole to the metabolites that were identified in rodent urine.

Investigation of the fate of 1-(4'-methoxyphenyl)propan-3-ol revealed that a large proportion of the dose of this compound was excreted as 4-methoxyhippuric acid. This evidence supports the theory that the 4-methoxybenzoic acid excreted after p-propylanisole administration may have arisen via ω-hydroxylation, then oxidation to 4-methoxyphenylpropionic acid and convertion via β-oxidation to 4-methoxybenzoic acid (Dakin, 1909).

Another route of metabolism of p-propylanisole to 4-methoxybenzoic acid involving the metabolites identified in the urine of rodents, namely 1- and 2-hydroxylated compounds would implicate 1-(4'-methoxyphenyl)propan-1,2-ol as an intermediate (figure 4.6.). This compound

Figure 4.6. Proposed routes of metabolism of p-propylanisole in rodents



was detected in the urine of animals administered high doses of p-propylanisole (chapter 5) and in the urine of all animals administered trans-anethole (0.05-1500mg/kg). However evidence from the latter studies suggested that this route of metabolism to 4-methoxybenzoic acid was less favoured, although a further possible intermediate 1-(4'-methoxyphenyl)-1-oxo-propan-2-ol was also a minor metabolite of trans-anethole in rodents (figure 2.19. chapter 2).

p-Propylanisole has been shown to be completely absorbed at a dose of 50mg/kg in two rodent species (rat and mouse), with less than 2% of the dose in the faeces and no radioactivity remaining in the carcass after 48 hours or excreted in the expired air as 'volatiles'. p-Propylanisole was completely metabolised at this dose level to metabolites consistent with previously reported data on alkyl substituted benzenes.

CHAPTER 5

Dose-dependent variations in the metabolism of p-propylanisole in the rat and mouse

5.1. INTRODUCTION

p-Propylanisole, the saturated analogue of trans-anethole and estragole, has been investigated in acute and chronic toxicity tests in doses up to 5g/kg/day.

Jenner et al. (1964) and Taylor et al. (1964) reported the LD_{50} of p-propylanisole in rodents to be in the range 2-9g/kg, and the major toxic consequences to be scrawny appearance and porphyrin-like deposits around the eyes. Daily administration of 1,470mg/kg p-propylanisole to rats for 4 days caused mild hepatic lesions (Taylor et al., 1964) and administration of 2g/kg/day rising to 5g/kg/day for 32 days (Hagan et al., 1967) resulted in damage to the stomach and osteoporosis.

The doses used in the toxicity tests were very large, in an attempt to amplify any possible toxicity resulting from low dose exposure to p-propylanisole. However, in metabolism studies where [^{14}C]-trans anethole (chapter 3) and [^{14}C] estragole (Zangouras et al., 1981) were administered over the range 0.05-1500mg/kg to rodents, the major route of metabolism of both these food flavours, namely oxidative O-demethylation became saturated at higher doses, resulting in larger amounts of methoxy-substituted metabolites being excreted in rodent urine and less $^{14}CO_2$ being excreted in the expired air. The significance of increased urinary elimination of radioactivity after estragole administration at high doses has been discussed in chapters 1 and

3, with particular reference to an increased elimination of the presumed proximate carcinogenic metabolite, 1'hydroxyestragole at high doses.

These studies on trans-anethole and estragole metabolism highlight the problem of extrapolating high dose toxicity tests to low exposure risks. The exposure to a compound at high doses is not the same exposure experienced at low doses, as different relative percentages of metabolite(s) and unchanged drug may be present.

p-Propylanisole was shown in the last chapter to be extensively metabolised by oxidative O-demethylation, and to a lesser extent by side chain oxidation and cleavage. This has similarities to the disposition of trans-anethole and estragole (Solheim and Scheline, 1973). Therefore it is very likely that p-propylanisole would show similar 'dose-dependent' variations in metabolism.

This chapter describes the variations in disposition of p-propylanisole in the rat and mouse at various doses in the range 0.05-1500mg/kg. The lowest dose is some 20 times larger than the average daily human exposure, but the highest dose was within the range of the doses used in toxicity tests.

The rate of oxidative O-demethylation was compared in the mouse at 0.05mg/kg and 500mg/kg exposure to examine whether this pathway was saturated or inhibited at higher doses.

Elimination of radioactivity in the expired air and urine was measured at each dose level, and urinary metabolites isolated 50mg/kg (chapter 4) were quantified for every dose level.

The implications of these results were discussed with respect to interpretation of high dose toxicity data for a possible risk to man.

5.2. MATERIALS AND METHODS

The materials and methods used in this study were described in chapters 2 and 4, where the procedures differ they are described below.

Animals and dosing

Rats and mice were administered [^{14}C-methoxy]-p-propylanisole diluted as appropriate with unlabelled p-propylanisole dissolved in trioctanoin in the dose range 0.05-1500mg/kg (5ml/kg). Each animal received 2µCi of radioactivity, except the low dose animals when rats administered a 0.05mg/kg dose received 0.6µCi and mice 0.06µCi and mice administered a 0.5mg/kg dose received 0.6µCi. The animals were dosed, housed and maintained as described in chapter 2. Urine and faeces were collected every 24 hours. Samples of $^{14}CO_2$ trapping solutions were moved every 30 minutes for the first 7 hours post-dosing from mice given 0.05mg/kg and 500mg/kg p-propylanisole and there after changed every 24 hours. Trapping solutions from all other animals were changed after every 24 hours only.

Extraction and quantification of ^{14}C-metabolites in the urine of rodents administered [^{14}C-methoxy]-p-propylanisole

Samples of 0-24 hour urine (5ml from rats and the total sample from mice) or 0-48 hour urine after high doses (500mg/kg and 1500mg/kg) were analysed. The samples were treated and extracted as described in chapter 4, thus resulting in a pH5.0 extract of

untreated urine, a pH5.0 extract after β-glucuronidase treatment and a pH1.0 extract (a portion of which had been acid hydrolysed) from each urine sample.

The pH5.0 extracts were analysed by tlc (system A) and hplc (system 5) and ^{14}C was quantified as previously described. Analysis of the pH5.0 extracts of untreated urine of high dose animals (500 and 1500mg/kg) revealed the presence of a third major component at R_f 0.00 in tlc system A. This band was eluted into methanol, concentrated and subjected to further tlc (system G). The band resolved into two radioactive bands R_f 0.50 and R_f 0.90, which were again eluted into methanol and concentrated. The radioactive band R_f 0.50 gave a single band in solvent system B (R_f 0.10) while that at R_f 0.90 further divided into two bands R_f 0.34 and 0.51 in this system. All three bands were eluted and concentrated and analysed by ms.

The pH1.0 extracts were analysed by tlc (system B) and the acid hydrolysed pH1.0 extracts by hplc (system 6). ^{14}C was quantified as previously described.

^{14}C-Urea content of the aqueous residues was estimated as before.

5.3. RESULTS

Elimination of radioactivity from rats and mice after [^{14}C-methoxy]-p-propylanisole at doses in the range 0.05-1500mg/kg

In both species and at all doses the recovery of radioactivity was greater than 80% of the dose and generally in excess of 85% of the dose (tables 5.1. and 5.2.). The excretion data is illustrated in figure 5.1. for rats and 5.2 for mice.

Pulmonary elimination was the major route of excretion of radioactivity, and the percentage of the dose eliminated via this route showed significant dose dependency, 78% of the dose being eliminated after a 0.05mg/kg dose (82% from rats and 75% from mice) and only 49% of the dose after a 1500mg/kg dose (47% from rats and 50% from mice).

Urinary elimination was as a consequence a less important route of elimination of radioactivity. The percentage of the dose eliminated by this route still however, showed dose-dependency. 12% of a dose of 0.05mg/kg was excreted in the urine (8% in rat urine and 15% in mouse urine) and this increased to 38% of a 1500mg/kg dose (37% in rat urine and 38% in mouse urine). After a 1500mg/kg dose of [^{14}C]-p-propylanisole to rats the urine volume produced per 24 hours was in excess of 35ml, and also the rate of elimination of the dose was slower than at lower doses, taking up to 72 hours.

Table 5.1. Excretion of ^{14}C after the administration of [^{14}C]-p-propylanisole to rats orally at various doses

Dose (mg/kg)	% ^{14}C dose excreted in 72 hours			
	Urine	^{14}C in expired air	faeces	Total
0.05	8.0(6.2-93)*	81.6(80.1-85.0)	<1.0	89.5(88.1-89.5)
0.5	13.7(10.4-16.7)	74.3(68.2-82.3)	3.6(2.3-4.7)	91.5(85.0-101.5)
5	22.5(20.8-22.9)	65.4(61.7-68.8)	1.5(1.4-2.1)	89.1(84.6-93.3)
50	26.0(25.2-27.1)	56.2(53.9-57.8)	2.0(1.1-3.0)	84.2(82.1-84.5)
500	28.7(26.0-30.9)	50.7(42.3-52.9)	2.1(1.2-3.3)	81.5(73.1-84.6)
1500	37.1(35.3-39.6)	47.2(43.6-50.9)	1.3(0.4-2.7)	85.6(81.6-89.3)

* figures are means with ranges in parentheses

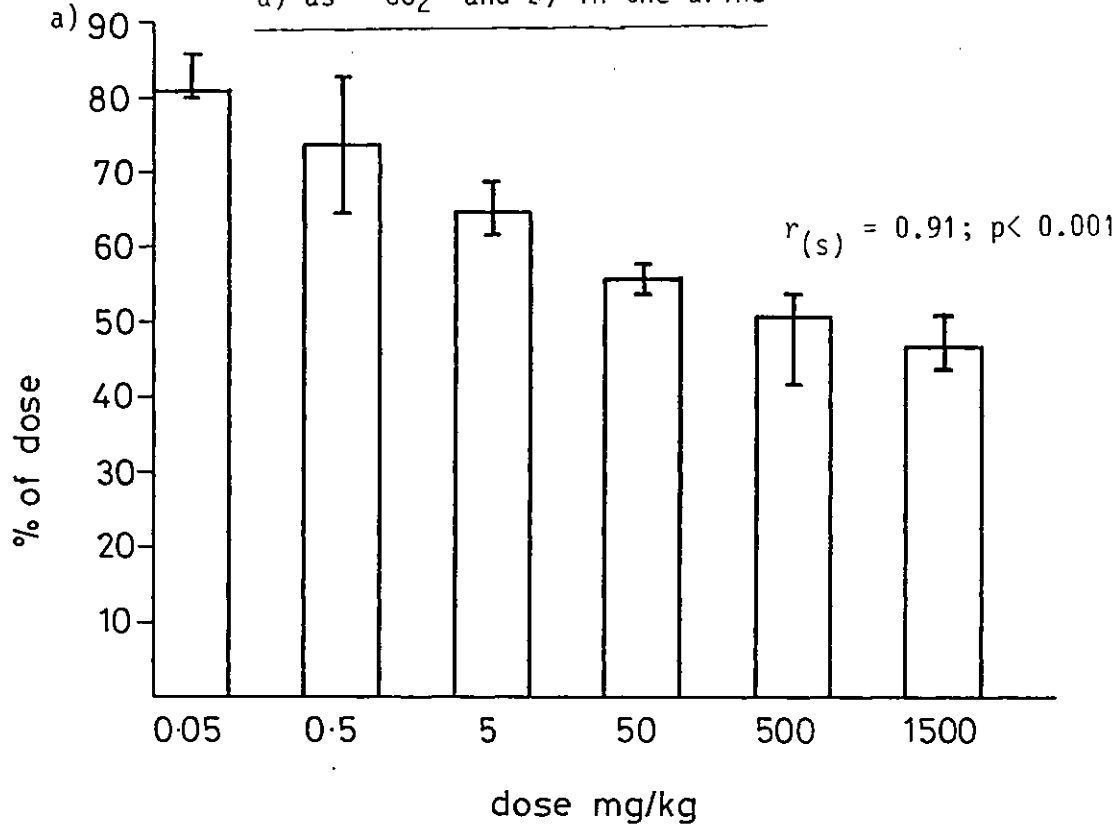
Table 5.2. Excretion of ^{14}C after the administration of [^{14}C]-p-propylanisole to mice i.p. at various doses

Dose (mg/kg	% ^{14}C dose excreted in 72 hours			
	Urine	^{14}C in expired air	faeces	Total
0.05	15.1(14.6-15.6) *	74.5(69.9-79.1)	n.d.	89.6(85.5-93.7)
0.5	18.3(13.2-21.7)	67.7(67.0-68.3)	0.3	86.0(81.1-92.0)
50	27.6(23.2-30.0)	59.0(50.8-69.1)	0.8	86.6(80.4-98.8)
500	32.9(30.9-34.5)	57.6(52.0-68.2)	1.1	90.5(83.4-101.6)
1500	38.0(32.8-40.6)	49.9(47.1-51.6)	0.9	87.9(83.9-92.2)

n.d. - none detected

* figures are means with ranges in parentheses

Figure 5.1. Variation in the excretion of radioactivity from rats after the oral administration of [^{14}C-methoxy]-p-propylanisole over the dose range 0.05-1500mg/kg.
a) as $^{14}CO_2$ and b) in the urine



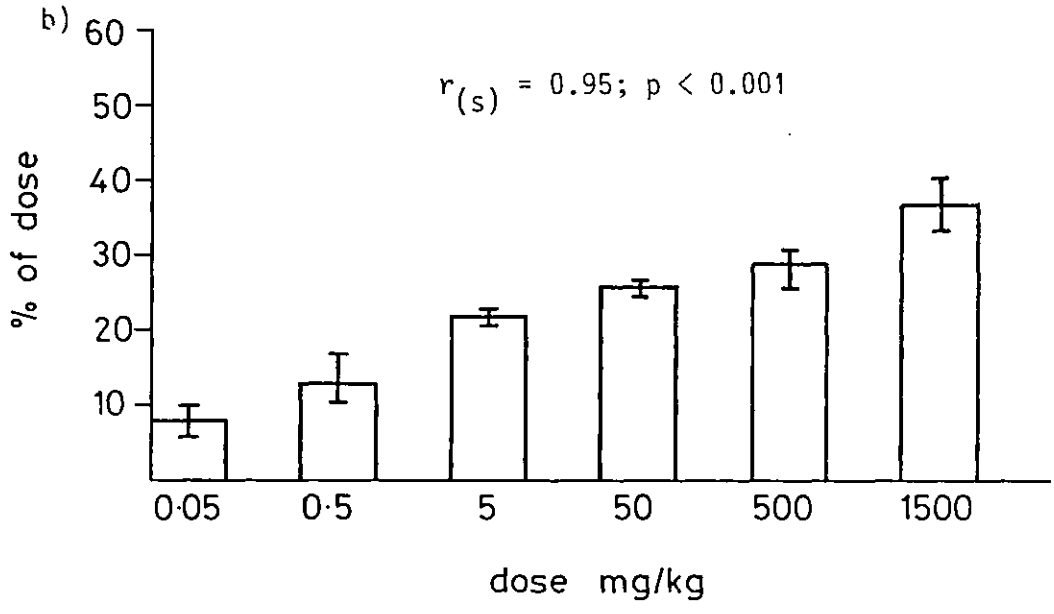
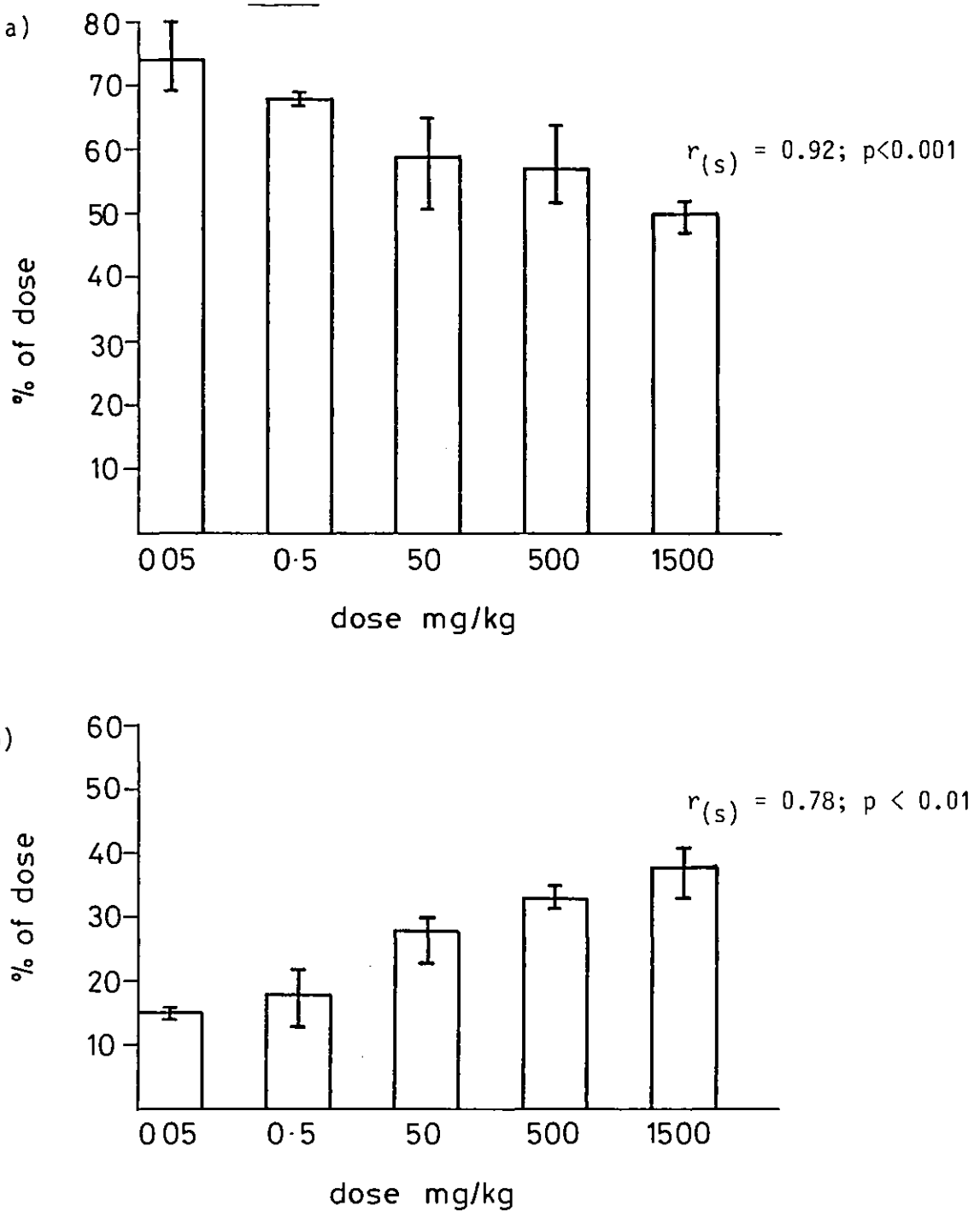


Figure 5.2. Variation in the excretion of radioactivity from mice after the i.p. administration of [^{14}C-methoxy]-p-propylanisole over the dose range 0.05-1500mg/kg a) as $^{14}CO_2$ and b) in the urine



In both species faecal radioactivity accounted for less than 4% of the dose at all doses, and there was no correlation between dose size and faecal excretion.

Variation in metabolism of p-propylanisole with dose size in the rat.

As illustrated in figure 5.1. urinary elimination of radioactivity in the rat increased four fold over the dose range studied (Spearman rank correlation $r_{(s)} = 0.91$; $p < 0.001$). Consequently the percentage of the dose converted to each of the major urinary metabolites also increased significantly with increasing dose (table 5.3). These changes are illustrated in figure 5.3. Table 5.4. shows that 1-(4'-methoxyphenyl)propan-1-ol excretion rose from 0.7% of the dose to 7.5% of the dose ($r_{(s)} = 0.93$; $p < 0.001$) and 1-(4'-methoxyphenyl)propan-2-ol from 1.8% of the dose to 9.8% the dose ($r_{(s)} = 0.95$; $p < 0.001$) over the dose range 0.05-1500mg, and 4-methoxyhippuric acid excretion rose from 4.0% to 12.8% of the dose over a similar range ($r_{(s)} = 0.91$; $p < 0.001$).

At all doses the hydroxylated metabolites were excreted as glucuronides, and at doses of 500 and 1500mg/kg they were also detected in the urine unconjugated.

Table 5.3. Extraction characteristics of urine from rats after [^{14}C-methoxy]-p-propylanisole at various doses

Dose mg/kg	% of dose extractable		% of dose aqueous residue
	pH5.0*	pH1.0	
0.05	2.6(2.0-3.1)**	4.0(3.8-4.2)	1.6(1.2-2.0)
0.5	5.2(3.7-6.3)	5.3(3.4-6.5)	2.9(1.7-4.1)
5.0	10.7(9.6-11.5)	7.6(7.1-8.4)	3.9(3.3-4.8)
50	14.5(13.7-16.1)	7.8(7.0-8.7)	3.8(2.9-4.1)
500	17.2(14.8-20.4)	8.5(6.1-10.3)	2.7(1.5-4.0)
1500	21.6(19.6-24.5)	12.8(11.7-13.4)	3.2(2.4-3/7)

* after β-glucuronidase treatment

** figures are means with ranges in parentheses

Figure 5.3. Changes in the excretion of ^{14}C metabolites of [^{14}C-methoxy]-p-propylanisole in the urine of rats over the dose range 0.05-1500mg/kg a) 1-(4'-methoxyphenyl)propan-1-ol, b) 1-(4'-methoxyphenyl)propan-2-ol and c) 4-methoxyhippuric acid

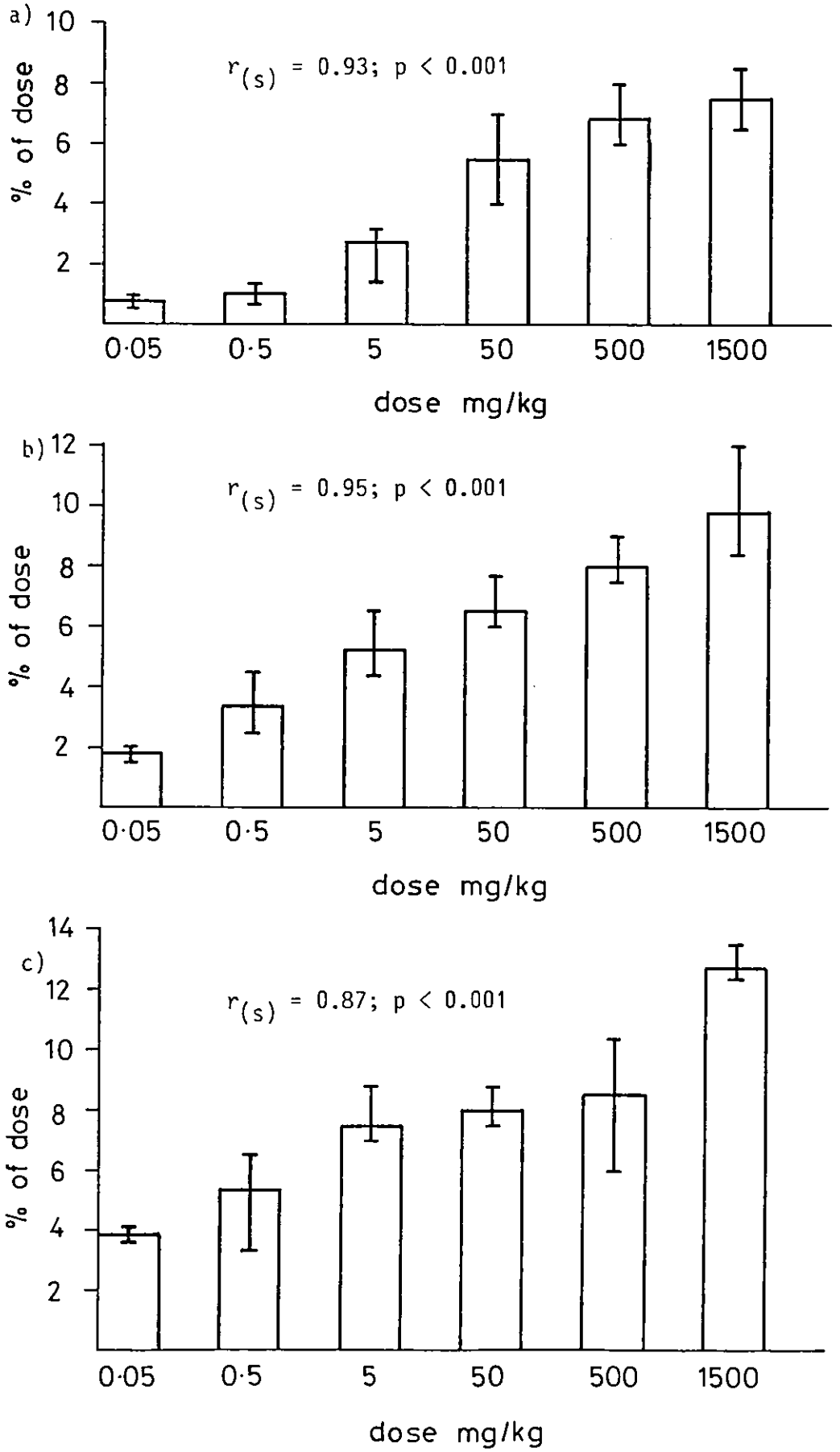


Table 5.4. Metabolites extractable at pH5.0 from rat urine after [^{14}C-methoxy]-p-propylanisole at various doses

Dose mg/kg	polar metabolites	1-(4'-methoxyphenyl)propan-1-ol	1-(4'-methoxyphenyl)propan-2-ol
0.05	0.1(0.1-0.2)	0.7(0.4-0.9)	1.8(1.5-2.0)
0.5	0.5(0.3-0.8)	1.0(0.6-1.4)	3.3(2.4-4.3)
5	1.6(1.4-1.9)	2.7(1.9-3.4)	5.3(4.3-6.5)
50	2.0(0.9-2.8)	5.6(4.1-7.1)	6.6(6.0-7.7)
500	1.6(1.5-2.0)	6.8(6.0-8.0)	8.0(7.5-9.1)
1500	3.4(3.0-3.6)	7.5(6.3-8.4)	9.8(8.2-11.8)

figures are percentage of the dose with ranges in parentheses

At the highest dose studied 5% of the urinary radioactivity was extractable at pH5.0 prior to β-glucuronidase treatment. This extract separated into three radioactive components after tlc (system A). Two were the alcohols 1-(4'-methoxyphenyl)-propan-1-ol and 1-(4'-methoxyphenyl)propan-2-ol, and the third, on further tlc fractionation, proved to have three minor components. An interpretable mass spectrum was obtained for the band at R_f 0.34 (system B), which showed a molecular ion at $m/_Z$ 182, and high resolution ms gave a molecular weight of 182.0943 amu, corresponding to the empirical formula of $C_{10}H_{14}O_3$ (requires 182.0943 amu). This formula indicates the addition of two oxygen atoms to p-propylanisole. Comparison of the mass spectrum of authentic 1-(4'-methoxyphenyl)-propane-1,2-diol (appendix 2.7) with that of this metabolite (figure 5.4.) shows that this metabolite is indeed the diol and accounts for 0.5% pf the dose. The stereochemistry of this metabolite was not further investigated (see chapter 2). One of the other minor metabolites gave a mass spectrum which is reproduced in figure 5.5. There is a molecular ion at $m/_Z$ 195, and the base peak is $m/_Z$ 121. The molecular ion suggests the addition of up to three oxygen atoms into the p-propylanisole molecule but as yet the spectrum has not been interpretable. It should be noted that two of the metabolites of trans-anethole (2A and 6) showed addition of three oxygen atoms, one in the benzene ring.

The percentage of the dose that could not be extracted at pH5.0 or pH1.0 remained relatively constant over the dose range at about 3% of the dose. Approximately 1% of the dose was excreted as ^{14}C-urea at every dose level.

Figure 5.4. Mass spectrum of 1-(4'-methoxyphenyl)propane-1,2-diol extracted from the urine of rats after 1500mg/kg [^{14}C-methoxy]-p-propylanisole

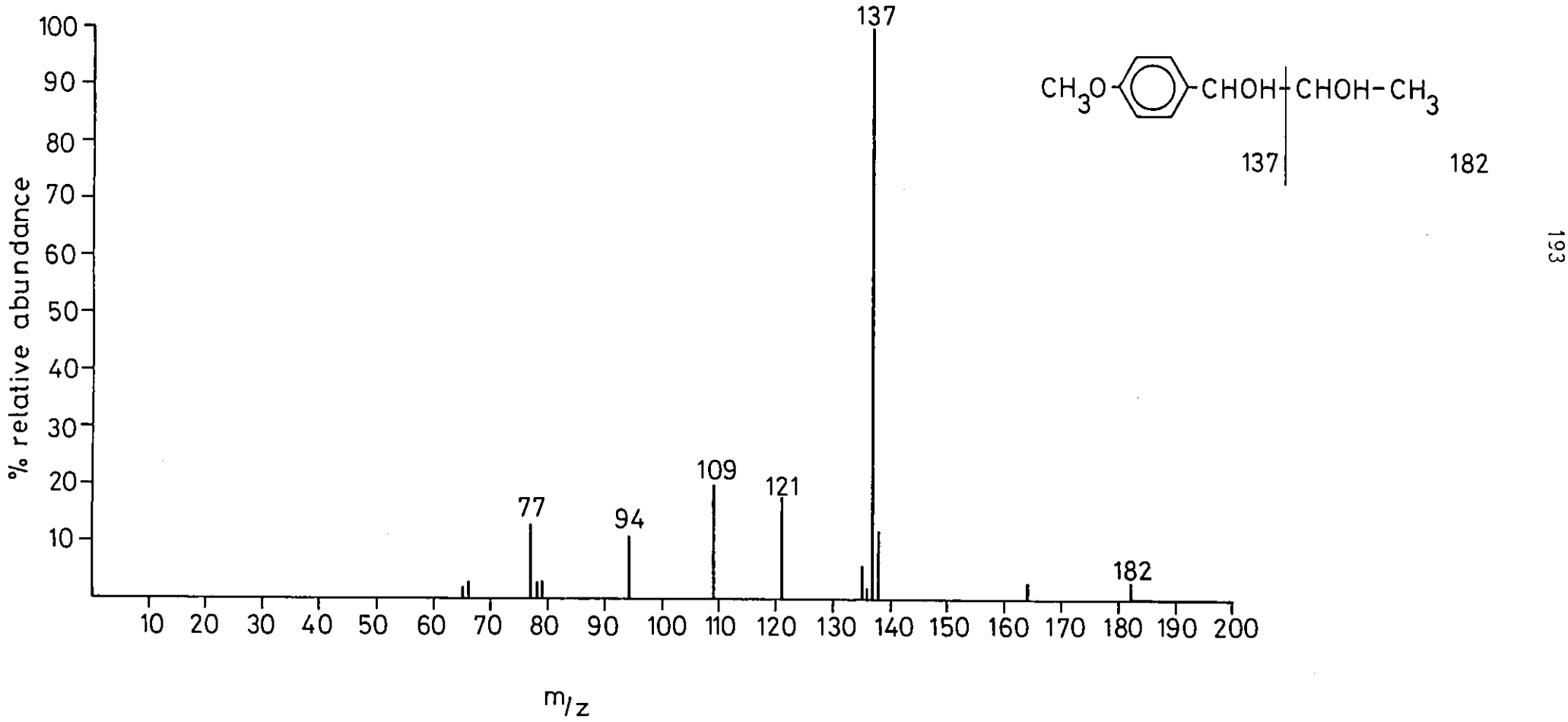
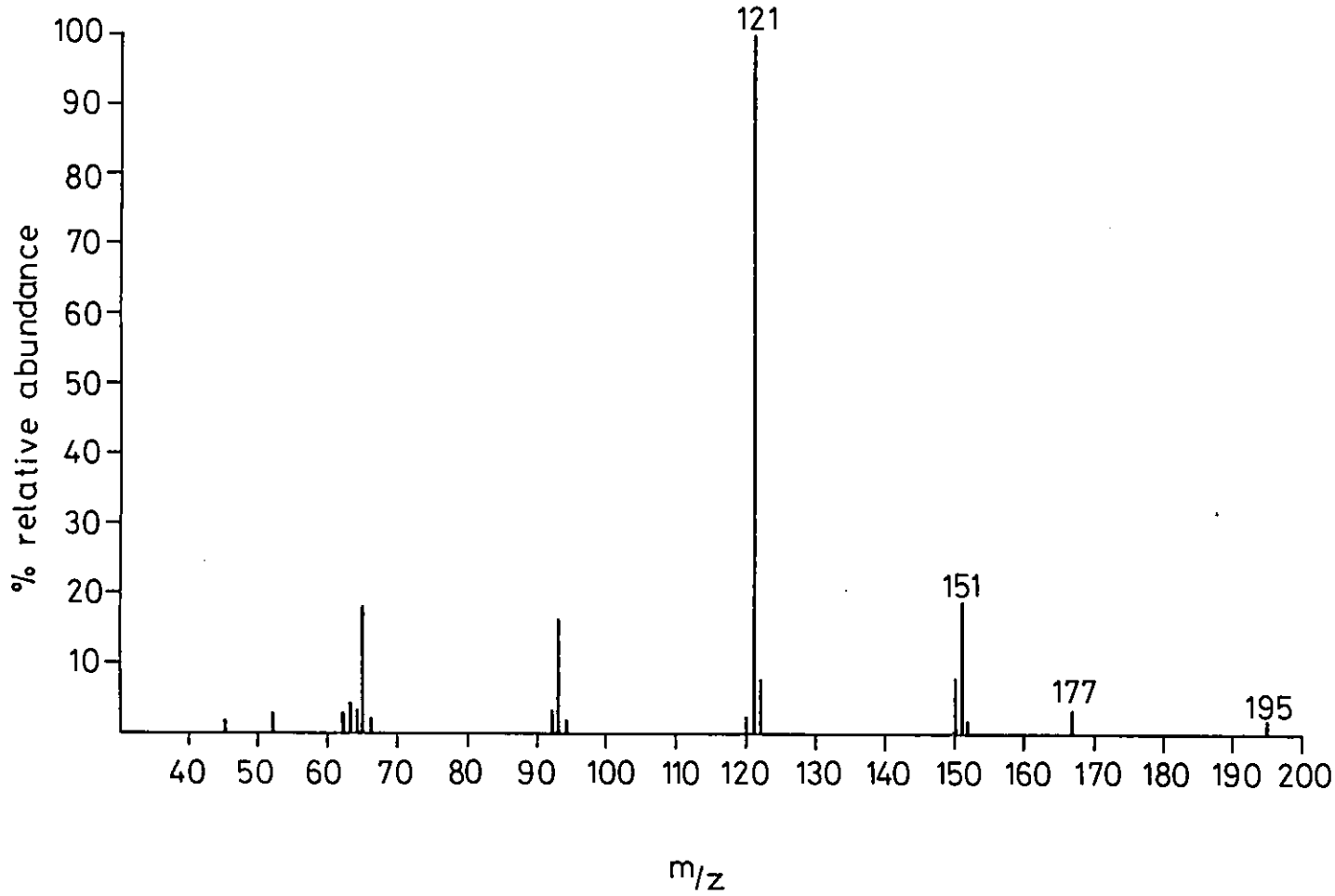


Figure 5.5. Mass spectrum of a ^{14}C-labelled unidentified metabolite of [^{14}C-methoxy]-p-propylanisole extracted from rat urine



Variation in metabolism of p-propylanisole with dose in the mouse

As with the rat, the urinary elimination of [^{14}C]-p-propylanisole in the mouse increased with dose ($r_{(s)}$ = 0.92; $p < 0.002$). The percentage of the dose extractable at pH5.0 and pH1.0 increased with increasing dose (table 5.5.) and consequently the major urinary metabolites, 1-(4'-methoxyphenyl)propan-1-ol, 1(4'-methoxyphenyl)propan-2-ol and 4-methoxyhippuric acid also increased as a percentage dose in the urine as the dose rose (figure 5.6.). These increases with dose were all statistically significant by Spearman ranking. The two alcohols were excreted as glucuronides at all doses and at doses of 500mg/kg or higher they were also detected in the urine unconjugated. The minor metabolites in the pH5.0 extract (table 5.6.) and the unextractable radioactivity (table 5.5.) were not further investigated.

Rate of elimination of $^{14}CO_2$ from mice administered [^{14}C-methoxy]-p-propylanisole at 0.05 and 500mg/kg.

The elimination of $^{14}CO_2$ over the first 7 hours post-dosing in mice administered 0.05 and 1500mg/kg [^{14}C-methoxy]-p-propylanisole is summarised in table 5.7. and illustrated in figure 5.7.

Over the first 1½ hours there is no significant difference in the rate of elimination of $^{14}CO_2$ between the two levels of exposure as calculated as percentage of the dose. However, after

Table 5.5. Extraction characteristics of urine from mice after [^{14}C-methoxy]-p-propylanisole at various doses

Dose mg/kg	%of the dose extractable		
	pH5.0*	pH1.0	Aqueous residue
0.05	7.7(7.2-8.2)**	4.8(4.3-5.3)	2.6(2.1-3.1)
0.5	7.3(6.1-10.7)	5.7(4.0-7.1)	3.6(3.1-4.0)
50	13.6(12.0-15.8)	8.1(6.8-9.5)	5.9(4.4-8.7)
500	17.4(16.6-18.6)	9.8(9.0-11.1)	5.8(5.3-6.4)
1500	21.1(17.9-22.8)	12.0(10.8-13.7)	5.0(3.5-7.2)

*after β-glucuronidase treatment

**figures are means of at least 3 animals with ranges in parentheses

Table 5.6. Metabolites extractable at pH5.0 from mouse urine after [^{14}C-methoxy]-p-propylanisole at various doses

Dose mg.kg	polar metabolites	1-(4'-methoxyphenyl)propan-1-ol	1-(4'-methoxyphenyl)propan-2-ol
0.05	0.4	2.1 (1.9-2.3)	5.2 (5.0-5.5)
0.5	0.6(0.5-0.7)	1.7 (1.1-2.6)	4.7 (4.2-5.5)
50	0.7(0.4 -0.9)	3.8 (3.6-4.0)	8.2 (7.0-9.3)
500	0.9(0.7-1.1)	6.9 (6.3-7.9)	9.0
1500	1.6(1.3-2.1)	10.3 (9.4-11.7)	8.3 (6.7-10.1)

figures are percentage of the dose with ranges in parentheses

Figure 5.6. Changes in the excretion of ^{14}C-metabolites of [^{14}C-methoxy]-p-propylanisole in the urine of mice over the dose range 0.05-1500mg/kg, a) 1-(4'-methoxyphenyl)-propan-1-ol, b) 1-(4'-methoxyphenyl)propan-2-ol and c) 4-methoxy-hippuric acid

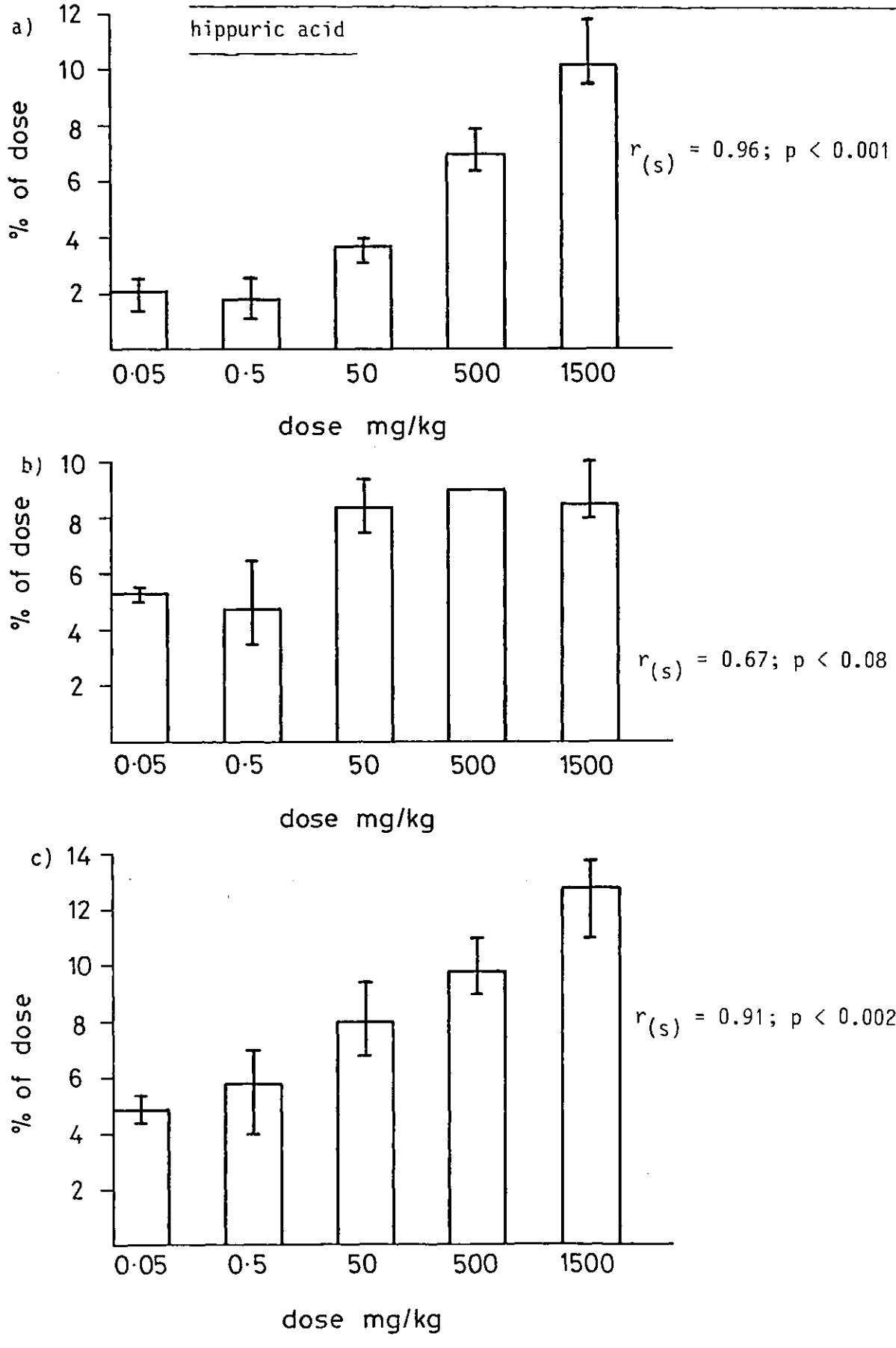
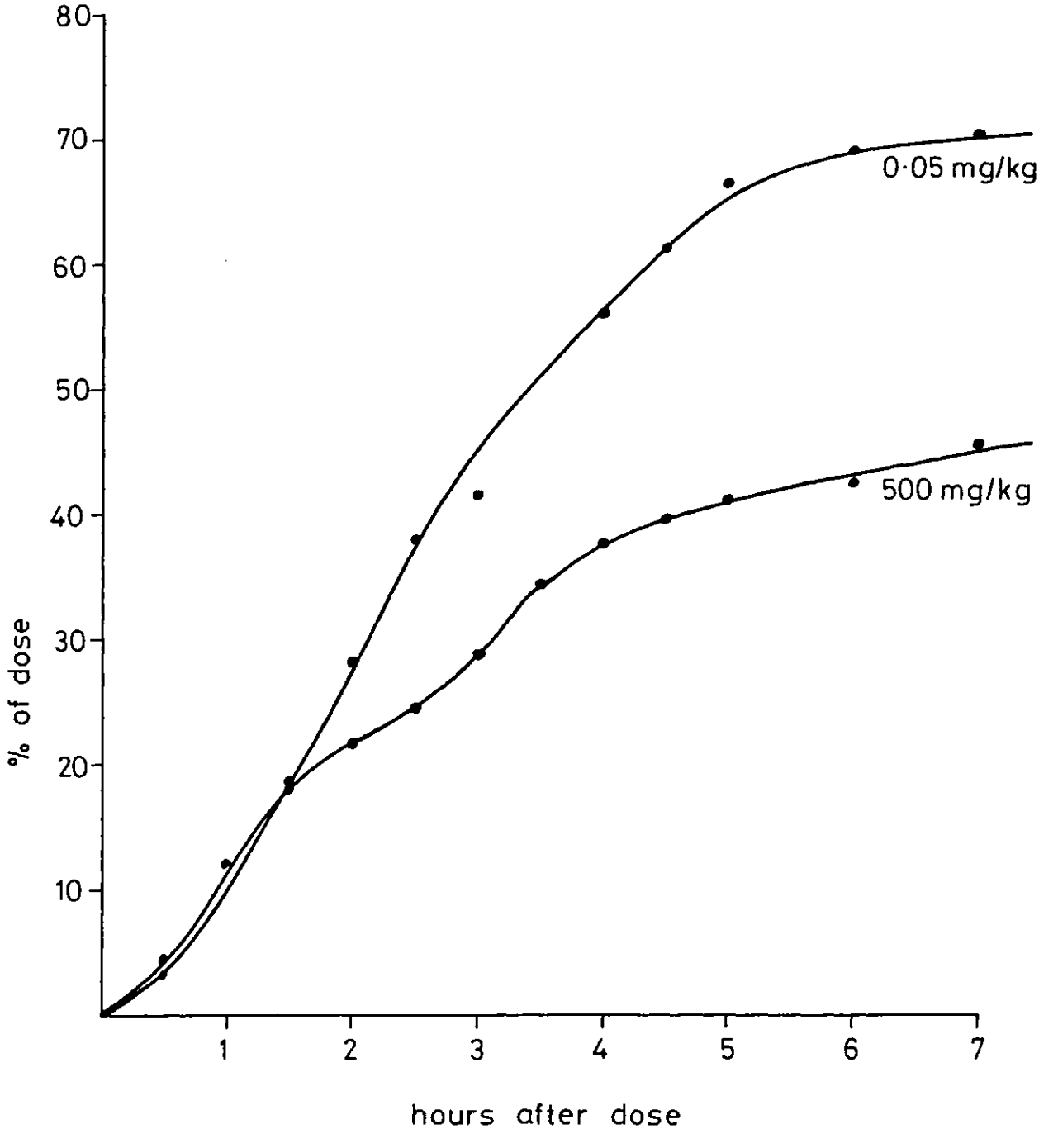


Table 5.7. Excretion of $^{14}CO_2$ in the expired air of mice administered [^{14}C-methoxy]-p-propylanisole at 0.05 and 1500mg/kg i.p.

time since dose (hours)	% of the dose eliminated as $^{14}CO_2$	
	0.05mg/kg*	500mg/kg
0.5	3.2	4.7
1.0	12.2	12.4
1.5	18.9	18.3
2.0	28.4	21.8
2.5	38.3	24.8
3.0	41.6	28.4
3.5	-	34.5
4.0	56.3	37.6
4.5	61.5	39.9
5.0	66.8	4±.3
6.0	69.2	42.7
7.0	70.5	45.8

* at least 4 animals at each dose level

Figure 5.7. Excretion of $^{14}CO_2$ in the expired air of mice administered [^{14}C-methoxy]-p-propylanisole at 0.05mg/kg and 500mg/kg levels



this initial period the percentage of the dose eliminated as $^{14}CO_2$ after a 500mg/kg dose fell behind the amount eliminated after the lower dose. After 7 hours, 70% of the 0.05mg/kg dose had been eliminated as $^{14}CO_2$ and only 45% of the 500mg/kg dose had been eliminated by this route.

5.4. DISCUSSION

The metabolic patterns of p-propylanisole disposition in both rats and mice varied considerably with dose. At the lowest doses, elimination of radioactivity as $^{14}CO_2$ predominated with only small amounts of radioactivity (10%) present in the urine. At the highest doses, urinary elimination rose to 40% of the dose and elimination as $^{14}CO_2$ fell to 50% of the dose. The total recovery of radioactivity was not dependent upon dose size.

Oxidative O-demethylation was a far more important route of metabolism for p-propylanisole than for trans-anethole or estragole. After a 50mg/kg dose 65% of p-propylanisole was metabolised by this pathway, whereas only 43% of a similar dose of trans-anethole of estragole underwent O-demethylation. This indicates that p-propylanisole is either a better substrate for the O-demethylation enzyme system, or a poorer substrate for the side chain oxidation enzymes than the two unsaturated analogues.

Investigation of the rate of oxidative O-demethylation of p-propylanisole in mice showed that this route of metabolism was independent of dose size until 1.5 hours post dosing, when $^{14}CO_2$ elimination at the higher dose fell behind that of the lower dose.

As the rate of $^{14}CO_2$ elimination at a dose of 0.05mg/kg was not faster than at the higher dose from the beginning of the experiment, it suggests that the O-demethylation enzyme system may have been inhibited by a metabolite at the higher dose, which by its formation decreased or inhibited the rate of oxidative O-demethylation, rather than the enzyme system simply being overloaded by the high dose of substrate.

The inhibitor could either be an O-demethylated metabolite or a side chain oxidation metabolite. In either case at the higher dose there is the potential for some 10,000 times the amount of metabolite available than at the lower dose.

If the O-demethylation enzyme system had been inhibited by a side chain oxidation metabolite it would explain the 4-fold increase in urinary elimination of radioactivity, because more unchanged p-propylanisole would become available for side-chain oxidation resulting in the increased elimination of radioactivity in the urine and further inhibition of O-demethylation.

At low doses of p-propylanisole, 4-methoxyhippuric acid and 1-(4'-methoxyphenyl)propan-2-ol were the major urinary metabolites At higher doses there were significantly larger amounts of these

metabolites in the urine, and in addition 1-(4'-methoxyphenyl)-propan-1-ol became a major metabolite. In the rat it was present in amounts approximately equal to those of 1-(4'-methoxyphenyl)propan-2-ol, while in the mouse 1-hydroxylation exceeded 2-hydroxylation at the highest dose (figures 5.3. and 5.6.). In both species the alcohols were detected unconjugated in the urine at doses of 500 and 1500mg/kg, indicating that glucuronyltransferase may have become overloaded. It is highly unlikely that glucuronic acid could have been depleted, although this has been reported for paracetamol (Jollow et al., 1974).

The higher concentration of the unconjugated metabolites at the high doses of p-propylanisole led to dihydroxylation taking place, and small amounts of 1-(4'-methoxyphenyl)-propane-1,2-diol were detected in the urine at a 1500mg/kg dose. This finding supports the theory that one possible route of metabolism of p-propylanisole to 4-methoxybenzoic acid may be via a diol intermediate (figure 4.6., chapter 4 and cf. chapter 2, figure 2.19. for proposed routes of metabolism of anethole).

One so far unidentified polar metabolite of p-propylanisole was extracted from the urine of rats after a 1500mg/kg dose. Its mass spectrum is illustrated in figure 5.5. and suggests the possible introduction of up to 3 oxygen atoms into

p-propylanisole. This type of metabolite was identified after trans-anethole administration to rodents, 1-(3'-hydroxy-4'-methoxyphenyl)propane-1,2-diol. The same type of metabolite may arise from p-propylanisole, as the diol was identified as a minor metabolite.

One of the major toxicity problems with p-propylanisole at high doses is osteoporosis. This condition may have been responsible for the increased diuresis observed in rats at high doses, because the large amount of calcium salts resulting from bone breakdown would require increased urine production for elimination. Another possible cause of high diuresis at a 1500mg/kg exposure is that the large concentration of p-propylanisole metabolites would also require a larger volume of urine to dissolve them. Increased diuresis was also observed after administration of trans-anethole at 1500mg/kg and 1-(4'-methoxyphenyl)propan-3-ol at 50mg/kg, the only common metabolite being large amounts of 4-methoxyhippuric acid.

The purpose of these studies in rodents at various levels of exposure were to help interpretation of high dose animal toxicity data with respect to the risk to man from low level exposure to chemicals in his environment.

It is well known that extrapolation of metabolic data from animals to man may cause problems due to species variations in respect of preferred routes of metabolism (Dring, 1977)

Extrapolation of high dose animal toxicity data to the low exposure human situation introduces more complicated considerations.

With trans-anethole, estragole (Zangouras et al., 1981) and now p-propylanisole it has been shown that over the dose range required for extrapolation of animal toxicity data to levels of human exposure, that all three compounds show marked variation in route of metabolism. Most strikingly oxidative O-demethylation becomes saturated/inhibited and elimination can no longer be described by first order pharmacokinetics. All the consequences of saturation of metabolism are observed as described by Wagner (1974) (see chapter 3) and the pharmacokinetics of these compounds very likely follow Michaelis-Menten kinetics.

Therefore in considering the risk from related chemical structures based upon the known toxicity data, consideration must be taken of the routes of metabolism of the compound, and whether the doses involved in the test may have altered the linearity of the pharmacokinetics of the test compound.

CHAPTER 6

The metabolism and disposition of *trans*-anethole and *p*-propylanisole in man

6.1. INTRODUCTION

The present procedure for assessing the toxic risk to man from food additives principally involves long term feeding studies in rodent species such as the mouse and rat. However the exposure levels employed in these tests are usually many thousand times the levels to which man is exposed. The intention of using the high doses is to counteract the poor sensitivity of the test methods and allow statistical evaluation of toxicity incidences found in treated and control animals. Administration of a dose comparable to human dietary exposure would decrease the toxicity incidences and therefore necessitate the use of very large number of animals.

A major criticism of using high doses is that primary detoxication mechanisms may become overloaded, and either alternative pathways may increase in importance or high levels of unchanged compound may accumulate in the body. Therefore the high dose exposure may not be a good model for the exposure during normal intake, because relative levels of metabolites and unchanged compound may be different at high and low doses.

The situation may not affect the toxicity of the test compound itself but it may be of concern if one of the alternative pathways leads to the formation of a toxic/carcinogenic metabolite, as

has been shown to occur with estragole (chapter 1) and the common analgesic phenacetin. This drug is metabolised largely by oxidative O-deethylation and deacetylation and to a lesser extent by aromatic hydroxylation and cysteine conjugation (Smith and Timbrell, 1974). There is strong circumstantial evidence that susceptibility to phenacetin toxicity is related to the ability to form a minor metabolite of aromatic hydroxylation 2'-hydroxyphenetidine (Devonshire et al.,1983).

The ability of individuals to O-deethylate phenacetin is partly under genetic control. In subjects whose capacity for this major reaction is reduced relative to the general population, the normally minor pathway leading to 2'-hydroxyphenetidine comes into greater prominence and toxic sequelae e.g. methaemoglobinaemia and signs of renal toxicity are observed after normal therapeutic doses of phenacetin.

The average daily intake (adult) of trans-anethole and p-propylanisole per capita for the population of the United States of America has been estimated to be 65µg and 15µg respectively (approximately 1µg/kg and 0.2µg/kg respectively; F.E.M.A., 1978). The doses used in the toxicity tests that have been conducted using these food flavours were up to a million times larger than the daily human intake. It might thus be suggested that this great difference in dose size will affect the pharmacokinetics of the test compounds.

p-Propylanisole disposition in man has not been investigated previously. However, that of trans-anethole has received attention (Le Bourhis, 1970; 1973b), in an attempt to determine acceptable daily intakes.

Oral administration of trans-anethole to five subjects in this study resulted in the recovery of a total of 57% of the dose in the urine within 24 hours, as 4-hydroxybenzoic (5% of the dose) and 4-methoxybenzoic acids (52% of the dose). Additionally analysis of blood samples at various intervals (not specified) following the ingestion of 1.0g of trans-anethole revealed no detectable blood levels of trans-anethole (Le Bourhis, 1973b). Based upon these results the author suggested that it may be possible to increase the 'Acceptable Daily Dose' of trans-anethole to 25mg/kg.

The metabolism of [^{14}C-methoxy]-trans-anethole and [^{14}C-methoxy]-p-propylanisole was investigated here in two volunteers at levels as close to the average daily exposure as the specific activities of the compounds and limits of detection of the analytical methods would allow (1mg and 100µg respectively). The elimination of ^{14}C in the urine and $^{14}CO_2$ in the expired air was determined and the urinary ^{14}C-metabolites isolated, identified and quantified.

It is anticipated that comparison of the metabolism of p-propylanisole and trans-anethole in man with their disposition in rodents will help to assess the suitability of the rodent model for the metabolism of the substituted anisoles in man, and hence aid the interpretation of the toxicity data for the risk to man, of these and other related compounds.

6.2. MATERIALS AND METHODS

Volunteers

Two healthy male volunteers (35 and 48 years) took by mouth on separate occasions after a normal breakfast 100µg [^{14}C-methoxy]-p-propylanisole (6.3µCi) or 1mg [^{14}C-methoxy]-trans-anethole (4.5µCi), both dissolved in trioctanoin, contained in a gelatine capsule. The labelled compounds were the products of the radiochemical syntheses described in chapters 2 and 4.

Sample collection

Urine was collected hourly up to 8 hours, and then from 8-12, 12-24 and 24-48 hours. Urine volumes were measured and samples assayed for ^{14}C as previously described (chapter 2), and the urine was stored at -20°C until analysed further.

$^{14}CO_2$ elimination was estimated every 30 minutes up to 8 hours, and then after 24 hours. Volunteers exhaled through a mouthpiece, via a drying trap containing anhydrous calcium chloride, into a trap containing 1M hyamine hydroxide (0.37ml) methanol (2ml) and thymolphthalein indicator (blue) until the solution became clear.

The hyamine hydroxide solution was standardised by titration against 1M HCl. 0.37 ml of hyamine hydroxide solution was neutralised by 0.34ml of 1M HCl (0.34 mmol), and thus 0.37ml of hyamine hydroxide solution will be neutralised by 0.34 mmol of exhaled CO_2.

It has been calculated based upon known physiological data (Passmore and Robson, 1967) that approximately 500mmol of CO_2 are exhaled every 30 minutes, and for this study it was assumed that this was excreted at a constant rate over the 30 minute period between $^{14}CO_2$ estimations.

The calculation of total $^{14}CO_2$ excreted is thus : if x dpm are collected in the hyamine hydroxide trapping solution, the total amount of $^{14}CO_2$ eliminated over the previous 30 minutes would be :-

$$x \quad X \frac{500}{0.34} \quad = 1471 \text{ x dpm /30 minutes}$$

from which the percentage of the dose eliminated as $^{14}CO_2$ was calculated.

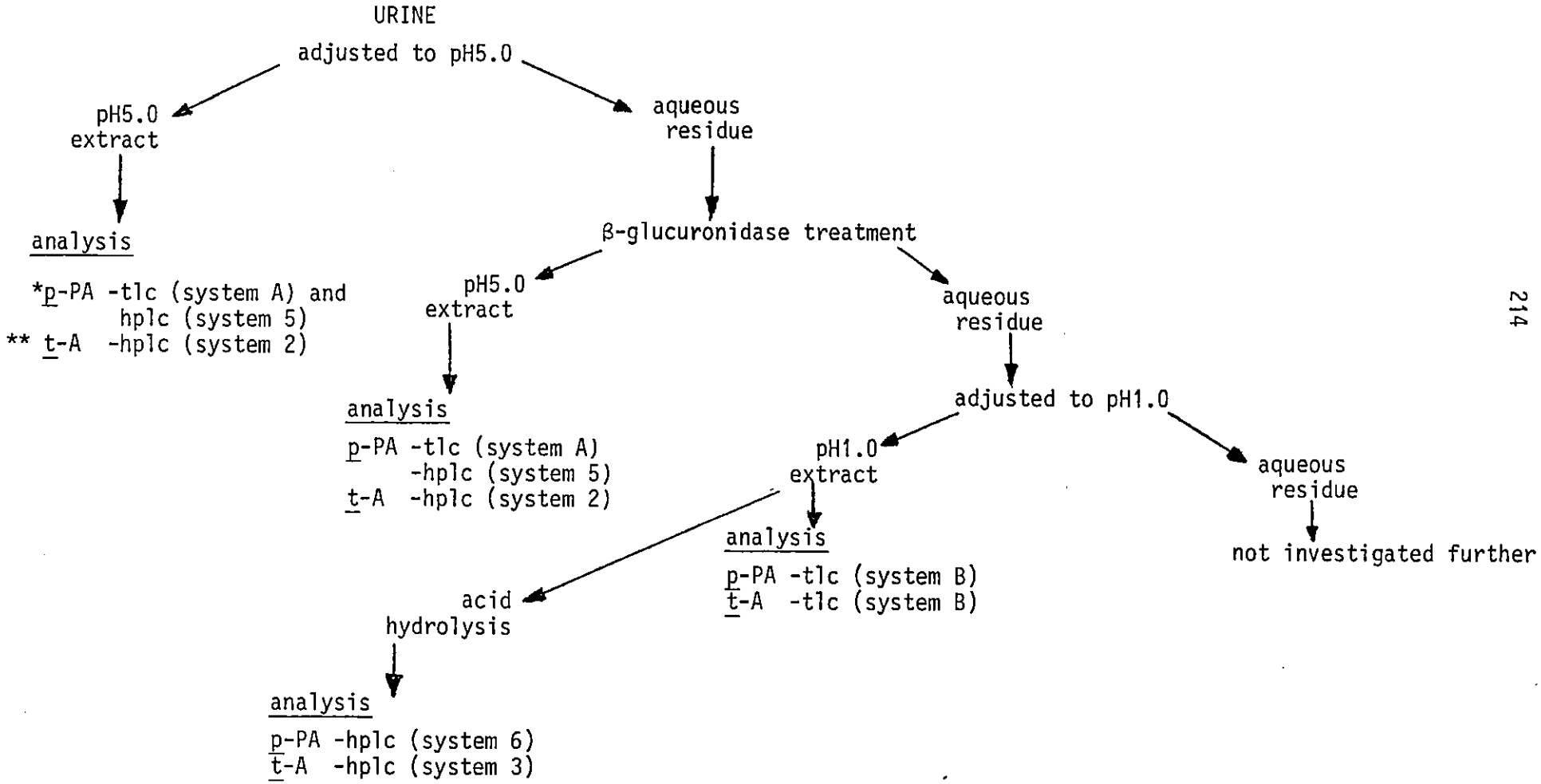
Analysis of Urinary Radioactivity

The urine sample containing the greatest percentage of the dose, and a 10% pool of the 0-24 hour urine were analysed for each subject in both studies. The urine samples were

treated and analysed as previously described for rodents (chapters 2 and 4) and summarised in figure 6.1.

The aqueous residues following the above treatments and extractions of urine from both studies contained very small amounts of radioactivity (< 5% of the original content) and because of this and the large volumes of residue were not further investigated.

Figure 6.1. Analysis of urinary radioactivity from the human study



*p-PA = p-propylanisole study

**t-A = trans-anethole study

6.3. RESULTS

The elimination of radioactivity from both subjects after [^{14}C-methoxy]-*trans*-anethole and [^{14}C-methoxy]-*p*-propylanisole is given in table 6.1. In both cases the majority of the dose was rapidly excreted in the urine and expired air within 8 hours of dosing and no further radioactivity was detectable in the excreta after 48 hours (figures 6.2. and 6.3.).

The major route of elimination differed between the compounds. After [^{14}C]-*trans*-anethole,65% of the dose was eliminated in the urine,with only 24% of the dose eliminated as $^{14}CO_2$ in the expired air. However,after administration of [^{14}C-methoxy]-*p*-propylanisole,43% of the dose was eliminated as $^{14}CO_2$ in the expired air and only 25% of the dose in the urine.

The total recovery achieved after [^{14}C]-*trans*-anethole administration amounted to 89% of the dose, while after [^{14}C]-*p*-propylanisole the corresponding figure was only 68% of the dose. This low value is probably the result of incomplete trapping of expired $^{14}CO_2$ or an under estimation of the rate of basal CO_2 elimination for the volunteers, and as this route is the major elimination pathway for [^{14}C-methoxy]-*p*-propylanisole, in either event the total recovery would be correspondingly lower.

Table 6.1. Elimination of radioactivity from volunteers after [^{14}C-methoxy]-*trans*-anethole and [^{14}C-methoxy]-*p*-propylanisole

Volunteer	JC	RLS	MEAN	JC	RLS	MEAN
Compound	[^{14}C-methoxy]-*p*-propylanisole			[^{14}C-methoxy]-*trans*-anethole		
%dose in urine	23.1	26.4	24.8	62.8	66.9	64.9
%dose in expired air	41.8	43.5	42.7	27.7	19.8	23.8
%total recovery	64.9	69.9	67.5	90.5	86.7	88.7

Figure 6.2. Cumulative elimination of radioactivity in the urine and expired air of volunteers after 1mg of [^{14}C-methoxy]-trans-anethole

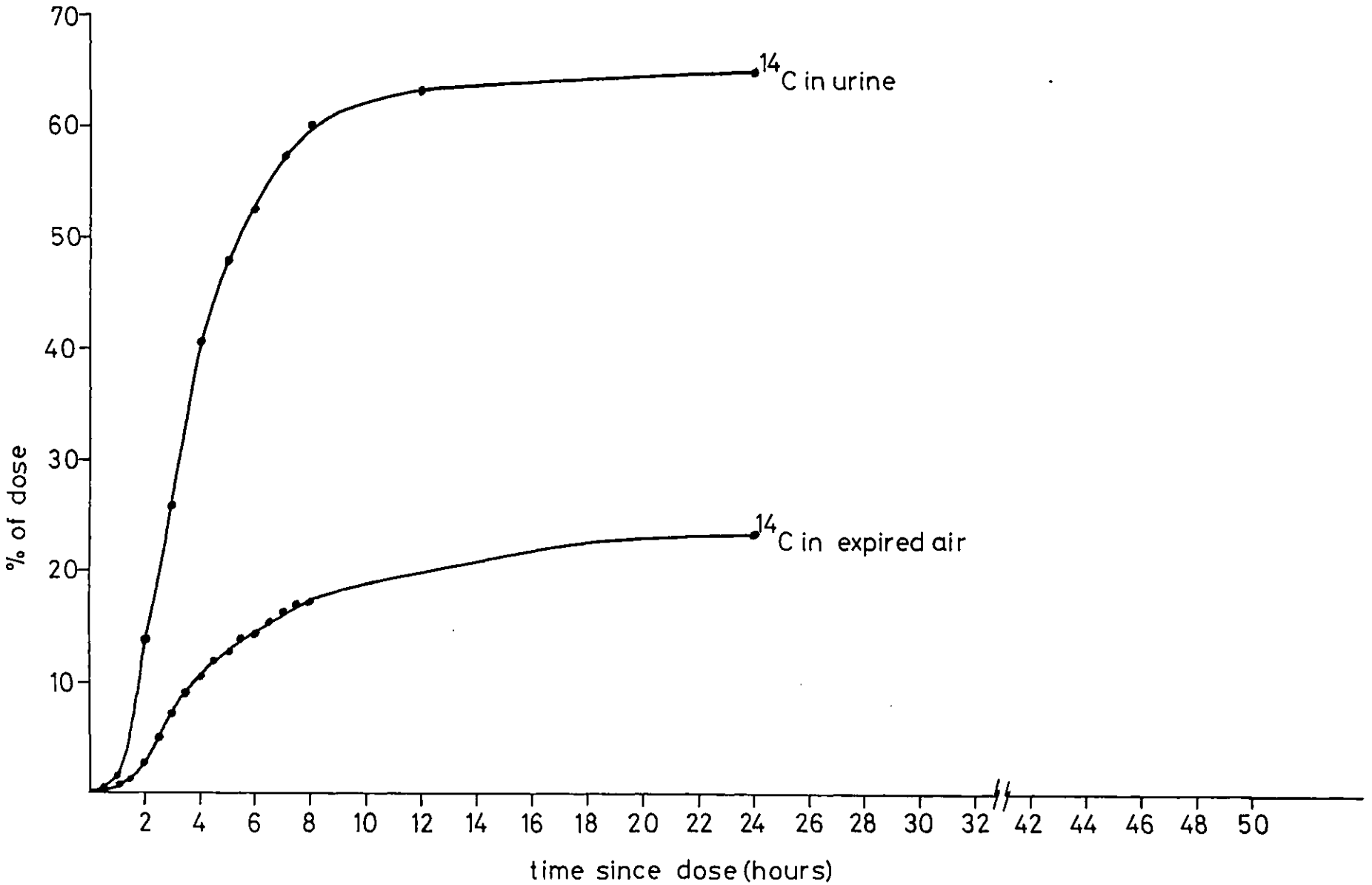
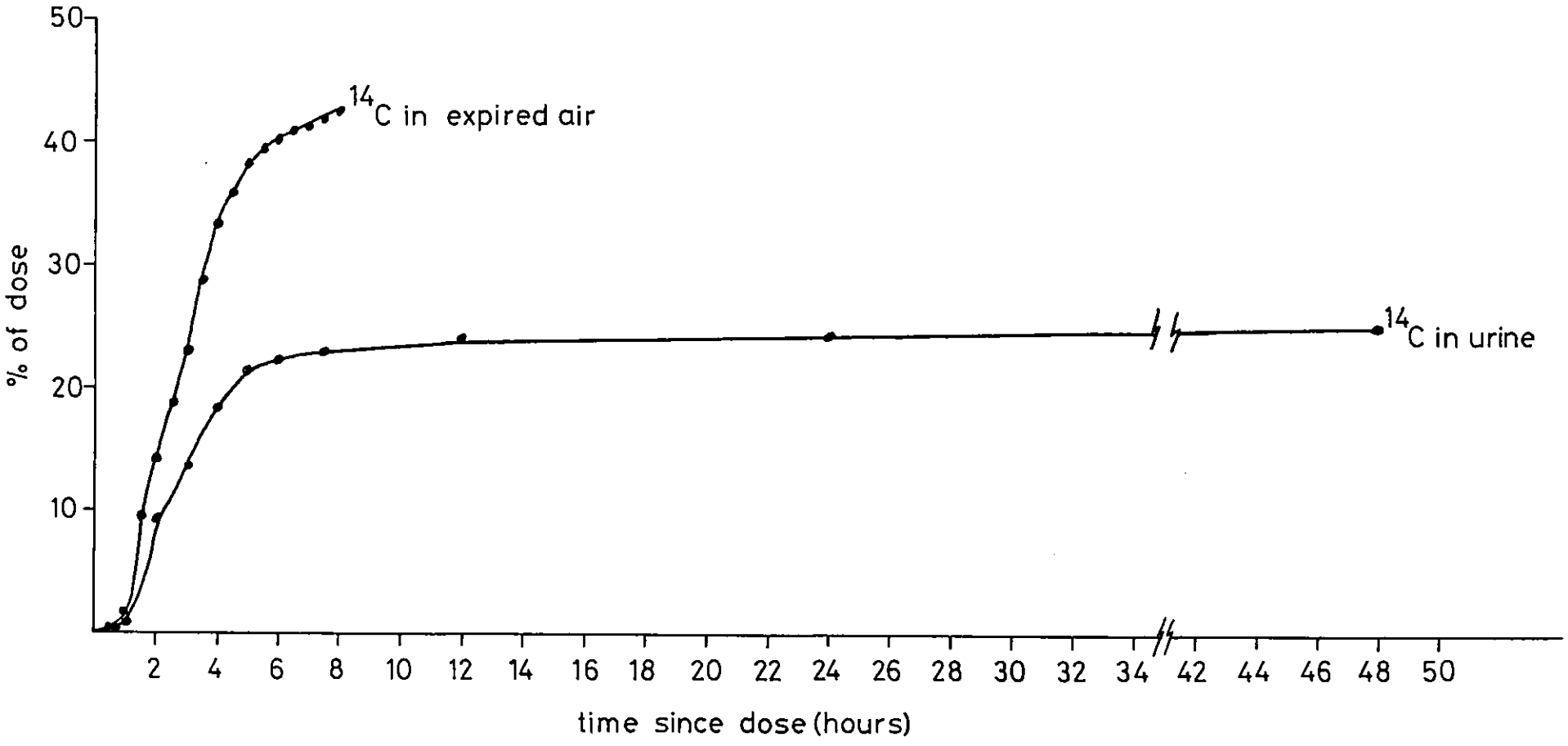


Figure 6.3. Mean cumulative elimination of radioactivity in the urine and expired air of volunteers after 100µg of [^{14}C-methoxy]-p-propylanisole



^{14}C-metabolites of [^{14}C-methoxy]-p-propylanisole in the urine of volunteers

The extraction characteristics of ^{14}C from the urine of volunteers after [^{14}C-methoxy]-p-propylanisole are given in table 6.2.

Very little radioactivity was extractable at pH5.0 prior to β-glucuronidase treatment (1% of dose), but after treatment a further 10% of the dose was extractable into ether at this pH. Analysis of these extracts by tlc (system A) and hplc (system 5) showed the presence of two major metabolites which had the same R_f values and retention times as 1-(4'-methoxyphenyl)-propan-1-ol (2% of the dose) and 1-(4'-methoxyphenyl)propan-2-ol (8% of the dose). A minor band that had the same chromatographic characteristics as 1-(4'-methoxyphenyl)-propane-1,2-diol was also identified. The two mono-hydroxylated metabolites were excreted as glucuronides in urine.

Adjustment of the pH of the residue to pH 1.0 made it possible to extract a further 12% of the dose into ether. This extract was shown by tlc (system B) and hplc (system 6) to contain one metabolite, 4-methoxyhippuric acid. Less than 2% of the dose remained unidentified in the aqueous residue (table 6.2.).

Table 6.2. Extraction of radioactivity from the urine of volunteers after [^{14}C-methoxy]-*trans*-anethole and [^{14}C-methoxy]-*p*-propylanisole

Volunteer	Compound	%dose in urine	%dose excreted at pH5.0	%dose excreted at pH5.0 after β-glucuronidase	%dose excreted at pH1.0	%dose in aqueous residue
JC	[^{14}C-methoxy]-*trans*-anethole	62.8	6.6	7.5	41.9	6.8
RLS	[^{14}C-methoxy]-*trans*-anethole	66.9	8.8	6.2	45.5	6.4
JC	[^{14}C-methoxy]-*p*-propylanisole	23.1	1.1	9.2	10.9	1.9
RLS	[^{14}C-methoxy]-*p*-propylanisole	26.4	1.1	10.5	13.2	1.7

Table 6.3. Metabolism of [^{14}C-methoxy]-*p*-propylanisole in volunteers

(results expressed as % of the dose)

Metabolites	Volunteers JC	RLS
p-propylanisole	0*	0
1-(4'-methoxyphenyl)propan-1-ol	1.9	2.5
1-(4'-methoxyphenyl)propan-2-ol	7.7	8.1
1-(4'-methoxyphenyl)propane-1,2-diol	0.6	0.8
4-methoxyhippuric acid	10.9	13.2
$^{14}CO_2$	41.8	43.5
aqueous residue	2.0	1.8
	64.9	69.9

* 0 = none detected

^{14}C-metabolites of [^{14}C-methoxy]-*trans*-anethole in the urine of volunteers

The extraction characteristics of ^{14}C from the urine of volunteers after [^{14}C-methoxy]-*trans*-anethole is summarised in table 6.2.

Prior to β-glucuronidase treatment 8% of the dose was extractable at pH5.0 into ether, and after treatment, a further 7% of the dose was extractable at this pH (table 6.2.).

Analysis of these extracts by hplc (system 2) revealed a similar pattern of metabolites to those found in rodent urine after the administration of [^{14}C]-*trans*-anethole, and Table 6.4 lists the metabolites. The two diastereoisomers of 1-(4'-methoxyphenyl)propane-1,2-diol were the major metabolites in these extracts, accounting together for 9% of the dose.

Some 45% of the dose was extractable at pH1.0 into ether. Hplc (system 3) and tlc (system B) analysis of this extract revealed one major metabolite, identified as 4-methoxyhippuric acid, together with a small amount of 4-methoxybenzoic acid (1% of the dose).

Table 6.4. Metabolism of [^{14}C-methoxy]-*trans*-anethole in volunteers

(results expressed as 1% of the dose)

Metabolites	Volunteers JC	RLS
trans-anethole	0*	0
1-(4'-methoxyphenyl)propane-1,2-diol (diastereoisomer 1)	4.3	5.1
1-(4'-methoxyphenyl)propane-1,2-diol (diastereoisomer 2)	4.0	3.8
4-methoxycinnamyl alcohol	<0.1	<0.1
4-methoxycinnamic acid	0.1	0.1
4-methoxybenzoic acid	3.4	4.5
4-methoxyhippuric acid	41.4	44.0
4-methoxyacteophenone	0.1	0.1
1-(3'-hydroxy-4'-methoxyphenyl)propane-1,2-diol 1-(4'-methoxyphenyl)-1-oxo-propan-2-ol	1.6	1.8
1-(3',4'dimethoxyphenyl)propane-1,2-diol	0.4	0.5
$^{14}CO_2$	27.7	19.8
aqueous residue	7.3	4.9
Total	90.5	84.7

*0 = none detected

6.4. DISCUSSION

[^{14}C-methoxy]-*trans*-Anethole and [^{14}C-methoxy]-*p*-propylanisole are completely metabolised and eliminated in man within 8 hours, when administered in doses approximating to, or slightly in excess of, the estimated average daily intake (figure 6.2 and 6.3.) (F.E.M.A., 1978).

A major route of metabolism of these substituted anisoles in man as in rodents is via oxidative O-demethylation. However, the data in this chapter shows that the extent of O-demethylation of these compounds in man is less than that observed in rodents. Comparison of results from studies of *trans*-anethole, estragole (Zangouras, 1982) and *p*-propylanisole metabolism at doses to man some 50 times lower than the lowest dose given to rodents (0.05mg/kg) with this rodent data, shows that man shows only 50% of the oxidative O-demethylation capacity of rats and mice (table 6.5). This may be because the substituted anisoles have a lower affinity for the enzyme system for oxidation O-demethylation in man, or they may have greater affinity for the enzymes responsible for side chain oxidations.

The corollary to the reduced pulmonary elimination of these compounds in man compared with rodents is that in all cases urinary elimination is higher in man than in rodents. The

Table 6.5. The relationship of the arrangement of the three-carbon side-chain to the oxidative O-demethylation* of [^{14}C-methoxy]-*trans*-anethole, [^{14}C-methoxy]-estragole*** and [^{14}C-methoxy]*p*-propylanisole in man, the mouse and rat

Species**	% of the dose O-demethylated after		
	p-propylanisole	*trans*-anethole	estragole
man	42.7	23.8	11.7
mouse	74.5	71.8	51.9
rat	81.6	56.3	55.2

* percentage O-demethylation based upon $^{14}CO_2$ in the expired air

** doses: man, *trans*-anethole 10µg/kg
estragole and *p*-propylanisole 1µg/kg
rat and mouse, all compounds 0.05mg/kg (50µg/kg)

*** Zangouras (1982)

data shows that with trans-anethole oxidative O-demethylation is so low that urinary elimination of radioactivity exceeds elimination of $^{14}CO_2$ in the expired air (table 6.1.). However the ^{14}C urinary metabolites in human urine showed the same patterns as were observed in rodent urine after administration of trans-anethole and p-propylanisole, but with different proportions of each metabolite (tables 6.3. and 6.4.).

The major urinary ^{14}C metabolites of p-propylanisole in human urine in this study were 1-(4'-methoxyphenyl)propan-2-ol and 4-methoxyhippuric acid, 1-(4'-Methoxyphenyl)propan-1-ol was a minor metabolite and the diol was detected in trace amounts. This pattern of relative proportions of metabolites was similar to that observed in the urine of rodents dosed with small amounts of p-propylanisole (chapter 5).

The pattern of urinary elimination of trans-anethole was dominated by the excretion of >45% of the dose as 4-methoxybenzoic acid or its glycine conjugate, 4-methoxyhippuric acid, which is in agreement with previous data (Le Bourhis, 1970), and only 9% of the dose was metabolised, presumably by epoxidation, to the two diastereoisomers of 1-(4'-methoxyphenyl)-propane-1,2-diol. These results indicate that in man low doses of trans-anethole are extensively metabolised by ω-oxidation as discussed in chapter 2. These relative proportions

of metabolites were not seen in the rodent studies at any dose. The most similar pattern is shown in the mouse at high doses when large amounts of 4-methoxybenzoic acid are excreted, but in this example only small amounts of diol are excreted, and the elimination of ^{14}C takes 3 days, not 8 hours as was the case in man.

The most important conclusion of this study is that, like rodents, man rapidly and completely metabolises and eliminates the substituted anisoles when administered a normal levels of dietary exposure. The routes of metabolism of these compounds are not species dependent, but the extent to which each route of metabolism is preferred is species and dose dependent.

From these studies one observes that man metabolises susbstituted anisoles in fashions qualitatively similar to those seen in rodents. Therefore in rodents studies the animals are exposed to a similar array of metabolites to that seen in man.

However, the quantitative aspects of metabolism in rodents are dose-dependent and the value of a rodent as a metabolic model for man is highly dependent upon the dose chosen.

CHAPTER 7

General Discussion

GENERAL DISCUSSION

From the point of view of safety evaluation and the extrapolation of high dose animal toxicity data to low exposure in man, the ideal situation would be one where the tissues of the animal are exposed to the same flux of chemicals (parent compound and metabolites) with respect to the qualitative, quantitative and kinetic aspects, as would occur in the human situation. Under these conditions the net balance of intoxication/detoxication pathways would be the same for both the human and the test species. However, this ideal situation is rarely, if ever, achieved. The two major factors that act against achieving this are :

i) the occurrence of inter-species differences in metabolism (qualitatively, quantitatively and kinetically), and

ii) the pattern of metabolism of a chemical in a test species is frequently dose-dependent i.e. the qualitative, quantitative and kinetic aspects of metabolism may alter with increasing dosage or exposure, and thereby also the relative balance of intoxication/detoxication pathways

Clearly in such high dose situations test animal species may be exposed to quite different arrays of metabolites compared with what would occur in the low dose animal experiment or in normal

human exposure. It would not be surprising, therefore, if the toxic effects evident at high doses were qualitatively different from those at low doses and this would be a direct consequence of saturation or metabolic overload.

The metabolism and disposition of the substituted anisole food flavours have been investigated at various doses, and both trans-anethole and p-propylanisole showed quantitative inter-species differences in metabolism. These compounds were metabolised by oxidative O-demethylation in man and rodents, but much less extensively in man than rodents.

From comparison of the metabolic data of trans-anethole, estragole (Zangouras et al., 1981) and p-propylanisole it becomes obvious that the affinity of a particular substituted anisole for oxidative O-demethylation was dependent on the nature of the three carbon side chain. trans-Anethole and estragole with unsaturated side chains are less extensively O-demethylated than the saturated analogue. 78% of a 0.05mg/kg dose of p-propylanisole in rodents underwent O-demethylation whereas only 55% of a similar dose of trans-anethole or estragole was metabolised by this route. Of the two unsaturated compounds, trans-anethole with the double bond in the 1,2-position has greater affinity for O-demethylation than estragole with the double bond in the 2,3-position. This pattern of extent of oxidative O-demethylation

of the substituted anisoles, dependent on the nature of the side chain, was similar in both rodents and man (Chapter 6, table 6.5.).

However, as oxidative O-demethylation was generally less extensive in man than in rodent species, man excreted more of the [^{14}C-methoxy]-labelled trans-anethole and p-propylanisole as ^{14}C-urinary metabolites than did rodents.

The same ^{14}C-urinary metabolites were identified after administration of [^{14}C-methoxy]-trans-anethole to man and to rodents and similarly after the administration of [^{14}C-methoxy]-p-propylanisole to rodents and man.

With regard to the second point raised above, the qualitative and quantitative aspects of the fate of trans-anethole and p-propylanisole varied with dose in rodents.

At doses up to 500mg/kg to rodents, both substituted anisoles were rapidly absorbed and eliminated (within 24 hours). Doses of 1500mg/kg (levels used in toxicity tests, Taylor et al., 1964) were only slowly eliminated in rodents taking up to 72 hours, but completely metabolised to yield the same ^{14}C urinary metabolites as were identified at lower doses. However these metabolites were excreted in significantly larger amounts at high doses

compared to low doses.

At 0.05mg/kg [^{14}C-methoxy]-trans-anethole and [^{14}C-methoxy]-p-propylanisole were extensively metabolised by oxidative O-demethylation (55% and 78% of the dose respectively) with low levels of ^{14}C-urinary metabolites. At the top dose administered (1500mg/kg) both compounds were less extensively O-demethylated (33% and 49% of the dose respectively), and much larger amounts of each of the ^{14}C-urinary metabolites were detected.

Therefore in the range of doses used for animal toxicity testing of trans-anethole and p-propylanisole the flux of unchanged compound and metabolites to which the animal is exposed will be quite different to the relative amounts of metabolites to which a low dose animal or man (dietary exposure) is exposed.

As a model for the human exposure to these substituted anisoles in the diet, the high dose animal toxicity tests can be considered in some respects adequate but in others poor:

i) Rodent species and man metabolise both trans-anethole and p-propylanisole by similar routes, and the same ^{14}C-urinary metabolites were identified. However,

ii) the relative quantities of each of these metabolites in rodents and man were different, most particularly after trans-anethole administration, and

iii) high doses in rodents were eliminated more slowly than low doses in rodents or the normal human dietary exposure in volunteers, indicating that perhaps the toxicity of these compounds is related to the accumulation of unchanged parent compound in the body awaiting metabolism, in a similar manner to the accumulation of high doses of safrole (Strolin-Benedetti et al., 1977).

There are many thousands of anutrient chemical substances present as natural constituents and as additives in food (Oser and Hall, 1977 and references therein). This multiplicity of substances can be subdivided into groups of similar chemical structures, for example the substituted anisoles discussed in this thesis, cinnamyl and cinnamate esters, furans, thiazoles etc. The use levels of individual members of these groups can vary very greatly from a few kilos to hundreds of tonnes per year. It is neither economically possible nor sensible to attempt to obtain the information needed to assess every imaginable toxic risk associated with every anutrient, and the pursuit of greater safety therefore demands the setting of priorities

as well as sensible limits of investigation.

There are significant philosophical differences in the requirements for the safety evaluation of drugs and anutrient additives in food.

Relatively few chemically distinct drug compounds are encountered by man during his lifetime. When they are encountered the patient is usually in ill health and the size of doses administered must be large enough to be of therapeutic value. However, the normal healthy individual is continuously exposed to a plethora of dietary anutrients, often at very low doses (F.D.A., 1983).

It is obviously impossible to test the individual and combined toxicity of all these anutrient dietary chemicals to the same extent as is legally required for the safety evaluation of possible therapeutic agents. Therefore for the prediction of any possible risk to man from such anutrients use must be made of the limited metabolic and toxicity data which is available, often by relating the compound in question to structural congeners which have been studied.

Prediction of metabolism based upon data from related compounds is not a novel idea. Examples of such predictions for the metabolism of previously uninvestigated drugs may be found in

the literature, and these examples serve to illustrate the drawbacks which may be encountered with such an approach (Caldwell, 1981).

The major metabolites of the arylacetic acids are amino acid and glucuronic acid conjugates. The relative extents of these metabolic options are determined principally by the structure of the acid and to a lesser extent by species.

From a knowledge of the behaviour of a number of structures related to arylacetic acids it would be predicted that 2-naphthylacetic acid would undergo amino acid conjugation to a greater extent than glucuronic acid conjugation. Studies in several species shows this to be the case but the nature of the amino acids used was radically different from expectations (Emudianughe et al., 1978; 1979).

The fate of clofibric acid the active metabolite of clofibrate was found to be exactly as would have been predicted from previous experience with related compounds (Caldwell et al., 1979). In particular its restriction of amino acid conjugation to carnivores and the utilisation of taurine for this purpose accords with precedents from related compounds.

The chemical structure of the amphetamine analogue chlorphentermine precludes all known routes of metabolism of amphetamine except attack on the primary amino group. Investigation of the fate of ^{14}C-chlorphentermine showed, as expected, that N-oxidation was

a major route of metabolism of this compound in most species (Caldwell et al., 1975). However, an unknown conjugate was also isolated, but not identified, in species unable to N-oxidize the amino group. This apparently represents a novel metabolic reaction.

This approach to the prediction of metabolism could be utilised in the estimation of toxic hazards from anutrients in the diet. Cramer et al (1978) included such an approach in compiling their 'decision tree' for use in the prediction of the toxic hazard for chemical substances. This is now used in the guidelines for toxicity testing requirements of food additives in the U.S.A. (F.D.A., 1983). The tree consists of 33 questions, each with a yes/no answer. Each answer leads to another question or to a final classification into one of three classes, reflecting low, intermediate or serious toxicity.

Classification according to presumptive toxicity can be combined with knowledge of human intake to provide for each substance a 'protection index' which can be used to establish priorities and define tentatively the extent of appropriate testing, as discussed in chapter 1.

The logic of the 'tree' rests heavily on the known data on metabolism and toxicity of groups of compounds. It is thus possible to see how the data described in this thesis

may be considered in the context of the 'decision tree' approach, to help assess toxicity requirements, by aiding the prediction of metabolism of compounds with structural similarities to the compounds which have been well studied.

From the study of the substituted anisoles one may speculate that any group of aromatic compounds with a methoxy moiety *para* to an alkyl side chain may be expected to be oxidatively O-demethylated, the extent of this metabolite route being influenced by the structure of the side chain.

Taking estragole as an example of the anisoles in flavour use today, it is possible to put the dose-dependent metabolic data in perspective. Animal studies have indicated that this compound may be carcinogenic (Drinkwater *et al.*, 1976; Miller *et al.*, 1983). Therefore, based upon this data and its similarity to safrole, estragole should perhaps be banned from use in human food? However, it is now known from metabolism studies in animals that the presumed proximate carcinogenic metabolite 1'hydroxyestragole is a very minor urinary metabolite at low doses and only excreted in significant amounts at high doses. Therefore taking this metabolic data into consideration should estragole be regarded as suitable for human consumption at normal dietary levels?

In scientific terms the safety evaluation of food additives is at the level of drug safety evaluation 20 years ago, before the essential contribution that mechanistic metabolism and toxicity studies could make to the interpretation of results of animal pathological studies, notably to the accuracy of extrapolation of such results to man,was appreciated.

Present legislation in most countries for drug licensing recognises the importance of studies of this nature.

Two major proposals emerge from this work in the context of safety evaluation of food additives. The first is that the metabolism of the compound should be assessed in the human situation; this allows the endorsement or otherwise of the animal model used in the chronic toxicity studies. The second point is the necessity to assess whether or not the compound under test exhibits dose-dependent metabolism at the dose levels used. If so, the significance of this needs to be considered and perhaps an additional dose level of exposure should be included in the protocol at which linear kinetics still occur as suggested in the current FDA 'Red Book' (1983).

Appendix 2.1. Chemicals and their sources

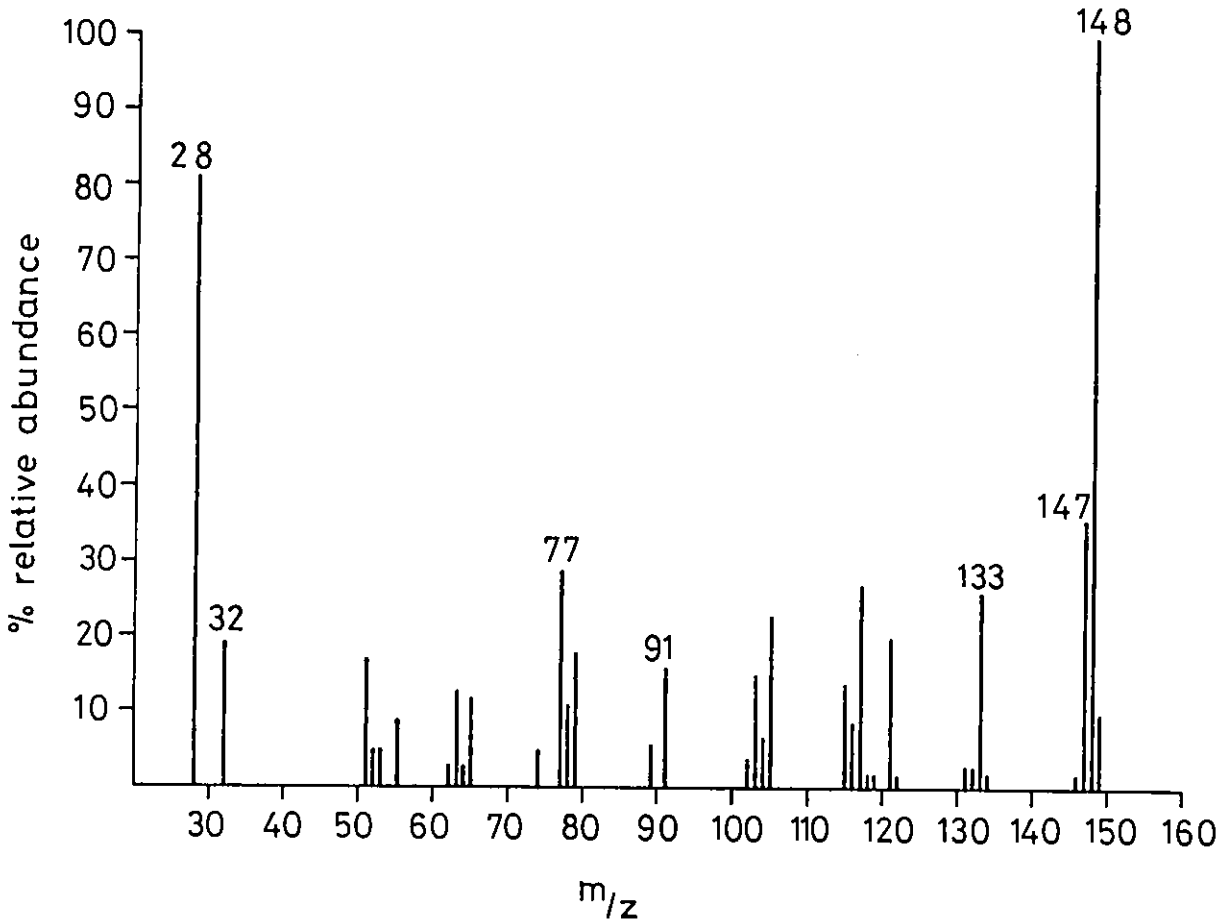
Enzymes	
Glucurase (β-glucuronidase)	Sigma Chemical Company, Poole, Dorset, U.K.
Jack Bean urease	
Trioctanoin (dosing vehicle)	
Chemicals	
Anethole (4-methoxypropenylbenzene)	Aldrich Chemical Company Ltd., Gillingham, Kent, U.K.
Estragole (4-methoxyallylbenzene)	
4-hydroxymandelic acid	
4-hydroxyphenyllactic acid	
4-hydroxyphenylpropane	
4-hydroxyphenylpyruvic acid	
4-methoxyacetophenone	
4-methoxybenzoic acid	
4-methoxycinnamic acid	
4-methoxyphenylacetic acid	
4-methoxyphenylacetone	
4-methoxypropriophenone	
1-(4'-methoxyphenyl)propan-3-ol	
4-methoxyphenylpropionic acid	
4-methoxybenzoyl chloride	
p-propylanisole (4-methoxyphenylpropane)	Life Sciences Group, Plainsview, N.Y., U.S.A. and International Flavours and Fragrances, 1515 Highway 36, Union Beach, N.J.07735, U.S.A.
1-(4'-methoxyphenyl)propane-1,2-diol	Gift from E. Solheim, Univ. of Bergen, Bergen, Norway.

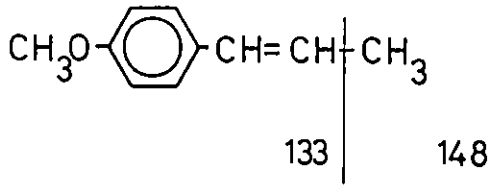
continued over page

Appendix 2.1. In continuation

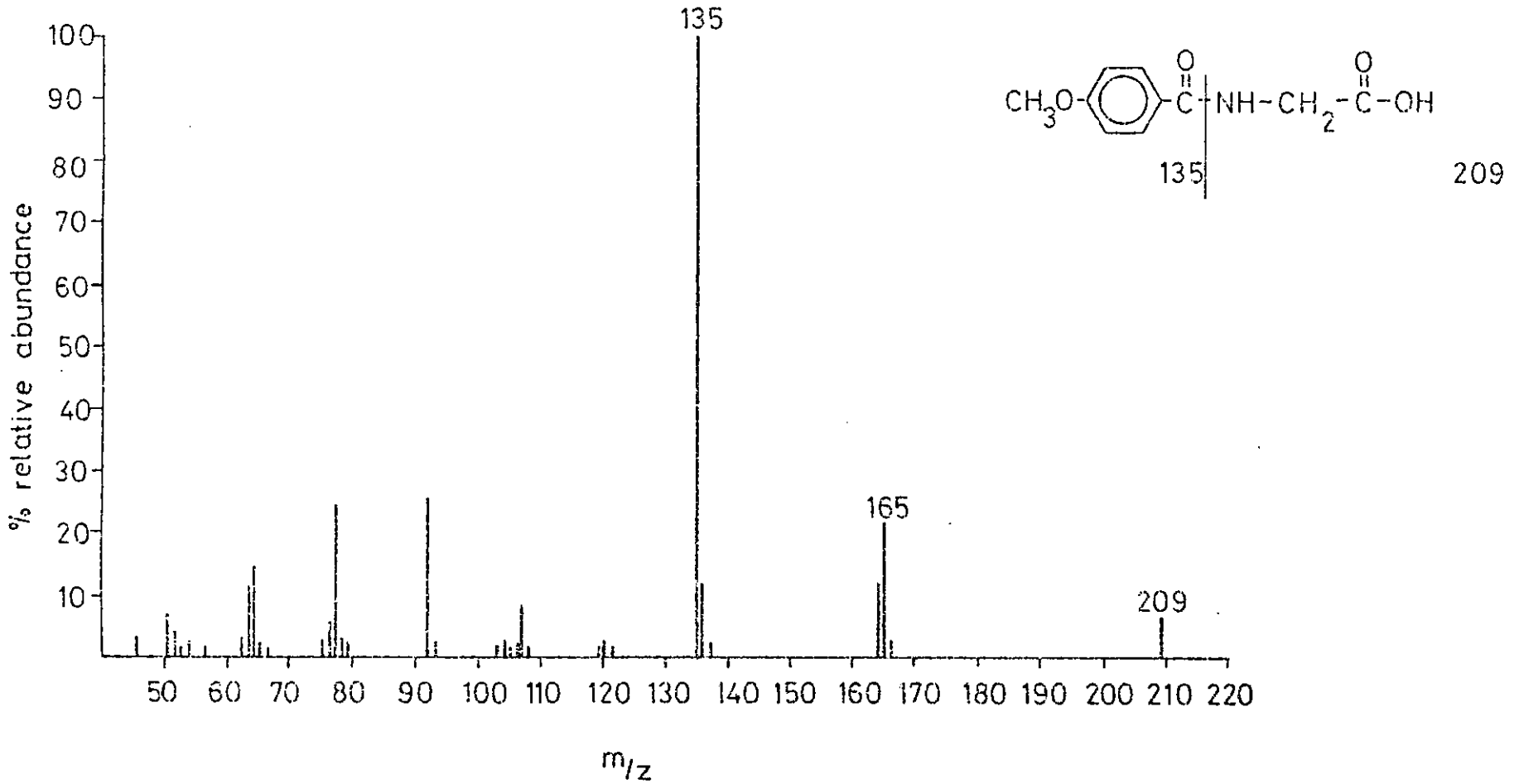
diphenyloxazole (PPO)	Fisons Scientific Apparatus. Leicestershire, UK.
1,4 di-2'-(5"-phenyloxazolyl)benzene (POPOP)	
4-methoxycinnamyl alcohol	Synthesised in the Department.
4-methoxycinnamoylglycine	
4-methoxyphenaceturic acid	
1-(4'-methoxyphenyl)propan-2-ol	

Appendix 2.2. Mass spectrum of trans-anethole

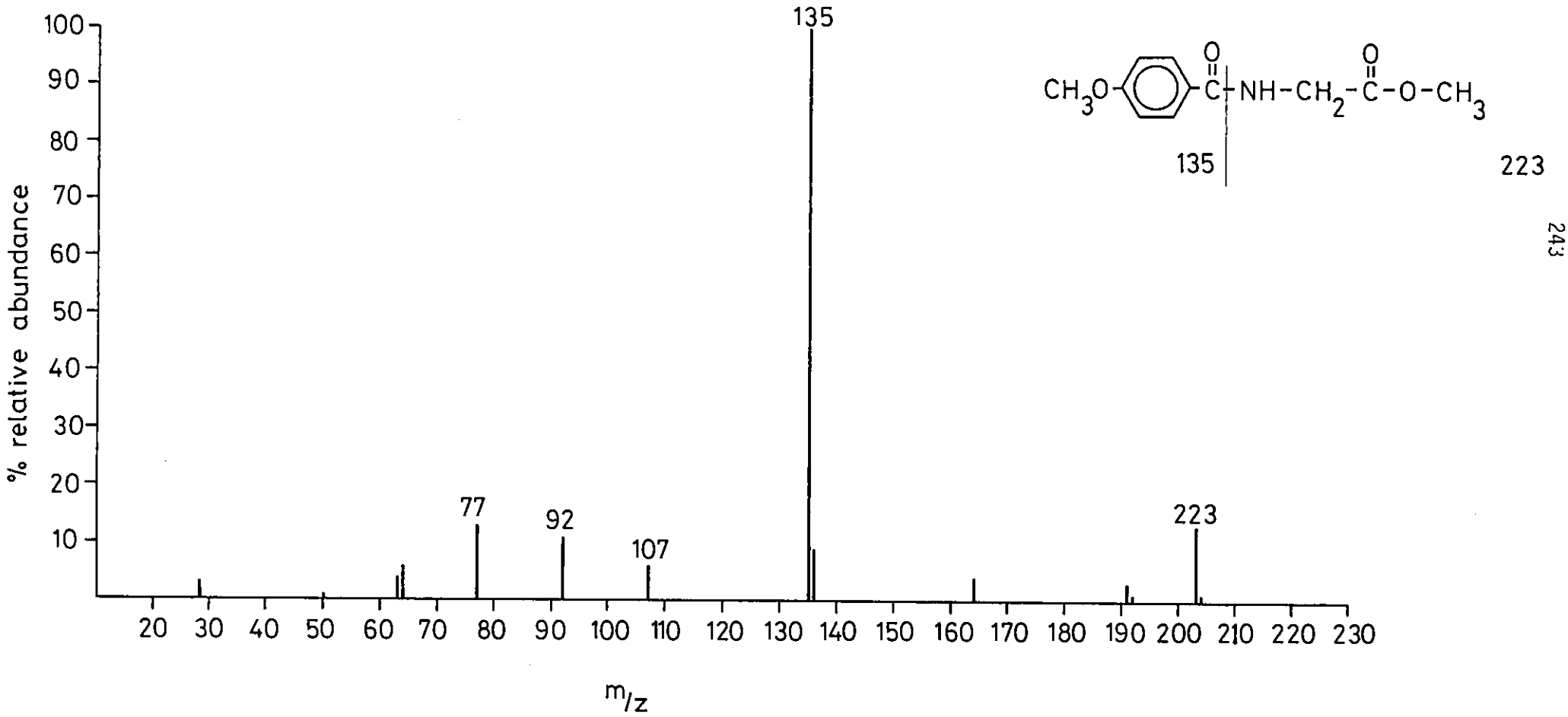




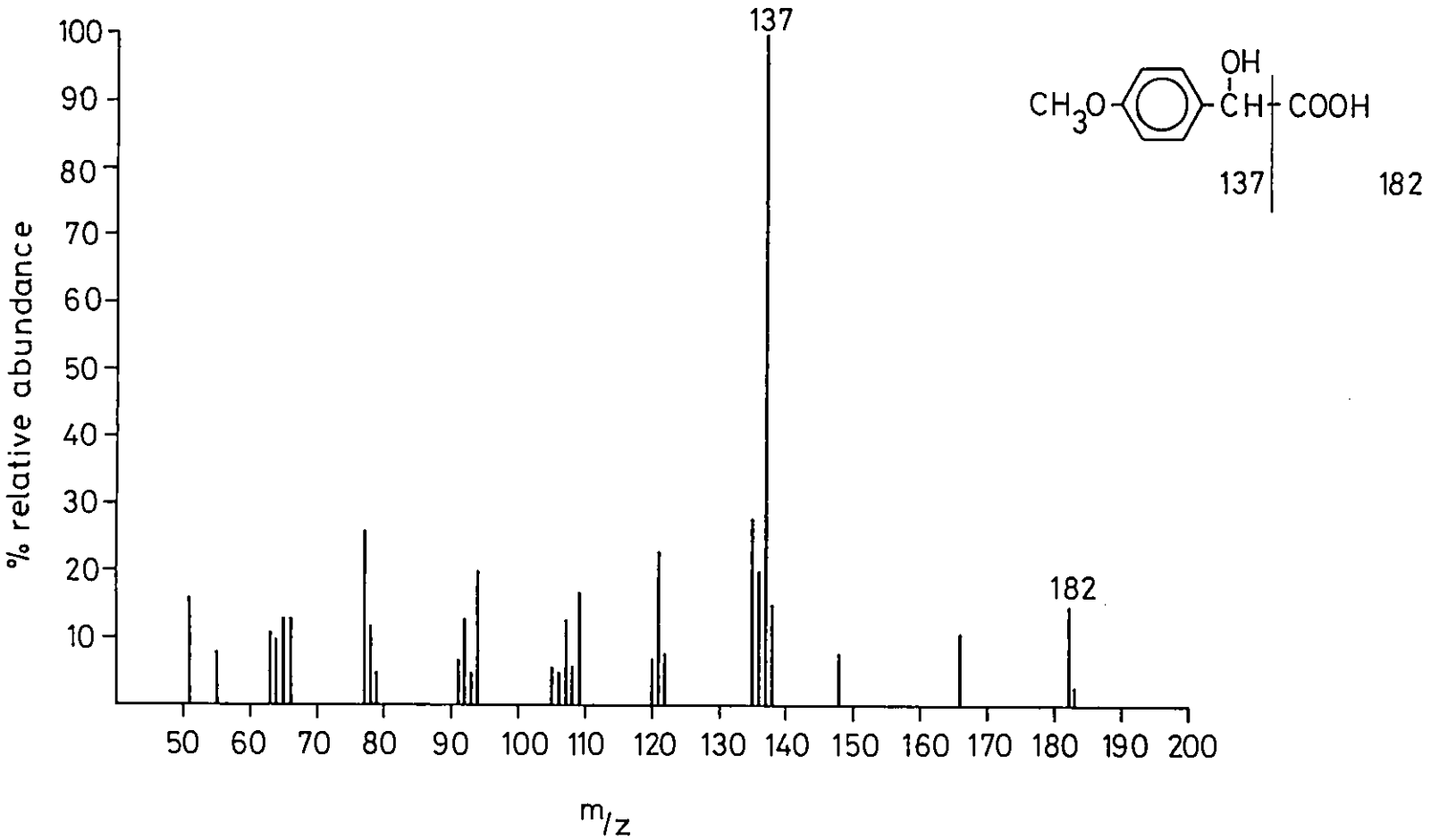
Appendix 2.3a.a)Direct probe mass spectrum of 4-methoxyhippuric acid and b) mass spectrum of 4-methoxyhippuric acid - methyl ester by gc-ms.



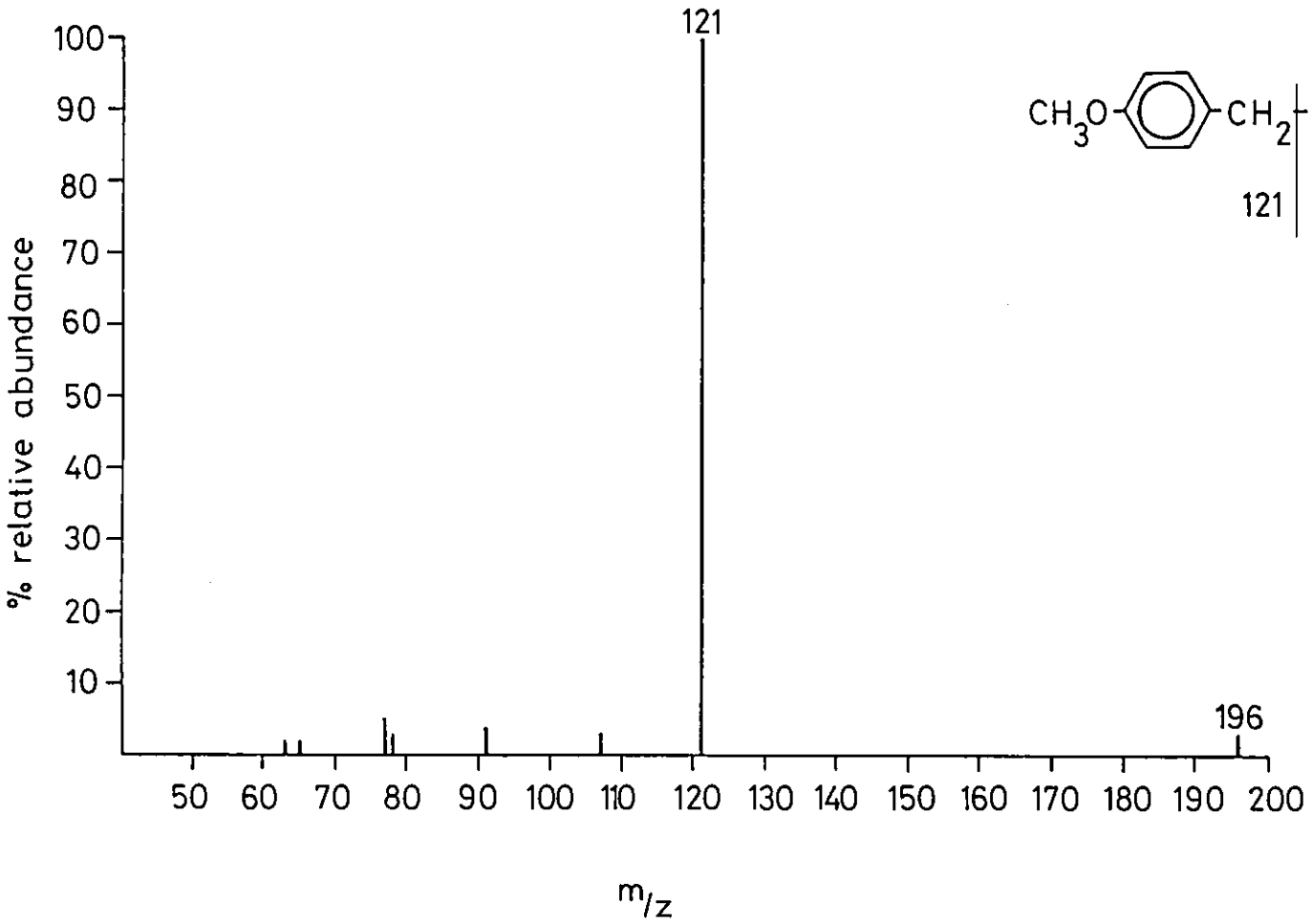
Appendix 2.3b.



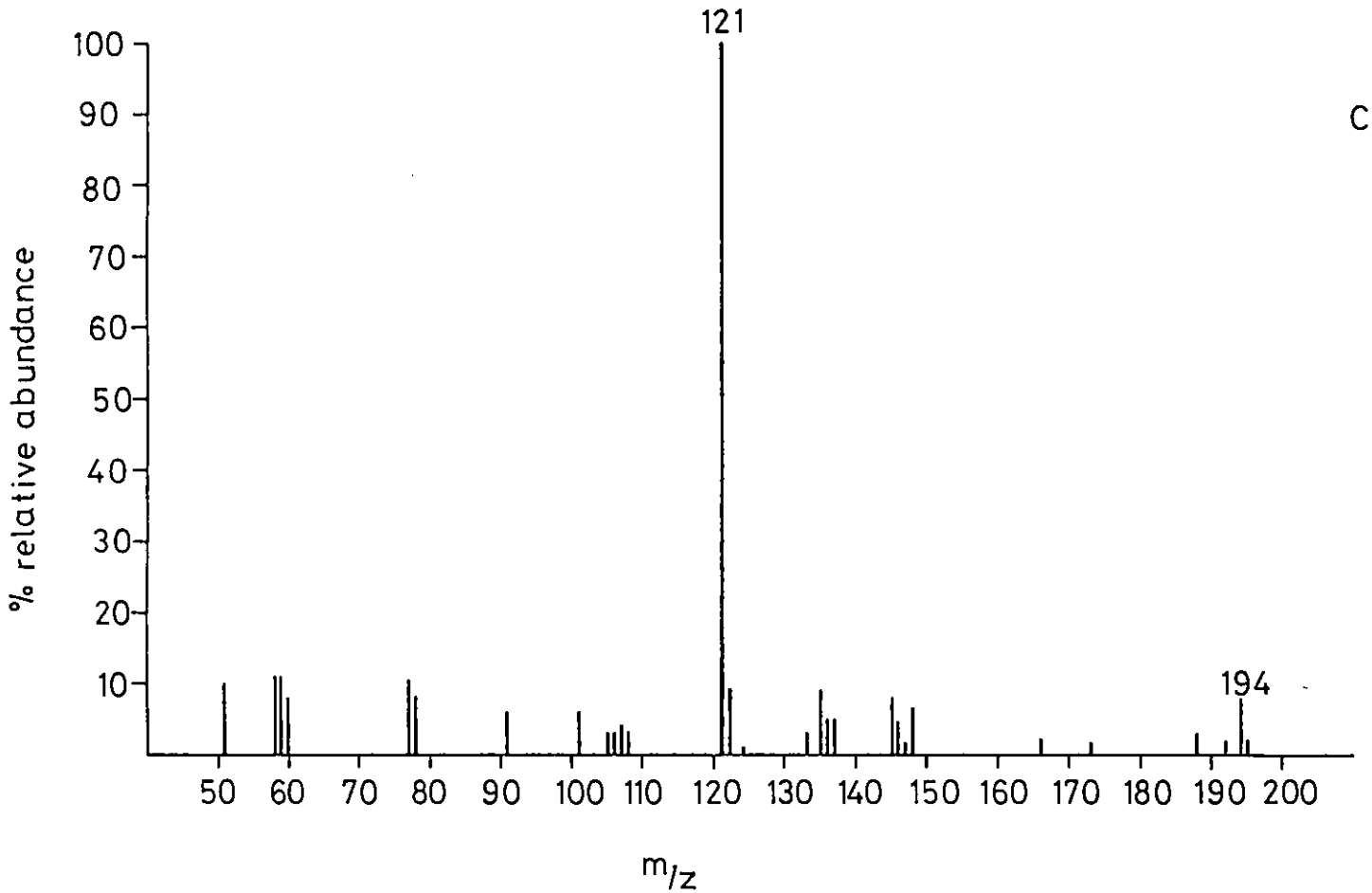
Appendix 2.4. Mass spectrum of 4-methoxymandelic acid obtained by gc-ms.

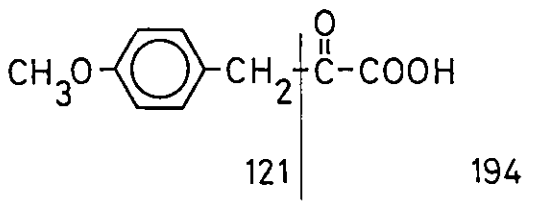


Appendix 2.5. Direct probe mass spectrum of 4-methoxyphenyllactic acid



Appendix 2.6. Direct probe mass spectrum of 4-methoxyphenylpyruvic acid

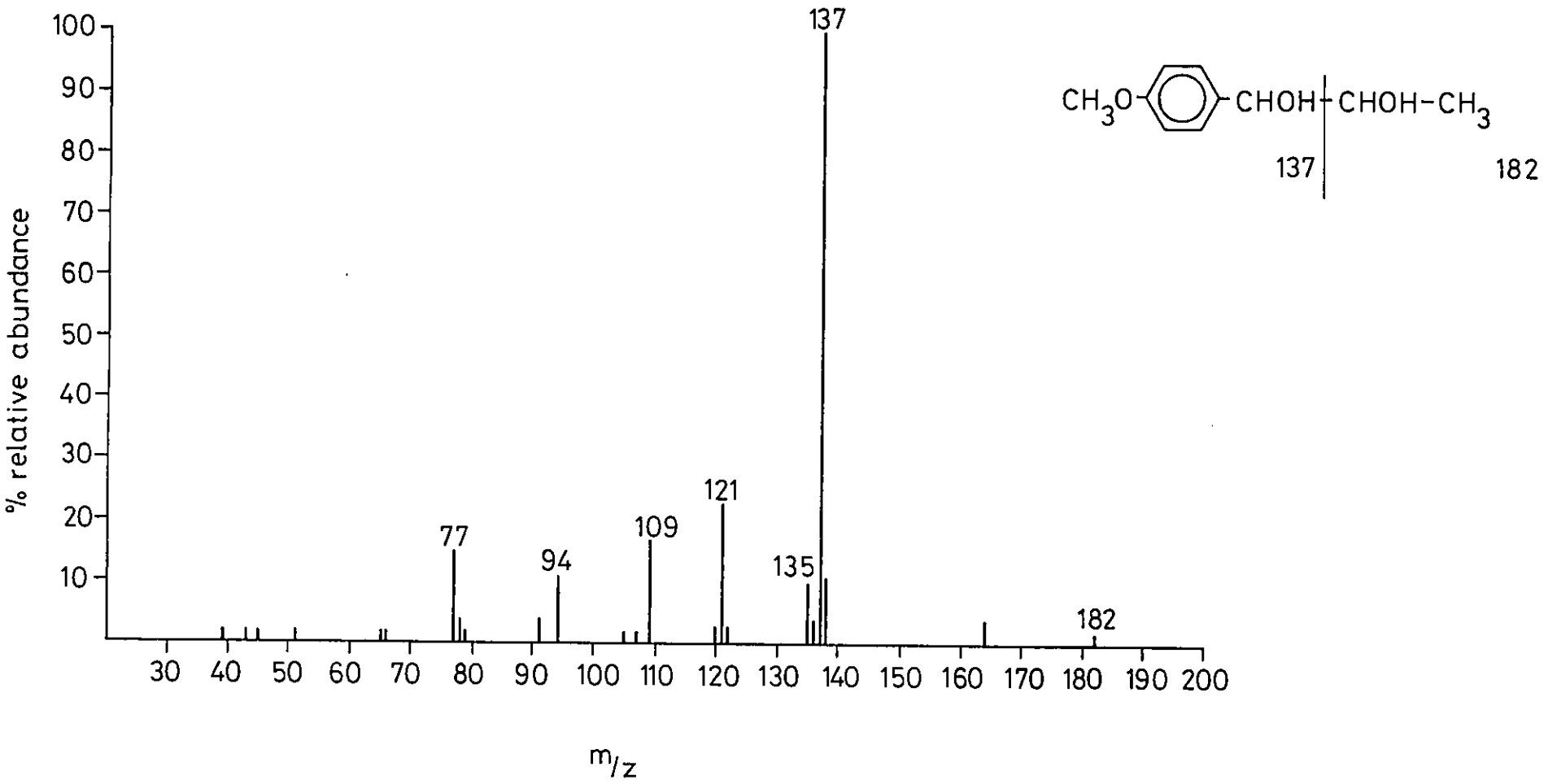




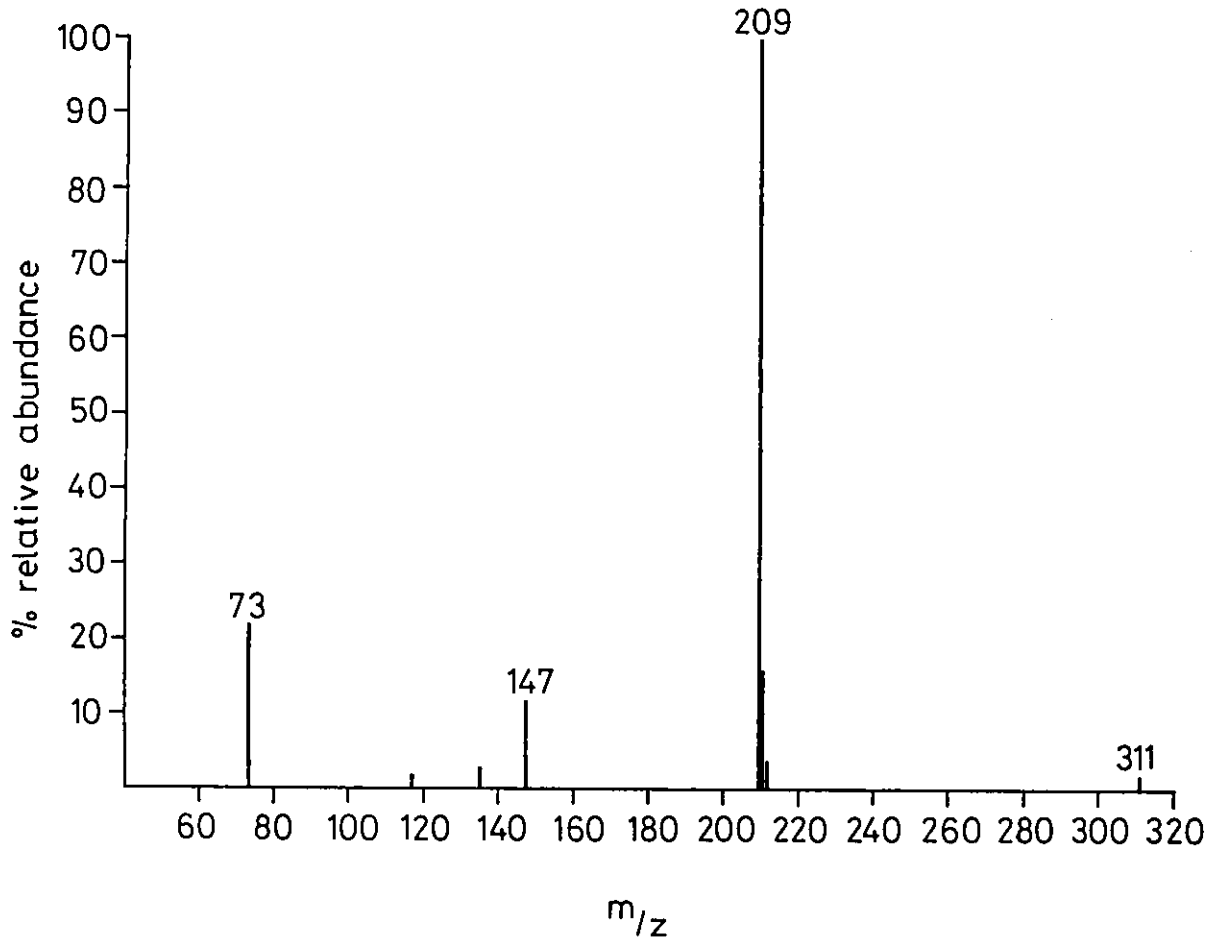
Appendix 2.7. Mass spectra of authentic 1-(4'-methoxyphenyl)propane-1,2-diol and the two metabolites isolated from rat urine both with similar characteristics, metabolite 1 and 2, after *trans*-anethole

Sample	$^{m}/_{z}$ values with % abundance
1-(4'-methoxypheny)propane-1,2-diol	182(2.3), 164(2.3), 138 (11.8), 137 (100), 135 (7.1) 122 (3.1), 121 (14.1), 109 (24.2), 107 (3.1), 94 (16.5), 77 (31.3)
Metabolite 1	182 (1.7), 164 (0.9), 138 (12.1), 137 (100), 135 (6.0), 122 (2.6), 121 (13.8), 109 (39.7), 107 (1.7), 94 (26.7), 77 (27.6).
Metabolite 2	182 (2.7), 164(2.3), 138 (12.7), 137 (100), 135 (8.0), 122 (4.0), 121 (24.0), 109 (38.7) 107 (9.3), 95 (25.4), 77 (34.7).

Appendix 2.8a. a)Mass spectrum of authentic 1-(4'-methoxyphenyl)propane-1,2-diol and b) the TMS derivative of 1-(4'-methoxyphenyl)propane-1,2-diol, both by gc-ms.



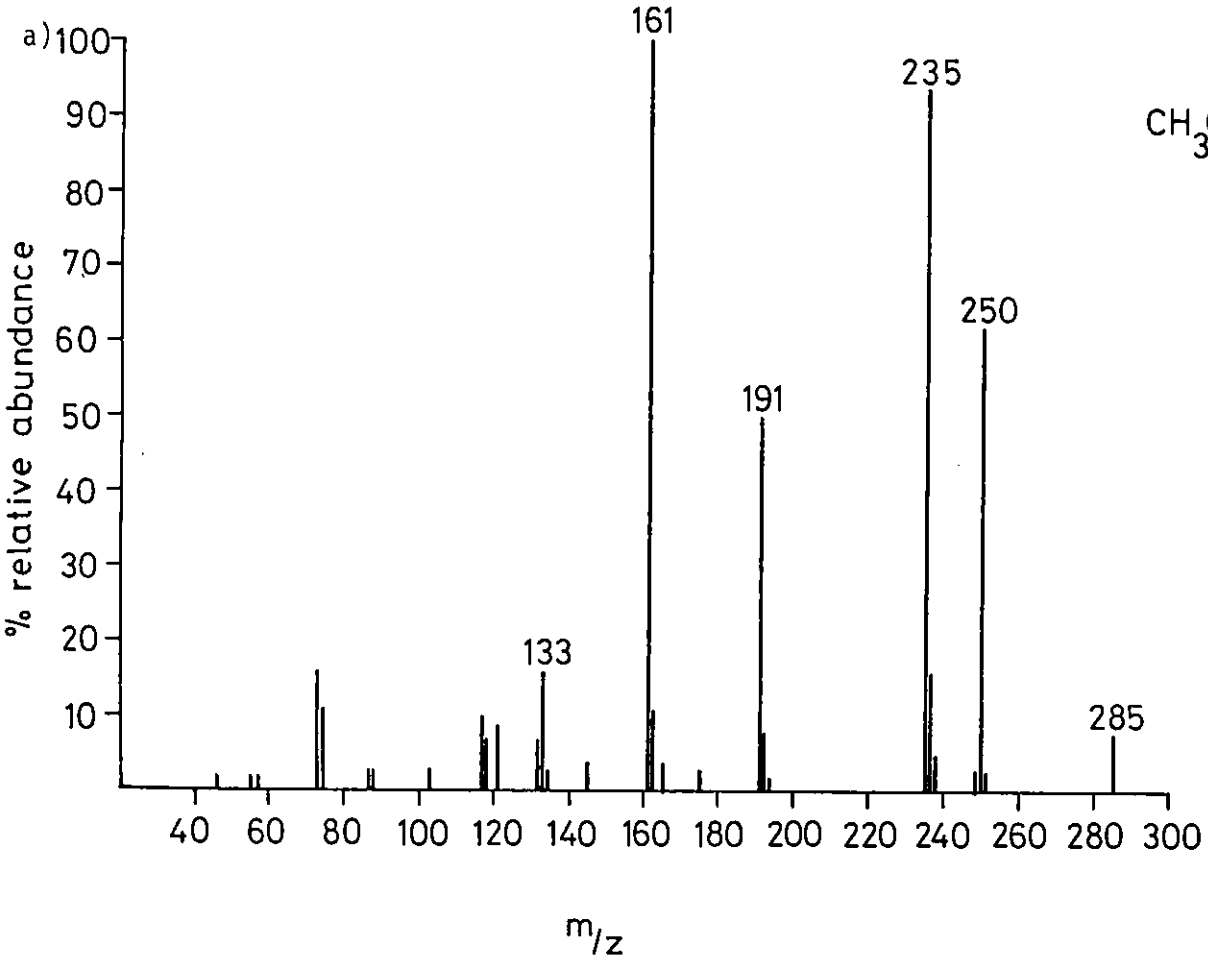
Appendix 2.8b

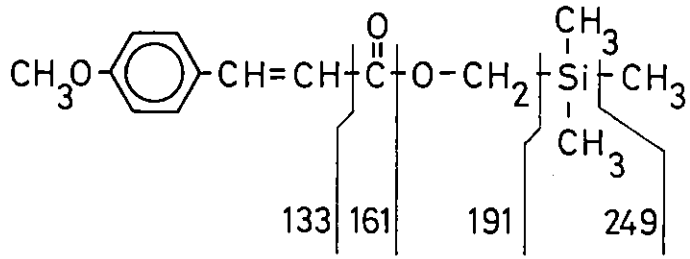


Appendix 2.9. Mass spectra by gc-ms of a) 4-methoxybenzoic acid, b) 4-methoxyacetophenone and c) 4-methoxycinnamyl alcohol

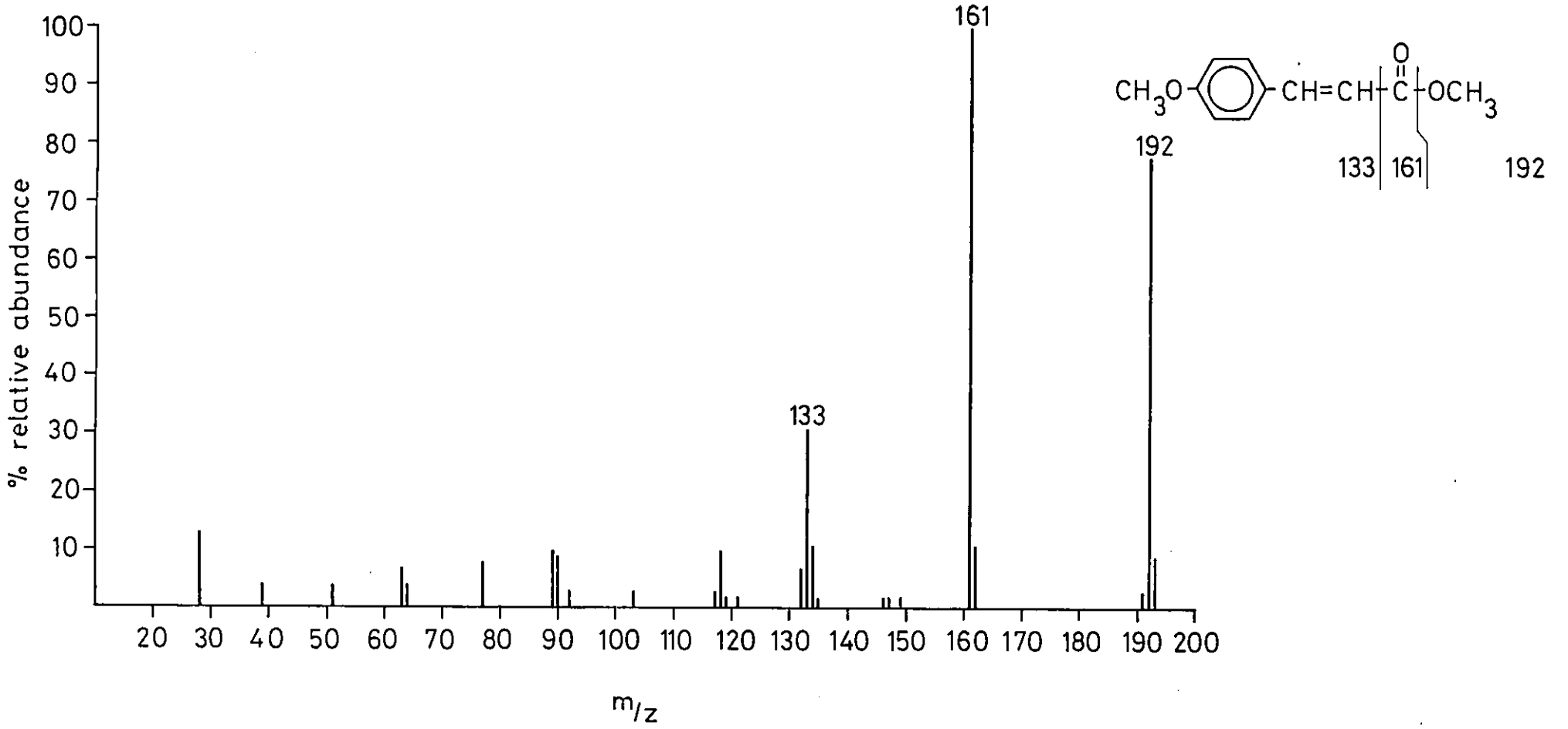
Standard	$^{m}/_{z}$ value with % relative abundance
4-methoxybenzoic acid	153 (7.9), 152 (92.5), 136 (8.6), 135 (100), 107 (13.5), 92 (11.3), 77 (21.2)
4-methoxyacetophenone	151(3.2), 150 (36.9), 136 (9.2), 135 (100), 107 (11.9), 92 (15.4), 77 (24.2)
4-methoxycinnamyl alcohol	165 (4.0), 164 (35.0), 121 (100), 108 (30.2), 103 (9.3), 91 (13.0), 77 (13.5)

Appendix 2.10. a) The mass spectra of the TMS derivative of 4-methoxycinnamic acid and b) trans-4-methoxycinnamic acid methyl ester by gc-ms.

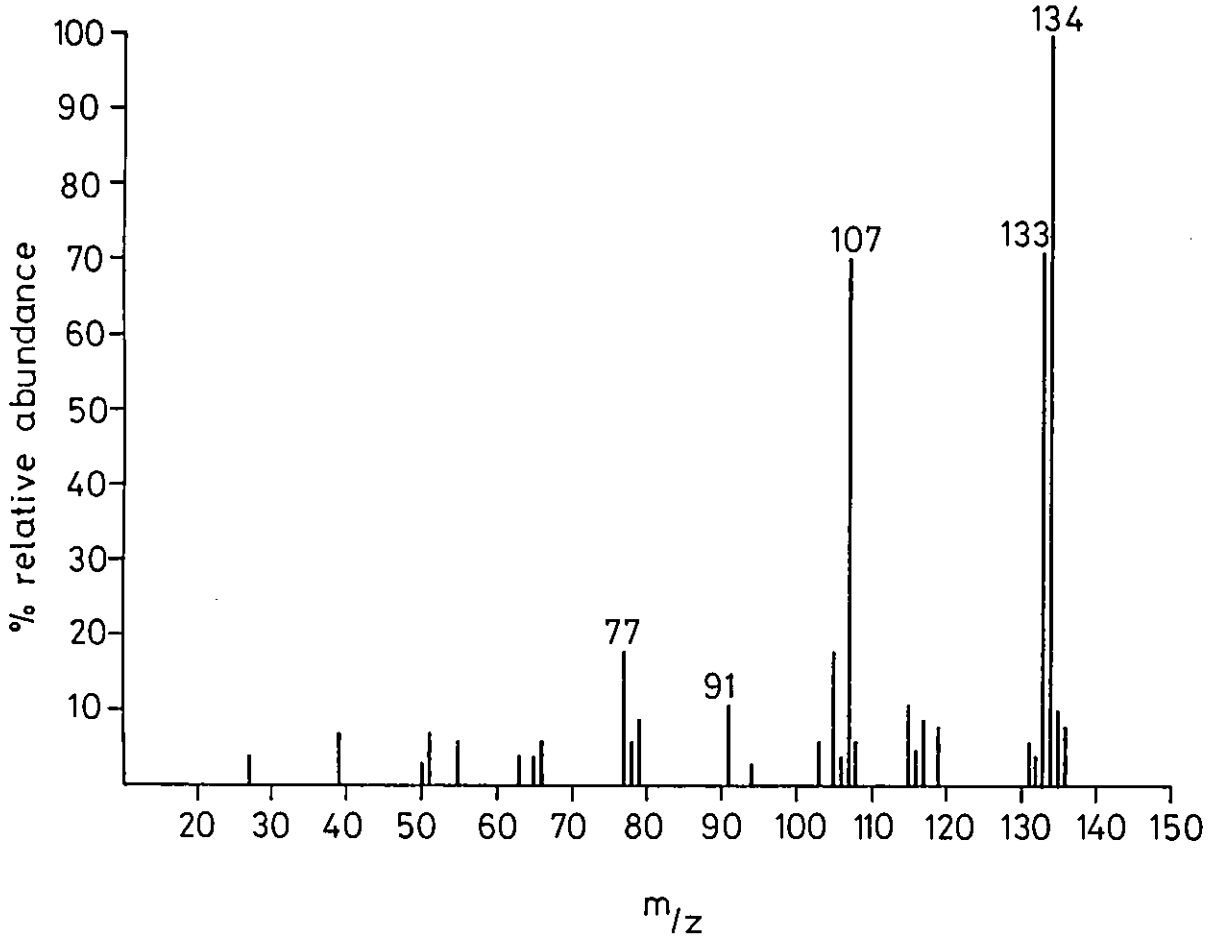


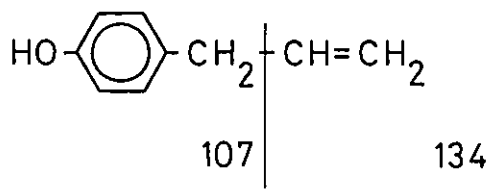


Appendix 2.10b.

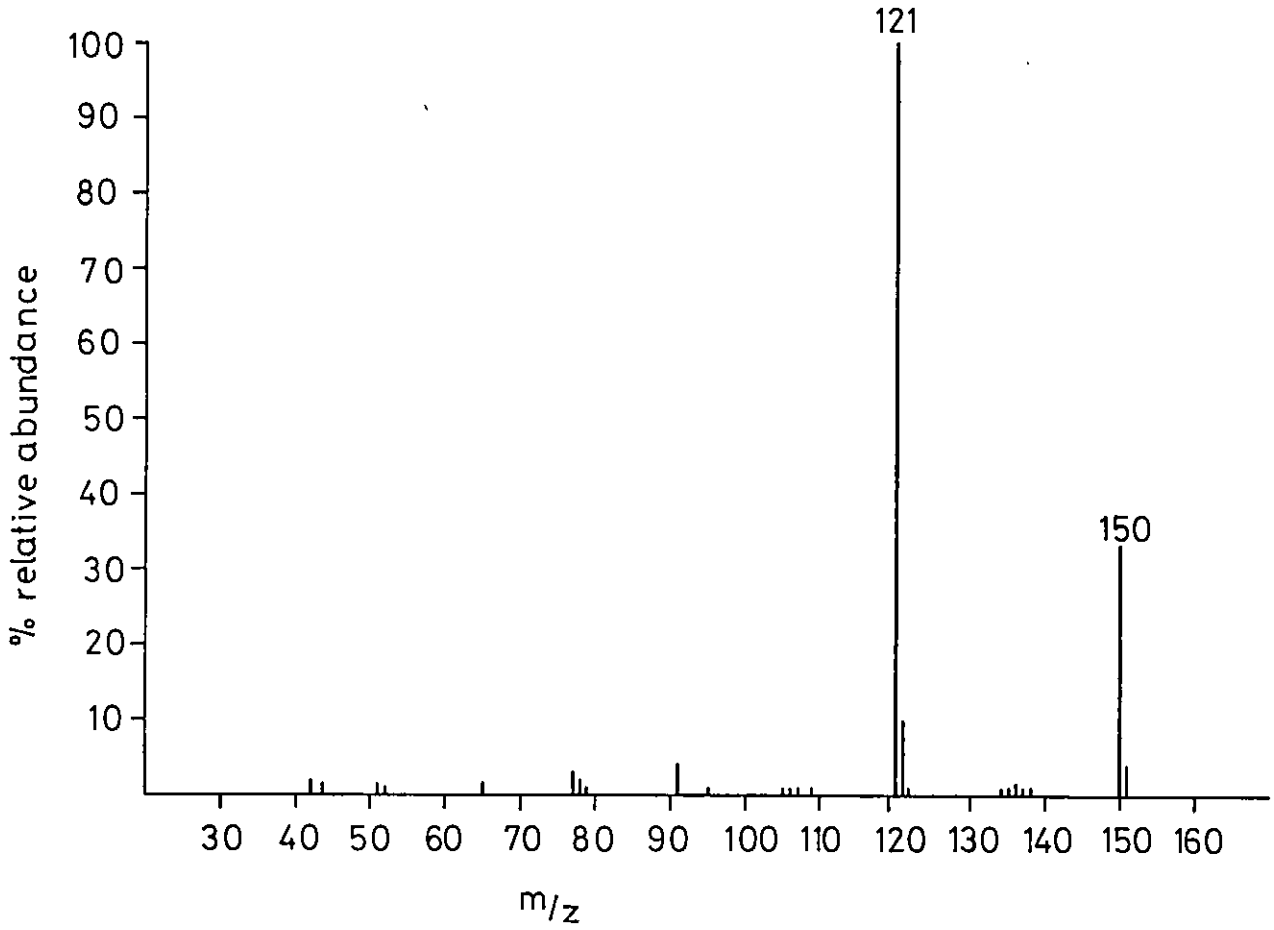


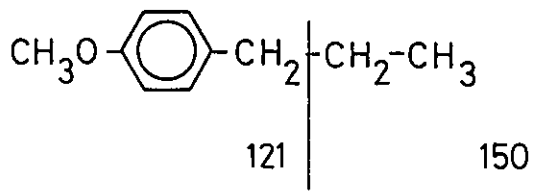
Appendix 2.11. Mass spectrum of chavical (4-hydroxy-allylbenzene) by gc-ms.





Appendix 4.1. Direct probe mass spectrum of p-propylanisole





Appendix 4.2. Direct probe mass spectra of 1 -(4'-methoxyphenyl)propan-1-ol, 1 (4'-methoxyphenyl)propan-2-ol and 1 -(4-methoxyphenyl)propan-3-ol

Compound	$^{m}/_{z}$ with % relative abundance
a. 1-(4'-methoxyphenyl)propan-1-ol	166(10.6), 148 (19.8), 137 (100), 13.5 (5.8), 121 (2.3), 77 (17.6)
b. 1-(4'-methoxyphenyl)propan-2-ol	166 (17.7), 122 (61.1), 121 (100), 107 (17.7), 91 (8.8), 77 (11.0).
c. 1-(4'-methoxyphenyl)propan-3-ol	166(25.3), 122 (12.0), 121 (100), 107 (2.1), 91 (7.1), 77 (8.2)

Appendix 4.3. Nmr spectra of a) 1-(4'-methoxyphenyl)propan-1-ol, b) 1-(4'-methoxyphenyl)propan-2-ol and c) 1-(4'-methoxyphenyl)propan-3-ol

a)

δ 0.90 (t,3,J = 7.4 Hz, CH_2 $C\underline{H}_3$)
δ 1.58 (broad 2, O<u>H</u>)
δ 1.77 (m,2, $C\underline{H}_2CH_3$)
δ 3.81 (s,3,$C\underline{H}_3O$)
δ 4.55 (t,1,J=6.6 Hz, C<u>H</u> OH)
δ 6.86 - 7.30 (M,4, Ar <u>H</u>)

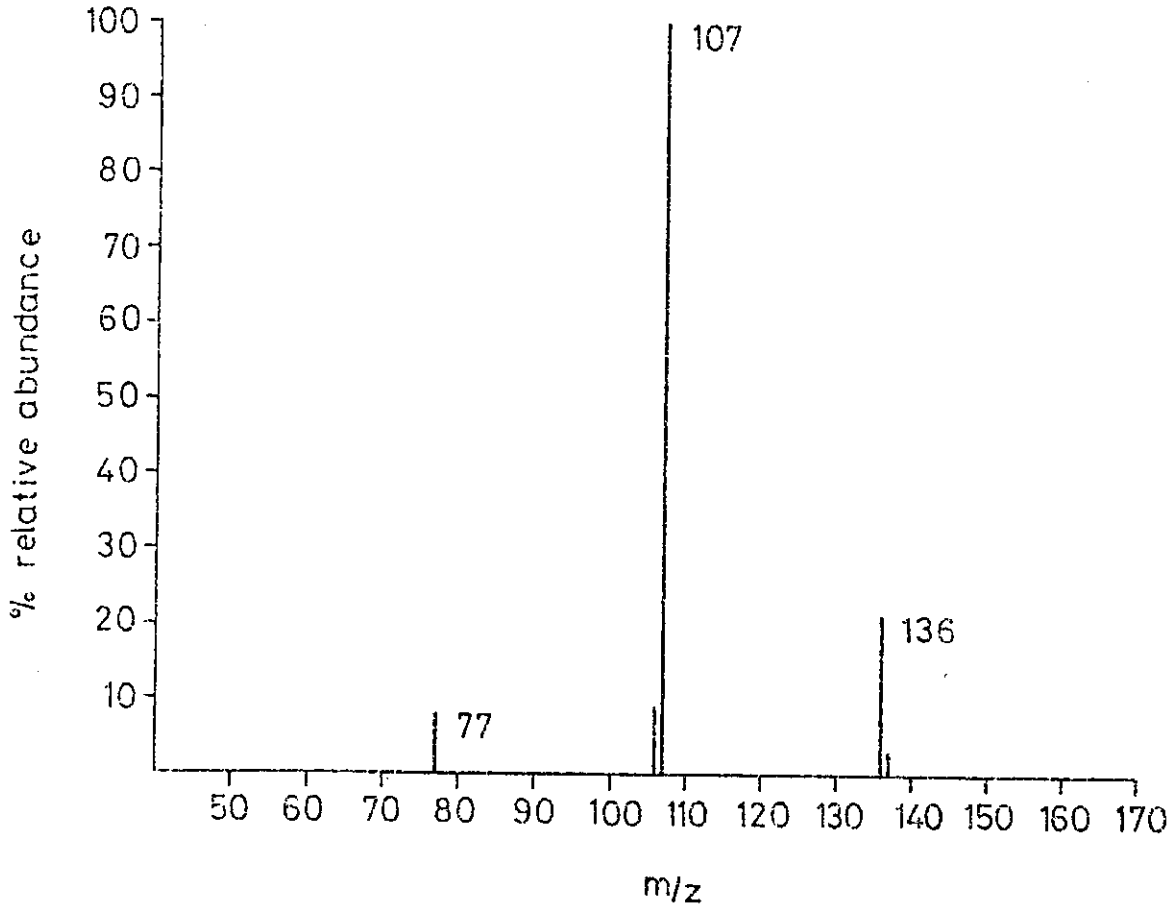
b)

δ 1.23 (d,3,J = 6.3Hz, $CHOHC\underline{H}_3$)
δ 1.66 (broad s, O<u>H</u>)
δ 2.67 (m,2,$C\underline{H}_2CHOH$)
δ 3.79 (s,3,$C\underline{H}_3O$)
δ 3.96 (M, 1, C<u>H</u>=H)
δ 6.83-7.26(M,4,Ar<u>H</u>)

c)

δ 1.85 (m,2,$C\underline{H}_2CH_2OH$)
δ 2.65 (t, 2, J = 7.5 Hz, $ArC\underline{H}_2CH_2$)
δ 3.67 (t, 2, J = 6.4Hz, $C\underline{H}_2OH$)
δ 3.79 (s, 3, $C\underline{H}_3O$)
δ 6.82-7.26 (m, 4, Ar<u>H</u>)

Appendix 4.4. Direct probe mass spectrum of 4-hydroxypropylbenzene



References

The style of references and abbreviations used are those recommended by the Biochemistry Society.

Ames, B.N., Durstan, W.E., Yamasaki, E. & Lee, F.D. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2281-2285.

Beaud, P. & Ramaz, A. (1975). Trav. Chim. Hyg. 66, 384-389.

Berenblum, I. (1974). Carcinogenesis as a Biological Problem. North-Holland Publishing Co. Amsterdam.

Blackburn, D. & Burghard, G. (1965). J. Pharm. Sci. 54, 1586-1590.

Bodley-Scott, R. (1981). Cancer. Oxford University Press.

Boissier, J.R., Simon, P & Le Bourhis, B. (1967). Therapie. 22, 309-323.

Booth, J. Boyland, E., Sato, T. & Sims, P. Biochem. J.(1960) 77, 182-186.

Borchert, P., Wislocki, P.G., Miller, J.A. & Miller, E.C. (1973a) Cancer Res. 33, 575-589.

Borchert, P., Miller, J.A., Miller, E.C. & Shires, T.K., (1973b). Cancer Res. 33, 590-600.

Boutwell, R.K. (1974). C.R.C. Crit. Rev. Toxicol. 2, 419-443.

Boutwell, R.K. (1978). In Origins of Human Cancer, Book B (eds. Hiat, H.H., Watson, J.D. & Winsten, J.A.) pp 773-783, Cold Spring Harbour, N.Y.

Boyland, E. & Sims, P. (1960). Biochem. J. 77, 175-181.

Boyland, E. & Williams, K. (1965). Biochem. J. 94, 190-197.

Bray, H.G. Ryman, B.E. & Thorpe, W.V. (1948). Biochem. J. 43, 561-567.

Bray, H.G. Thorpe, W.V. & White, K. (1951). Biochem. J. 48, 88-96.

Burke, M.D. & Orrenius, S. (1979). Pharmacol. Ther. 7, 549-599.

Butler, W.H. & Barnes, J.M. (1963). Br. J. Cancer. 17, 699-710.

Caldwell, J. (1980). In Concepts in Drug Metabolism Part A (ed. Jenner, P. and Testa, B.) pp.211-250, Marcel Dekker Inc.

Caldwell, J. (1981). Drug Metabolism Reviews, 12, 221-237.

Caldwell, J., Dring, L.G. & Williams, R.T. (1972). Biochem. J. 129, 11-22.

Caldwell, J., Idle, J.R. & Smith, R.L. (1970). In Extrahepatic Metabolism of Drugs and other Foreign Compounds (ed. Gram, T.E.) p.p. 453-492. Spectrum Publ. Holliswood, N.Y.

Caldwell, J. Köster, U., Smith, R.L. & Williams, R.T. (1975) Biochem. Pharmacol. 24, 2225-2232.

Caldwell, J., Lancaster, R., Monks, T.J., Smith, R.L. (1977). Brit. J. Clin. Pharmacol. 4, 637P-638P.

Capel, I.D., French, M.R., Millburn, P., Smith, R.L. & Williams, R.T. (1972). Xenobiotica 2, 25-34.

Caujolle, F., Meynier, D. (1958a). C.R. Acad. Sci. 246, 1465-1468.

Caujolle, F., Meynier, D. (1958b). C.R. Acad. Sci. 247, 1916-1917.

Central Statistical Office Annual Abstract of Statistics (1983). No. 119. (ed. Lawrence, E.) H.M.S.O., London.

Chain, E., Florey, H.W., Gardner, A.D., Heatley, N.G., Jennings, M.A., Orr-Ewing, J. & Sanders, A.G. (1940). Lancet, ii, 226-228.

Colombo, E. & Manitto, P. (1971). Parf. Cosm. Savons. France 1, 350-356.

Council of Europe (1970). Natural and Artifical Flavouring Substances. List A (1) Series 1. No. 184, p.58. Strasbourg.

Cramer, G.M., Ford, R.A. & Hall, R.L. (1978). Fd. Cosmet. Toxicol. 16, 255-276.

Crampton, R.F. & Charlesworth, F.A. (1975). Br. Med. Bull. 31, 209-213.

Dakin, H.D. (1909). J. Biol. Chem. 6, 203-219 and 221-233.

Daly, J.W., Jerina, D.M., Witkop, B. (1972). Experimentia, 28, 1129-1149.

Delbressine, L.P.C., Van Bladeren, P.J., Smeets, F.L.M., Seutter-Berlage, F. (1981). Xenobiotica 11, 589-594.

Devonshire, H.W., Kong, I., Cooper, M., Sloan, T.P., Idle, J.R. & Smith, R.L. (1983). Br. J. Clin. Pharmac. 16, 157-166.

Domagk, G. (1935). Deut. Med. Woch. 61, 250-260.

Dring, L.G. (1977). In Drug Metabolism from Microbe to Man (eds. Parke, D.V. & Smith, R.L.) pp.281-298. Taylor and Francis Ltd., London.

Drinkwater, N.R., Miller, E.C., Miller, J.A. & Pitot, H.C. (1976). J. Natl. Cancer Inst. 57, 1323-1331.

Drucker, M.M., Blondheim, S.H., Wislocki, L. (1964). Clin. Sci. 27, 133-141.

Dumas, J. (1832) Ann. Chim. Phys. 50,225-240.

ECETOC (1982). Hepatocarcinogenesis in laboratory animals: Relevance for Man. Monograph No. 4. Brussels.

El Masri, A.M., Smith, J.N. & Williams, R.T. (1956). Biochem. J. 64, 50-56.

El Masri, A.M., Smith, J.N. & Williams, R.T. (1958).
Biochem. J. 68, 199-204.

Emudianughe, T.S., Caldwell, J., Dixon, P.A.F. & Smith, R.L. (1978).
Xenobiotica 8, 525-534.

Emudianughe, T.S., Caldwell, J. & R.L. Smith
Biochem. Soc. Trans. (1979) 7, 522-523.

Erhlich, P. (1913). Lancet, ii, 445-451.

Farber, E. (1981). New Engl.J. Med. 305, 1379-1389

Fieser, L.F. & Fieser, M. (1967). Reagents for Organic Synthesis
2nd edn. p.136. John Wiley and Sons Inc. New York.

Fleming, A. (1929). Brit. J. Exp. Path. 10, 226-236.

Florey, M.E. & Florey, H.W. (1943). Lancet i, 387-397.

Food and Extract Manufacturers' Association (FEMA) Report 1970.
Washington, D.C.

Food and Extract Manufacturers' Association (FEMA) (1978)
Scientific Literature Review of Anisole and Derivatives in Flavor
Usage. Washington, D.C.

Food and Drug Administration (1982). Interim Report, Washington, D.C.

Food and Drug Administration (1983) "Red Book".

Fritsch, P., de Saint Blanquat, G., Derache, R. (1975). Fd. Cosmet. Toxicol. 13, 359-363.

Gerber, N. & Wagner, J.G. (1972). Res. Commun. Chem Pathol. Pharmacol. 3, 455-466.

Gerhardt, C. (1942). Lieb. Ann. 44, 318. Cited by Naves, Y.R. (1958b). Parf. Cosm. Savons 1, 1-3.

Gessner, P.K., Parke, D.V. & Williams, R.T. (1961) Biochem. J. 79, 482-489.

Gurdon, J.B. (1974). The Control in Gene Expression in Animal Development. Cambridge, Mass. Havard University Press.

Hagan, E.C., Hansen, W.H., Fitzhugh, O.G., Jenner, P.M., Jones, W.I., Taylor, J.M., Long, E.L., Nelson, A.A. & Brouwer, J.B. (1967). Fd. Cosmet. Toxicol. 5, 141-157.

Hernandez, O., Walker, M., Cox, R.H., Foureman, G.L., Smith, B.R. & Bend, J.R. Biochem. Biophys. Res. Commun. 96 1494-1502.

Homburger, F., Kelly, T., Friedler, G. & Russfield, A.B. (1961). Med. Exptl. 4, 1-11.

Illensee, K. and Mintz, B. (1976). Proc. Natl. Acad. Sci. U.S. 73, 549-553.

Inglis, B. (1981). The Diseases of Civilisation. Hodder and Stoughton Ltd.

James, S.P. & White, D.A. (1967). Biochem. J. 104, 914-921.

Janiaud, P., Delaforge, M., Levi, P., Maume, B.F. & Padieu, P. (1977). Coll. Int. CNRS No. 286, p.431.

Jaffray, H. & Alvarez, J. (1961). Analyt. Chem. 33, 612-615.

Jeffray, A.M. & Jerina, D.M. (1975). J. Am. Chem. Soc. 97, 4427-4428.

Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L. & Fitzhugh, O.G. (1964) Fd. Cosmet. Toxicol. 2, 327-343.

Jerina, J.M., Ziffer, H. & Daly, J.W. (1970). J. Am. Chem. Soc. 92, 1056-1060.

Joint FAO/WHO Expert Committee on Food Additives (1967). FAO Nutr. Mtg. Rep. Ser. No. 44A. Geneva.WHO/Food Add./68.33.

Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davies, D.S., Gillette, J.R. & Brodie, B.B. (1973). J. Pharmacol. Exp. Ther. 187, 195-202.

Jollow, D.J., Thorgeirsson, S.S., Potter, W.Z., Hashimoto, M. & Mitchell, J.R. (1974). Pharmacology 12, 251-271.

Jones, G. (1981). In Testing for Toxicity (ed. Gorrod, J.W.) Taylor and Francis Ltd., London.

Kakemi, K., Arita, T., Sezaki, H. & Hanano, M. (1963). J. Pharm. Soc., Japan. 83, 871-874.

Kapp, E.M. & Coburn, A.F. (1942). J. Biol. Chem. 145, 549-565.

Kings I. Ch. 10 v. 10. Old Testament. Authorised King James Version.

Knight, R.H. & Young, L. (1958). Biochem. J. 70,111-119.

Knoop, F. (1905). Beitr. Chem. Physiol. u. Path. 6 150-161.

Le Bourhis, B. (1968). Ann. Biol. Clin. 26 (5-6), 711-715.

Le Bourhis, B. (1970). Annls. Pharm. Fr. 28, 355-361.

Le Bourhis, B. (1973a). Propriétés Biologiques de Anéthole. Maloine S.A. Paris.

Le Bourhis, B. (1973b). Parfum. Cosm. Sav. France. 3, 450-456.

Le Bourhis, B. & Soene, A.M. (1973). Fd. Cosmet. Toxicol. 11, 1-9.

Leibman, K.C. & Ortiz, E. (1968). Pharmacologist 10, 203.

Leibman, K.C. & Ortiz, E. (1970). J. Pharm. Exp. Ther. 173, 242-246.

Leung, A.Y., (1980). Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics. John Wiley and Sons, N.Y.

Levy, G. & Matsuzawa, T. (1967). J. Pharmacol. Exp. Ther. 156, 285-293.

Levy, G. & Procknal, J.A. (1976). Clin. Chem. 22, 395.

Levy G. Tsuchiya, T. & Amsel, L.P. (1972). Clin. Pharmacol. Ther. 13, 258-268.

Levy, G. & Yamada, H. (1971). J. Pharm Sci. 60, 215-221.

Long, E.L. Nelson, A.A., Fitzhugh, O.G. & Hansen, W.H. (1964). Archs. Path. 75, 595-604.

Lundquist, F., Wolthers, H. (1958). Acta. Pharmacol. Toxicol. 14, 265-270.

McKeown, T. (1979). The Role of Medicine pp.29-44. Basil Blackwell, Oxford.

McKinnell, R.G., Deggins, B.A. & Labat, D.D. (1969). Science, 165 394-396.

Marsh, M.V., Caldwell, J., Hutt, A.J. & Smith, R.L. (1982). Biochem. Pharmacol. 31, 3225-3230.

Martin, E. & Berner, C. (1972). Mitt. Geb. Lebenmittelunters. Hyg. 63, 127-132.

Matsushima, T., Kakizoe, T., Kawachi, T., Hara, K., Sugimura, T., Takeucki, T. & Umezawa, H. (1976). In:Fundamentals in Cancer Prevention (eds. Magee, P.N., Takayama, S., Sugimura, T. & Matsushima, T.) pp. 57-68. Baltimore University Park Press.

Miller, E.C. (1978). Cancer Res. 38, 1479-1496.

Miller, J.A., Miller, E.C. (1981). Cancer, 47, 2327-2345.

Miller, J.A., Swanson, A.B. & Miller, E.C. (1979). In: Naturally Occurring Carcinogens - Mutagens and Modulators of Carcinogenesis (eds. Miller, E.C., Miller, J.A. Takayama, S., Sugimura, T. & Hirono, I.) pp.111-125. Baltimore University Park Press.

Miller, E.C., Swanson, A.B., Phillips, D.H., Lloyd-Fletcher, T. Liem, A. & Miller, J.A. (1983). Cancer Res. 43, 1124-1134.

Ministry of Agriculture Fisheries and Food (1976). Memorandum on Procedures for Submissions on Food Additives and on Methods for Toxicity Testing. H.M.S.O., London.

Mintz, B. & Illensee, K. (1977). Proc. Natl. Acad. Sci. U.S. 72. 3583-3589.

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R. & Brodie, B.B. (1973). J. Pharmacol. Exp. Ther. 187, 211-217.

Mitchell, J.R., Thorgeisson, S.S., Potter, W.Z., Jollow, D.J. & Keiser, H. (1974). Clin. Pharmacol. Ther. 16, 676-684.

Muir, C.S. & Kirk, R. (1960). Brit. J. Cancer 14, 597-608.

Naves, Y.R. (1958a). C.R. Ac. Sci. 246, 1734-1736.

Naves, Y.R. (1958b). Parf. Cosmet. Savon. 1, 1-3.

Naves, Y.R., Ardizio, P. & Favre, C. (1958). Bull. Soc. Chim. Fr. 4, 566-569.

Oesch, F., Jerina, D.M. & Daly, J. (1970). Biochim. Biophys. Acta. 227, 685-691.

Offices of Health Economics (1980). Medicines: 50 years of Progress (1930-1980).

Ohtsuji, H. & Ikeda, M. (1971). Toxicol. Appl. Pharmacol. 18, 321-328.

Opdyke, D.L.J. (1973). Fd. Cosmet. Toxicol. 11, 863-864.

Oser, B.L. & Hall, R.L. (1977). Fd. Cosmet. Toxicol. 15, 457-466.

Ota, K. & Hammock, B.D. (1980). Science, 207, 1479-1481.

Pachecka, J., Gariboldi, P., Cantoni, L., Belvedere, G., Mussini, E. & Salmona, M. (1979). Chem. Bio. Interact. 27, 313-321.

Passmore, R. & Robson, J.S. (1976). A Companion to Medical Studies. 2nd edn. Blackwell Scientific Publications.

Pearl, I.A. & Beyer, D.L. (1951). J. Org. Chem. 16, 221-224.

Peele, J.D. & Oswald, E.O. (1977). Biochim. Biophys. Acta. 497, 598-607.

Phillips, D.H., Miller, J.A., Miller, E.C. & Adams, B. (1981). Cancer Res. 41, 176-186.

Piper, W.N., Rose, P.Q., Leng, M.L., & Gehring, P.J. (1973). Toxicol. Appl. Pharmacol. 26, 339-351.

Potter, W.Z., Thorgeisson, S.S., Jollow, D.J. & Mitchel, J.R. (1974). Pharmacology 12, 129-145.

Purchase, I.F.H., Longstaff, E., Ashby-Styles, J.A., Andersen, D., Lefevre, R.A. & Westwood, F.R. (1976). Nature (London) 264, 624-627.

Quick, A.J. (1928). J. Biol. Chem. 77, 581-593.

Quick, A.J. (1931). J. Biol. Chem. 92, 65-85.

Quick, A.J. (1933). J. Biol. Chem. 101, 475-485.

Quilley, E. & Smith, M.J.H. (1952). J. Pharm. Pharmac. 4, 624-630.

Rowland, M. (1977). In: Drug Metabolism from Microbe to Man (eds. Parke, D.V. & Smith, R.L.) pp. 123-145. Taylor and Francis., London.

Ryan, A.J. & Bend, J.R. (1977). Drug Metab. Dispos. 5, 363-367.

Schachter, D. & Taggart, J.V. (1954). J. Biol. Chem. 208, 263-275.

Scheline, R.R. (1978). Mammalian Metabolism of Plant Xenobiotics. Academic Press.

Shelanski, M.V. (1958). Industrial Biology Research and Testing Laboratories (Unpublished Report). see F.E.M.A., 1978.

Smith, J.N., Smithies, R.H. & Williams, R.T. (1954). Biochem. J. 56, 317-320.

Smith, R.L. (1960). Ph.D. Thesis, University of London.

Smith, R.L. & Timbrell, J.A. (1974). Xenobiotica 4, 489-501.

Smoking and Health (1964). Report of the Advisory Committee to the Surgeon General of the Public Health Service. Public Health Service Publication No. 1103, pp.149-196. Washington, D.C. U.S. Government Printing Office.

Solheim, E. & Scheline, R.R. (1973). Xenobiotica 3, 473-510.

Stilwell, W.G., Carman, M.J., Bell, L., Horning, M.G. (1974). Drug Metab. Dispos. 2, 489-498.

Strolin-Benedetti, M. & Le Bourhis, B. (1972). C.R. Acad. Sci. Paris Series D. 274, 2378-2381.

Strolin-Benedetti, M. Malnoë, A. & Louis-Broillet, A. (1977). Toxicology 7, 69-83.

Sugimura, T., Wakabayashi, K., Yamada, M., Nagao, M. & Fujina, T. (1980). In: Mechanisms of Toxicity and Hazard Evaluation (eds. Holmstedt, B., Lauwerys, R., Mercier, M. & Roberfroid, M.) pp. 205-217.

Swanson, A.B., Chambliss, D.D., Blomquist, J.C., Miller, E.C. & Miller, J.A. (1979). Mutation Res. 60, 143-153.

Tanon, L. (1938). see Le Bourhis, B., (1973a).

Taylor, J.M., Jenner, P.M. & Jones, W.I. (1964). Toxicol. appl. Pharmac. 6, 378-387.

Toennis, G. & Kolb, J.J. (1951). Analyt. Chem. 23, 823-826.

Tomatis, L. (1979). Ann. Rev. Pharmacol. Toxicol. 19, 511-530.

Tomatis, L., Agthe, C., Bartsch, H., Huff, J., Montesano, R., Saracci, R., Walker, E. & Wilbourne, J. (1978). Cancer Res. 38, 877-885.

Toth, L. (1967). Planta Medica. 15, 371-389.

Troll, W. (1976). In: Fundamentals of Cancer Prevention (eds. Magee, P.N., Takayama, S., Sigimura, T. & Matsushima, T.) pp.41-55. Baltimore University Park Press.

Vignoli, L., Cristeau, B. & Carboni, M. (1949). Med. Tropic. 6, 991-997.

Vignoli, L. Morel, M.C. & Simon, O. (1965). Rev. Pathol. Comparee. 65, 281-287.

Wagner, J.G. (1974). In: Pharmacology and Pharmacokinetics (eds. Teorell, T., Dedrick, R.L. & Condliffe, R.G.) p.27. Plenum Press, N.Y.

Watabe T., Hiratsuka, A., Ozawa, N. & Isobe, M. (1981). Biochem. Pharmacol. 30, 390-392.

Watabe, T. & Maynert, E.W. (1968). Pharmacologist 10, 203.

Watabe, T., Ozawa, N. & Hiratsuka, A. (1983). Biochem. Pharmacol. 32, 777-785.

Watabe, T., Sawahata, T. & Horie, J. (1979). Biochem. Biophys. Res. Commun. 87, 469-475.

Wendt, A.S. (1971). The Flavor Industry 2, (8), 455-459.

White, A., Handler, P. & Smith, E.L. (1973). Principles of Biochemistry 5th End. McGaw-Hill.

Wislocki, P.G. Borchert, P., Miller, J.A., Miller, E.C. (1976). Cancer Res. 36, 1686-1695.

Wislocki, P.G., Miller, E.C., Miller, J.A., McCoy, E.C. & Rosenkranz, H.Z. (1977). Cancer Res. 37, 1883-1891.

Young, Y.D., Braun, W.H., Le Beau, J.E. & Gehring, P.J. (1976). Toxicol. Appl. Pharmacol. 37, 138.

Zangouras, A. (1982). Ph.D. Thesis. University of London.

Zangouras, A., Caldwell, J., Hutt, A.J. & Smith, R.L. (1981). Biochem. Pharmacol. 30, 1383-1386.