THE EFFECTS OF ETHANOL ON CATECHOLAMINE AND SEROTONIN METABOLISM IN MAN

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ABSTRACT

Chemical and gas chromatographic methods for the estimation of catecholamine and serotonin metabolites in normal urine have been investigated with the aim of applying them to the study of the effects of ethanol on biogenic amine metabolism. It was concluded that both methods would be incapable of accurately demonstrating any changes in urinary metabolite levels that were expected to occur as a consequence of ethanol ingestion.

A GCMS technique for quantitating five acidic catecholamine and serotonin metabolites was developed, and was found to exhibit excellent specificity and sensitivity. When applied to the analysis of alcoholic metabolites, the technique was subject to interference from extraneous compounds, and further development is required.

The GCMS technique was applied to the analysis of catecholamine and serotonin metabolites in the urine of normal male adults who had ingested ethanol. It was concluded that ethanol induces a shift in metabolism away from oxidative toward reductive pathways for adrenaline, noradrenaline and serotonin, but not for dopamine. An increased HVA excretion observed after ethanol ingestion was shown to be possibly due to the diuretic effect of ethanol.

This work provides a clarification of the diverse results previously reported in the literature, but it was, however, concluded that there are still several aspects of this field of alcohol research that require estensive investigation before a complete understanding of the ways in which ethanol influences catecholamine and serotonin metabolism can be achieved.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

GENERAL

GC, GLC	gas chromatography, gas-liquid chromatography
MS	mass spectrometry
GCMS	gas chromatography-mass spectrometry
FID	flame ionisation detection
ECD	electron capture detection
MID	multiple ion detection
SIM	selected ion monitoring
HPLC	high performance liquid chromatography
REA	radioenzymatic assay

CHEMICALS, METABOLITES, ENZYMES

CA	catecholamine, catecholamine metabolites
IA	indoleamine, indoleamine metabolites
PG	propyl gallate
Res	rescorcinol
HGA	homogentisic acid
TMS	trimethylsilyl

All other metabolite and enzyme abbreviations are either explained in the text or given in the key to Figs. 1(b) and 1(c).

STATISTICAL

$\bar{x}$	mean
s.d.	standard deviation
s.e.	standard error
a	ordinate (y axis) intercept
b	regression coefficient
s_b	standard deviation of the regression coefficient (b)
n	sample number

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The Aim of This Study

The primary aim of this study is to evaluate the various methods used for the determination of urinary catecholamine and indoleamine (serotonin) metabolites, and to develop techniques for the analysis of samples obtained from human volunteers after consuming alcohol. The present investigation is part of a research programme that has been initiated to provide a greater understanding of the ways in which ethanol elicits changes in metabolic pathways.

1.2 The Physiological and Behavioural Roles of the Catecholamines and Serotonin

The distribution and the diverse behavioural and physiological roles that these compounds play, both as neurotransmitters and as neuroendocrine effectors, are outlined in Table 1(i). Because of the important part that the catecholamines and serotonin seem to play in the CNS, several speculations have arisen as to their role in modifying behaviour. It was, therefore, not surprising that in view of the effects of ethanol on behaviour, studies on the catecholamines and serotonin would be undertaken, hence this field of alcohol research currently represents one of the most intensive areas investigated.

1.3 The Mechanism of Neurotransmission by Catecholamines and Serotonin

1.3.1 Storage (Molinoff and Axelrod (1971), Seiden and Dykstra (1977))

Catecholamines in sympathetic nerves or in the adrenal medulla are stored in membrane-bound chrommaffin granules. This serves to inactivate the amines temporarily and to protect them from enzymatic destruction until they are released by an appropriate stimulus. The granules contain predominantly noradrenaline, but can also take up adrenaline, dopamine and serotonin. In serotonergic nerve cells serotonin is believed to be bound to similar dense-core granules. Evidence suggests that the granules are synthesized in the cell body, and migrate along the axon to the synaptic ending.

Table 1(i) Distribution and Physiological and Behavioural Roles of the Catecholamines and Serotonin

Compound	Distribution	Physiological and Behavioural Roles
Dopamine	Found in both the central (striatum, limbic structures, terminals ending in the functional neocortex) and peripheral nervous systems. Is a precursor of both NA and A.	Involved in prolactin secretion, is deficient in Parkinson's disease and is suggested to be involved in the pathogenesis of schizophrenia.
Dopamine and Noradrenaline		Involved in motor behaviour, aggression, food intake and schedule controlled behaviour.
Noradrenaline	Found in the adrenal medulla and in chrommaffin cells scattered throughout the body. NA is highly localized in peripheral postganglionic sympathetic nerves and is found in several areas of the CNS.	NA release from neuroeffector sites increases blood pressure, heart rate and stroke volume. Dilates the blood vessels supplying skeletal muscle, constricts those supplying the gut and skin with an overall increase in peripheral resistance. Get decreased GI tract motility and mydriasis (enlargement of pupils). Increases secretion from salivary and sweat glands and causes A and NA secretion from the adrenal medulla.
Noradrenaline and Adrenaline		Inhibit insulin secretion, increase mobilization of free fatty acids, stimulate the metabolic rate. NA and A both produce increased alertness, in humans A usually evokes more excitement and fear. Increased CA secretion is an important endocrine response to cold.
Adrenaline	Functions mainly as a hormone, being released into the circulation primarily from the adrenal medulla. Small amounts have also been found in mammalian brain and heart. Is found in chrommaffin cells.	Decreases peripheral resistance. Causes glycogenolysis by activating phosphorylase enzyme.
Serotonin	Centrally, is found mostly in a small part of the pons called the raphé system. Also found in many regions of the brain, and in cerebellar and spinal projections. Peripherally, 5-HT is found in blood platelets and in the GI tract (in enterochrommaffin cells and the mysenteric plexus).	Is a powerful smooth muscle stimulant, and a vasoconstrictor. 5-HIAA, the major serotonin metabolite is excreted in excessive quantities in metastatic carcinoid syndrome. Centrally, is purported to be involved in temperature regulation, sleep, seizure disorders, extrapyramidal function, mental deficiency, aggression and hypersexuality, affective disorders, pain perception and narcotic analgesics, and psychotic behaviour.

Ref: Seiden and Dykstra (1977), Molinoff and Axelrod (1971), Ganong (1975).

1.3.2 Release (Kelly et al. (1979))

It now appears likely that the catecholamines and serotonin are released directly from the granules by exocytosis. This release occurs as an all-or-none phenomenon with respect to any granule and all of the soluble contents are released. Calcium has been shown to have a role in release from vesicles in the adrenal medulla and in peripheral nerve terminals.

1.3.3 Synaptic Transmission (Seiden and Dykstra (1977))

Synaptic transmission by the neurotransmitters dopamine, noradrenaline and serotonin is represented diagramatically in Fig. 1(a). Neurotransmitters released by the nerve endings following presynaptic stimulation diffuse across the synaptic gap and interact with receptor sites on the post-synaptic membranes of nearby neurons. As a result of these interactions, a post-synaptic potential is developed.

1.3.4 Reuptake and Inactivation (Molinoff and Axelrod (1971), Seiden and Dykstra (1977))

Reuptake is the major mechanism for inactivation, particularly in tissues with rich adrenergic innervation. In sparsely innervated tissues, catechol-O-methyl transferase (COMT) has been shown to be important in terminating the effects of adrenaline. Catecholamines discharged into the blood stream either from the adrenal gland or by overflow from neuronal release are primarily inactivated by liver and kidney COMT and monoamine oxidase (MAO), or by reuptake by sympathetically innervated organs. Reuptake and oxidation by MAO are also thought to be involved in serotonin inactivation.

1.4 Effects of Ethanol on Catecholamine and Serotonin Turnover and Secretion

1.4.1 Animal Studies

Corrodi et al. (1966) using α-methyl-p-tyrosine to inhibit catecholamine biosynthesis, found that ethanol accelerates depletion of the noradrenaline content of rat brain, but not that of dopamine. These workers suggested that ethanol acts as a specific activator of noradrenergic neurons. In a further study, Carlsson et al. (1973) examined the synthesis of $[^3H]$-catecholamines from $[^3H]$-tyrosine in brain. They found an increase in $[^3H]$-dopamine and $[^3H]$-noradrenaline, and that

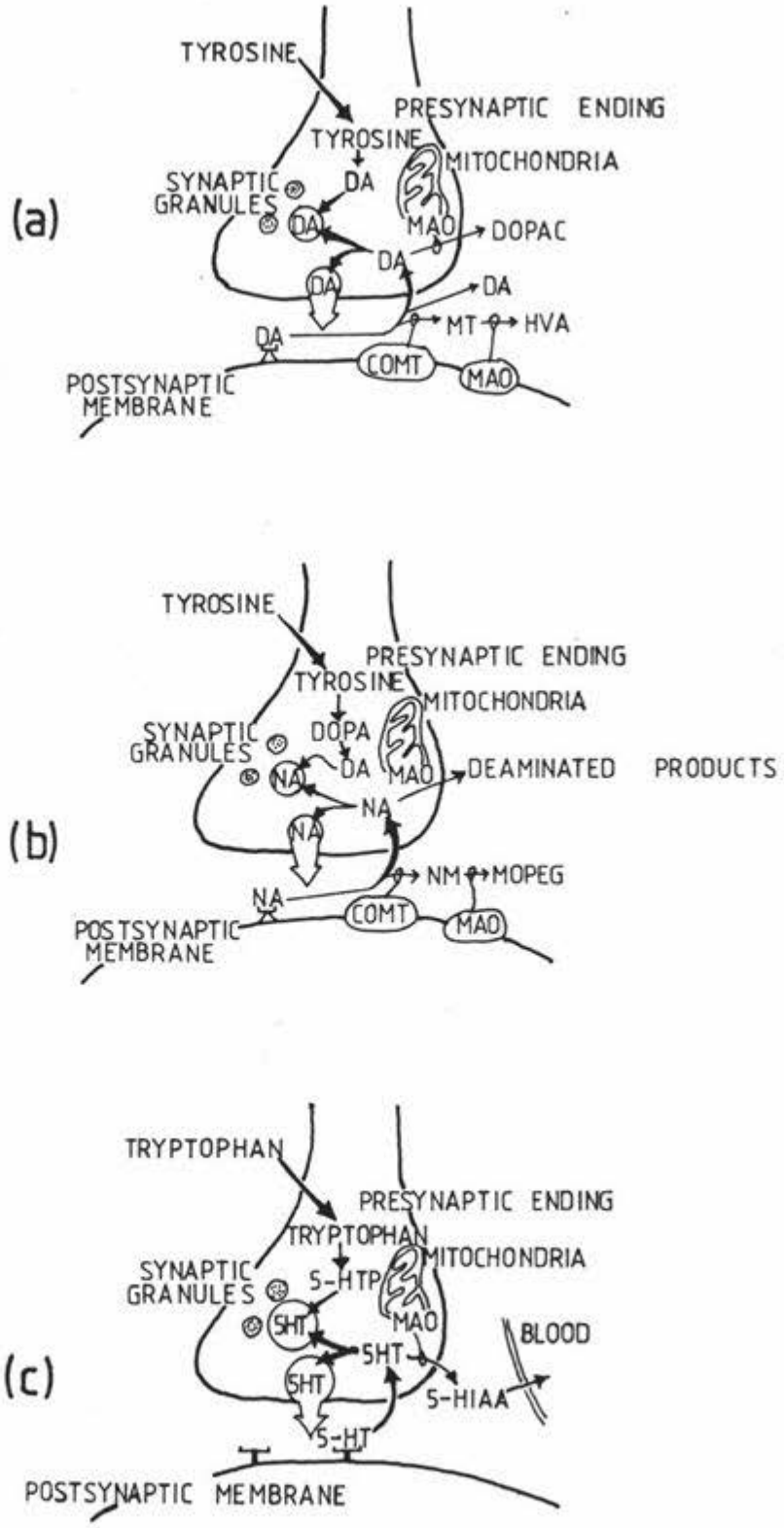


Fig. 1(a) Schematic models of the neuron; (a) dopamine, (b) noradrenaline and (c) serotonin, illustrating synthesis, storage in granules, release, and inactivation through reuptake and enzymatic degradation (from Seiden and Dykstra (1977)).

the ratio of [^{3}H]-dopamine/[^{3}H]-noradrenaline was significantly increased by ethanol. Hunt and Majchrowicz (1974a) found that in animals given a single dose of ethanol, noradrenaline turnover was increased, while dopamine turnover was unaffected during the first few hours of treatment. Afterwards, the turnover of both noradrenaline and dopamine was reduced, which they speculated may be in accordance with the known biphasic behavioural and physiological effects of ethanol on the CNS ie. initial stimulation and later depression.

Although the effects of ethanol on brain serotonin have received considerable attention, few consistent findings are available. Levels of brain serotonin following acute administration of ethanol have been measured by several investigators. Some have reported decreases (Gursey and Olsen (1960), Bonnycastle et al. (1962)), others have found increases (Reichle et al. (1971), Palaić et al. (1971)), and others have described unchanged levels (Efron and Gessa (1961), Tyce et al. (1968)). There is also conflicting data on the rate of biosynthesis following acute ethanol administration. Some show decreased rates, (Hunt and Majchrowicz (1974b)), others, increased rates (Palaić et al. (1971), while other investigators found no effects on brain serotonin turnover (Frankel et al. (1974)).

1.4.2 Studies on Normal Human Subjects

Kinzius (1950) reported transient elevations in blood noradrenaline levels 15-30 mins after ethanol ingestion. Abelin et al. (1958) noted that the urinary excretion of adrenaline increased 12-fold and noradrenaline 3-4 fold during the first hour after alcohol ingestion. Perman (1958), after giving normal subjects ethanol, noted that the rate of urinary catecholamine excretion increased relative to baseline values. He noted increased excretion of adrenaline, but not of noradrenaline. Anton (1965) reported that ethanol significantly increased urinary dopamine, noradrenaline and metanephrine, and slightly increased urinary adrenaline.

1.4.3 Studies on Alcoholic Subjects

Giacobini et al. (1960a) noted that hospitalized alcoholic subjects had significantly elevated excretions of adrenaline and noradrenaline during withdrawal. In a further study, Giacobini et al. (1960b) determined urinary catecholamine concentrations of sixteen male alcoholic patients in 24hr collections before , during and after ethanol

consumption. Despite markedly elevated blood ethanol levels, no change in the excretion of catecholamines was observed. The lack of adrenal medullary activation by ethanol in alcoholic subjects (in a convalescent phase), in contrast to the response seen in acute studies in normal non-alcoholic volunteers or experimental animals, may be a manifestation of the "tolerance" to alcohol seen in alcoholics (Gordon and Southren (1977)).

Carlsson and Haggendal (1967) noted increased arterial noradrenaline levels in 36 alcoholics studied at intervals from 6hr to 21 days after ethanol withdrawal. Withdrawal symptoms developed at about 12hr and elevated noradrenaline levels were noted between 13 and 24hr. The more striking withdrawal symptoms were associated with the highest levels of adrenaline.

Ogata et al. (1971) studied 4 alcoholic subjects who consumed ethanol on a "free-choice" programme (ad lib) and a programmed dose protocol. During free-choice ingestion, but not during programmed ethanol use, there was a significant increase in urinary adrenaline. There were no significant changes in noradrenaline excretion during the alcohol phase of the programmed study, although a small increase was observed. There were no significant changes in dopamine excretion. The authors noted that in the free-choice study, the progressive increase in adrenaline and noradrenaline values paralleled the upward movement of blood ethanol levels, and that during the post withdrawal period in a subject who experienced a symptomatically significant withdrawal syndrome, adrenaline and noradrenaline were maximally elevated. These studies suggest that enhanced urinary catecholamine excretion occurs throughout the course of long-term ethanol intake in alcoholics without any adaptational response (ie. sympathetic-adrenal medullary tolerance) (Gordon and Southren (1977)).

1.5 Biosynthesis and Metabolism of the Catecholamines and Serotonin

The biochemistry of catecholamines has been extensively reviewed by Sandler and Ruthven (1969), Weiner (1970), Molinoff and Axelrod (1971) and McIlwain and Bachelard (1971), and of serotonin by McIlwain and Bachelard (1971).

1.5.1 Biosynthesis

The pathway by which the catecholamines are derived from the dietary amino acids phenylalanine and tyrosine is shown in Fig. 1(b),

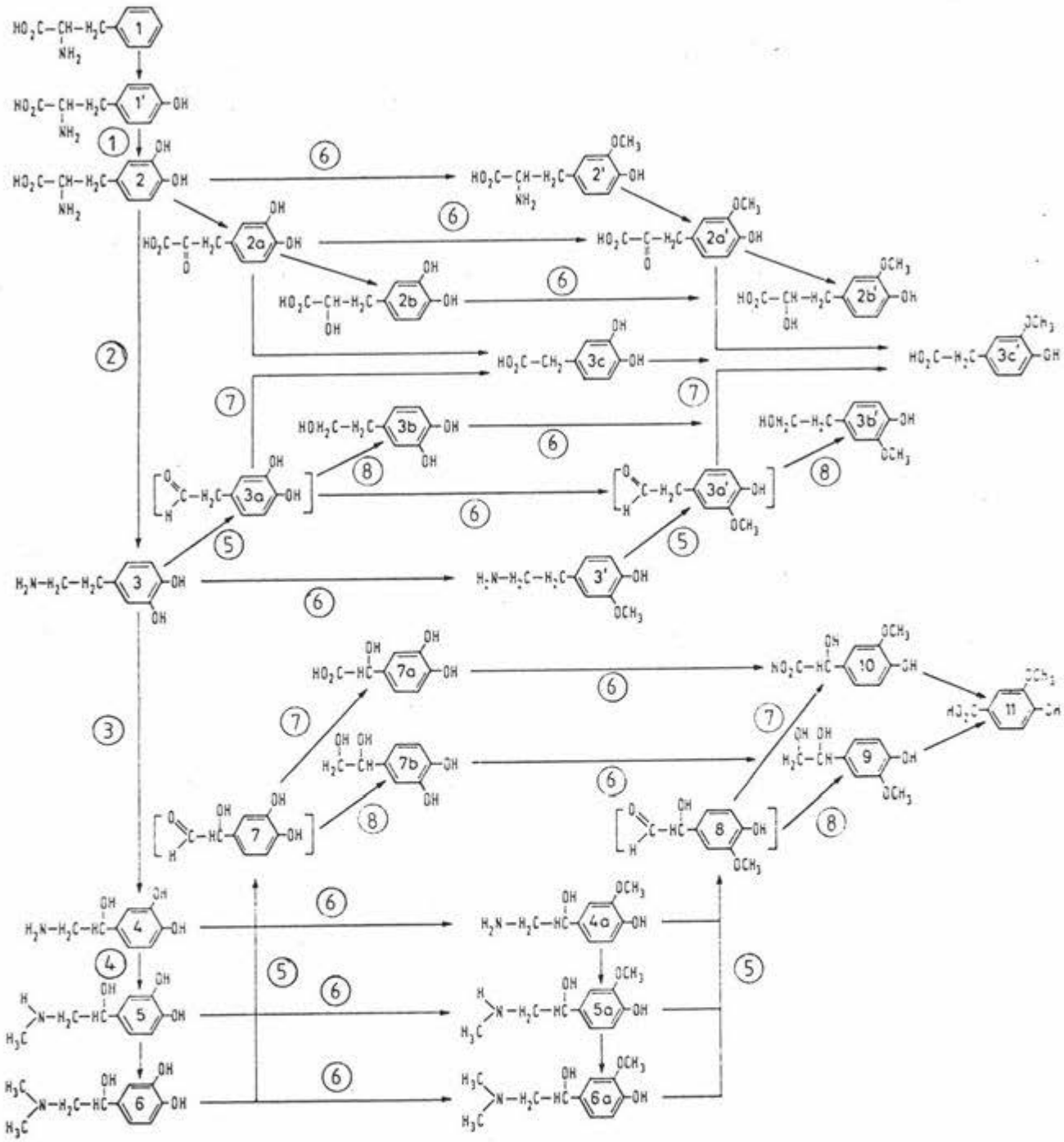


Fig. 1(b) The Biosynthesis and Metabolism of Catecholamines, (from Wisser and Knoll (1973)).

contd...

Fig. 1(b) Key

Index	Systematic Name	Trivial Name	Abbrev.
1	Phenylalanine	-	Phe
1'	4-Hydroxyphenylalanine	Tyrosine	Tyr
2	3,4-Dihydroxyphenylalanine	-	DOPA
2'	3-Methoxy-4-hydroxyphenylalanine	3-Methoxytyrosine	3-MTyr
2a	3,4-Dihydroxyphenylpyruvic Acid	-	DOPPA
2a'	3-Methoxy-4-hydroxyphenylpyruvic Acid	Vanilpyruvic Acid	VPA
2b	3,4-Dihydroxyphenyllactic Acid	-	DOPLA
2b'	3-Methoxy-4-hydroxyphenyllactic Acid	Vanillactic Acid	VLA
3	3,4-Dihydroxyphenylethylamine	Dopamine	DA
3'	3-Methoxy-4-hydroxyphenylethyl-amine	3-Methoxytyramine	3-MT
3a	3,4-Dihydroxyphenylacetaldehyde	-	-
3a'	3-Methoxy-4-hydroxyphenylacet-aldehyde	-	-
3b	3,4-Dihydroxyphenylethanol	-	DOPEt
3b'	3-Methoxy-4-hydroxyphenylethanol	Vanilethanol	MOPEt
3c	3,4-Dihydroxyphenylacetic Acid	Homoprocatechuic Acid	DOPAC
3c'	3-Methoxy-4-hydroxyphenylacetic Acid	Homovanillic Acid	HVA
4	1-(3,4-Dihydroxyphenyl)-2-amino-ethanol	Noradrenaline	NA
4a	1-(3-Methoxy-4-hydroxyphenyl)-2-aminoethanol	Normetanephrine	NM
5	1-(3,4-Dihydroxyphenyl)-2-methyl-aminoethanol	Adrenaline	A
5a	1-(3-Methoxy-4-hydroxyphenyl)-2-methylaminoethanol	Metanephrine	M
6	1-(3,4-Dihydroxyphenyl)-2-dimeth-ylaminoethanol	N-Methyladrenaline	N-MA
6a	1-(3-Methoxy-4-hydroxyphenyl)-2-dimethylaminoethanol	N-Methylmetanephrine	N-MM
7	3,4-Dihydroxyphenylglycolaldehyde	-	-
7a	3,4-Dihydroxymandelic Acid	-	DOMA
7b	3,4-Dihydroxyphenylethyleneglycol	-	DOPEG
8	3-Methoxy-4-hydroxyphenylglycol-aldehyde	-	-

contd...

Fig. 1(b) Key (contd.)

Index	Systematic Name	Trivial Name	Abbrev.
9	3-Methoxy-4-hydroxyphenylethylene-glycol	Vanilglycol	MOPEG
10	3-Methoxy-4-hydroxymandelic Acid	Vanilmandelic Acid	VMA
11	3-Methoxy-4-hydroxybenzoic Acid	Vanillic Acid	VA

Enzymes

1 Tyrosine Hydroxylase

2 DOPA Decarboxylase

3 Dopamine-β-Hydroxylase

4 Phenylethanolamine-N-Methyl Transferase (PNMT)

5 Monoamine Oxidase (MAO)

6 Catechol-O-Methyl Transferase (COMT)

7 Aldehyde Dehydrogenase

8 Aldehyde Reductase (Alcohol Dehydrogenase)

steps ①-④. Although this pathway represents the major route for catecholamine biosynthesis, the lack of specificity of several enzymes involved permits several alternative biosynthetic routes (Sandler and Ruthven (1969)). Serotonin biosynthesis from the dietary amino acid tryptophan is shown in Fig. 1(c), steps ①-②.

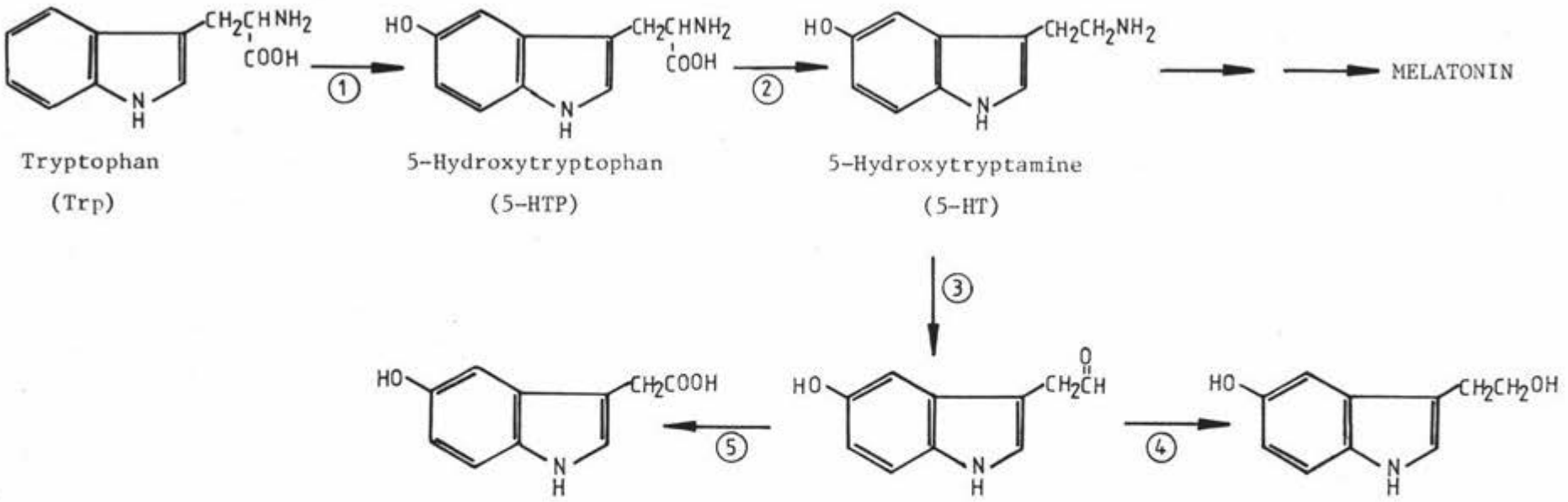
1.5.2 Metabolism

The metabolism of catecholamines (Fig. 1(b), steps ⑤-⑧) involves primarily two enzymes: catechol-O-methyl transferase (COMT, Fig. 1(b) ⑥) and monoamine oxidase (MAO, Fig. 1(b) ⑤). Both act on a wide variety of substrates and each is fully active on the products of the other. Thus an entire spectrum of catecholamine metabolites can be identified in urine - some acted on by MAO, COMT, or both. In addition to these two major enzymes, two others - an aldehyde dehydrogenase and an aldehyde reductase (Fig. 1(b) ⑦ and ⑧ respectively) are present, which act on the aldehyde products of MAO. The metabolism of serotonin (Fig. 1(c) steps ③-⑤) is much simpler than that of the catecholamines. Serotonin is acted on by the same MAO involved in catecholamine metabolism to give rise to an aldehyde product, which is in turn modified by either aldehyde dehydrogenase or aldehyde reductase.

Monoamine oxidase deaminates compounds in which the amine group is attached to the terminal carbon atom. N-methylation and β-hydroxylation decrease the susceptibility of phenylethylamines to MAO. Thus dopamine and tyramine are metabolized more readily than noradrenaline and adrenaline. MAO is widely distributed and, in addition to liver and the adrenal gland, has been shown to occur in skin, heart, kidney, salivary glands, intestine, and sympathetic nerve terminals where it is present mainly in the outer membrane of mitochondria.

The enzyme COMT is responsible for the 3-O-methylation of the catechol group using S-adenosylmethionine as a methyl donor. The enzyme can methylate catechols, but not monohydroxy derivatives of phenylethylamine. The enzyme preferentially O-methylates the 3-hydroxyl group of 3,4-dihydroxycatechols, although it does have activity on the 4-hydroxyl group to form 4-methoxy metabolites. It is broadly distributed in mammalian tissues, but the highest activity is found in the liver and kidney.

Aldehyde dehydrogenase is a NAD-dependent enzyme found in brain, liver and kidney. It oxidizes a wide range of aldehydes including those derived from catecholamines, tryptamines, tyramine and serotonin, to acid



Enzymes:

① Tryptophan-5-Hydroxylase

② 5-Hydroxytryptophan Decarboxylase

③ Monoamine Oxidase

④ Aldehyde Reductase

⑤ Aldehyde Dehydrogenase

Fig. 1(c) The Biosynthesis and Metabolism of Serotonin (5-HT).

end products.

Aldehyde reductase is also found in liver, brain and kidney, and has an equilibrium far to the side of alcohol production. There are two enzymes, a NAD oxidoreductase and a NADP oxidoreductase which have different substrate specificities. The NADP-linked enzyme will not reduce short chain aliphatic aldehydes while the NAD-linked enzyme will oxidize ethanol. The NADP-linked enzyme will reduce aldehydes derived from catecholamines, octopamine and tyramine.

In the central nervous system of man and throughout the body of rat and other species, the aldehydes derived from phenylethylamines (eg. dopamine) and indoleamines (serotonin) are oxidized to acids by NAD-dependent aldehyde dehydrogenase, whereas the aldehydes derived from β-hydroxylated phenylethylamines (eg. adrenaline, noradrenaline) are reduced to alcohols by NADP-dependent aldehyde reductase. However, in the liver and other peripheral tissues of man the aldehydes of adrenaline and noradrenaline are oxidized rather than reduced.

1.6 Excretion of Catecholamine and Serotonin Metabolites

1.6.1 Metabolite Conjugation

Catecholamines and their basic and neutral metabolites can be conjugated with sulphuric or glucuronic acids in position 4 (conjugation with sulphuric seems to be the predominant reaction in man) (Weil - Malherbe (1971)). This process occurs mainly in the liver, but conjugating enzymes and conjugated metabolites have also been found in the brain. The conjugated fraction of the acidic metabolites, if it exists, is small, and is usually disregarded during the assay of these metabolites.

There exists an enzyme - PAPS: serotonin sulphotransferase - found in brainstem, which catalyzes the transfer of sulphate from 3'-phospho-adenosine-5'-phosphosulphate to serotonin (Hidaka et al. (1969)). Sulphate conjugates of serotonin have been detected in urine, but conjugation does not appear to occur to a significant degree with serotonin metabolites.

1.6.2 Normal Levels of Urinary Catecholamine and Serotonin Metabolites

The normal physiological concentrations of neurotransmitter metabolites in urine vary widely amongst normal individuals. Tietz (1976) has described normal levels for 3 major metabolites using chemical

estimation techniques.

Metabolite	Normal Urinary Concentration ($\mu g\ mg^{-1}$ creatinine)
VMA	1.5 - 7.0
HVA	1.0 - 40
5-HIAA	0.8 - 7.3

Such wide ranging normal values may be partially due to the unreliability of the techniques used for their estimation. Probably more reliable normal values have been described in the literature using highly specific GCMS techniques for catecholamine (Muskiet et al. (1978b)) and serotonin (Domino et al. (1979)) metabolites in normal male subjects (Table 1(ii)).

Table 1(ii) Normal Levels of Urinary Catecholamine and Serotonin Metabolites Determined by GCMS Techniques

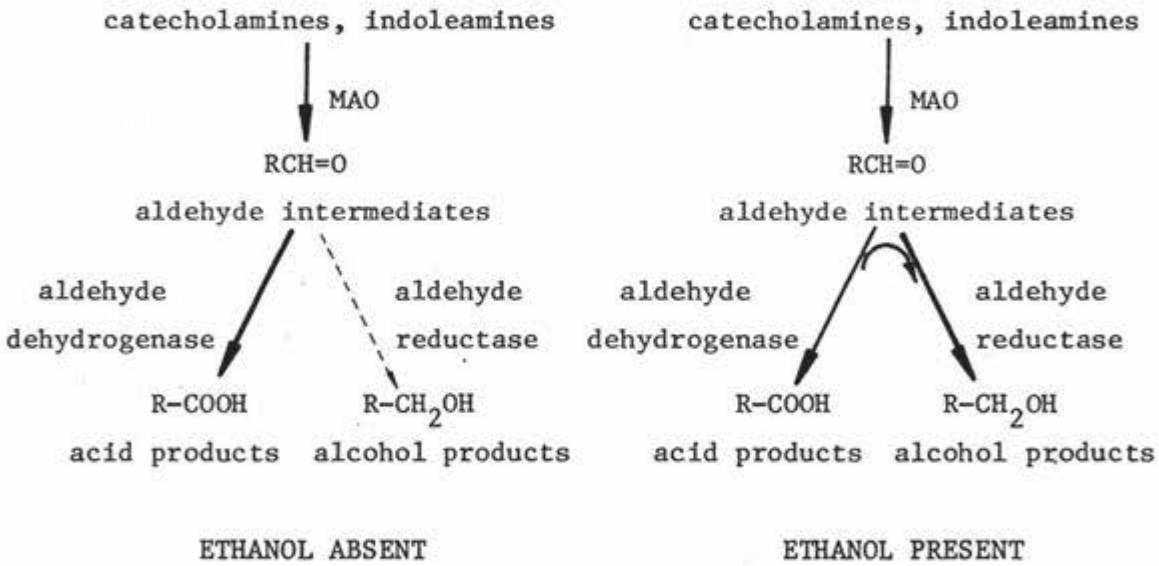
Metabolite	Normal Urinary Concentration		
	n	mean $\pm$ 1 s.d.	range
DOPPA	4	n.m.	-
VPA	6	0.74 $\pm$ 0.14	0.53 - 0.95
DOPLA	4	n.m.	-
VLA	5	0.18 $\pm$ 0.13	0.08 - 0.40
DOPAC	5	1.5 $\pm$ 0.5	0.6 - 2.0
HVA	25	4.0 $\pm$ 1.6	1.7 - 8.1
DOPEt	4	0.016 $\pm$ 0.009	0.005 - 0.026
MOPEt	4	0.032 $\pm$ 0.006	0.027 - 0.037
DOMA	4	0.118 $\pm$ 0.042	0.071 - 0.165
VMA	25	1.9 $\pm$ 0.8	0.8 - 4.4
DOPEG	7	0.33 $\pm$ 0.15	0.16 - 0.60
MOPEG	14	1.5 $\pm$ 0.5	0.7 - 2.5
5-HIAA	7	4.87 $\pm$ 0.32	3.88 - 5.64

Concentrations expressed as μg metabolite mg^{-1} creatinine, n.m. indicates not measurable.

1.7 Effects of Ethanol on Catecholamine and Serotonin Metabolism

The ingestion of significant quantities of ethanol alters the metabolism of the cell. Some of the alterations are due directly to the metabolism of ethanol (eg. a change in the lactate/pyruvate ratio, Papenberg (1971)), whereas others are due to its mere presence (eg. an increase in the corticosteroids, Jenkins and Connolly (1968)).

Ethanol, in addition to altering basic cellular biochemistry, affects the behaviour of the drinker. Behavioural events can possibly be related to neurotransmitter levels and metabolism. In the presence of ethanol, not only are the levels of transmitters altered, but the basic metabolic pathways are also changed (Feldstein (1971), Truitt and Walsh (1971)). In the presence of ethanol, the biogenic amine metabolites are diverted away from their oxidative pathways (which give rise to acidic products) into reductive pathways (which give rise to alcoholic products).



Effect of Ethanol on Catecholamine and Indoleamine Metabolic Pathways

Alterations in human biogenic amine metabolism due to ethanol have been investigated almost exclusively in urine samples due to their accessibility (as opposed to, for instance, CSF samples) and the fact that the immediate fate of the metabolised products is their excretion in the urine. The exact effects that an acute dose of ethanol has on the urinary levels of catecholamine and indoleamine metabolites in normal subjects so far described in the literature have been diverse and sometimes contradictory. This has been caused, in part, by the variety of ethanol doses, of distribution of dose over time, as well as the

inclusion or absence of controls for the diuretic effects of ethanol. The questionable adequacy of some of the methods used for the separation and identification of the amines and their metabolites is certainly one of the major contributing factors to these inconsistent findings (Hawkins and Kalant (1972)). The different results reported, and the various techniques employed are outlined in Table 1(iii).

The purpose of this study was to critically evaluate techniques previously used for the estimation of urinary catecholamine and serotonin metabolites. The techniques ultimately resolved as being the most accurate were then to be applied to alcohol studies in normal human volunteers in an attempt to resolve the diverse results of previous workers.

Table 1(iii) Techniques Used, and Results Attained by Previous Authors Investigating the Effects of Ethanol on Urinary Catecholamine and Serotonin Metabolites

Reference	Technique	Results
Smith et al (1960)	^{14}C-labelled precursors	Decreased VMA excretion
Perman (1961)	Chemical estimation	No significant change in 5-HIAA excretion
Feldstein et al. (1964)	^{14}C-labelled precursors, solvent extraction	Decreased 5-HIAA excretion
Anton (1965)	Chemical estimation	Increased VMA excretion, decreased 5-HIAA excretion
Davis et al. (1967a)	^{14}C-labelled precursors, solvent extraction	Decreased 5-HIAA excretion, increased 5-HTPh excretion
Davis et al. (1967b)	^{14}C-labelled precursors, column chromatography	Decreased VMA excretion, increased MOPEG excretion. No change in DOMA, DOPEG excretion
Feldstein et al. (1967)	^{14}C-labelled precursors, solvent extraction	Decreased 5-HIAA, postulated increased 5-HTPh excretion
Walsh et al. (1970)	^{14}C-labelled precursors, column chromatography	Acetaldehyde causes decreased VMA, DOMA excretion, increased MOPEG excretion in rats
Ogata et al. (1971)	Chemical estimation	Decreased VMA excretion, increased MOPEG excretion
Gitlow et al. (1976)	Paper chromatography (VMA), GLC (MOPEG, HVA)	Decreased VMA excretion, increased MOPEG excretion. Slightly increased HVA excretion

CHAPTER 2

THE CHEMICAL ESTIMATION OF CATECHOLAMINE AND SEROTONIN METABOLITES

2.1 Introduction

Chemical techniques for the estimation of the catecholamines and their metabolites in body fluids have been reviewed by Weil - Malherbe (1968, 1971), and for serotonin and its metabolites by Lovenberg and Engelman (1971). Although there have been several modifications and alternatives published since these reviews, the techniques described are still extensively employed in routine clinical analysis (Tietz (1976)). Methods for the estimation of the catecholamine metabolites VMA and HVA, and the serotonin metabolite 5-HIAA will be examined in this chapter.

2.2 The Estimation of VMA in Urine

2.2.1 Introduction to the Method

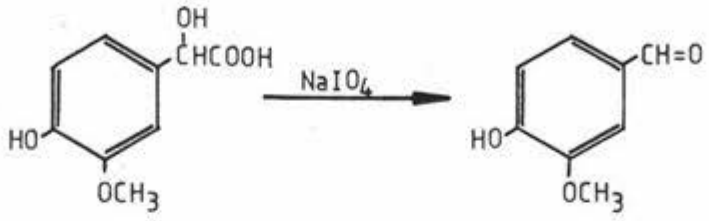
Of the many methods available for the estimation of VMA in urine, colorimetric techniques based on the oxidation of VMA to vanillin have found the widest application. Since the first introduction of such a procedure a great number of modifications have been described (Weil - Malherbe (1968)). Ferricyanide (Sunderman et al. (1960), periodate (Pisano et al. (1962)), an alkaline copper reagent (Weil - Malherbe (1964) and catalytic oxidation under pressure (Sandler and Ruthven (1961)) have all been used to bring about this conversion. Of these, the periodate method of Pisano et al. is probably the most widely used, but Weil - Malherbe (1971) found it to be subject to inhibition with some urine samples.

In the method of Pisano et al., VMA, along with other phenolic acids, is extracted from acidified urine into ethyl acetate. It is then extracted from the organic solvent with aqueous potassium carbonate solution. Weil - Malherbe (1971) has suggested an alternative extraction into phosphate buffer, pH 7.0, to separate VMA from unconjugated MOPEG, an interfering compound which would remain in the organic phase. However unconjugated MOPEG seems to be absent or is, at most, a small proportion of the total MOPEG found in normal urine, and is therefore of little consequence. After VMA oxidation, vanillin is separated from contaminating urinary phenolic acids by extracting into toluene, a process which also extracts *p*-hydroxybenzaldehyde, the oxidized product of *p*-hydroxymandelic acid (Sunderman et al. (1960)). Vanillin is determined spectrophotometric-

ally at a wavelength of 360nm, and not at its absorption maximum of 348nm to avoid interference by ρ-hydroxybenzaldehyde.

Urine is preserved prior to analysis by acidification, VMA is stable in this condition for several days at room temperature (Winsten (1965)).

The method of Pisano et al. (1962) for VMA estimation (Fig. 2(a)) is evaluated below.



VMA Oxidation to Vanillin

2.2.2 Results

The standard curve (Fig. 2(b)) showed good linearity over the range 1.25-10.0 μg VMA ml^{-1}. Below this range linearity deteriorated and resulted in a high lower limit of detection (approx. 1.25 μg ml^{-1}). The reproducibility and recovery of the method were satisfactory (Table 2(i)), and VMA estimations in six normal urines were all found to be within the normal range of 1.5 - 7.0 μg mg^{-1} creatinine (Tietz (1976)).

Table 2(i) VMA Determination by the Method of Pisano et al. (1962)

	n	$\bar{x}$	range	s.d.	s.e.
Reproducibility:	6	2.44	1.81 - 3.05	0.41	0.167
Normal Levels:	6	2.59	1.72 - 4.01	1.04	0.423
Recoveries (%):	6	89.8	69.2 - 104.4	9.1	3.72

Concentrations expressed as μg VMA mg^{-1} creatinine

Reproducibility was determined by repeatedly assaying the same sample 6 times. Recoveries were determined by adding a known amount of VMA to a different aliquot of each sample analysed.

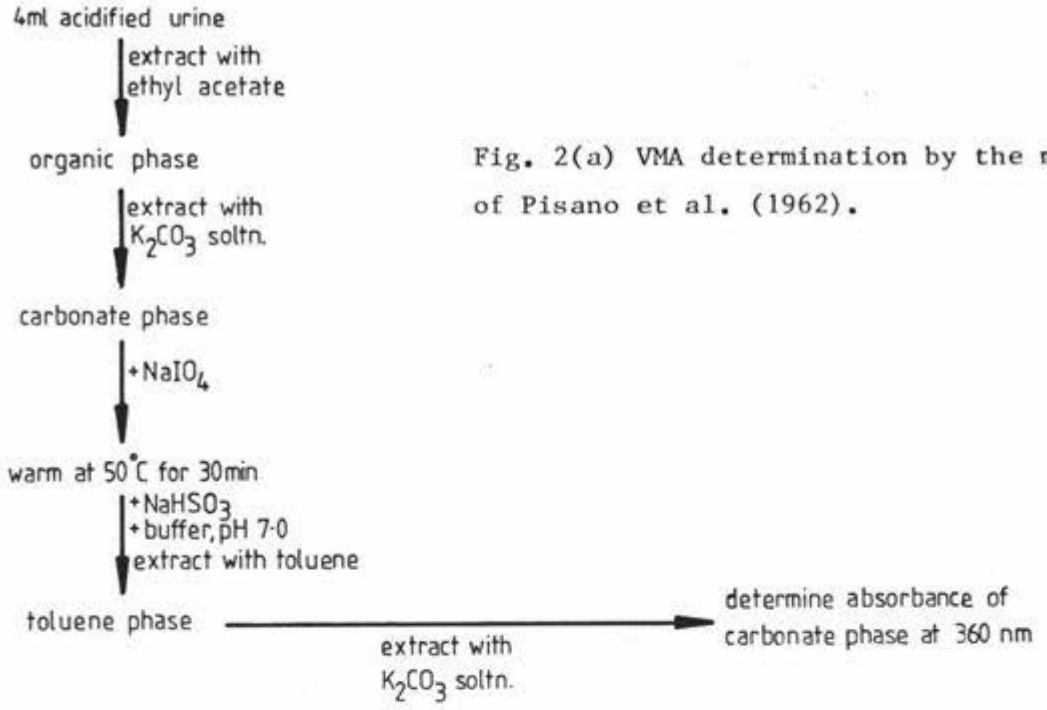


Fig. 2(a) VMA determination by the method of Pisano et al. (1962).

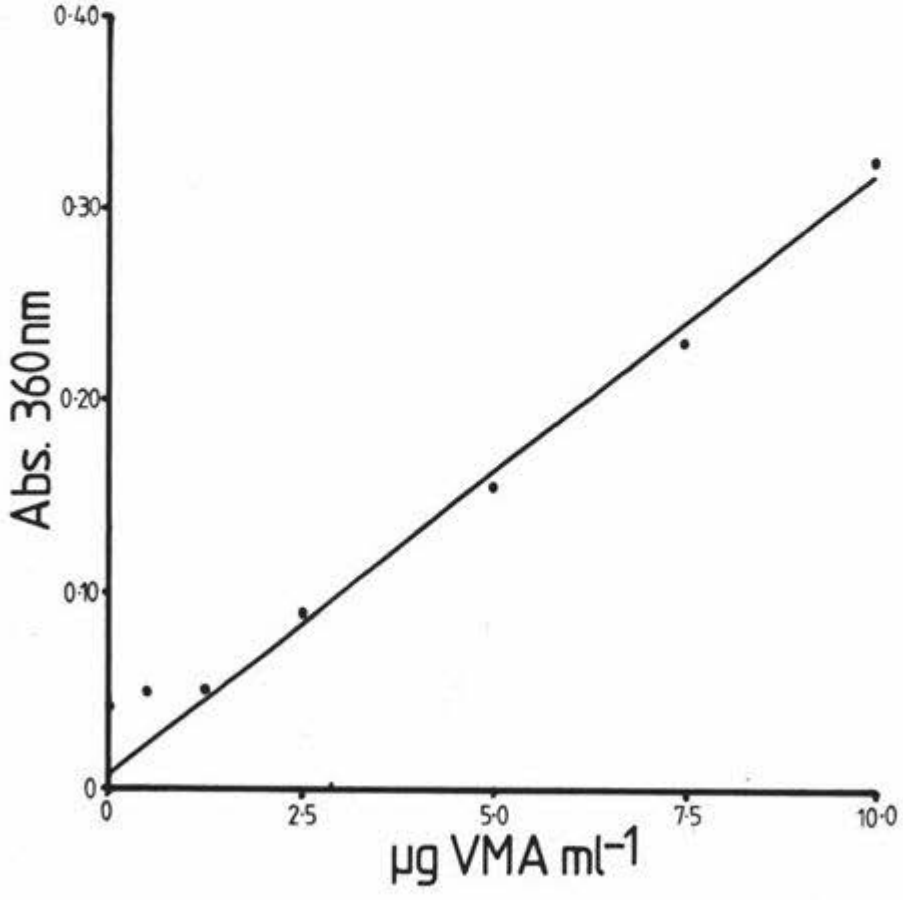


Fig. 2(b) Standard curve for the colorimetric determination of VMA a=-0.283, b=32.8 $\pm$ s_b=1.31; where y=a + bx, y=µg VMA ml^{-1}, x=abs. 360 nm

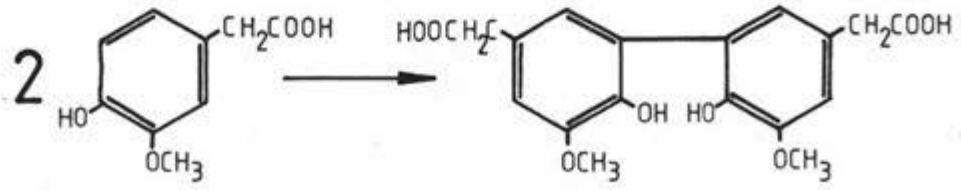
2.3 The Estimation of HVA in Urine

2.3.1 Introduction to the Method

Andén et al. (1963) found that HVA is oxidized to a fluorescent compound by treatment with ferricyanide in ammoniacal solution, the reaction being stopped by the addition of cysteine. Corrodi and Werdinius (1965) identified the product and determined the fluorescence maxima. Sato (1965) determined the optimum conditions for fluorescence, tested 13 structurally similar compounds commonly found in urine and showed that they had minimal fluorescence development. Gjessing et al. (1967) examined the specificity of the reaction and concluded that the structure required was a 3-methoxy-4-hydroxyphenyl compound with a side chain containing at least two carbon atoms, the first of which must be a CH_2 group. A positive reaction is therefore also given by vanilalanine and vanillactic acid, substances that may appear in urine (Gjessing (1963)).

When assayed in urine, the purification of HVA on an ion-exchange resin (Sato (1965), Geissbühler (1969), Kahane and Vestergaard (1971)) is necessary to remove interfering substances. Sato (1965) showed that up to 100% of the HVA could be eluted from the ion-exchange column using the appropriate concentration and volume of NaCl. Furthur purification of the column extract is usually necessary and for this Sato used a toluene - chloroform mixture since it gave good recovery of HVA without extracting interfering substances. Kahane and Vestergaard (1971) suggested extracting HVA into methylene chloride prior to column chromatography since the extraction is essentially quantitative and selective, leaving behind many of the impurities present in the aqueous phase. From the organic phase, the HVA is back extracted into tris buffer (Sato (1965)), the fluorescent compound formed, and determined by its fluorescent emission at 420 nm after excitation at 320 nm.

The method described by Sato (1965) (Fig. 2(c)) is investigated below.



Formation of the Fluorophore 2,2'-Dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic Acid from Homovanillic Acid.

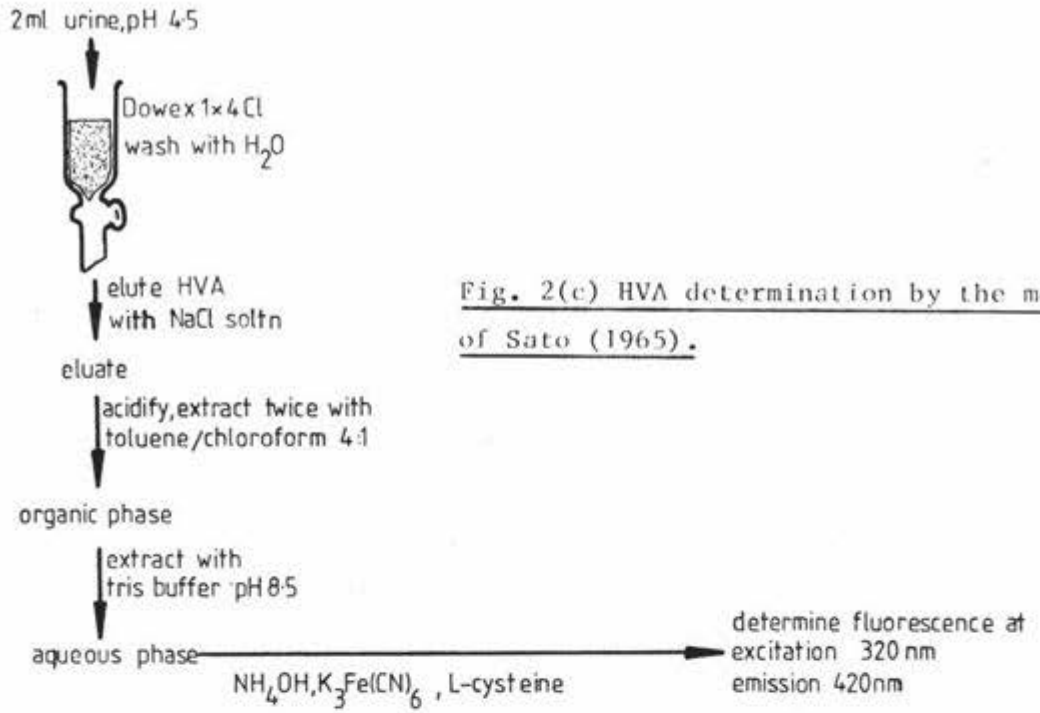


Fig. 2(c) HVA determination by the method of Sato (1965).

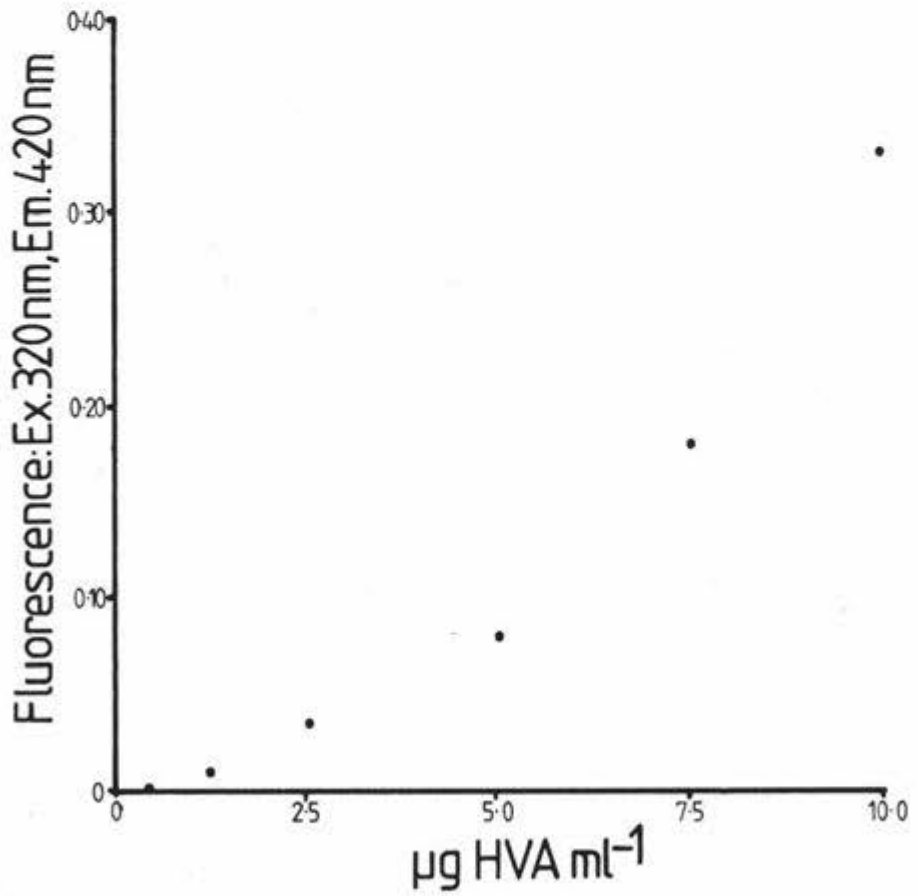


Fig. 2(d) Standard curve for the fluorometric determination of HVA

2.3.2 Results

The standard curve (Fig. 2(d)) was found to be nonlinear over the concentration range investigated and the method was not furthur examined in any detail. A single urine showed a concentration of 3.6 $\mu g\ mg^{-1}$ creatinine, within the normal range of 1-40 $\mu g\ mg^{-1}$ creatinine (Tietz (1976)).

2.4 The Estimation of 5-HIAA in Urine

2.4.1 Introduction to the Method

The colorimetric method of Udenfriend et al. (1955) still remains the most practical and extensively used method for 5-HIAA estimation (Tietz (1976)). The procedure involves the preliminary treatment of the urine with dinitrophenylhydrazine to react with any ketoacids that may interfere. The urine is then extracted with chloroform to remove indoleacetic acid which will also form a coloured product during the colour reaction. After furthur purification and extraction into an aqueous phase, the 5-HIAA is reacted with nitrosonaphthol reagent to form a coloured derivative that may be determined spectrophotometrically at 540 nm.

Urine samples are preserved prior to analysis by acidification, in which state 5-HIAA has been shown to be stable for at least 8 days at 4 °C (Geeraerts et al. (1980)).

The method of Udenfriend et al. (1955) (Fig. 2(e)) is investigated below.

2.4.2 Results

The standard curve (Fig. 2(f)) showed good linearity over the concentration range investigated, but failed to pass through the origin, having an intercept on the abscissa of 0.63 μg 5-HIAA ml^{-1}. The reproducibility and recovery (Table 2(ii)) were satisfactory, and 5-HIAA determinations of six normal urines were all found to be within the normal range of 0.8-7.3 $\mu g\ mg^{-1}$ creatinine (Tietz (1976)).

Table 2(ii) 5-HIAA Determination by the Method of Udenfriend et al. (1955)

	n	$\bar{x}$	range	s.d.	s.e.
Reproducibility:	6	2.93	2.67 - 3.37	0.175	0.071
Normal levels:	6	3.17	1.42 - 5.52	1.04	0.423
Recoveries (%):	6	94.1	84.4 - 105.6	6.5	2.65

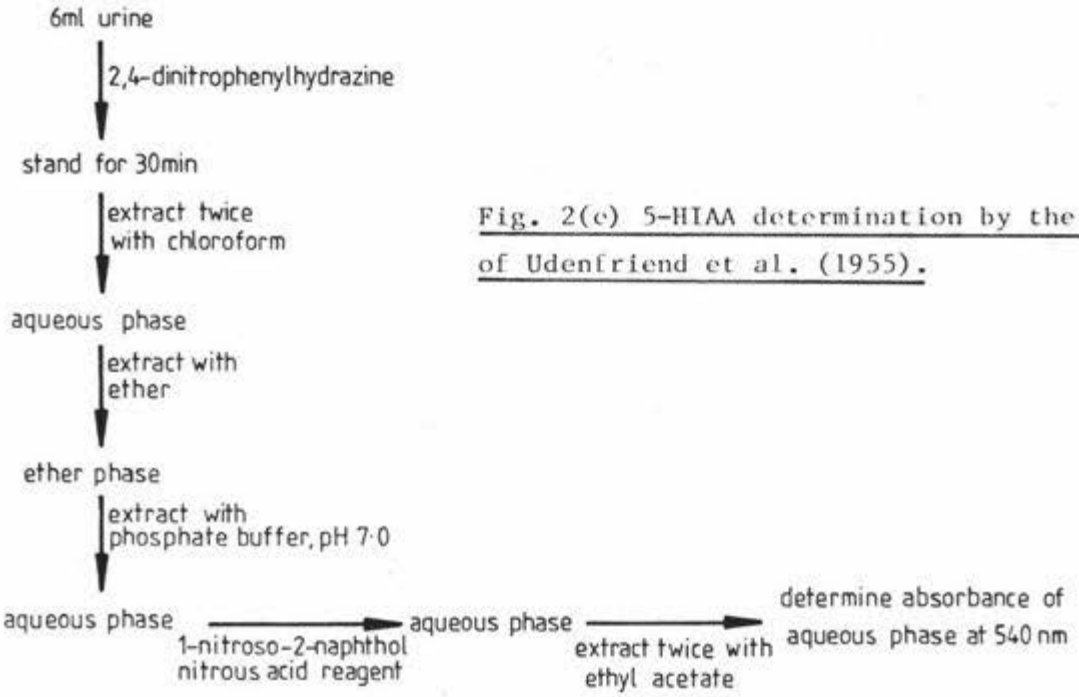


Fig. 2(e) 5-HIAA determination by the method of Udenfriend et al. (1955).

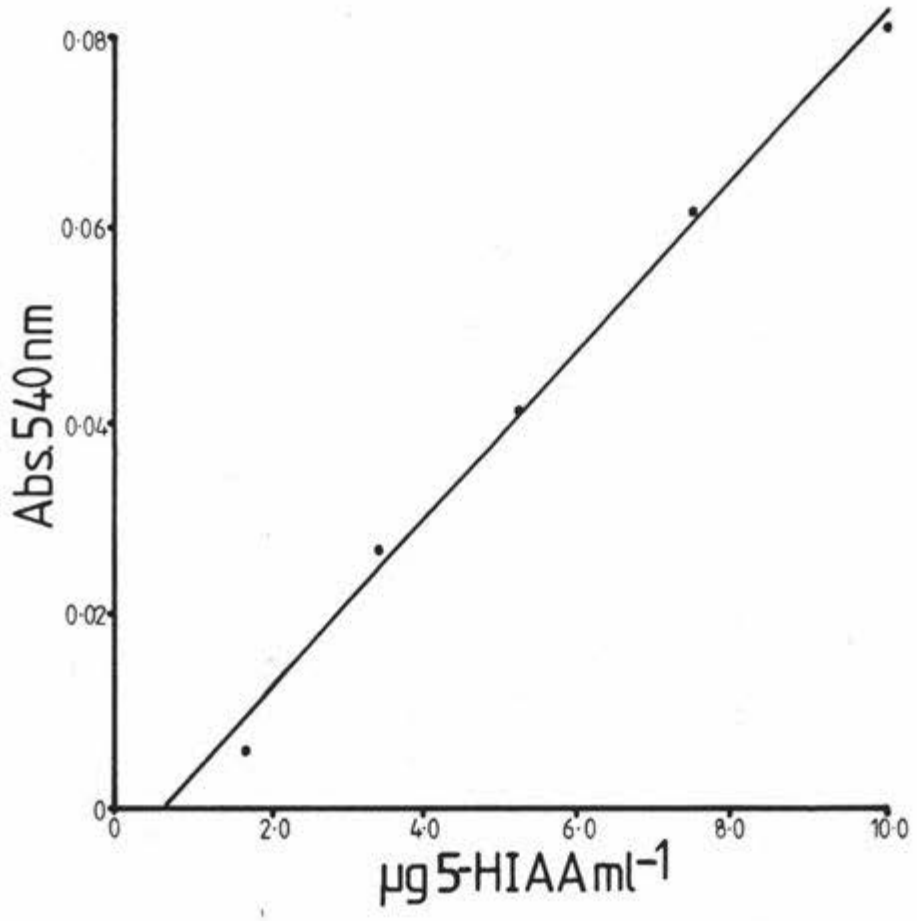


Fig. 2(f) Standard curve for the colorimetric determination of 5-HIAA

a= 0.631, b= 114.0 $\pm$ s_b= 6.12; where y=a + bx, y=µg 5-HIAA ml^{-1}, x= abs. 540 nm

Table 2(ii) contd.

Concentrations expressed as μg 5-HIAA mg^{-1} creatinine.

Reproducibility and recoveries determined as for Table 2(i).

2.5 Discussion

A cursory examination of chemical techniques for the estimation of some catecholamine and indoleamine metabolites has been carried out in an effort to determine the feasibility of applying these techniques to the study of ethanol/neurotransmitter interactions.

The techniques were found to display adequate linearity at higher metabolite concentrations in all cases except one (HVA). However, the procedures examined generally showed a lack of sensitivity resulting in poor linearity at lower metabolite concentrations and subsequently high lower limits of detection. It seemed unlikely that these techniques would be able to accurately demonstrate any minor changes in metabolite concentration that might occur with ethanol loading.

The fact that each method only estimated one metabolite would mean that the analysis of a large number of metabolites in a single urine sample would necessitate the application of the same number of techniques; a very lengthy procedure.

Although not investigated in this study, it is well known that chemical techniques for the estimation of catecholamine and indoleamine metabolites are very susceptible to extraneous factors (Tietz (1976)). Young et al. (1975) have detailed the methodological interference by extraneous factors on the commonly employed chemical estimations for catecholamine and indoleamine metabolites.

It was concluded that the methods examined in this chapter would be inadequate for the intended study of ethanol/neurotransmitter interactions. Since these techniques have been thoroughly researched by several workers, it was presumed that they have achieved maximum sensitivity and selectivity. Subsequently, the chances of improvement to enhance their applicability to the proposed study were considered minimal, and chemical estimation techniques were not further investigated.

2.6 Other Techniques for the Estimation of Catecholamine and Serotonin Metabolites

Several methods have been described, and are still frequently used, that employ electrophoresis, paper or thin-layer chromatography for the estimation of catecholamine and serotonin metabolites in urine (Herman

(1964), Vahidi et al. (1971), Zawad and Brown (1976)). The need for a relatively elaborate pretreatment of samples, the lack of specificity and sensitivity of detection methods, and the length of analysis make these techniques unlikely candidates for regular analysis of these metabolites (Krstulovic (1979)) and subsequently they were not investigated.

Several radioenzymatic assays (REA) have been proposed (Engelman et al. (1968)) for catecholamine determination, and Argiolas et al. (1977) have described a REA technique for DOPAC estimation. These methods utilize catechol-O-methyl transferase (COMT) to transfer a labelled methyl group to the 3,4-dihydroxy group of the compound being measured, and are therefore inapplicable to the estimation of 3-methoxy metabolites.

The use of high performance liquid chromatography (HPLC) currently represents one of the most popular techniques for the simultaneous assay of catecholamines, indoleamines and their metabolites, and has been recently reviewed by Krstulovic (1979). Until recently, the use of HPLC was restricted due mainly to the lack of adequately sensitive detection methods - the most commonly used UV absorbance detector does not provide the sensitivity necessary for analysing trace amounts of catecholamines. The use of fluorescence monitors has improved the sensitivity, and this has been furthur enhanced by the advent of electrochemical detectors. The appropriate equipment was not available to allow the investigation of this technique in the present study.

Methods involving separations by gas-liquid chromatography (GLC) were considered to be promising alternatives, and are examined in the following chapter.

CHAPTER 3

GAS CHROMATOGRAPHY OF CATECHOLAMINE AND SEROTONIN METABOLITES

3.1 Introduction

Gas chromatographic (GC) techniques offer the advantages of simpler sample preparation and the potential for the simultaneous analysis of more than one metabolite with a single assay when compared with the chemical estimation procedures examined in the previous chapter. The GC analysis of catecholamine and indoleamine related compounds in biological samples was carried out as early as 1962, when Fales and Pisano (1962) studied the separation of free amines and Williams and Greer (1962) analysed urinary acid methyl esters for the diagnosis of neuroblastoma. Until that time, the fact that these compounds were highly polar and only slightly volatile had deterred workers from investigating their separation by this method.

Although Fales and Pisano showed the feasibility of GC separation of free amines, the peaks obtained were frequently marred by tailing because of the polar nature of the amino group. Since then, many different derivatives of these compounds have been examined in an attempt to meet the volatility requirements (Table 3(i)). With some of these methods, detection levels of 2-5 ng have been reported using flame ionisation detectors (FID). More recently, the use of electron-capture detection (ECD) has brought the quantitative evaluation down to the level of a few picograms.

The sample extraction procedure prior to gas chromatography varies depending on the nature of the metabolites being assayed. Acidic metabolites are usually extracted from salt saturated urine at pH 1 with either ethyl acetate (Muskiet et al. (1977)), or diethyl ether (Sprinkle et al. (1969)), or both of these solvents in successive steps (Horning and Horning (1970), Addanki et al. (1976), Brewster et al. (1977)). These procedures result in the removal of organic acids from the aqueous phase into the organic phase and strongly discriminate in favour of aromatic acids as opposed to polyhydroxy aliphatic compounds (Thompson and Markey (1975)). This gives rise to what has been described as an "aromatic acid profile" (Horning and Horning (1970)). Solvent extraction suffers from a lack of specificity due to the solubility of neutral compounds such as urea in both phases, as well as the partial solubility

Table 3(i) Derivatives Used for the GLC Study of Catecholamines, Indoleamines and Their Metabolites

Derivative	Reference	Comments
Trimethylsilyl	Sen and McGeer (1963) Horning et al. (1967) Maruyama et al. (1971) Addanki et al. (1976) Muskiet et al. (1971)	Easily formed, yields sharp, symmetrical peaks under FID, TMS derivatives of amino as well as hydroxyl groups are formed.
Acyl	Brooks and Horning (1964)	Have potential utility in the isolation of amines from dilute aqueous solution, resistant to hydrolysis, generally stable.
Ethyl ester	Narasimhachari (1974)	Used for the separation of isomeric 3- and 4- methoxy catechol amine metabolites.
Propionyl	Hiemke et al. (1978)	Easily formed in aqueous media, yields lipophilic derivatives therefore aids isolation from biological samples.
Trifluoroacetyl	Lombrozo et al. (1980) Dekirmenjian and Maas (1970) Bertani et al. (1970)	Yields increased sensitivity to electron-capture detection (ECD)
Heptafluorobutyryl	Sharpless (1977)	Increased sensitivity to ECD
Pentafluoropropionyl	Fellows et al. (1975) Nelson et al (1979) Wong et al (1973)	Increased sensitivity to ECD
Perfluorobenzoyl	Moffat et al (1972)	Increased sensitivity to ECD
2,4-Dinitrophenyl	Edwards and Blau (1972)	Increased sensitivity to ECD
Trifluoroacetylhexafluoroisopropyl	Chauhan and Darbre (1980)	Applied to capillary GC with ECD

of water (and its contents) in the organic phase (Thompson and Markey (1975)). Since these compounds could cause chromatographic interference, the removal of neutral substances by preliminary isolation of the urinary organic acids on ion-exchange resins prior to solvent extraction has been suggested (Melchert and Hoffmeister (1977), Horning and Horning (1970)).

For the analysis of alcoholic catecholamine and indoleamine metabolites it is necessary to include a hydrolysis step prior to extraction. This is usually achieved by incubating the buffered sample with glusalase, a mixed hydrolase preparation from Helix pomatia (Dekirmenjian and Maas (1970), Karoum et al. (1973), Fellows et al. (1975), Muskiet et al. (1977)). The alcoholic metabolites are then extracted from urine using the same solvents as for the acidic metabolites, but at a higher pH (pH 6.0-6.2).

The feasibility of GC procedures for the estimation of catecholamine metabolites is examined in this chapter.

3.2 Methods

3.2.1 Sample Preservation

Samples analysed for acidic metabolites were preserved by acidification as noted in the previous chapter, while those used for the analysis of alcoholic metabolites were preserved by the addition of 50 mg sodium metabisulphite per 100 ml of urine (Dekirmenjian and Maas (1970)). Samples were either assayed immediately or stored at 4 °C until analysis.

3.2.2 Extraction and Derivatization

Samples were prepared according to the method of Muskiet et al. (1977) (Fig. 3 (a)), adding propylgallate (PG) and resorcinol (Res) as internal standards. Alternative extraction (Thompson and Markey (1975)) and derivatization (Brewster et al. (1977)) procedures were also investigated (Fig. 3(a)).

3.2.3 Gas Chromatography

Gas chromatography was carried out on 1.0 µl of derivatized extract using a Varian Aerograph series 1700 instrument, on 2 metre glass columns packed with OV-1, OV-101, OV-17, XE-60 or OV-225, or a 40 metre open capillary column surface-coated with SE-30. Columns were preconditioned before use by temperature programming from 0 - 200 °C at a rate of 1 °C min^{-1}

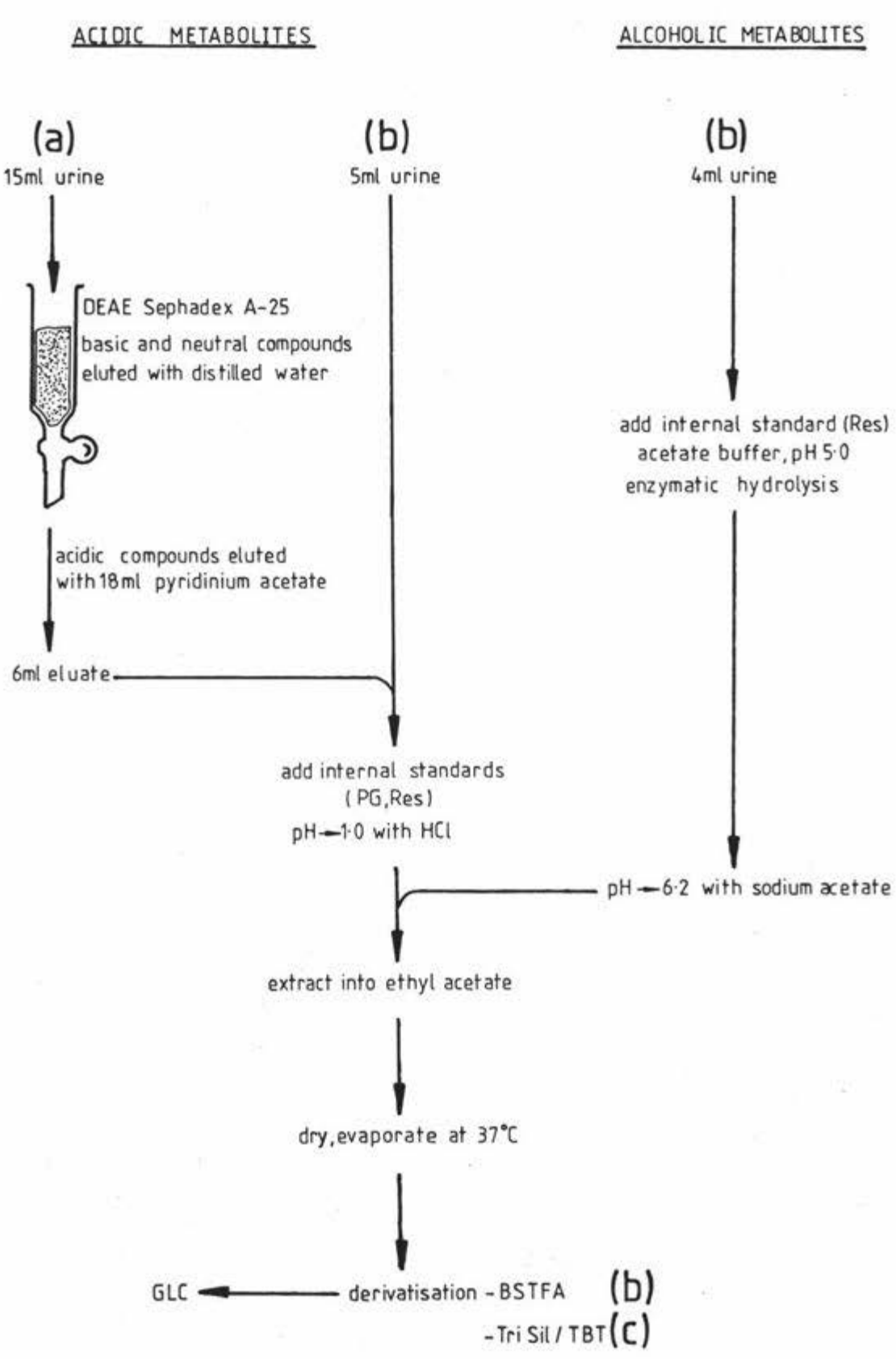


Fig. 3 (a) Methods investigated for the extraction and derivatization of catecholamine metabolites

Refs. (a) Thompson and Markey (1975)
(b) Muskiet et al. (1977)
(c) Brewster et al. (1977)

with a helium carrier gas flow rate of 30 ml min^{-1}, and then maintained at 200 °C until use. The column temperature was elevated to maximum for a period of 20 min between injections to ensure that all of the previous sample had eluted. Syringes were rinsed several times with chloroform between injections. The detector response was registered using a constant speed chart recorder at 1 mv full scale deflection.

3.3 Results

3.3.1 Extracts of Pure Solutions

The peak height ratios (metabolite/PG) from chromatograms of compounds extracted from pure aqueous solutions are given in Table 3(ii). The standard curves (Fig. 3(b)) were linear with the intercept of HVA and VMA occurring very close to the origin, but DOPAC had an intercept at 0.4 μg ml^{-1}, reflecting a greater deviation from regression.

Table 3(ii) Standard Curves from GC of Extracts of Pure Solutions

concn. (μg ml^{-1}):		1.0	2.0	4.0	8.0			
						a	b	$\pm s_b$
peak height ratio:	VMA:	0.054	0.118	0.208	0.425	-0.09	19.1	0.53
	HVA:	0.086	0.190	0.365	0.704	-0.09	11.4	0.25
	DOPAC:	0.052	0.095	0.183	0.463	0.45	16.7	1.36

Where $y = a + bx$, y = metabolite concentration, x = peak height ratio.

3.3.2 Injection Reproducibility

To determine the reproducibility of the GC method, a single derivatized extract containing VMA and internal standard was analysed with different injection volumes. The results were within ± 5% (2 s.d.) of the mean peak height ratio (Table 3 (iii)).

Table 3(iii) Injection Reproducibility of GC Procedure

Injection volume (μl) :	0.6	0.8	1.0	1.2	1.5		
Peak height (VMA) :	37	45	55	69	92.5		
Peak height (internal std.):	41	50	59	78.5	106	$\bar{x}$	± 1 s.d.
Peak height ratio :	0.902	0.900	0.932	0.879	0.873	0.897	0.023

GC protocol as described in the legend to Fig. 3 (b).

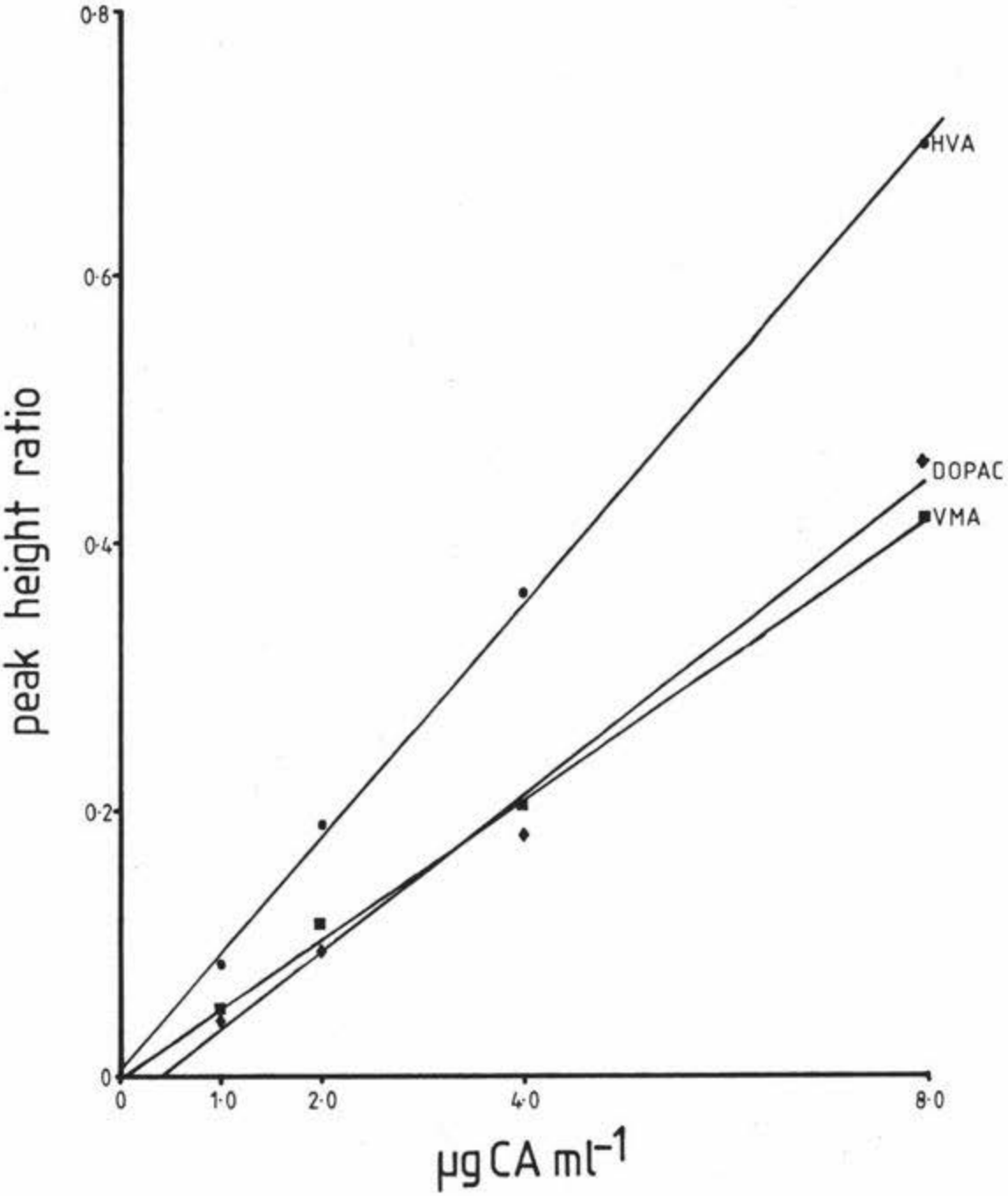


Fig. 3(b) Standard curves for VMA, HVA, DOPAC determination by gas chromatography.

Chromatographic conditions: injected isothermally onto OV-101 at 100 °C. After 4 min, programmed to 200 °C at a rate of 4 °C min^{-1}.

3.3.3 Urine Extracts

The results of urine extracted by the method of Muskiet et al. (1977) are shown in Fig. 3(c). Little improvement in resolution was obtained by a preliminary purification on a DEAE-Sephadex column (Fig. 3(d)). The peaks for VMA, HVA and DOPAC were identified by separately spiking a urine with small amounts of these metabolites prior to extraction (Fig. 3(e)). The effect of an alternative derivatization procedure utilising Tri Sil/TBT on the resolution of these metabolites is shown in Fig. 3(f).

The results of gas chromatography on OV-225, and on a SE-30 capillary column are shown in Figs. 3(g) and 3(h) respectively. Other liquid phases investigated did not show any improvement in resolution over those already described.

3.4 Discussion

Preliminary studies on extracts of pure aqueous solutions suggested that gas chromatography on OV-101 had good potential for quantitating catecholamine and indoleamine metabolites. The method investigated was found to have improved linearity and reproducibility when compared to the chemical estimation techniques examined in the previous chapter.

However, when the procedure was applied to urine extracts, a large contaminant peak was found to elute coincident with the metabolites being investigated (Fig. 3(e)). A major component of this peak was identified, using mass spectrometry, as the diTMS derivative of hippuric acid - the glycine conjugate of benzoic acid, a commonly used food preservative.

Hippuric Acid Hippuric Acid-diTMS

The fact that the gas chromatography of HVA and VMA is interfered with by other substances found in normal urine has been reported by many authors. Lanser et al. (1974), while investigating the method of Sprinkle et al. (1969), reported that normal urine extracts yielded a large disturbing peak with two small shoulders on either side where HVA and VMA were expected to elute. These workers tried four other extraction procedures but found no improvement. Coward and Smith (1969) demonstrated the difficulty of determining HVA and VMA on OV-1 (a liquid phase very similar to OV-101) due to the inherent heterogeneity of peaks. Melchert and Hoffmeister (1978) noted the interference by hippuric acid on the

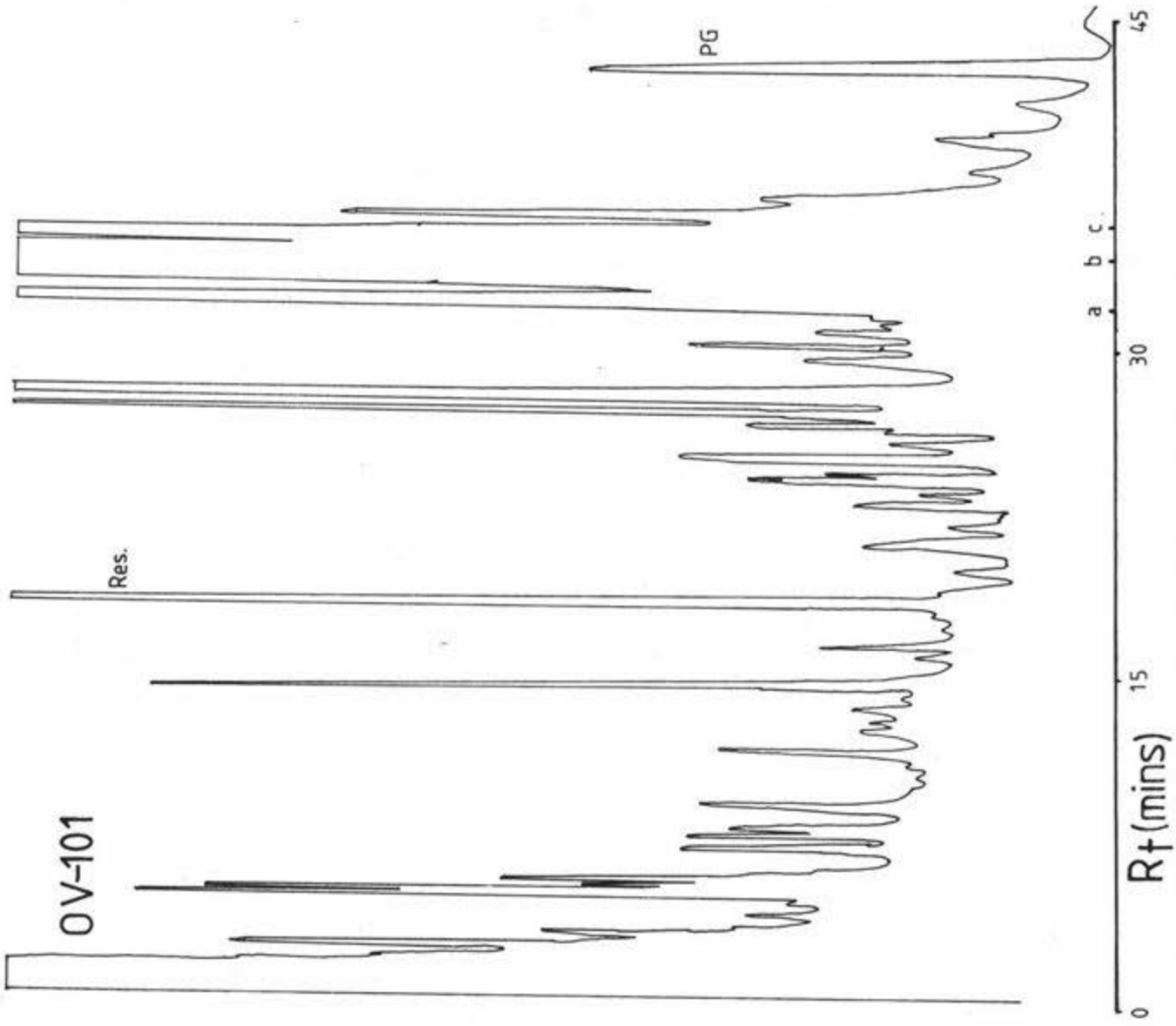


Fig. 3(c) Gas chromatography of urine extracted by the procedure of Muskiet et al. (1977).

Protocol: Injected onto OV-101 at 150 °C, programmed to 200 °C at a rate of 4 °C min^{-1} after 4 min.

Retention times: (a) HVA (b) DOPAC (c) VMA

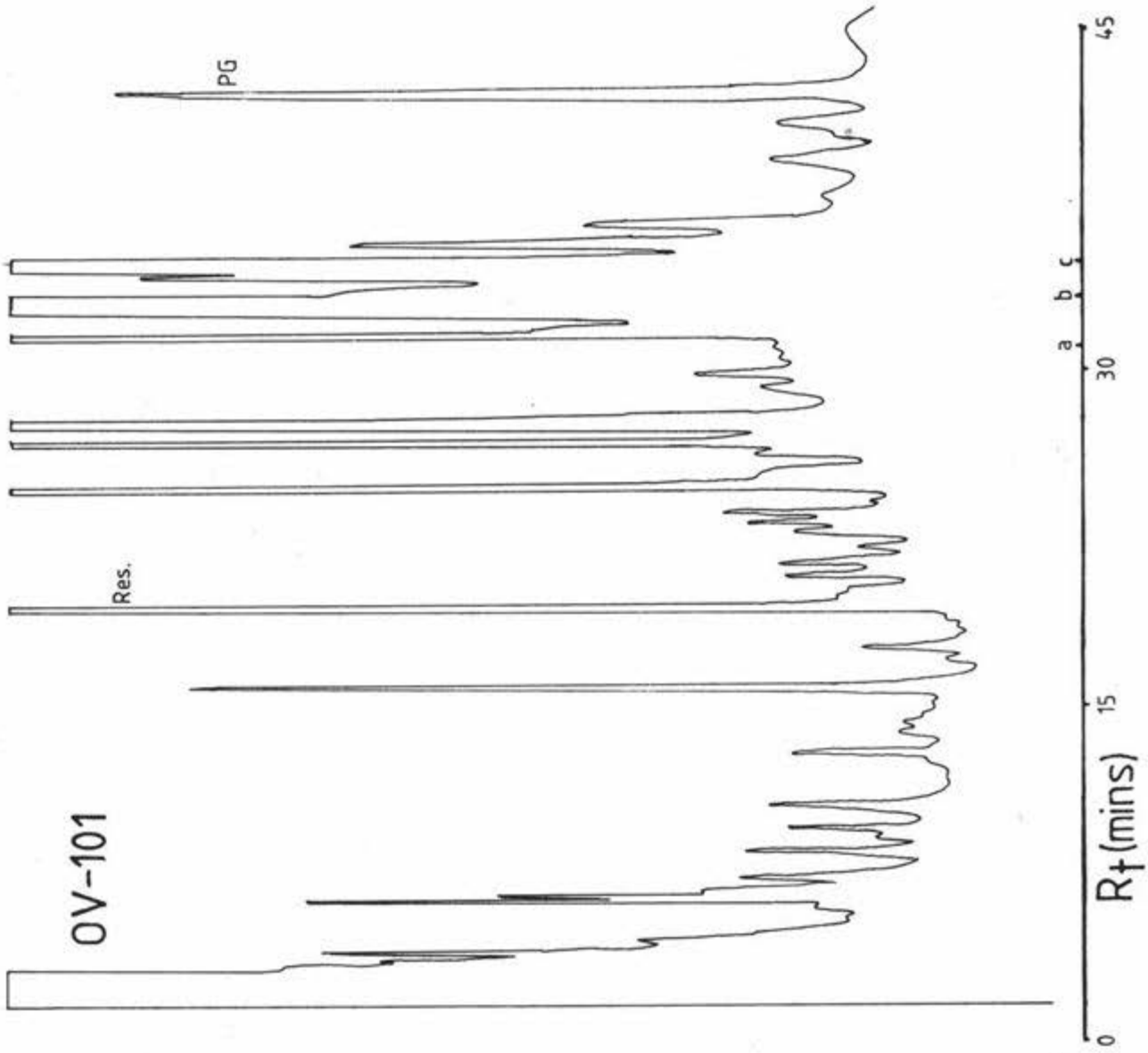


Fig. 3(d) Gas chromatography of urine extracted by the procedure of Thompson and Markey (1975).

The GC protocol was the same as that described in the legend to Fig. 3(c).

Retention times: (a) HVA (b) DOPAC (c) VMA

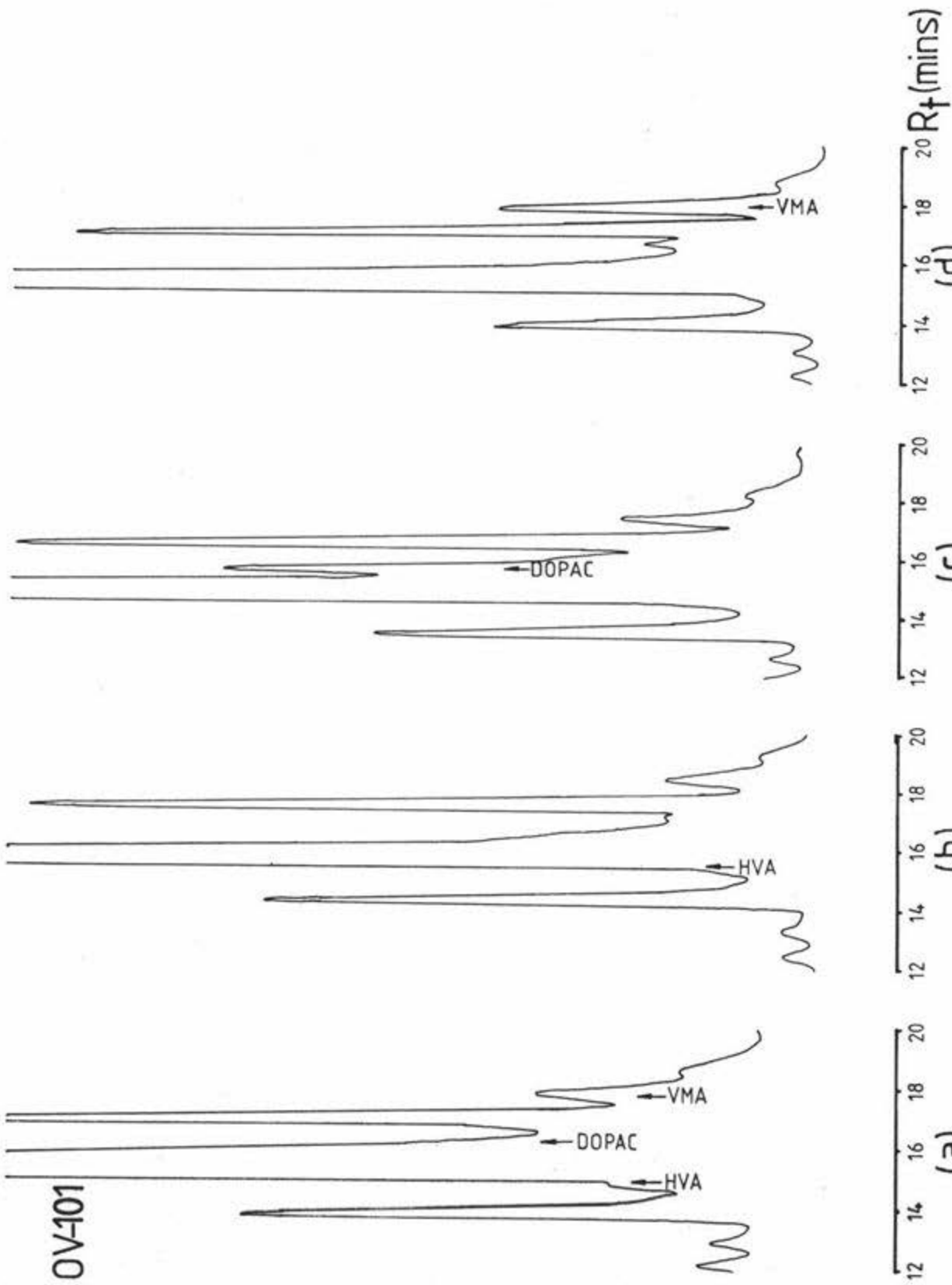


Fig. 3(e) Peak identification by urine spiking, ((a) unspiked, (b) urine + HVA, (c) urine + DOPAC, and (d) urine + VMA), showing the interfering peaks that eluted coincident with these metabolites.

The GC protocol was the same as that described in the legend to Fig. 3(c).

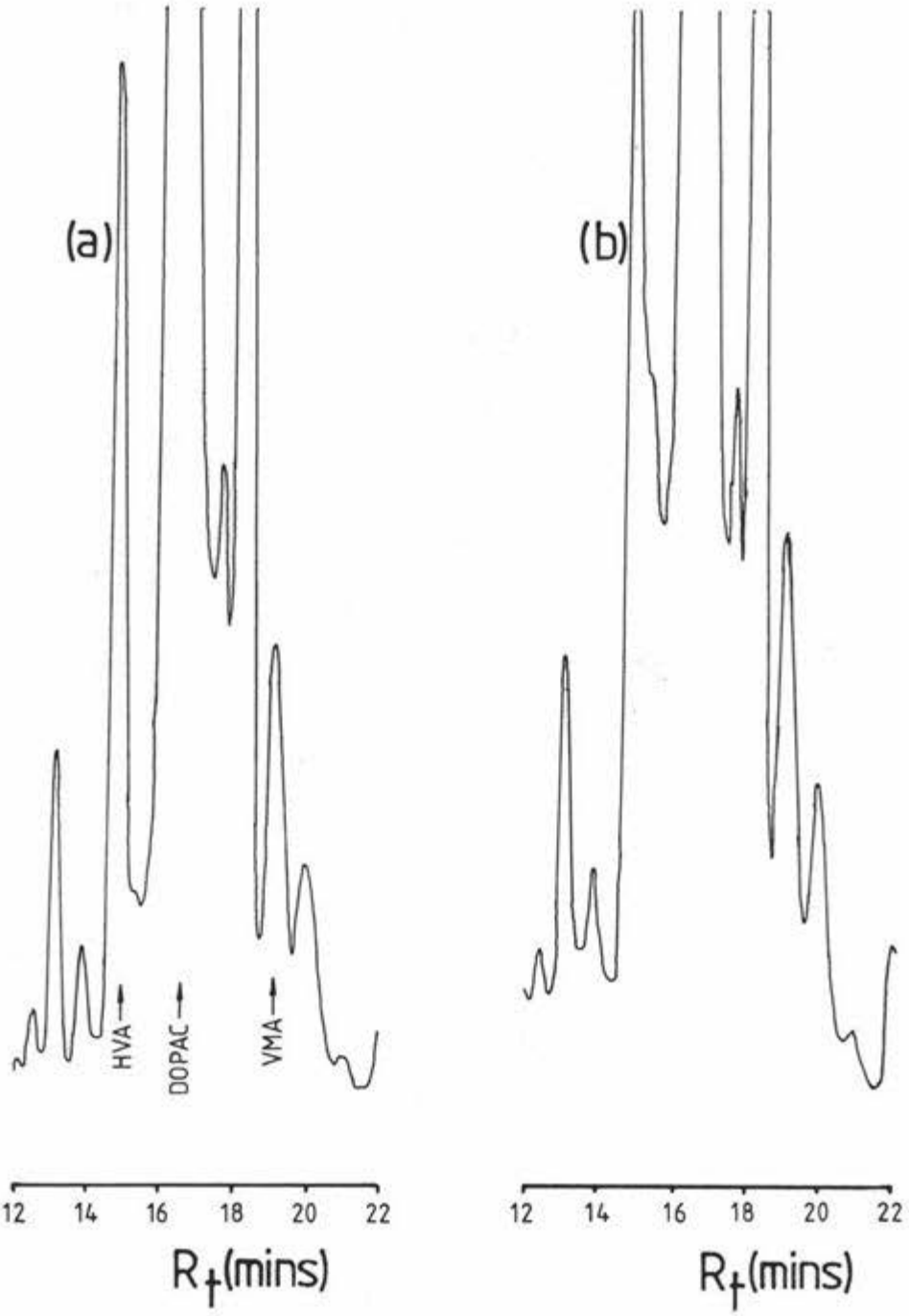


Fig. 3(f) Effect of derivatization procedures on peak resolution

(a) BSTFA (Muskiet et al. (1977))

(b) Tri Sil/TBT (Brewster et al. (1977)).

The GC protocol was the same as that described in the legend to Fig.3(c).

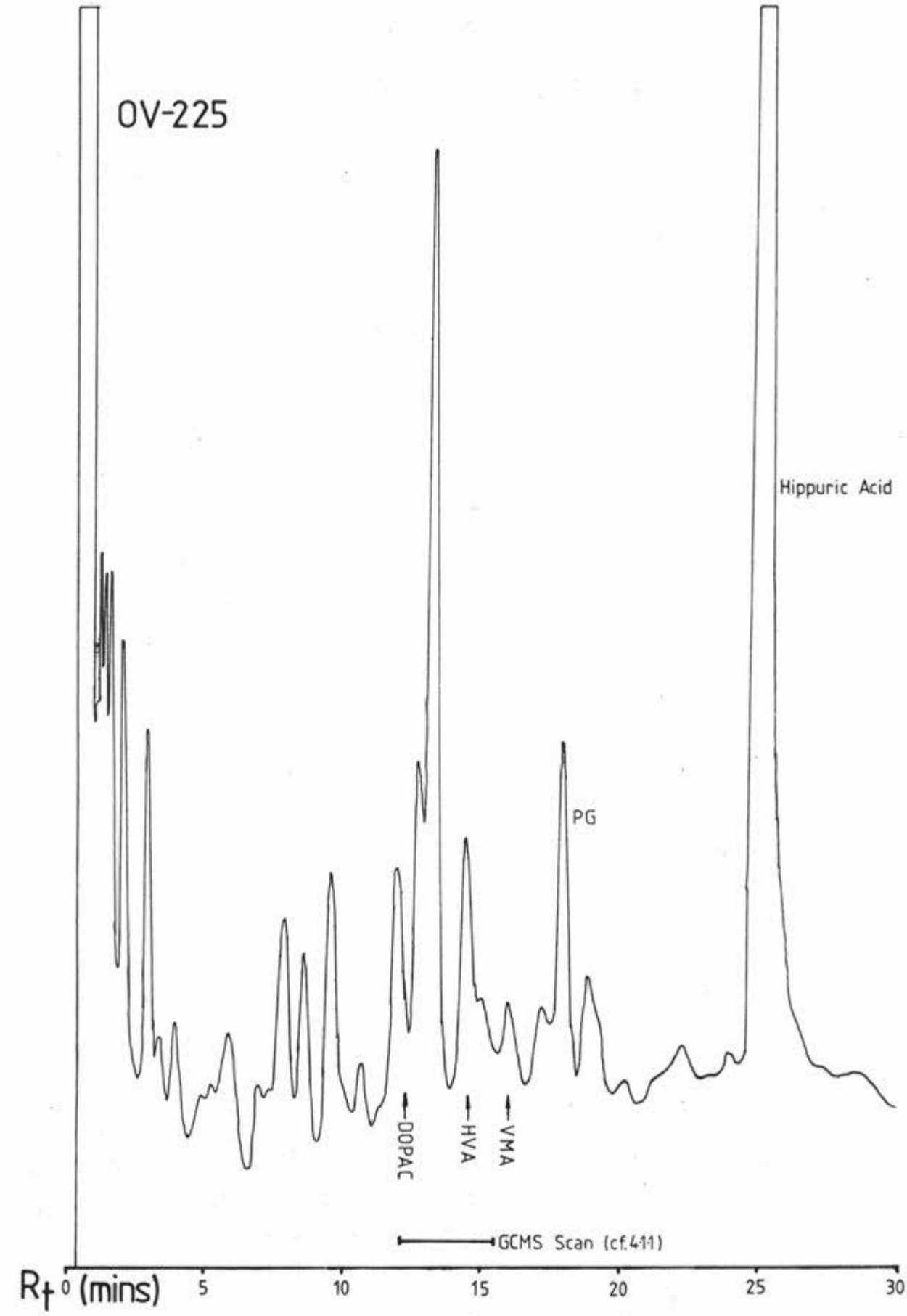


Fig. 3(g) Gas chromatography of extracted urine on OV-225.

Protocol: Injected isothermally onto OV-225 at 120 °C, programmed to 210 °C at a rate of 4 °C min after 4 min^{-1}.

The expected retention times for DOPAC, HVA and VMA are indicated by arrows.

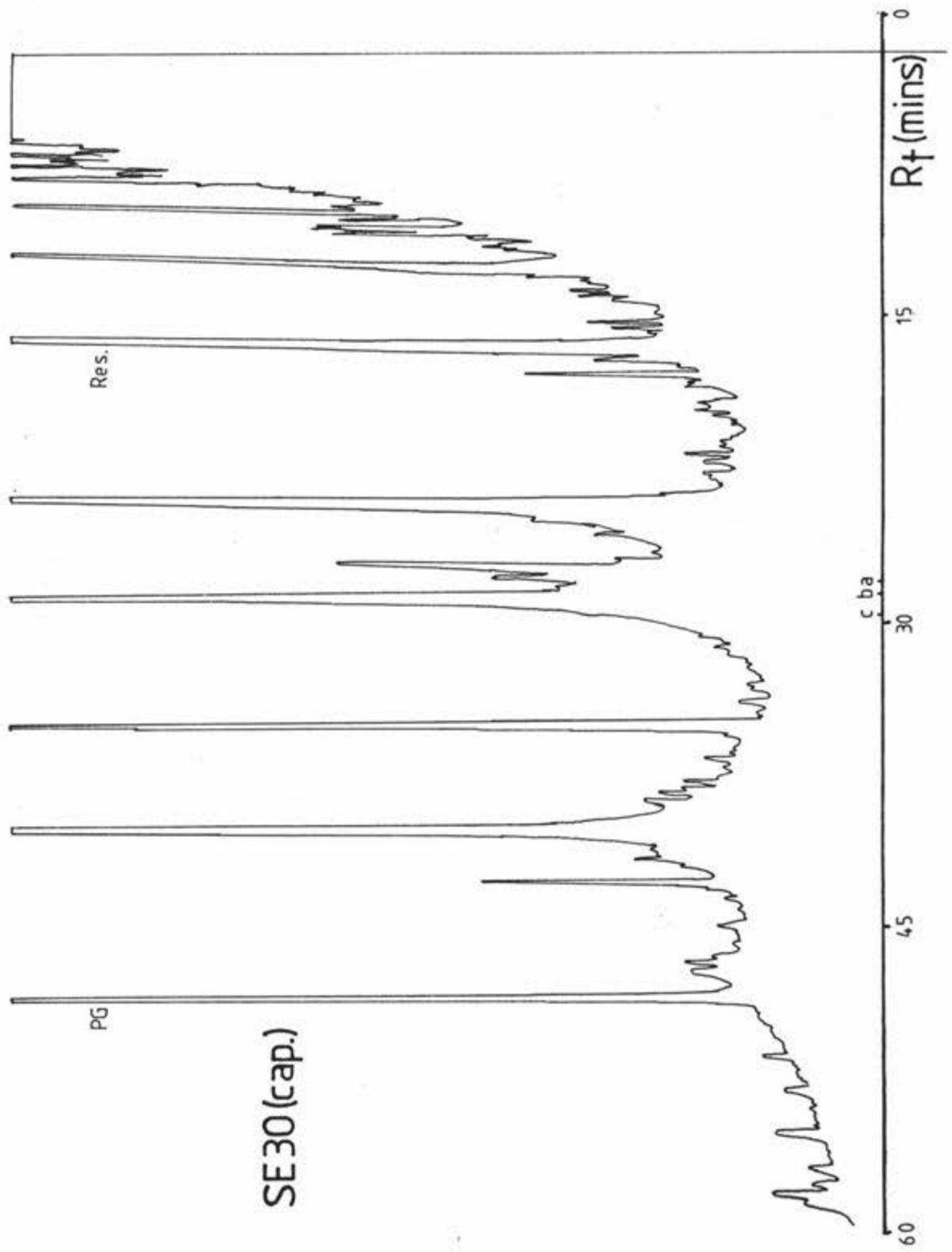


Fig. 3(h) Gaschromatography of extracted urine on a SE-30 capillary column.

Protocol: Injected at 140 °C, and then programmed to 220 °C at a rate of 2 °C min^{-1}.

Retention times: (a) DOPAC (b) HVA (c) VMA

method of Brewster et al. (1977).

The alternative extraction method of Thompson and Markey (1975) was found to have little effect on the resolution of DOPAC, HVA and VMA (Fig. 3(d)). Contrary to Brewster et al. (1977) and Brewster and Berry (1978), derivatization using Tri Sil/TBT was not found to have any significant effect on the hippuric acid peak (Fig. 3(f)).

Gas chromatography using different liquid phases showed greater potential for the improvement of the technique. Hippuric acid was found to elute later than HVA and VMA when chromatographed on OV-225 (Fig. 3(g)), however the resolution was still not considered to be sufficient to allow quantitation at low metabolite concentrations.

The GLC methods described by many authors (Sprinkle et al. (1969), Addanki et al. (1976), Brewster et al. (1977), and Muskiet et al. (1977)) have been designed primarily for the diagnosis, prognosis-assessment and followup of patients with tumours derived from the neural crest. In these subjects abnormally high amounts of catecholamine metabolites are excreted (up to 500 times the normal level, Muskiet et al. (1977)), therefore reducing the effect of interfering compounds and making quantitation by GLC using FID more feasible.

From this study, it was concluded that the estimation of normal levels of catecholamine and indoleamine metabolites in urine using GLC with FID was impracticable, and therefore would be unable to demonstrate any ethanol-induced changes in the excretion of these compounds. The possibility of improving the technique through more selective extraction procedures has been thoroughly researched by several authors with little progress made. It was subsequently decided to investigate the analysis of GC column effluents using a more selective detection method - mass spectrometry - and the application of this technique to the estimation of catecholamine and serotonin metabolites is discussed in the next chapter.

CHAPTER 4

GAS CHROMATOGRAPHY - MASS SPECTROMETRY OF CATECHOLAMINE AND SEROTONIN METABOLITES

4.1 Introduction

4.1.1 Gas Chromatography - Mass Spectrometry

From the GC studies in the previous chapter, the most promising result was a multicomponent chromatogram in which the resolution of DOPAC and HVA was subject to doubt (Fig. 3(g)). This section of the chromatogram was examined more closely using mass spectrometry (MS). The column effluent was diverted via a jet separator into the mass spectrometer as described later. A gas chromatogram was generated by total ion monitoring, and the area examined is indicated in Fig. 3(g). During DOPAC and HVA elution, 1 sec scans across the m/e range 145-400 were made every 10 sec and registered on an ultraviolet recording oscillograph (Fig. 4(a)). The mass numbers of individual spikes were determined by counting from the known background spikes, and the peak height (intensity) plotted against the scan number (Fig. 4(b)). The results showed that this area of the chromatogram contained multiple components of which DOPAC and HVA were only a minor contribution. It was concluded that attempts to quantitate these metabolites by GC alone would be susceptible to error.

However, the possibility of quantitation by using the mass spectrometer as a specific ion detector was appreciated and the method eventually developed is described in this chapter.

4.1.2 Mass Fragmentography

While the GC characterization of numerous volatile derivatives of catecholamine and indoleamine - related compounds has been reported in the literature, there have been relatively few detailed studies of their mass fragmentography. Hattox and Murphy (1978) and Francis et al. (1980) have studied the mass spectra of catecholamine and indoleamine - related compounds respectively, and discussed their fragmentation characteristics.

Compounds eluting from a GC column into the mass spectrometer fragment in a specific manner, giving rise to a spectrum of ions characteristic to each particular compound. Fragmentation patterns for the trimethylsilyl derivatives of HVA and VMA have been proposed by Lanser et al. (1974) (Figs. 4(c) and 4(d)). By selecting one of these

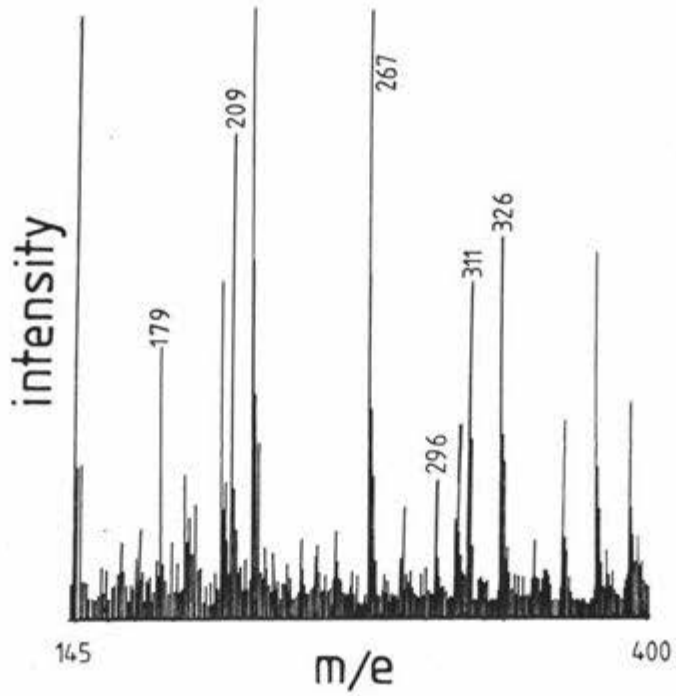


Fig. 4(a) UV recorder response to scan number 13 (refer Fig. 4(b)) of the repetitive scanning procedure used to investigate peak heterogeneity.

Numbered m/e values are ions known to be at least partially contributed by HVA.

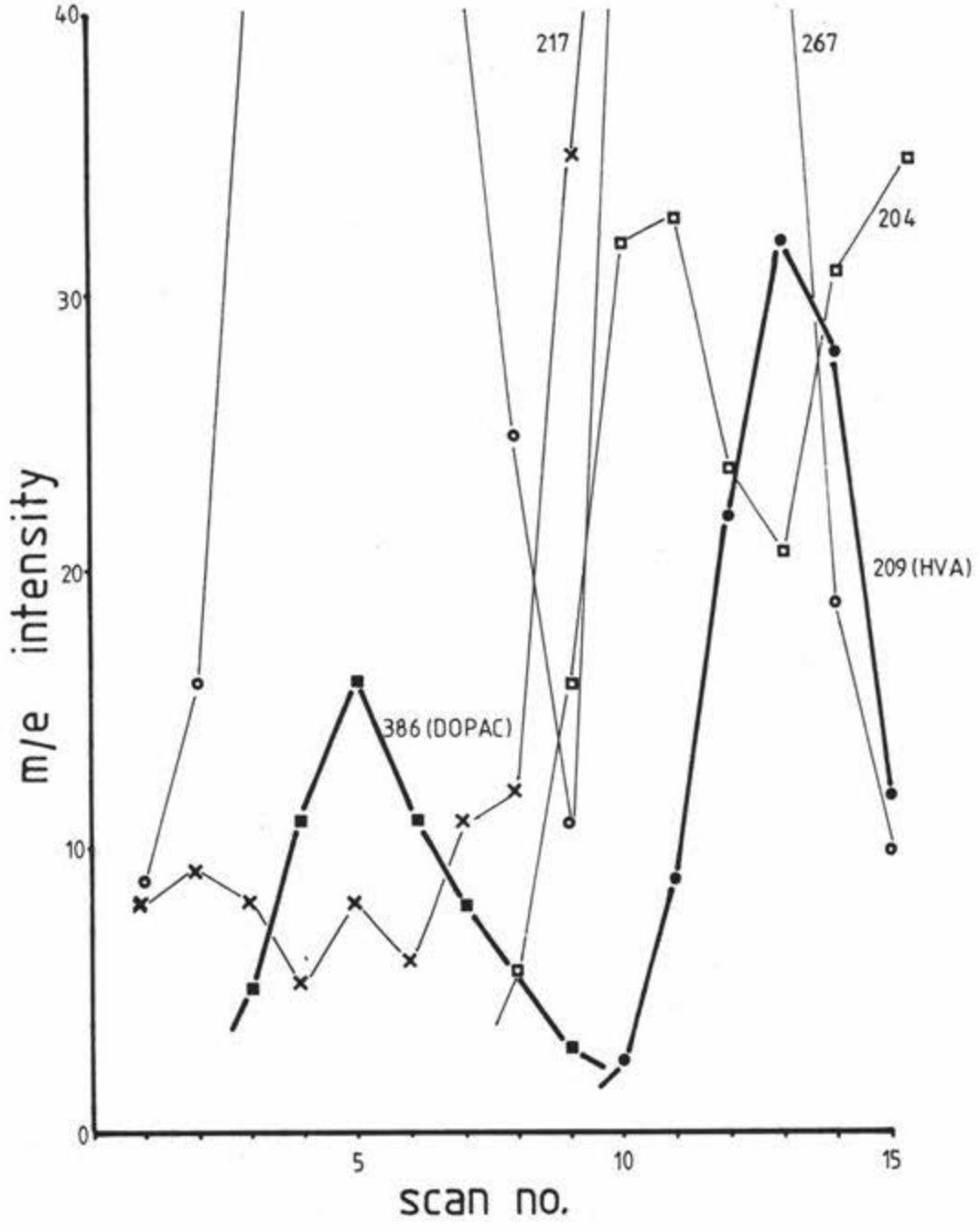


Fig. 4(b) Repetitive scanning of OV-225 column effluent to determine heterogeneity of DOPAC and HVA peaks.

Key: ▫ m/e 204
● m/e 209 (HVA)
× m/e 217
○ m/e 267
■ m/e 386 (DOPAC)

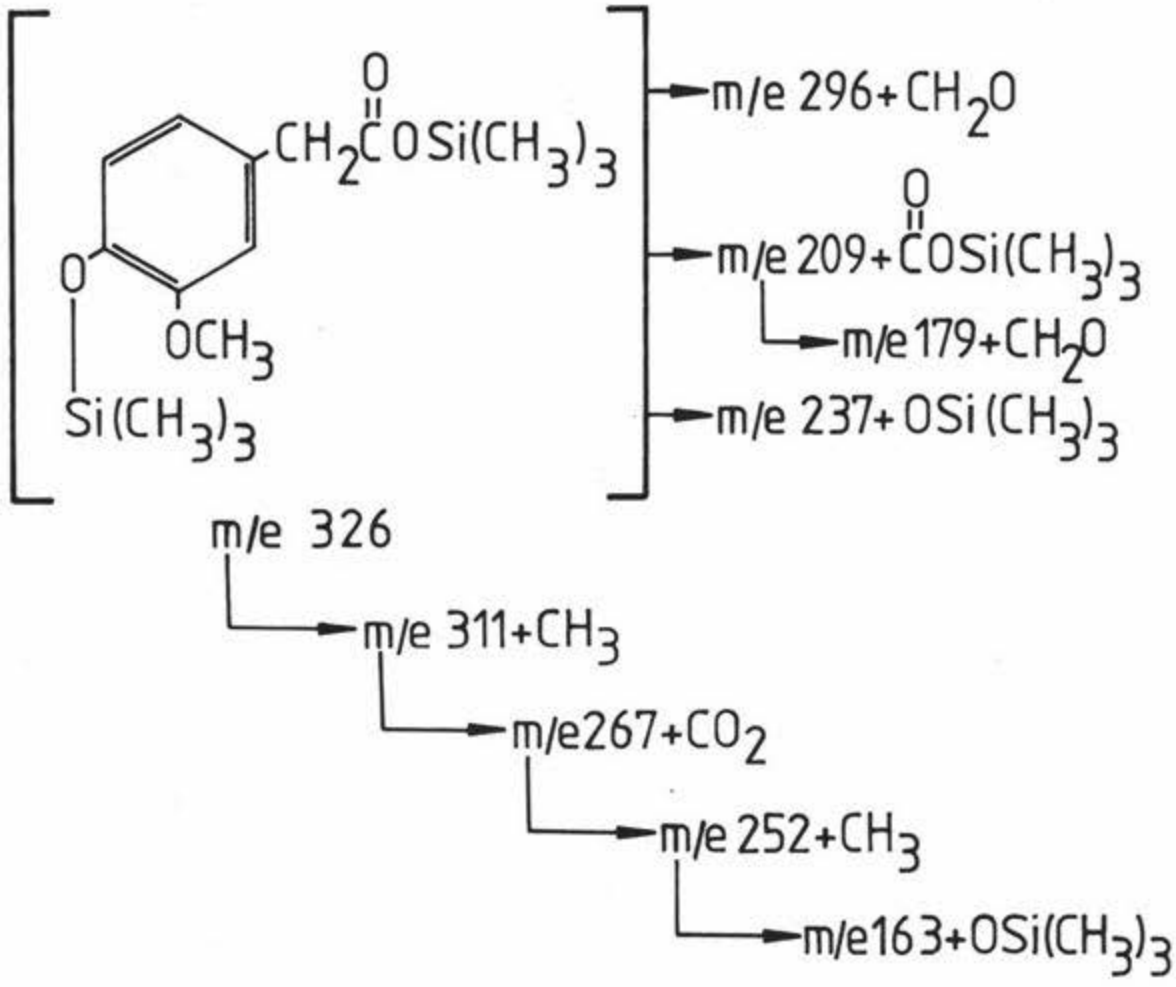


Fig 4(c) Proposed fragmentation pathways for the diTMS derivative of HVA (from Lanser et al. (1974)).

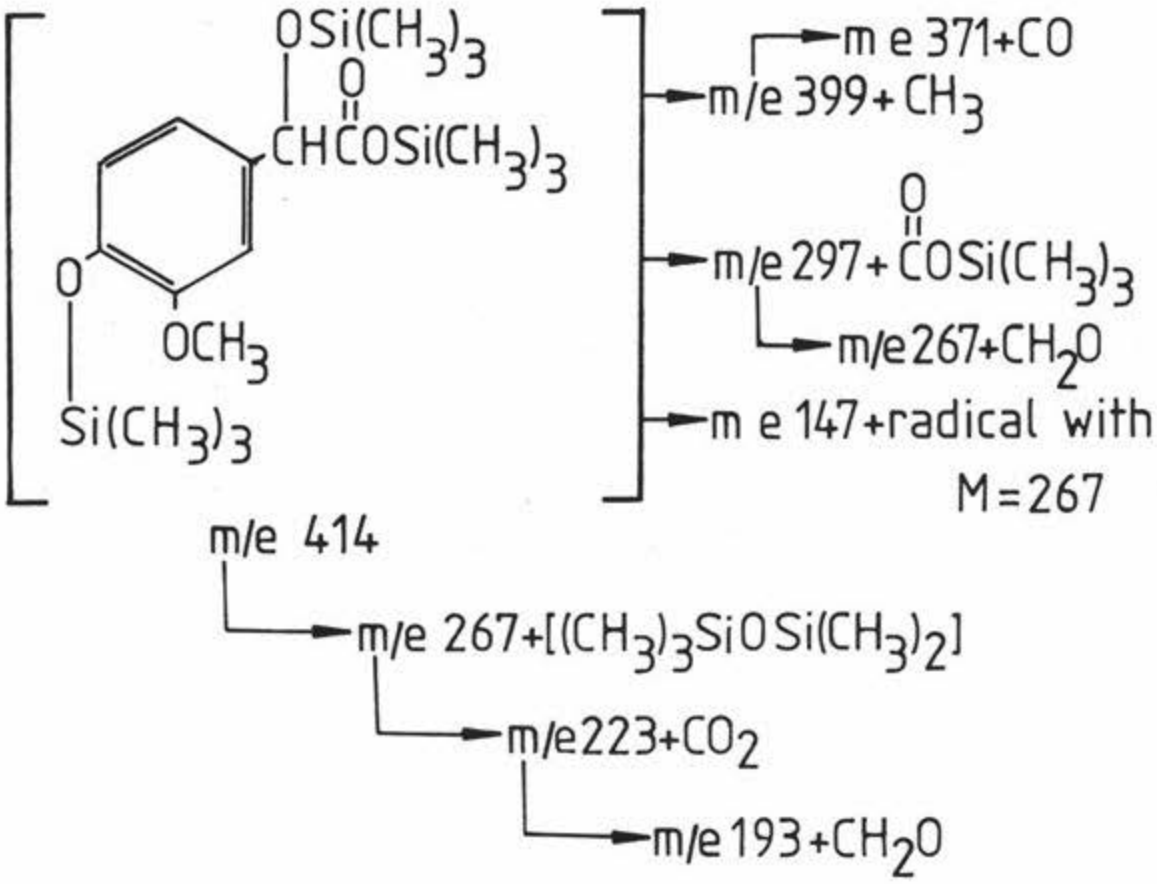


Fig 4(d) Proposed fragmentation pathways for the triTMS derivative of VMA (from Lanser et al. (1974)).

ions for specific detection it is possible to quantitatively estimate the parent compound, a technique called selected ion monitoring (SIM). The mass spectra of the TMS derivatives of all the metabolites investigated in this study are given in Appendix II, and it is apparent that several ions of outstanding relative abundance could be used for the monitoring of each compound. The basis on which an ion was selected for SIM consisted of three main criteria; (a) the ease with which each ion could be identified against the stationary phase background, (b) ions which showed conspicuous interference from other urine components were ruled out, and (c) high m/e values were given preference over low ones because the possibility of interference is greater at the lower part of the spectrum.

The use of SIM for the estimation of the TMS derivatives of catecholamine and indoleamine metabolites has been described by Domino et al. (1979), but their method required the use of a multiple ion detector (MID), a device with which most standard mass spectrometers, including the one used in this study, are not equipped. However, by controlling the magnet current, it was found to be possible to selectively monitor clusters of 4-5 ions, and a method utilising this technique for the estimation of catecholamine and indoleamine metabolites is described below.

4.2 Methods

4.2.1 Gas Chromatography - Mass Spectrometry

Acidic and alcoholic metabolites, extracted and derivatized as described in the previous chapter, were gas chromatographed on OV-225 or OV-101 liquid phases using a Varian Aerograph series 1700 instrument. This was connected to a VG 12F Micromass mass spectrometer via a jet separator with the connection line held at 250 °C. The mass spectrometer was operated in the electron impact mode with a source temperature of 250 °C, an electron energy of 70 eV, an emission of 100 μA, and an accelerating voltage of 4 kV.

4.2.2 Selected Ion Monitoring (SIM)

The ions selected for the SIM of each metabolite assayed are listed in Tables 4(i) and 4(ii) for acidic and alcoholic metabolites respectively. A group of 4-5 adjacent ions were continuously monitored at a rate of one

Table 4(i) SIM of Acidic Metabolites on OV-225

Compound	m/e selected	Intensity	Vh (Hall Probe)
DOPAC	384	41.3	1.0164
DOMA	355	100	0.9784
HVA	326	32.9	0.9397
VMA	297	100	0.8971
5-HIAA	290	56.0	0.8869
PG (internal standard)	428		1.0690
Calibration (column bleed)	207		0.7529

Table 4(ii) SIM of Alcoholic Metabolites on OV-101

Compound	m/e selected	Intensity	Vh (Hall Probe)
DOPEt	267	47.8	0.8520
DOPEG	355	100	0.9784
MOPEt	209	61.2	0.7564
MOPEG	297	40.0	0.8971
5-HTPh	290	100	0.8869
Res (internal standard)	255		0.8332
Calibration (column bleed)	281		0.8734

Intensities expressed as % of the peak with maximum intensity, catecholamine metabolite intensities are from Hattox and Murphy (1978) and indoleamine metabolite intensities are from Markey et al. (1974).

1 scan sec^{-1} by voltage scanning from 4.0 - 3.8 kV. The specific ion cluster was selected by switching a preset potentiometer device which varied the magnet current supply. The magnetic field, and consequently the ion to mass charge ratio (m/e), was monitored by a Hall probe situated on one pole of the electromagnet. Calibration of the magnet current was achieved by introducing heptacosafluorotri-n-butylamine via the direct inlet, and then correlating the known heptacosa m/e ions with the Hall probe readings (Vh values). In this way, the Vh value for any

m/e ratio being monitored could be determined from a graph of m/e vs. Vh (Fig. 4(e)). The output of the probe was monitored by a digital voltmeter enabling precise location of the requisite m/e from previous calibration. The calibration was checked at the beginning of each day's analyses by ensuring that the m/e of a known column bleed peak correlated with its predetermined Vh value.

The output of the electron multiplier on the mass spectrometer was integrated for each voltage scan and registered on a chart recorder at 50 mV full scale deflection. During a gas chromatographic run, the magnet current switching device was set to allow monitoring of the specific ion cluster selected for the first compound to elute. When this peak had been recorded, the magnet current was switched to detect the selected ion cluster of the next eluting compound. This process was repeated for each compound being monitored in the chromatographic run.

4.2.3 Quantitation

Quantitation was achieved by extracting a series of aqueous standards and a recovery sample (urine containing a known amount of each metabolite being estimated) together with the urine sample. Metabolite concentrations were estimated from the standard curves and then corrected for differences in recovery between aqueous and urine samples by determining recovery factors. Mean recoveries for the acidic metabolites (n=6) relative to the internal standard (PG), determined by GCMS - SIM, are given in Table 4 (iii).

Table 4(iii) Mean Recoveries of Acidic Metabolites from Urine Relative to the Internal Standard (PG), (n=6)

Metabolite	% Recovery ± 1 s.d.
DOPAC	94.5 ± 9.8 %
HVA	104.4 ± 15.4 %
VMA	107.4 ± 13.4 %
DOMA	97.6 ± 17.6 %
5-HIAA	83.6 ± 14.0 %

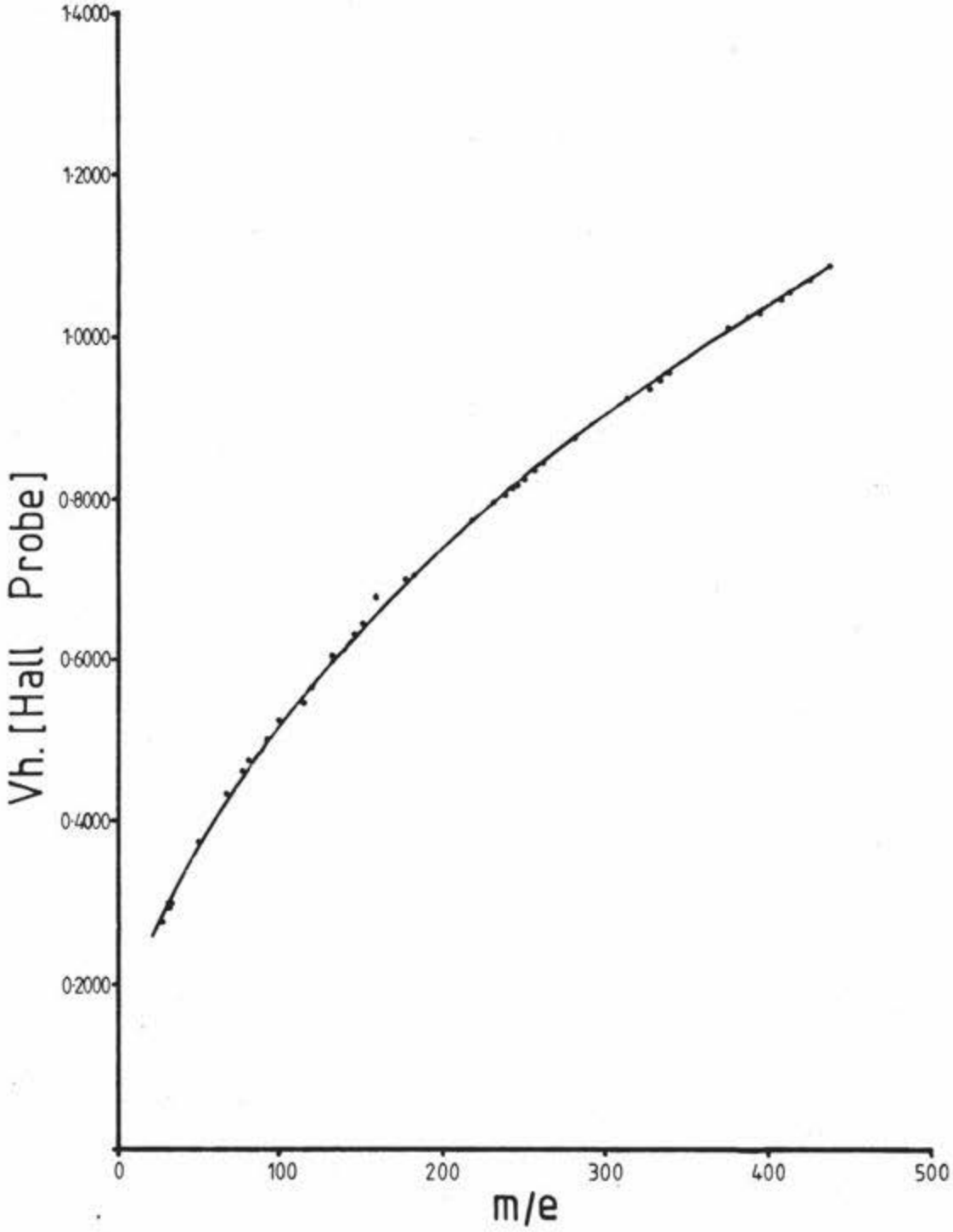


Fig. 4(e) Calibration of the magnet current using a Hall probe

$y = 8.6152 - 38.8574x + 401.5843x^2$

where y = m/e value, x = Hall probe reading (Vh).

4.3 Results

4.3.1 SIM of Metabolites Extracted from Pure Solutions

(a) Acidic Metabolites

Initial investigations on the GCMS-SIM estimation of the acidic metabolites showed that DOPAC, HVA and VMA could be estimated on a single chromatographic run (Fig. 4(f)a). Standard curves over their normal physiological ranges are given in Table 4(iv) and typically showed good linearity with an intercept on the abscissa usually between 0 - 0.5 μg metabolite ml^{-1}, (Fig. 4(g)).

Table 4(iv) Standard Curves for SIM Estimation of DOPAC, HVA and VMA

Concentration (μg ml^{-1})	1.0	2.0	4.0	8.0			
Peak Height Ratio:					a	b	$\pm s_b$
DOPAC/PG:	0.0310	0.0694	0.1473	0.3113	0.267	24.9	0.256
HVA/PG :	0.0197	0.0516	0.0717	0.1541	-0.226	53.5	4.87
VMA/PG :	0.0653	0.1553	0.3143	0.6429	0.163	12.2	0.112

Where $y = a + bx$ (y = metabolite concentration, x = peak height ratio)

Fig. 4(h) shows the chart recorder response for total ion monitoring of the metabolites DOPAC, HVA, VMA, DOMA and 5-HIAA together with the internal standard PG on a single chromatographic run on OV-225. It was found that the retention times of DOMA and VMA were identical. The metabolite 5-HIAA was found to have a long retention time and the fact that it eluted from the column at a high temperature meant that the peak was resolved on top of a shelf of column bleed. It was subsequently decided to estimate DOMA and 5-HIAA on a separate chromatographic run with a revised protocol (Fig. 4(f)b).

The resolution of 5-HIAA was found to be extremely poor with peaks showing extensive tailing. The injection of 10 - 20 μl of Silyl-8 column conditioner (Pierce Chemical Co.) at 200 - 250°C prior to sample injection was found to greatly reduce tailing and increase peak height (Fig. 4(i)). Column conditioning with Silyl-8 between injections was subsequently adopted as a routine procedure.

Standard curves for DOMA and 5-HIAA over their normal

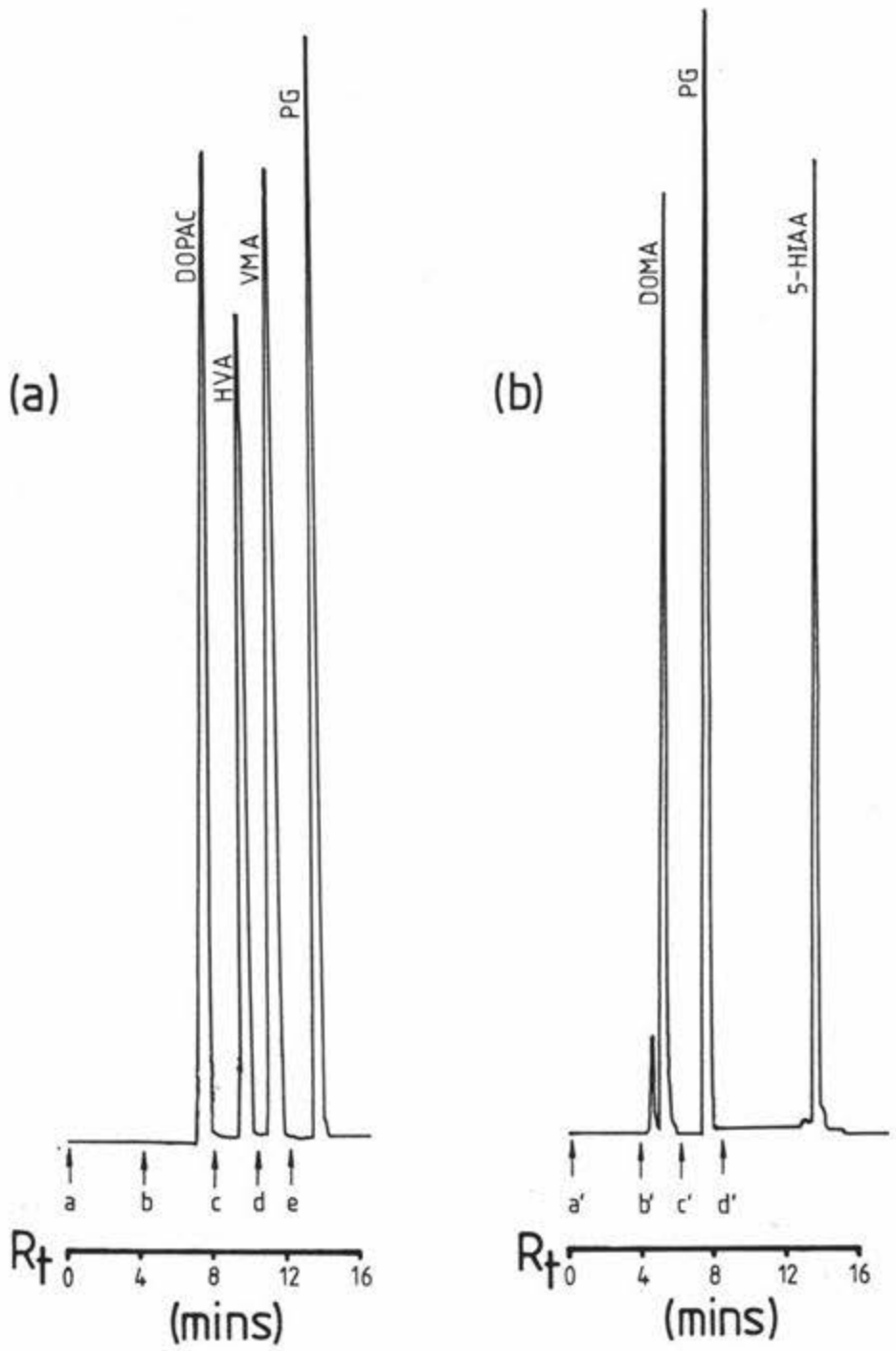


Fig. 4(f) SIM of acidic metabolites extracted from pure aqueous solution

(a) DOPAC, HVA, VMA, PG on OV-225. Protocol: (a) injected isothermally at 160 °C, dump valve closed (b) dump valve opened, SIM on m/e 384 (c) temperature programmed to 250 °C at 8 °C min^{-1}, SIM on m/e 326 (d) SIM on m/e 297 (e) SIM on m/e 428

(b) DOMA, PG, 5-HIAA on OV-225. Protocol: (a) injected isothermally at 180 °C, dump valve closed (b) temperature programmed to 250 °C at 8 °C min^{-1}, dumped valve opened, SIM on m/e 355 (c) SIM on m/e 428 (d) SIM on m/e 290

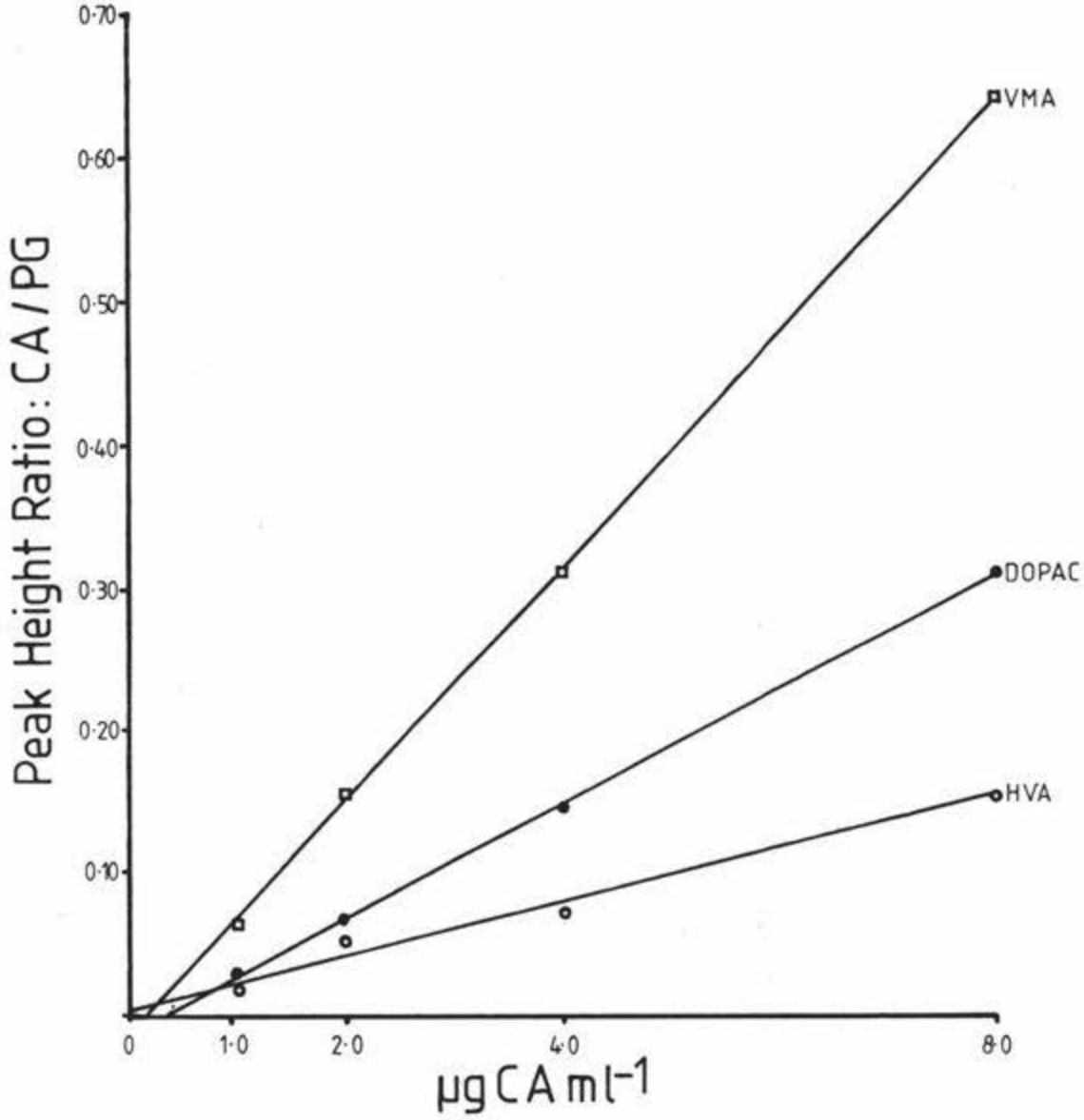


Fig. 4(g) Standard curves for SIM of DOPAC, HVA, VMA estimated by the protocol described in the legend to Fig. 4(f)a.

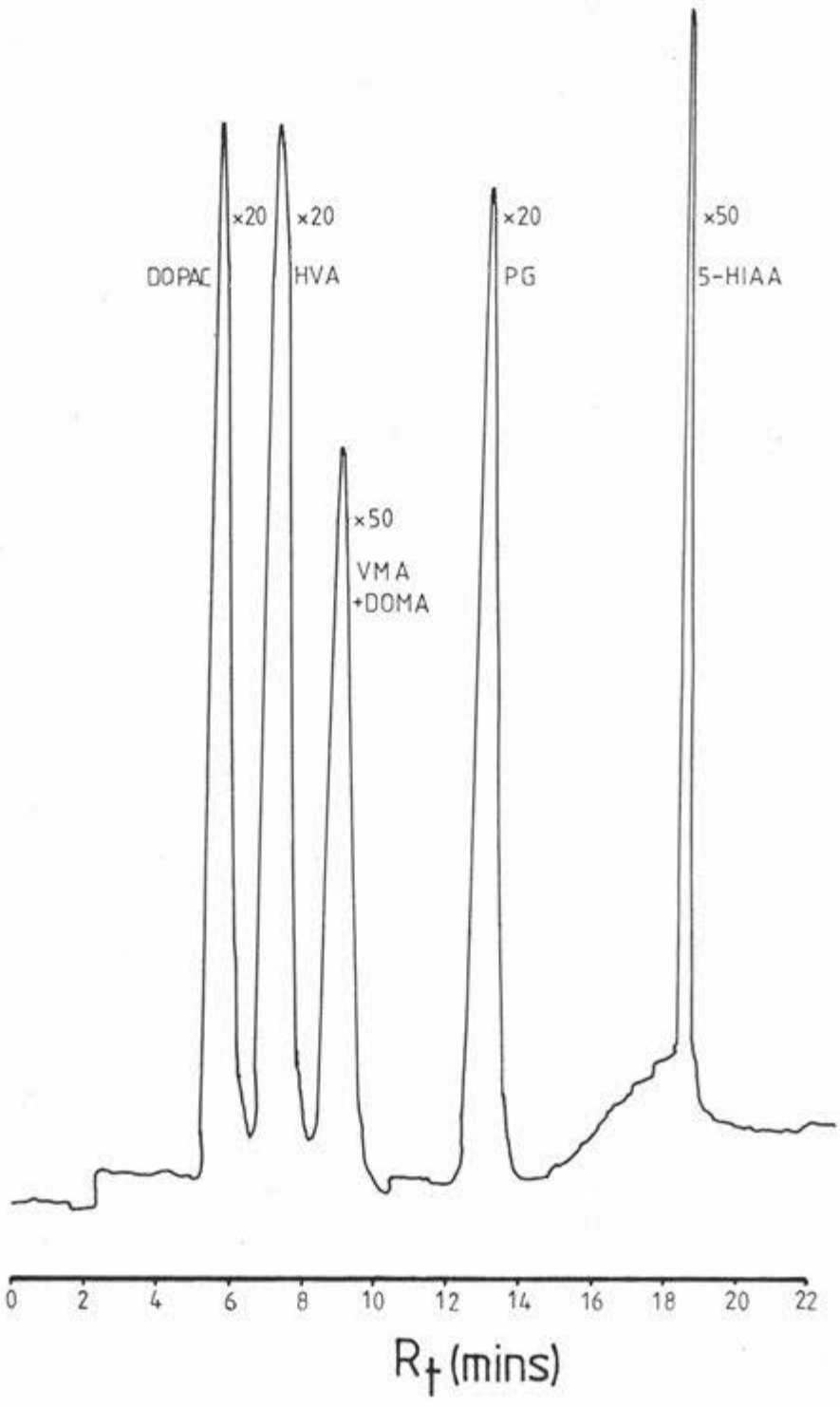


Fig. 4(h) Total Ion Monitoring (TIM) of an extract containing 100 µg of DOPAC, HVA, VMA, DOMA, PG and 5-HIAA by the GC protocol described the legend to 4(f)a showing coincident retention times (R_t) of VMA and DOMA on OV-225.

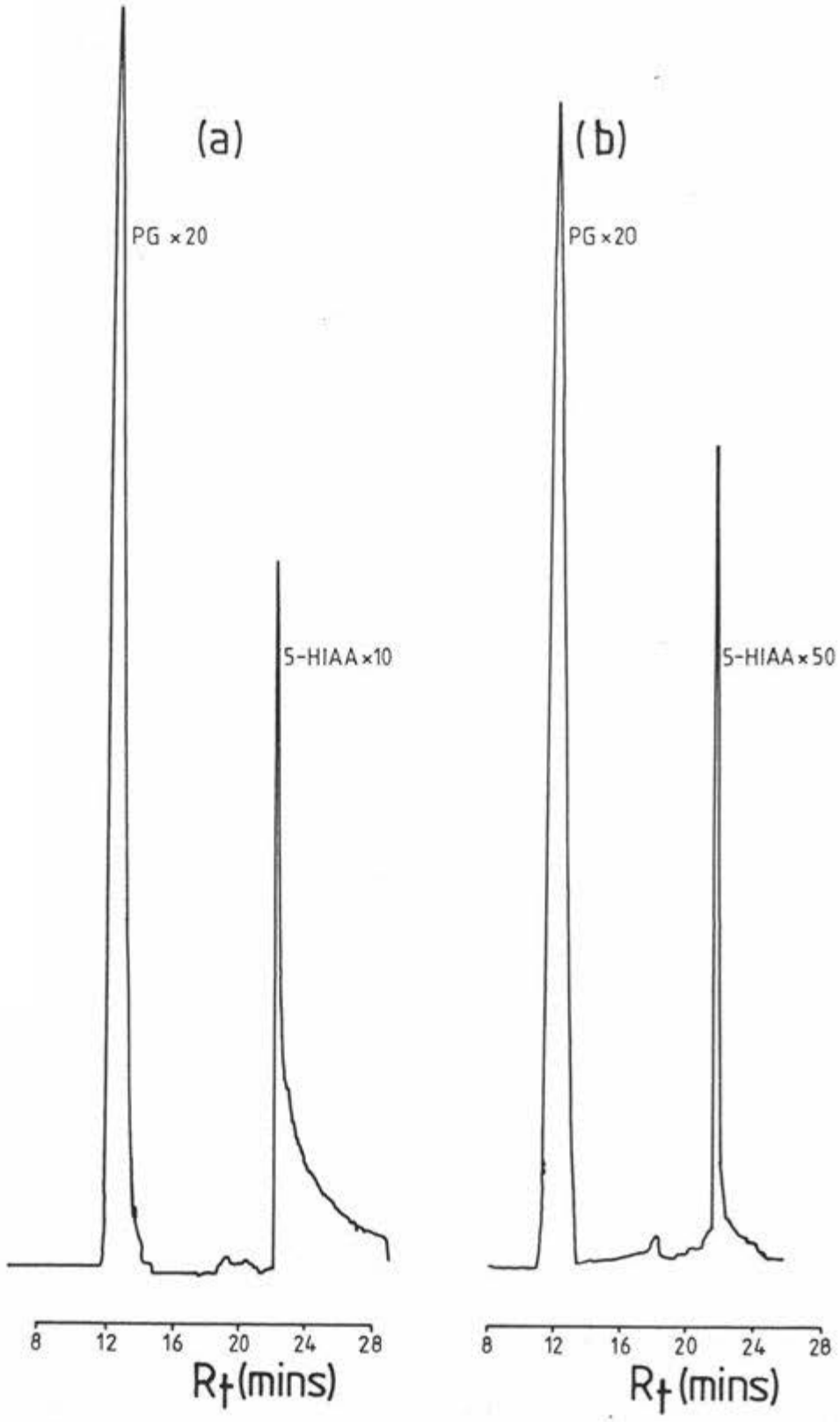


Fig. 4(i) Effect of Silyl-8 column conditioner on 5-HIAA peak resolution

(a) Before conditioning

(b) After conditioning with 20 µl Silyl-8 injected at 200 °C

physiological ranges are given in Table 4(v). They typically showed good linearity and a low abscissa intercept was consistently observed (Fig. 4(j)).

Table 4(v) Standard Curves for SIM Estimation of DOMA and 5-HIAA

Concentration ($\mu g\ ml^{-1}$)	0.2	0.4	0.8	1.6			
Peak Height Ratio:					a	b	$\pm s_b$
DOMA/PG:	0.0048	0.0104	0.0201	0.0419	0.019	37.9	0.607
Concentration ($\mu g\ ml^{-1}$)	2.0	4.0	8.0	16.0			
Peak Height Ratio:					a	b	$\pm s_b$
5-HIAA/PG:	0.017	0.307	0.966	2.200	1.75	6.48	0.142

(b) Alcoholic Metabolites

The metabolites DOPEt, MOPEt, DOPEG, MOPEG and 5-HTPh together with an internal standard Res were all found to be sufficiently resolved on OV-101 to allow their simultaneous quantitation on a single chromatographic run (Fig. 4 (k)a). Standard curves for these metabolites over their normal physiological ranges are given in Table 4(vi). They showed good linearity, and as with the acidic metabolites, typically showed a low intercept value on the abscissa (Fig. 4(l)).

Table 4(vi) Standard Curves for SIM Estimation of Alcoholic Metabolites

Concentration ($\mu g\ ml^{-1}$)	0.2	0.4	0.8	1.6			
Peak Height Ratio:					a	b	$\pm s_b$
DOPEt/Res:	0.0091	0.0195	0.0576	0.129	0.138	11.3	0.441
Concentration ($\mu g\ ml^{-1}$)	0.4	0.8	1.6	3.2			
Peak Height Ratio:					a	b	$\pm s_b$
MOPEt/Res:	0.08028	0.1452	0.2570	0.5429	-0.050	6.05	0.222
DOPEG/Res:	0.0074	0.0455	0.0994	0.2172	0.249	13.5	0.374
Concentration ($\mu g\ ml^{-1}$)	1.0	2.0	4.0	8.0			
Peak Height Ratio:					a	b	$\pm s_b$
MOPEG/Res:	0.1431	0.3423	0.7091	1.479	0.238	5.25	0.0369
5-HPTh/Res:	0.04765	0.1280	0.2909	0.5675	0.266	13.5	0.400

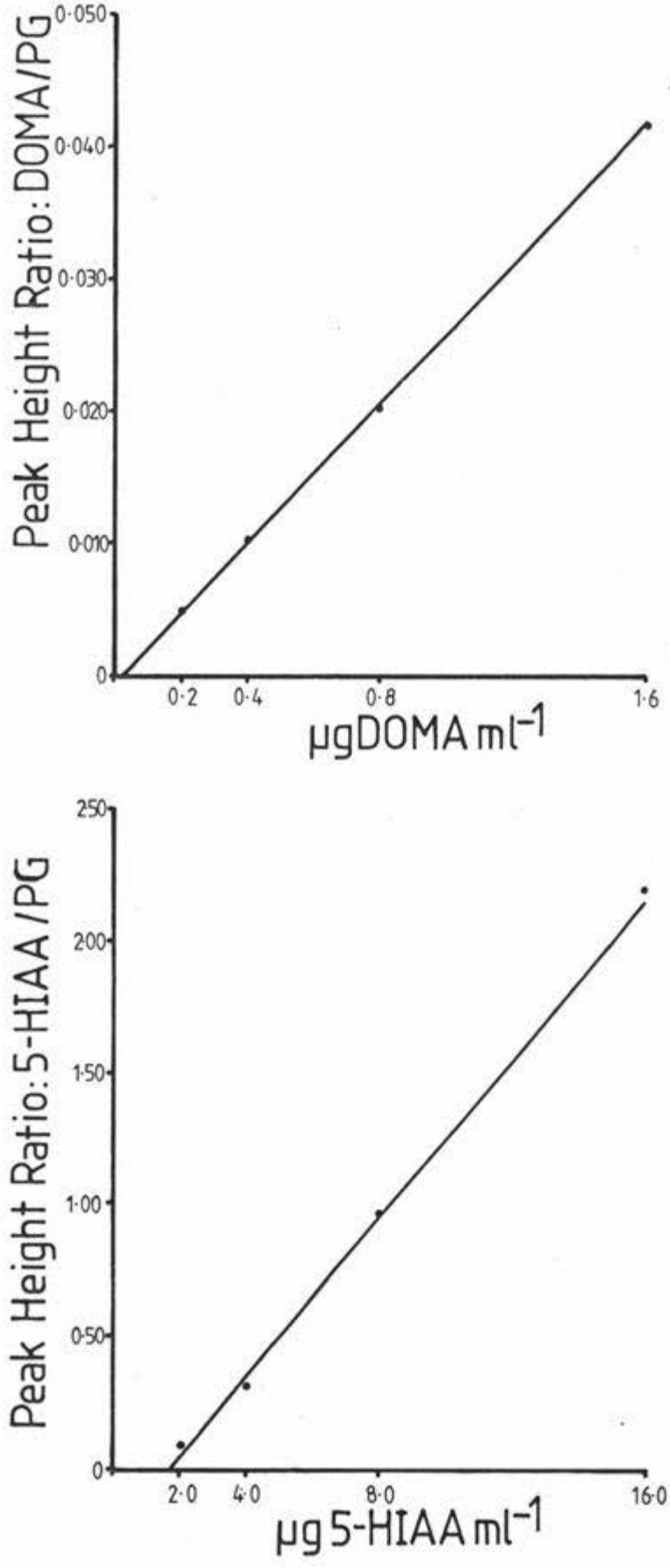


Fig. 4(j) Standard curves for SIM of DOMA and 5-HIAA estimated by the protocol described in the legend to Fig. 4(f)b.

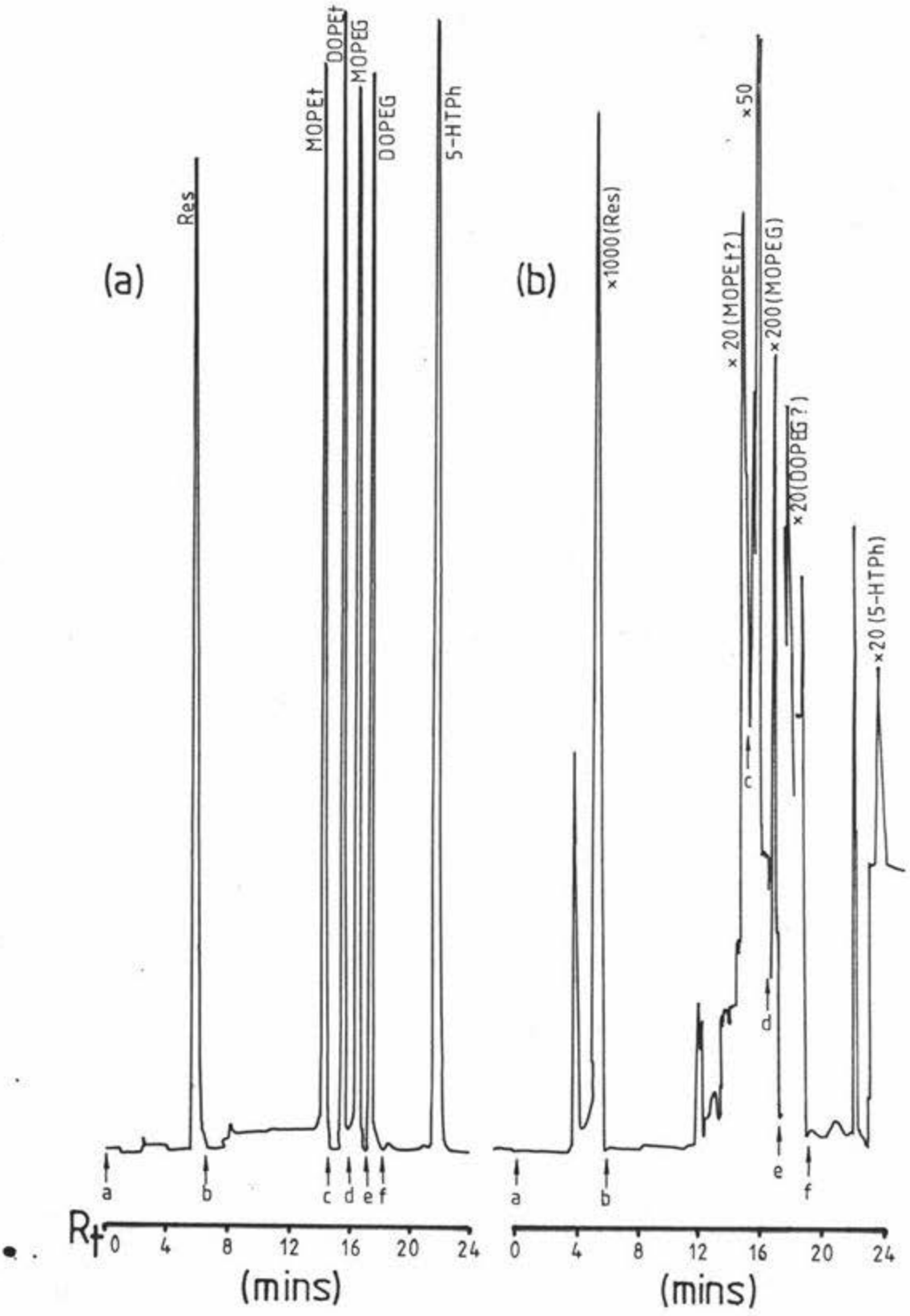


Fig. 4(k) SIM of alcoholic metabolites MOPEt, DOPEt, MOPEG, DOPEG, 5HPTh and Res (a) extracted from pure solution (b) extracted from urine. Protocol: (a) Injected isothermally at 150 °C, dump valve opened after 4 min, SIM on m/e 255 (b) Temperature programmed to 250 °C at 10 °C min^{-1}, SIM on m/e 209 (c) SIM on m/e 267 (d) SIM on m/e 297 (e) SIM on m/e 355 (f) SIM on m/e 290.

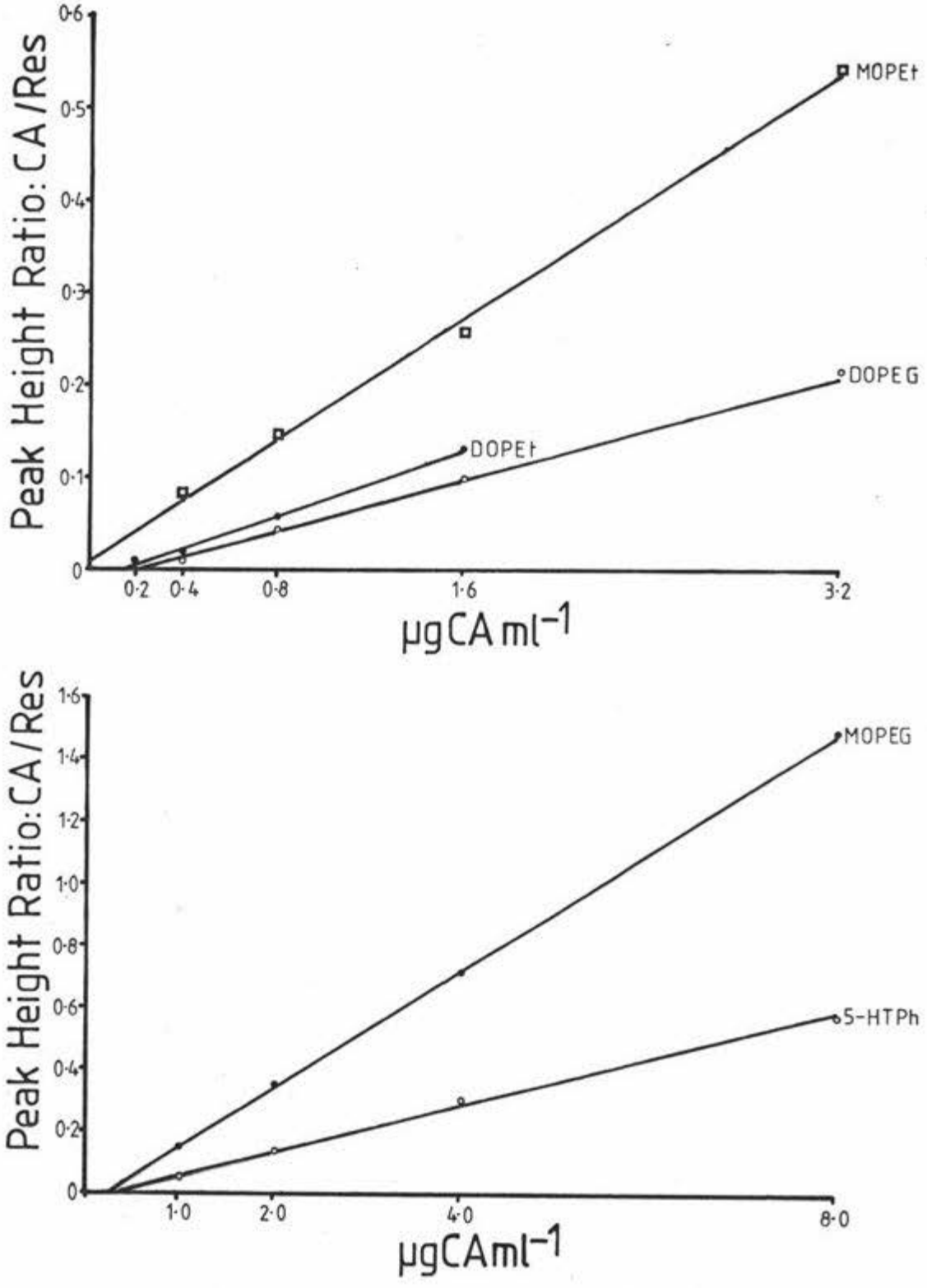


Fig 4(1) Standard curves for the alcoholic metabolites MOPEt, DOPEt, MOPEG, DOPEG, and 5-HTPh estimated by the protocol described in the legend to Fig. 5(k).

4.3.2 Peak Height Reproducibility

Peak heights for a derivatized sample containing DOPAC, HVA, VMA and PG repeatedly monitored by the protocol described in Fig 4(f)a are given in Table 4(vii). The peak height ratios showed good reproducibility, with 2 s.d. for DOPAC, HVA and VMA being 5.4%, 4.3% and 6.5% of their means respectively.

Table 4(vii) Peak Height Reproducibility of SIM Procedure

Peak Height				Peak Height Ratio: CA/PG		
DOPAC	HVA	VMA	PG	DOPAC/PG	HVA/PG	VMA/PG
60	47	85	105	0.571	0.448	0.810
68	52	89	114	0.596	0.456	0.781
74	58	102	125	0.592	0.464	0.816
88	71	125	148	0.595	0.474	0.845
61	47	84	101	0.604	0.465	0.832
67	50.5	92.5	108	0.620	0.466	0.856
			mean :	0.596	0.463	0.833
			± 1 s.d.:	0.016	0.010	0.027
			s.e. :	0.0065	0.0104	0.0110

4.3.3 Application to Derivatized Urine Extracts

(a) Acidic Metabolites

DOPAC, HVA and VMA were estimated in a single chromatographic run with no detectable interference (Fig. 4(m)a). The possibility of interference by homogentisic acid (HGA, 2,5-dihydroxyphenylacetic acid - a stoichiometric isomer of DOPAC) if present on the determination of DOPAC by SIM was investigated (Fig. 4(n)). Both compounds were found to have similar retention times and an abundant m/e 384 ion (which was used for DOPAC SIM), indicating the HGA would interfere with DOPAC determination if it was present at a significant concentration.

DOMA was found to elute on the shoulder of a large preceding peak with an ion at m/e 355 (Fig. 4(m)b), but was, however, sufficiently resolved to permit quantitation. When monitoring 5-HIAA, (Fig. 4(m)b), ions at m/e 290 were present after PG and before 5-HIAA eluted, but these extraneous compounds did not interfere with 5-HIAA determination, and were not recorded.

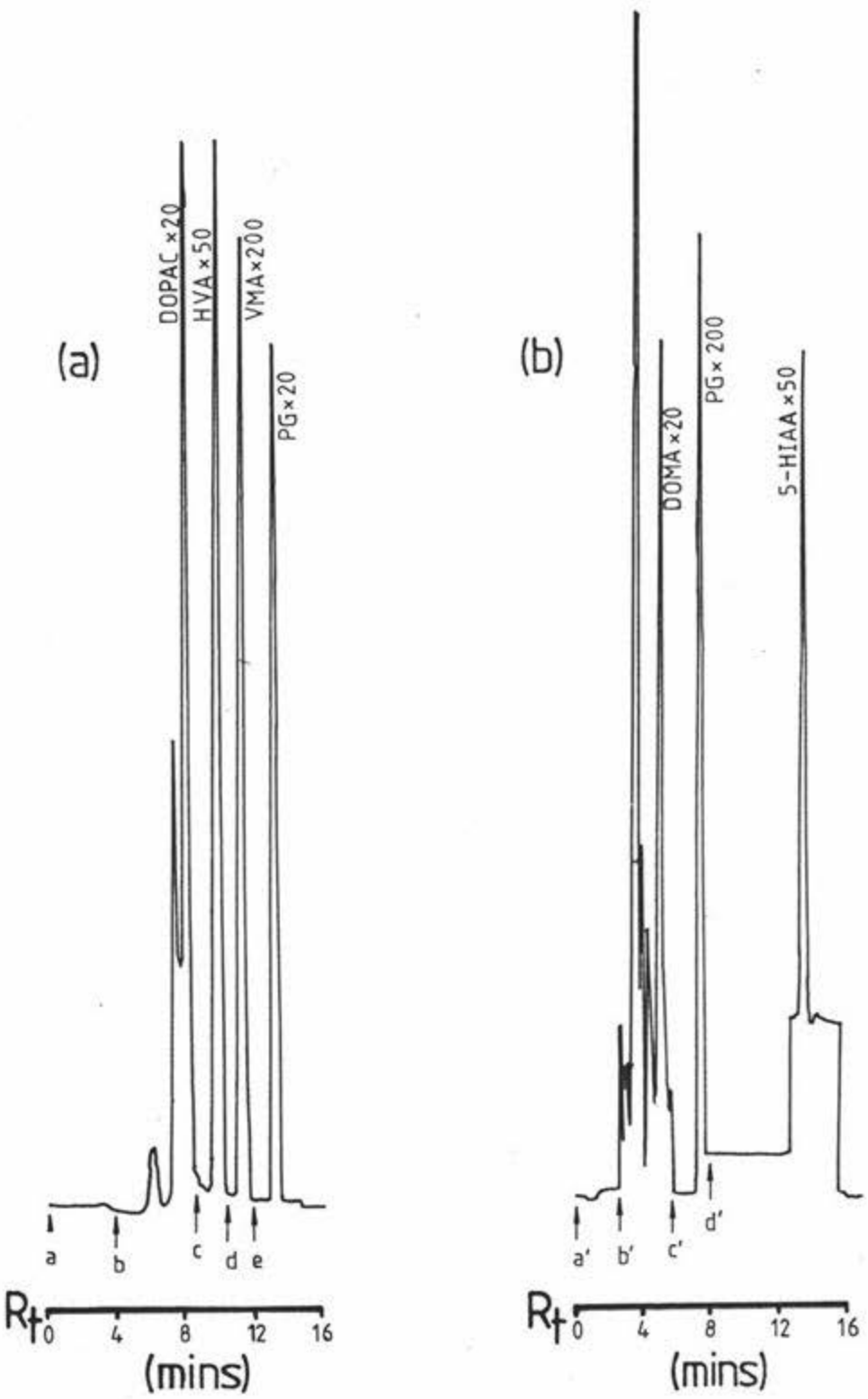


Fig. 4(m) SIM of acidic metabolites extracted from urine

(a) DOPAC, HVA, VMA, PG by the protocol described in the legend to Fig. 4(f) a.

(b) DOMA, PG, 5-HIAA by the protocol described in the legend to Fig. 4(f)b.

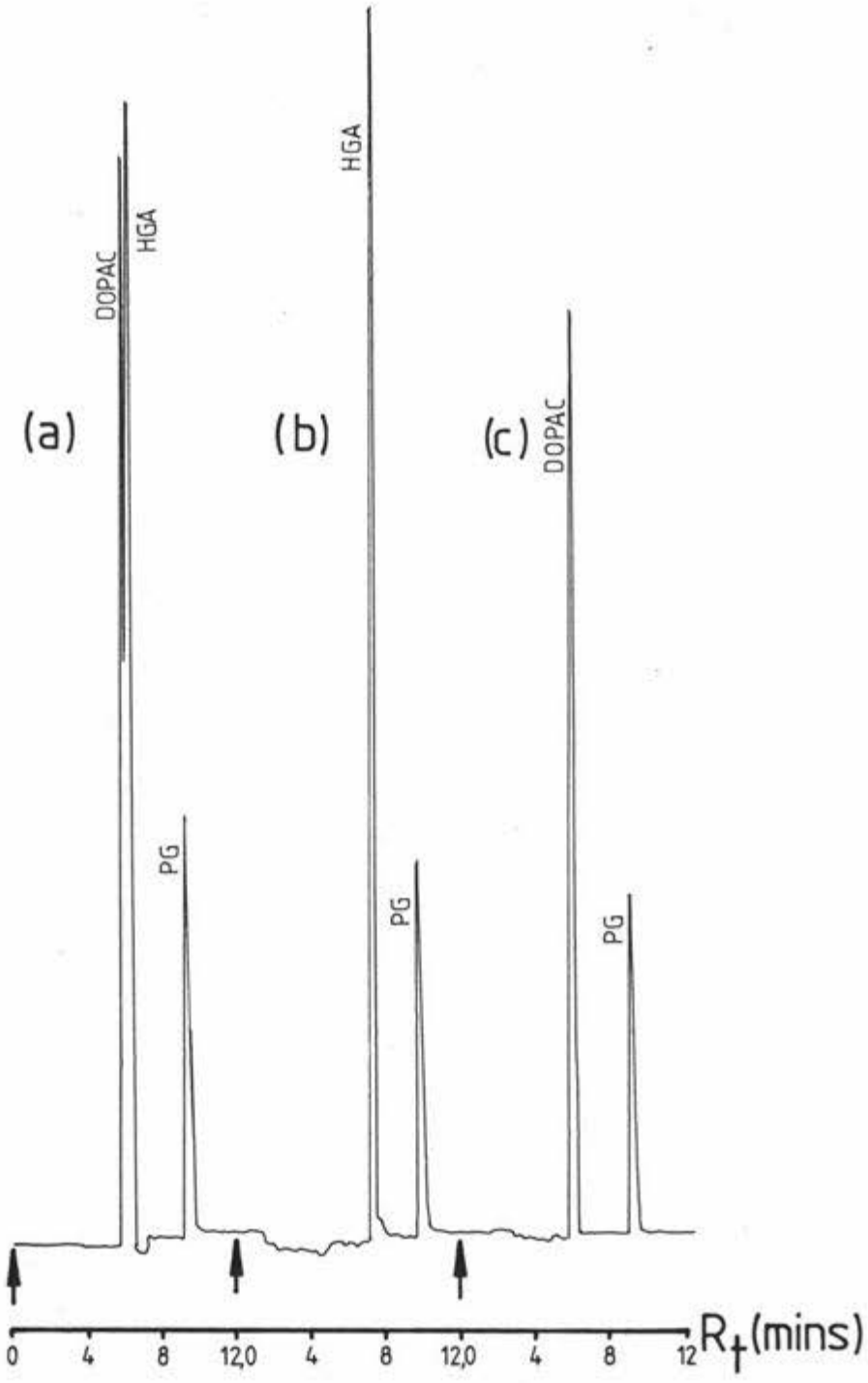


Fig. 4(n) Interference by HGA with the SIM estimation of DOPAC

(a) DOPAC, HGA, PG (b) HGA, PG (c) DOPAC, PG

DOPAC and HGA were both monitored at m/e 384.

(b) Alcoholic Metabolites

The metabolites DOPEt, MOPEt, DOPEG and 5-HTPh were all found to experience some degree of interference from extraneous compounds (Fig. 4(k)b), the former two to such an extent that they were inestimable. The fact that all four are found in normal urine at low concentrations (Table 1(ii))made their determination even more difficult.

4.3.4 Normal Urinary Levels

The concentrations of the acidic metabolites estimated in normal urines by GCMS-SIM are given in Table 4(viii). The levels of HVA, VMA and 5-HIAA were found to correlate well with literature values (Table 1(ii)). However, DOPAC and DOMA levels were found to be significantly higher than previously described, but both this study and the literature study had small sample numbers (n), so that the populations sampled may well have been different.

Table 4(viii) Normal Urinary Levels of Acidic Metabolites Using the GCMS-SIM Technique Described in this Study

metabolite	n	mean	range	1 s.d.	s.e.
DOPAC	5	3.15	1.36 - 4.05	1.12	0.499
HVA	5	3.97	2.23 - 6.78	1.83	0.818
DOMA	6	0.625	0.16 - 1.65	0.546	0.223
VMA	6	2.69	1.90 - 3.22	0.308	0.226
5-HIAA	5	4.65	3.51 - 8.20	2.00	0.895

Values expressed as μg metabolite mg^{-1} creatinine.

4.4 Discussion

In this chapter, a GCMS technique for profiling the acidic catecholamine and indoleamine metabolites DOPAC, HVA, DOMA, VMA and 5-HIAA in derivatized urine extracts has been described and evaluated. As well as good reproducibility and sensitivity, the technique exhibits excellent specificity, a feature that was found to prohibit the analysis of GC column effluents using FID. Although two chromatographic runs are required, mass spectrometers equipped with a multiple ion detector (MID) would require only one since VMA and DOMA could be estimated simultaneously in separate MID channels. A similar procedure to that

described in this study, utilising manual adjustments of the magnet current in the absence of MID, has been described by Peralta and Gelpi (1976). However these authors used a peak matching system (a device not available for this study) for the single ion monitoring of DOPAC, HVA and VMA as their pentafluoropropionyl derivatives.

It was demonstrated that DOPAC determination would be interfered with if significant levels of HGA were present in the urine. Urinary HGA levels are known to rise during the rare inborn error of metabolism known as alkaptonuria (La Du (1972)) and also after salicylate ingestion (Montgomery and Mamer (1978)), however Zoutendeim et al. (1976) have reported that HGA is not detectable in the urine of healthy individuals. In this study, all subjects investigated were non-alkaptonuric and restricted from salicylate ingestion, and therefore their HGA excretion was assumed to be nil.

The application of the SIM technique to the estimation of alcoholic metabolites in urine was subject to severe interference. Breakdown of the Hall probe and the limited time available prevented the further development of this technique. Gas chromatography on different liquid phases, alternative extraction procedures or SIM using alternative selected ions could possibly result in improvement. The use of alternative derivatives, especially those that introduce fluorine, could also reduce interference as they have the advantage of yielding more favourable fragmentation patterns with intense fragment-ions at higher m/e values (Muskiet et al. (1978b)). This normally reduces the chance of interference from other compounds which yield fragment-ions at lower m/e values. Chemical ionisation also yields intense ions at higher m/e values, and methods using this technique have been described for alcoholic metabolites (Edward et al. (1979), Mizuno and Ariga (1979)).

The use of deuterated internal standards in GCMS SIM yields a high degree of precision, and methods using this technique for the analysis of catecholamine and indoleamine metabolites have been described by Karoum et al. (1975) and Muskiet et al. (1978a, 1978b, 1979). These procedures were not investigated in this study, as a MID is required to allow the estimation of both the endogenous compound and its corresponding deuterated analogue simultaneously, as they both elute together.

It was concluded that the GCMS procedure described offered greater

specificity and sensitivity than the chemical and GLC techniques previously investigated. Since the method would be able to accurately detect small changes in metabolite concentrations, it was subsequently applied to the alcohol loading studies described in the next chapter.

CHAPTER 5

ALCOHOL LOADING STUDIES

5.1 Introduction

To the knowledge of this author no report has yet appeared in the literature that has utilized the high specificity of GCMS techniques for the detection of ethanol - induced changes in catecholamine and serotonin metabolism. The method described in the previous chapter was therefore applied to alcohol loading studies with a view to resolving the conflicting results previously reported (Table 1(iii)), and to show any changes in metabolism that were not detected by previous authors.

In this chapter, the effect of a standard ethanol dose on the catecholamine and serotonin metabolism of normal male adults has been examined. By estimating the levels of those metabolites that are formed by the action of either aldehyde dehydrogenase or aldehyde reductase on the aldehyde intermediates formed in the metabolic pathways of these biogenic amines, it was hoped that a greater understanding of how ethanol influences the oxidative and reductive pathways of catecholamine and serotonin metabolism could be achieved.

5.2 Methods

5.2.1 Breath Ethanol Analysis

Blood ethanol levels were determined from breath tests made on a Carle AGC 211 portable gas chromatograph as described by Couchman (1979). The subject blew for a period of six seconds into a breath sampling system in which a portion of the exhaled air passed through a 1.0 ml heated gas sampling loop within the gas chromatograph. This loop was switched into the carrier gas flow (20 ml min^{-1} of 40:60 hydrogen/nitrogen mixture) of a 150 mm porapak Q column at 160 °C. The retention time of the ethanol component was 15 seconds. The peak heights were proportionated to the alcohol concentration in the vapour obtained from a Stephenson Breath Ethanol Simulator (Smith and Wesson) containing a standard ethanol solution. Blood ethanol concentration was determined from the accepted blood/breath ratio of 2,100.

5.2.2 Urinary Creatinine Determination

Creatinine was determined by the Jaffe reaction which results in the production of a red tautomer of creatinine picrate after addition of alkaline picrate solution (Tietz (1976)). Urines were diluted within the range 1/100 - 1/800 with distilled water before analysis. A 4 ml aliquot was used for the colour reaction, and the absorbance was determined at 500 nm against a reagent blank. Concentrations were determined from a standard curve over the range 1.25 - 20.0 μg creatinine ml^{-1} diluted urine, and then corrected for dilution factors. The assay demonstrated excellent linearity and sensitivity, with standard curves consistently passing through the origin (Fig. 5(a)).

5.2.3 Alcohol Loading Experiments

All subjects were non-smoking, male adults that had not consumed alcohol for at least 24 hours prior to the experiment. They were requested to have a light breakfast without excessive fluid intake before experiments. The bladder was voided to provide a basal sample prior to an ethanol dose of 0.35 gm kg^{-1} body weight (with or without diluent), which was ingested within a 30 min period. Urine samples were taken at approximately 30 min intervals and blood ethanol levels determined at approximately 10 min intervals. All experiments were started between 9.00 and 10.00 am and subjects maintained a low level of physical activity and refrained from eating during the course of the experiment. The specifications for the alcohol loading experiments are given in Table 5(i).

Table 5(i) Subject and Dose Specifications for Alcohol Loading Experiments

Subject	NC	KA	KC	RG	AB	PB
Age	22	21	49	49	21	22
Body weight (kg)	78	64	85	60.5	63	71
Dose (gm ethanol)	27.3	22.4	29.8	21.2	22.1	24.9
Beverage	gin	vodka	rum	vodka	bourbon	vodka
Volume (ml)	80	66	93	63	69	73
Diluent	-	coke	coke	lemonade	coke	-

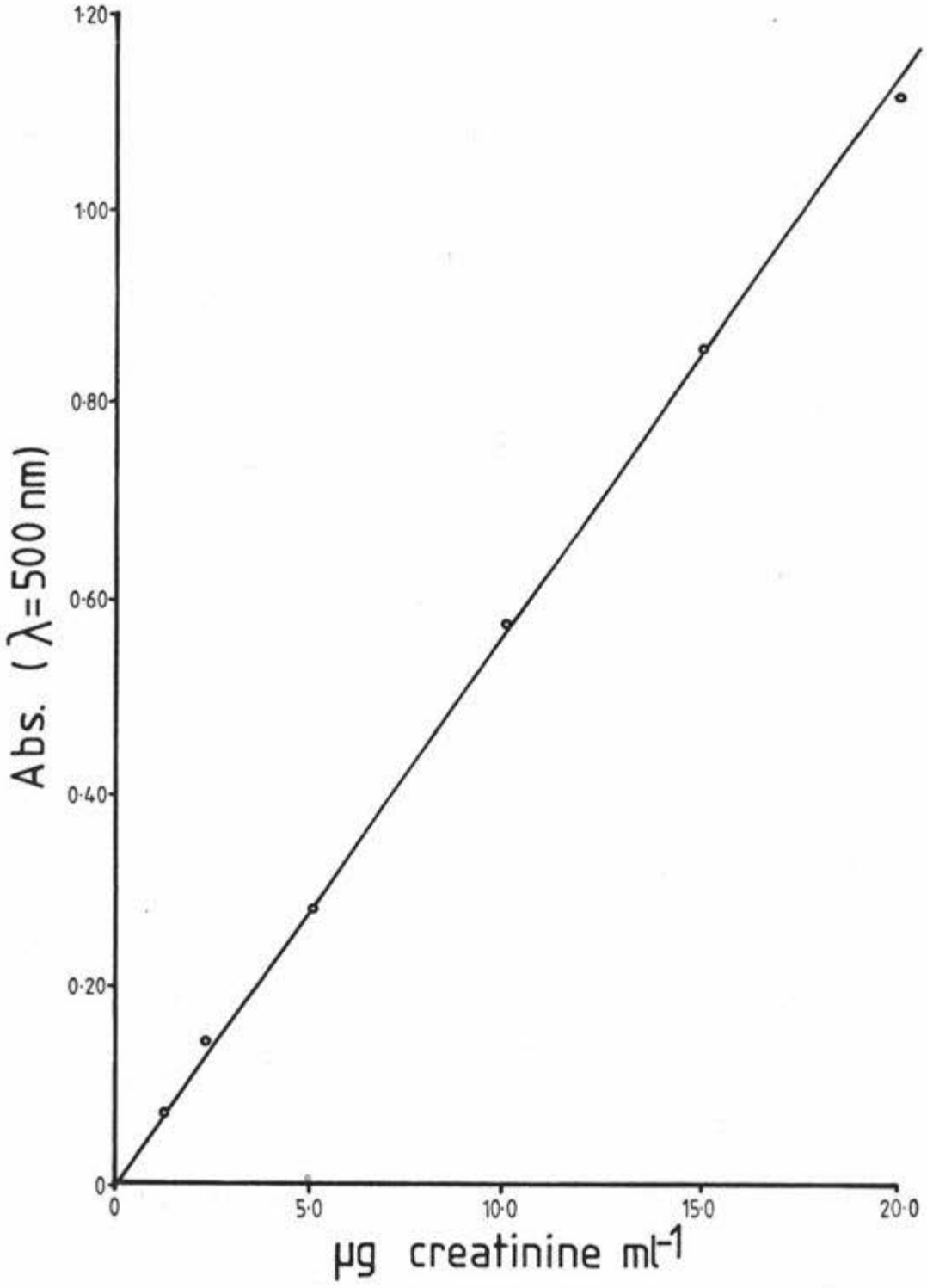


Fig. 5(a) Standard curve for creatinine determination

$a = -0.11$, $b = 19.2 \pm s_b = 0.126$ where $y = a + bx$

y = µg creatinine ml^{-1}, x = abs at 500 nm.

5.2.4 Metabolite Estimation

Catecholamine and serotonin metabolites were estimated by the GCMS techniques described in the previous chapter. Instead of 5 ml of urine as previously used, an aliquot containing 5 mg of creatinine diluted to 5 ml with distilled water was analysed. Where the volume of one or more samples from a subject containing 5 mg of creatinine exceeded 5 ml, the other samples, together with the standards, were diluted to the volume of the most dilute sample before analysis to ensure a consistent recovery. Alcoholic metabolites were assayed in only one subject due to the necessity for further development of the method.

5.3 Results

5.3.1 Blood Ethanol Levels

Blood ethanol concentrations derived from breath analysis are given in Appendix III(a) and Fig. 5(b). Most subjects had attained a maximum blood concentration of 4.0 - 7.9 mM 40 - 80 mins after drinking had commenced. Blood levels then declined at apparently linear rates of 2.35 - 3.00 mM hr^{-1} to below 0.9 mM before the final urine sample was taken.

5.3.2 Urine Volumes and Creatinine Concentrations

The times when urine was voided are shown in Fig. 5(b) and recorded together with their volumes and creatinine concentrations in Table 5(ii). Samples from subjects KG, RG and AB necessitated dilution to the volume of the most dilute sample containing 5 mg of creatinine. Samples from KG, RG and AB increased in volume to about 200 ml within 60 mins of the commencement of ethanol ingestion, while those from NC, KA and PB remained at 15 - 30 ml.

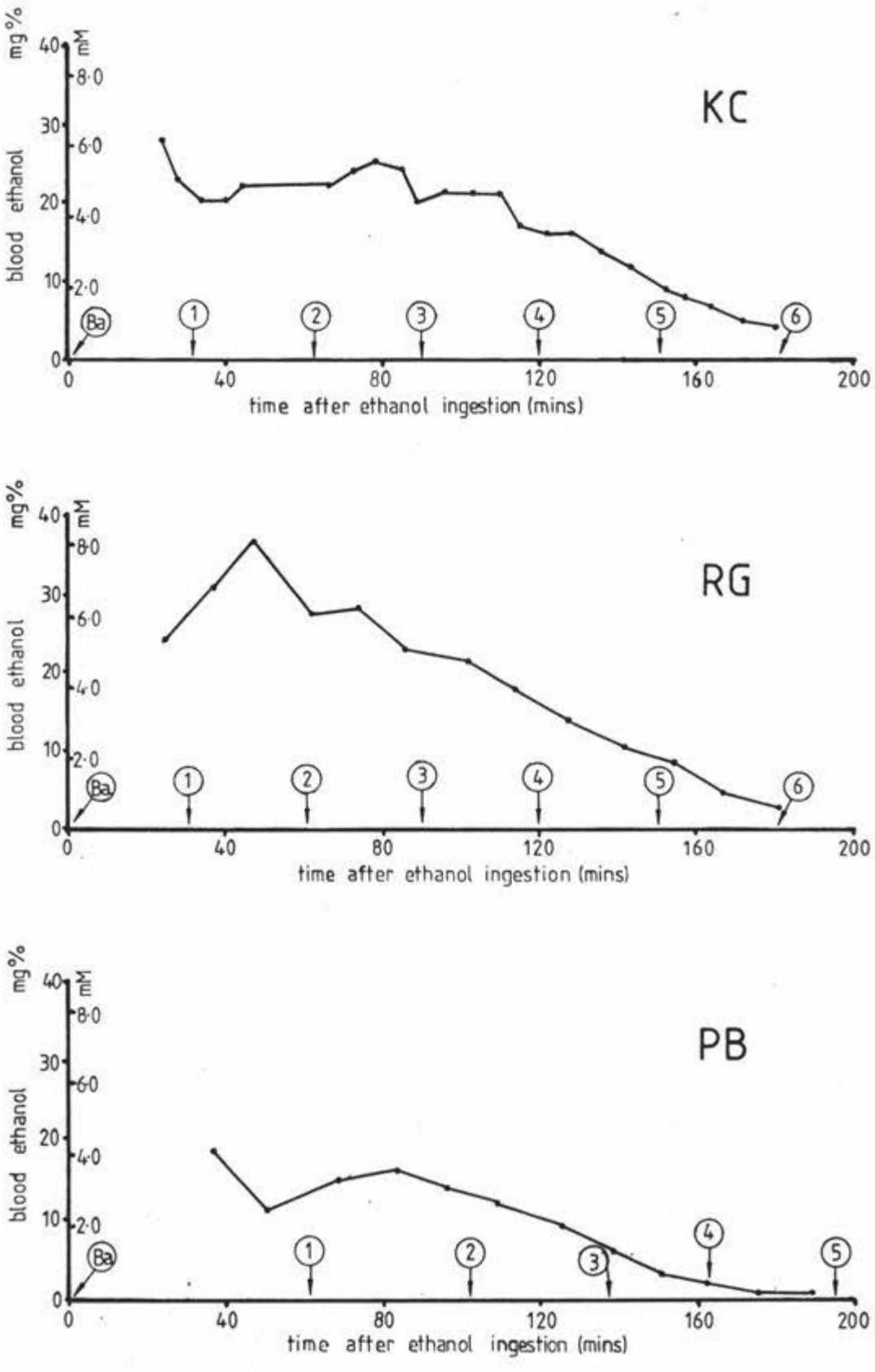


Fig. 5(b) Blood ethanol concentrations during alcohol loading experiments. Circled numbers indicate times when urine samples were taken.

Refer appendix III(a) for data.

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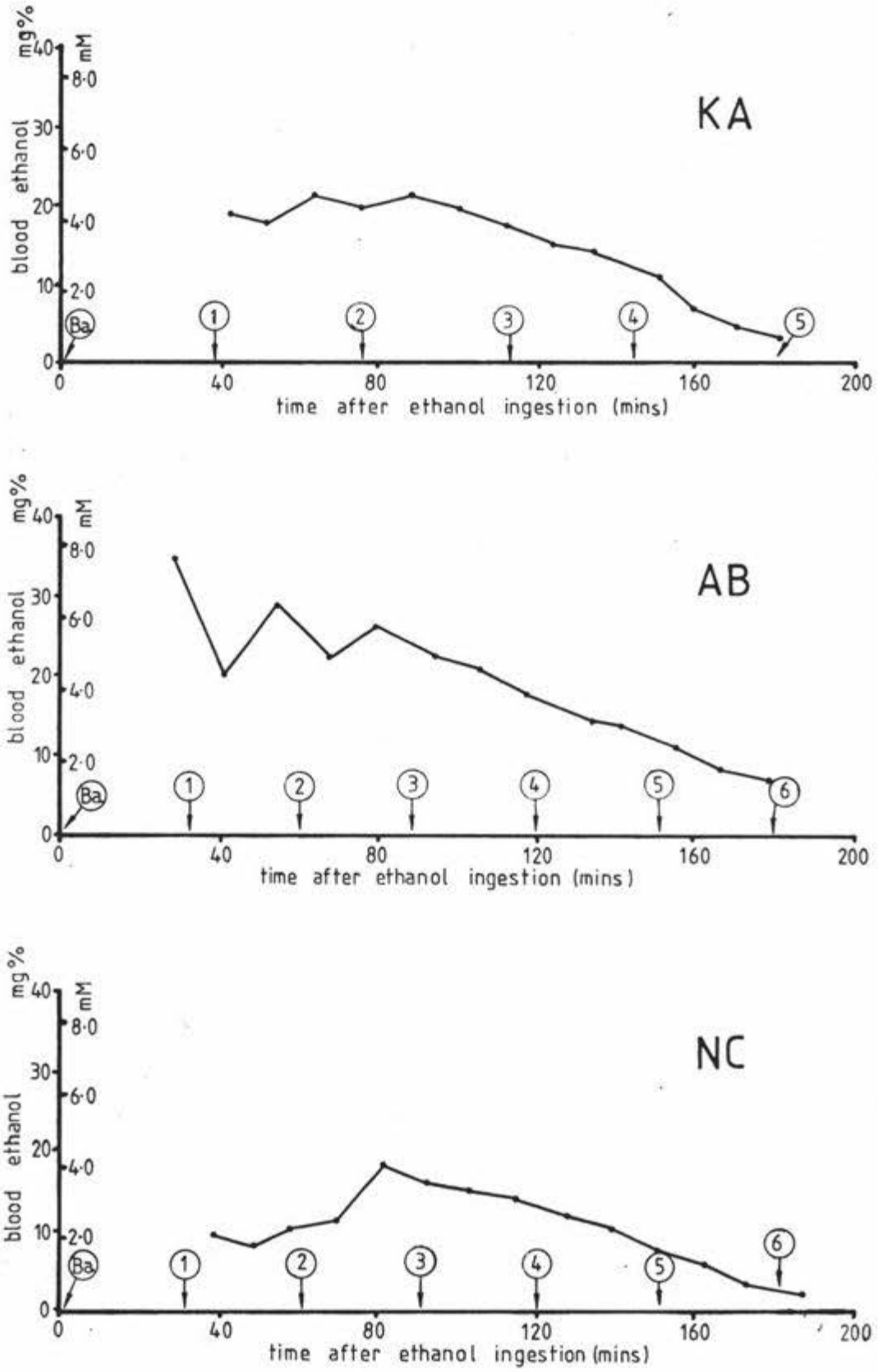

KA
AB
NC
blood ethanol
mg%
mM
8·0
6·0
4·0
2·0
Ba
1
2
3
4
5
6
0
40
80
120
160
200
time after ethanol ingestion (mins)

Fig. 5(b) contd.

Table 5(ii) Sample Volumes and Creatinine Concentrations

	NC			KA			KC		
	time	vol.	creat.	time	vol.	creat.	time	vol.	creat.
Ba.	-	-	1.97	-	-	1.14	-	-	2.86
①	30	23	1.79	38	30	1.65	30	91	0.37
②	60	25	1.54	76	38	1.23	60	246	0.14
③	90	24	1.60	113	30	1.38	90	272	0.12
④	120	24	1.55	144	23	1.38	120	169	0.19
⑤	150	25	1.68	180	33	1.32	150	53	0.57
⑥	180	21	1.60				180	26	1.09

	RG			AB			PB		
	time	vol.	creat.	time	vol.	creat.	time	vol.	creat.
Ba.	-	-	1.36	-	-	0.20	-	-	1.99
①	30	38	0.98	30	115	0.18	66	23	2.72
②	60	197	0.15	60	226	0.16	103	24	2.96
③	90	104	0.27	90	108	0.33	135	13	2.85
④	120	54	0.54	120	96	0.31	163	15	2.34
⑤	150	38	0.59	150	30	0.88	195	20	1.35
⑥	180	38	0.56	180	24	1.26			

time = mins after drinking commenced

vol. = mls

creat. = mg creatinine ml^{-1} urine

5.3.3 The Effect of Diuresis on the Excretion of Catecholamine and Serotonin Metabolites

It is well known that diuresis usually occurs as a response to rising blood ethanol levels (Ogata et al. (1968)) and this is evident in the increased urine volumes exhibited by subjects KC, RG and AB during alcohol loading. The effect of diuresis on the excretion of catecholamine and serotonin metabolites was examined by inducing a water diuresis in subject KC with the diluent used by this subject in the alcohol loading experiment (750 ml coke). Urine sampling and experimental conditions were maintained as for the alcohol loading experiments. Urines were found to increase to similar volumes as those

produced in the alcohol loading experiments, and results for the determination of acidic metabolites in these samples are given in Appendix III(b) and Fig. 5(c).

5.3.4 Metabolite Excretion During Alcohol Loading

The metabolites VMA and DOMA were determined in samples from all six subjects, while DOPAC, HVA and 5-HIAA were determined in five. The concentrations of these metabolites together with the mid-point of the time span which the sample represents are given in Appendix III(c) - (g), and shown in Figs. 5(d) - (h). The alcoholic metabolites DOPEG, MOPEG and 5-HTPh were estimated in urines from one subject (PB) and are given in Appendix III(h) and Fig. 5(i).

The ratio of these compounds to their corresponding acidic metabolites of common origin (ie. DOPEG/DOMA, MOPEG/VMA and 5-HTPh/5-HIAA) during alcohol loading are given in Fig. 5(j).

5.4 Discussion

5.4.1 The Effects of Ethanol on Urinary Metabolite Levels

(a) Noradrenaline and Adrenaline Metabolites

Ethanol ingestion resulted in a slight rise in the DOMA excretion of all subjects except one (KA), who displayed a slight decrease. All subjects showed a pronounced decrease in VMA excretion, with levels dropping to 15 - 62 % of the pre-ethanol level. In subject PB, the excretion of MOPEG showed a 3-fold increase from the pre-ethanol level, while DOPEG excretion remained relatively constant throughout the experiment. There was a substantial increase in the MOPEG/VMA ratio after ethanol, while the DOPEG/DOMA ratio increased slightly and then decreased to a ratio significantly lower than that of the pre-ethanol sample.

These results for the 3-methoxy metabolites confirm the findings of Smith et al. (1960) and Smith and Gitlow (1967) who were the first to demonstrate that ethanol shifted the metabolism of infused noradrenaline in man away from the oxidative pathway toward the reductive pathway. Davis et al. (1967b, 1967c) confirmed that ethanol induced a shift from VMA to MOPEG after the intravenous infusion of noradrenaline in man. These workers also reported that

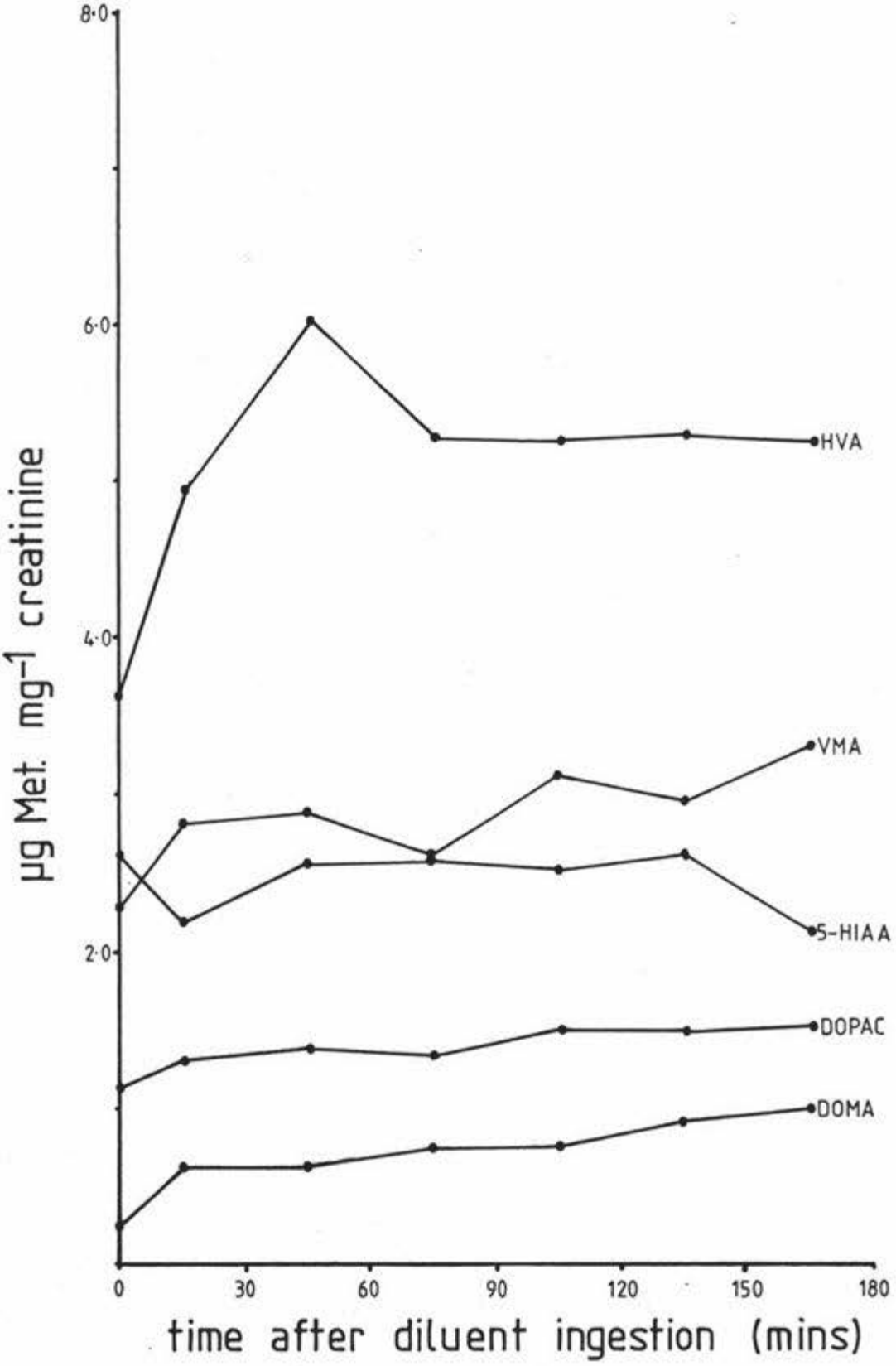


Fig. 5(c) Diuresis experiment, subject KC. Refer Appendix III(c) for data.

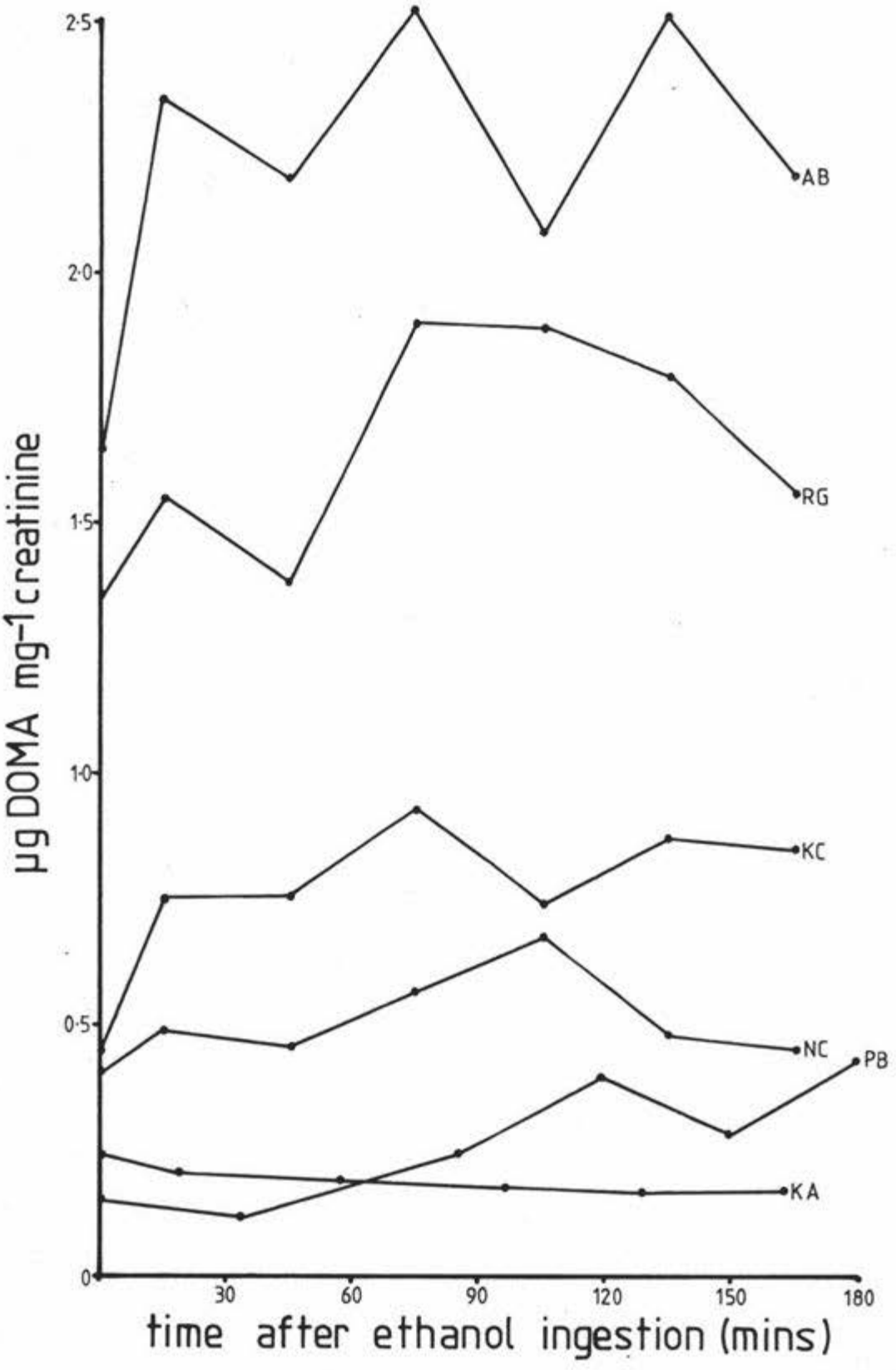


Fig. 5(d) DOMA levels during alcohol loading experiments. Refer Appendix III(c) for data.

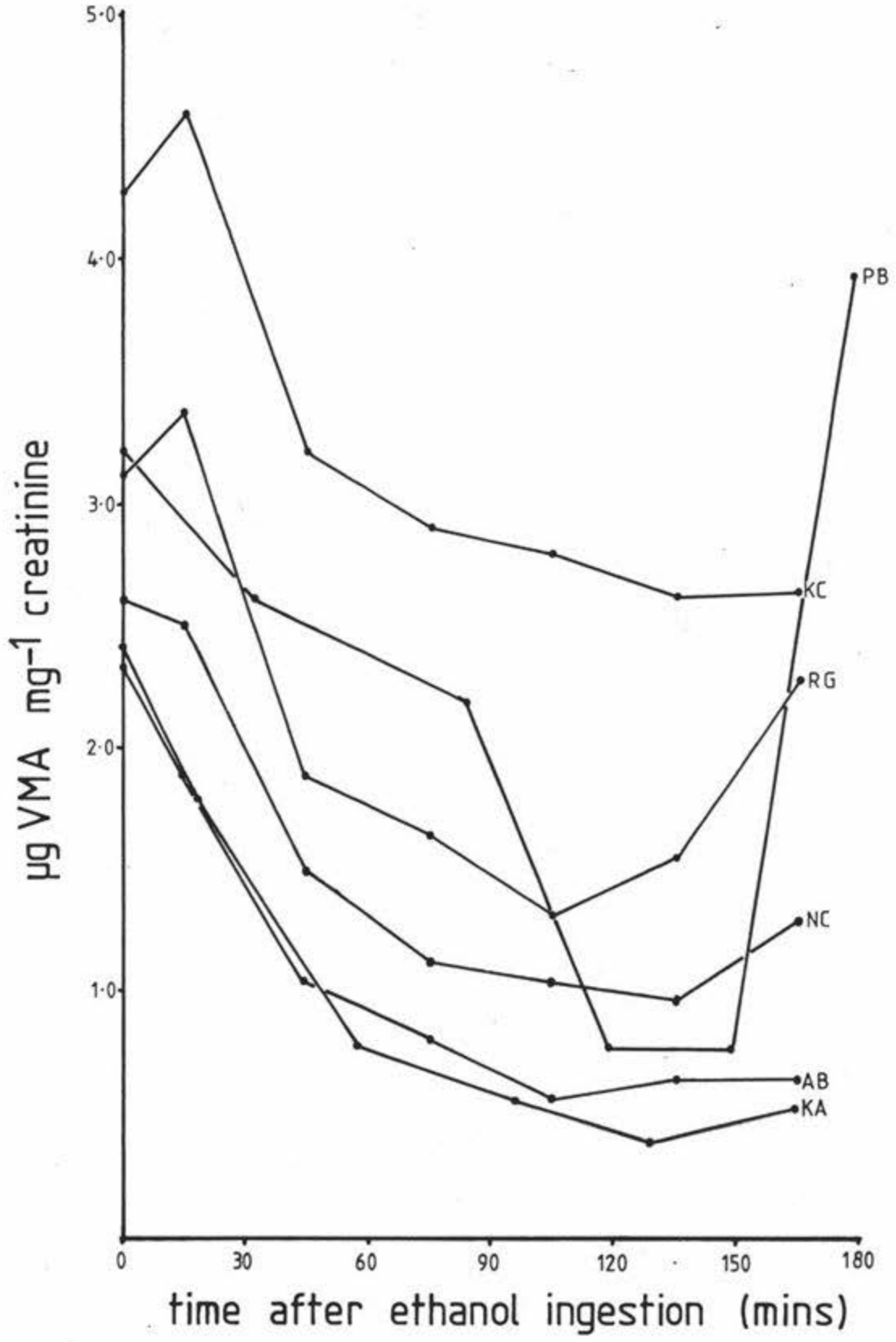


Fig. 5(e) VMA levels during alcohol loading experiments. Refer Appendix III(d) for data.

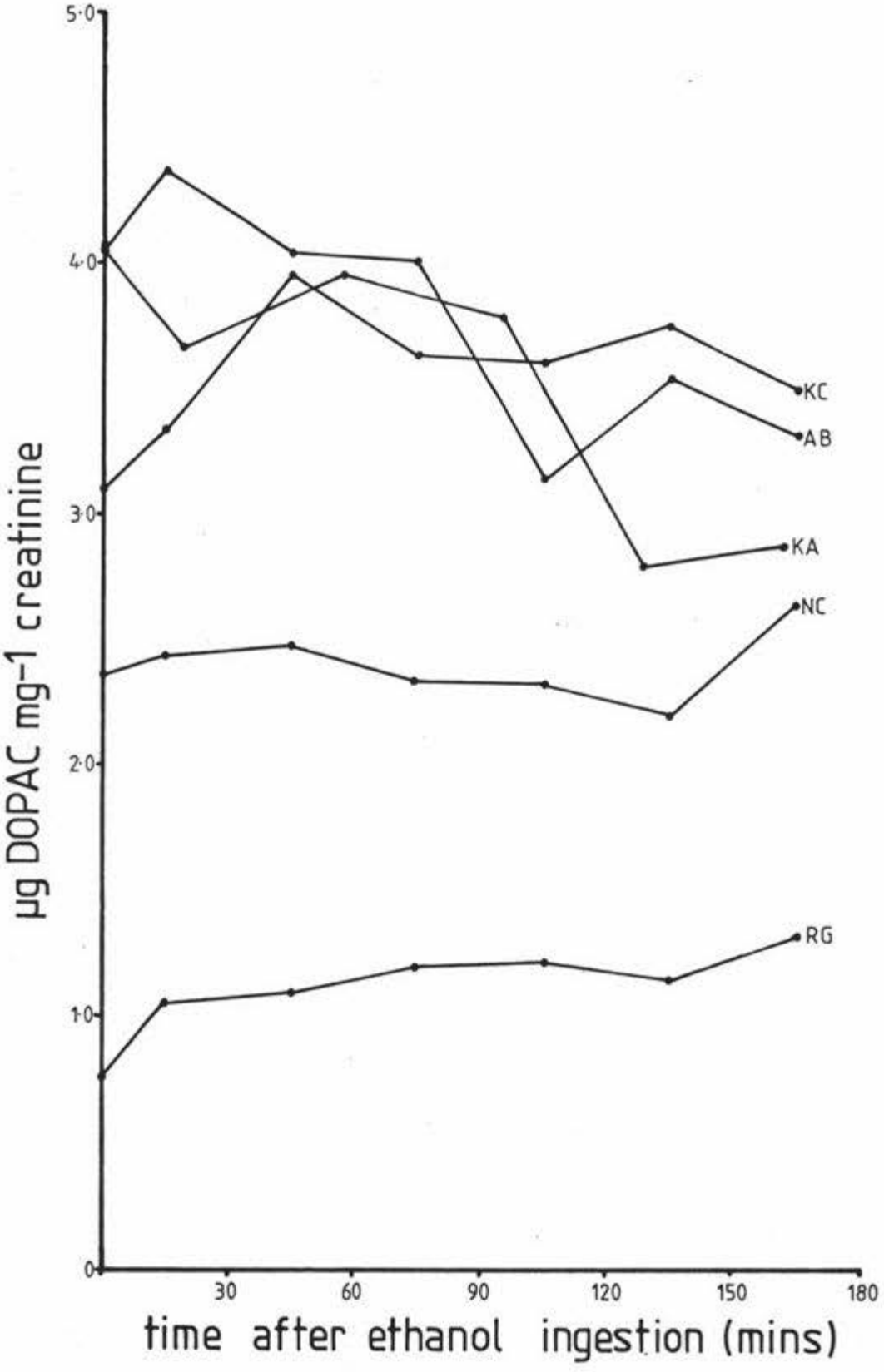


Fig. 5(f) DOPAC levels during alcohol loading experiments. Refer Appendix III(e) for data.

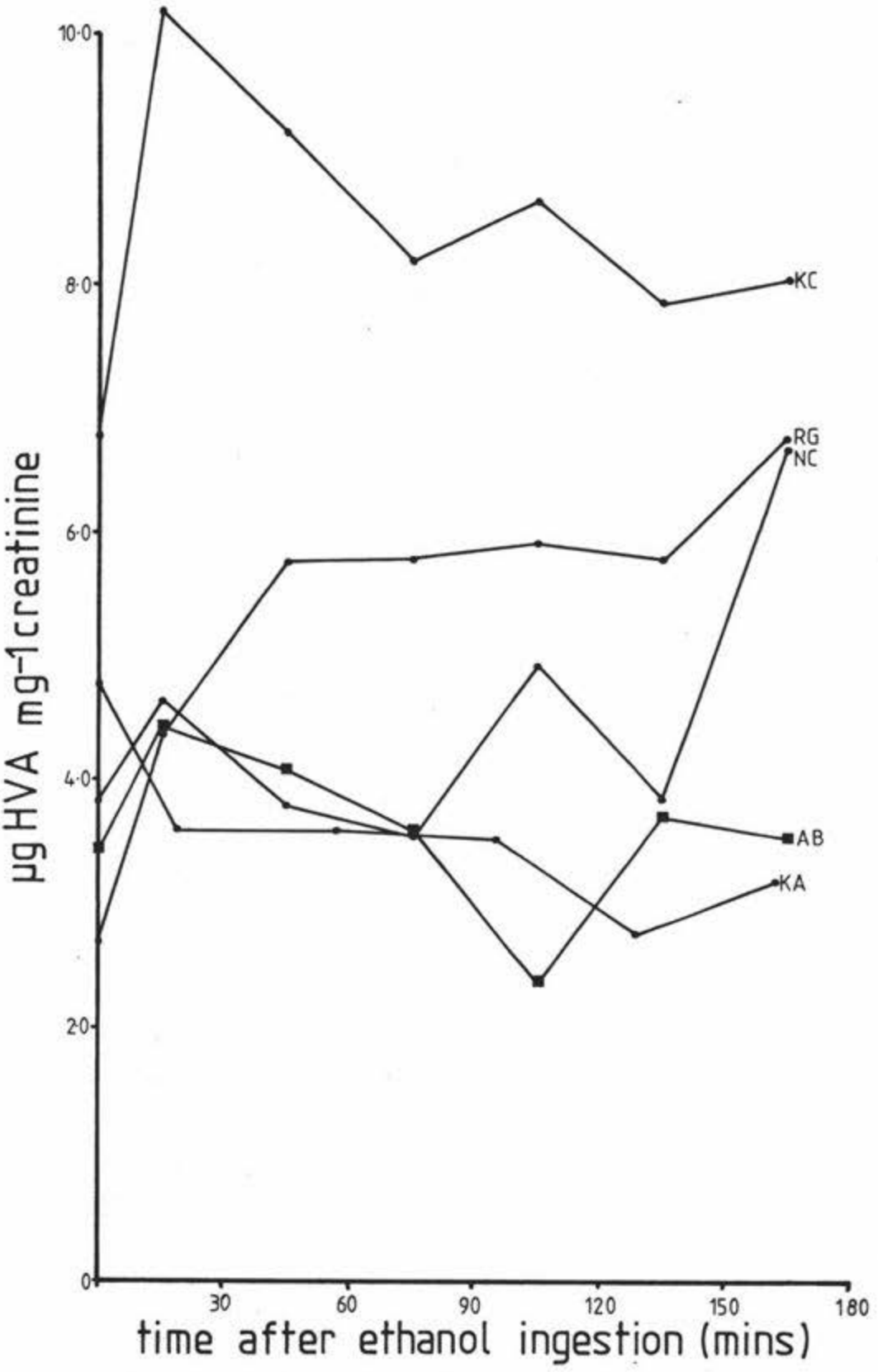


Fig. 5(g) HVA levels during alcohol loading experiments. Refer Appendix III(f) for data.

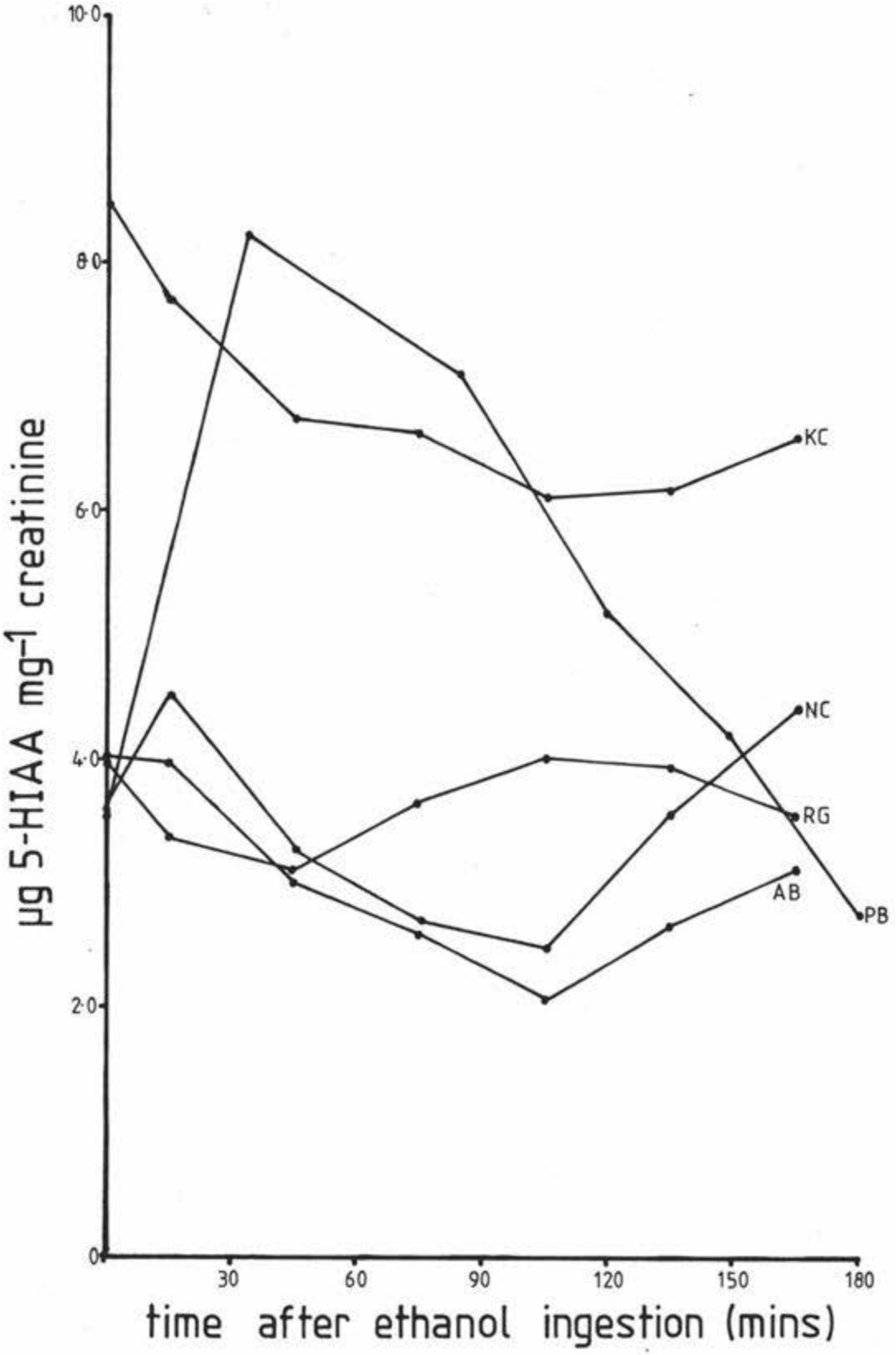


Fig. 5(h) 5-HIAA levels during alcohol loading experiments. Refer Appendix III(g) for data.

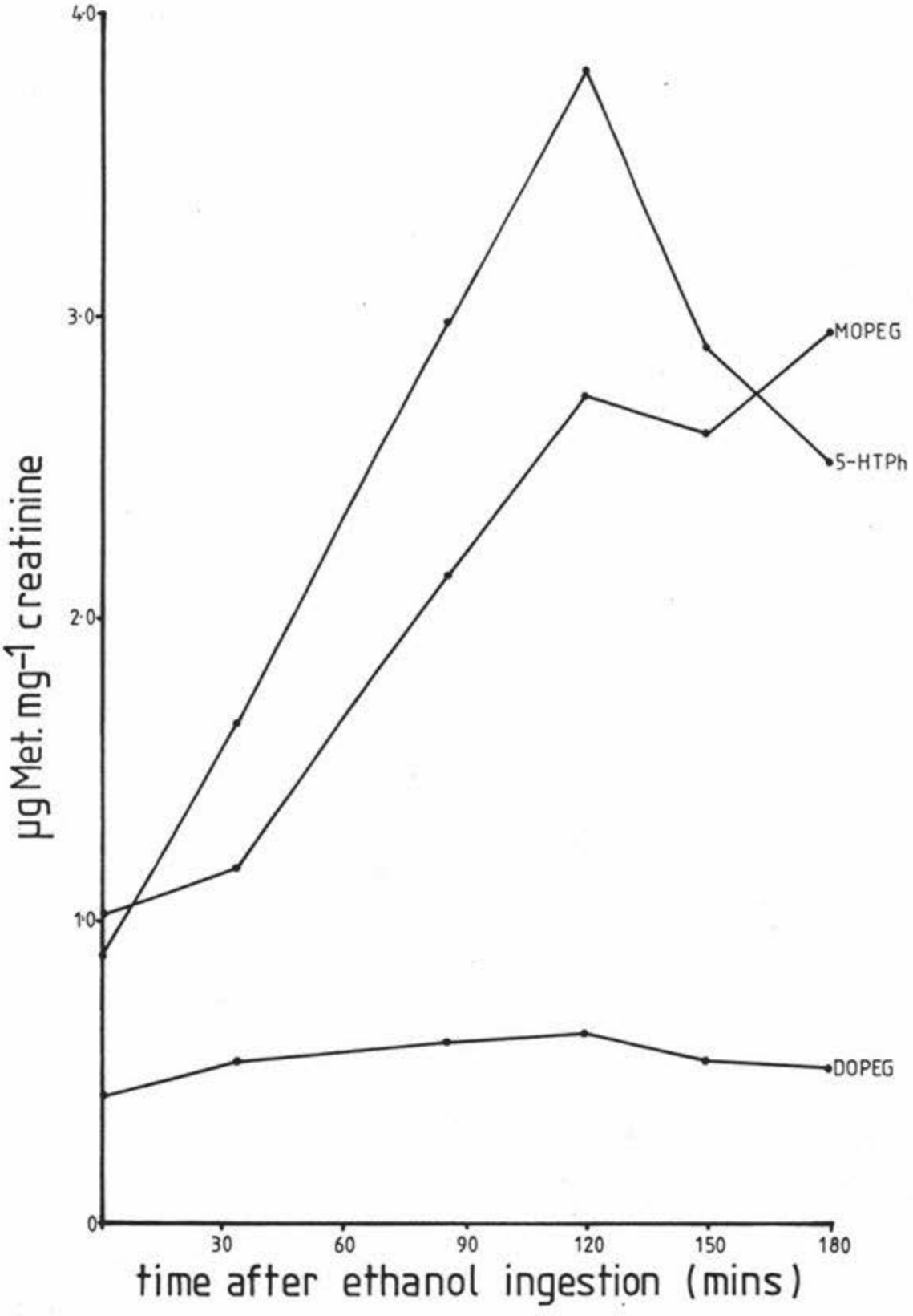


Fig. 5(i) Alcoholic metabolite levels during PB alcohol loading experiment. Refer Appendix III(h) for data.

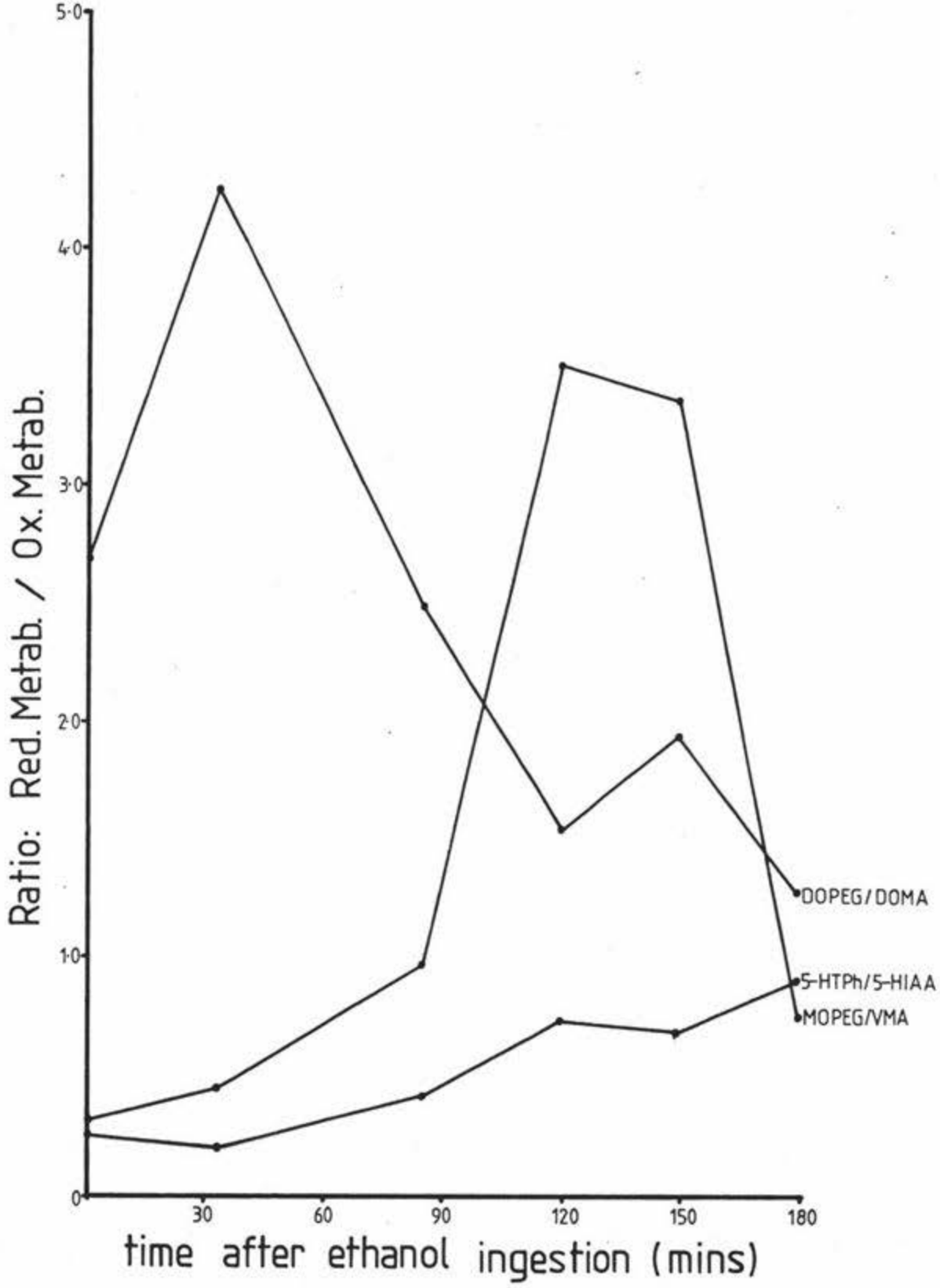


Fig. 5(j) Ratios of reduced metabolites (ie. alcoholic) to their complementary oxidized metabolites (ie. acidic) of common origin during PB alcohol loading experiment.

ethanol did not alter the ratio of DOPEG to DOMA, (as was evident in this study), but they suggested that this was possibly due to the timing of urine collections. Walsh et al. (1970) observed decreased VMA and DOMA excretion, and increased MOPEG excretion after acetaldehyde was given intraperitoneally to rats. Ethanol alone did not produce these changes to such an extent as it does in man, but these authors attributed this to species differences ; man has a greater propensity to excrete noradrenaline metabolites as acidic products than the rat, and it is possible that this pathway would therefore be more sensitive to the effects of agents that competitively inhibit the oxidation of the aldehyde. Other workers have described both increased and decreased levels of VMA in urine after ethanol ingestion (Table 1(iii)).

It was subsequently concluded that ethanol does elicit a shift in metabolism from oxidative to reductive pathways for the 3-methoxy metabolites of adrenaline and noradrenaline, but no similar shift is evident for the 3,4-dihydroxy metabolites.

(b) Dopamine Metabolites

The ingestion of ethanol caused a slight decrease in the DOPAC excretion of two subjects, a slight increase in two others and no change in the fifth. In three subjects HVA was increased, while two others showed slight decreases.

Gitlow et al. (1976) reported significant elevations in the excretion of the dopamine metabolites HVA and 3-MT after ethanol ingestion. It was concluded that ethanol fails to induce a change in dopamine metabolism from oxidative to reductive pathways as is evident for adrenaline and noradrenaline metabolism. It was, however, appreciated that the estimation of the reduced metabolites DOPEt and MOPEt in the urine of alcohol loaded subjects would provide important clarification.

(c) Serotonin Metabolites

Ethanol ingestion was found to cause a significant decrease in the 5-HIAA excretion of all subjects. This occurred after an initial rapid rise to a level more than twice that of the pre-ethanol level in one subject (PB), and in a further subject (RG), the decreased level was found to rapidly return to the original pre-ethanol level. The excretion of 5-HTPh determined in one subject

increased markedly in response to ethanol, as did the 5-HTPh/5-HIAA ratio.

These results are in agreement with an extensively reported shift in metabolism away from the formation of oxidized products toward reduced products after ethanol ingestion (Perman (1961), Anton (1965), Feldstein et al. (1964, 1967), Davis et al. (1967a)).

5.4.2 Differences in the Effects of Ethanol on Peripheral and Central Catecholamine and Serotonin Metabolism

A change in the urinary level of a particular metabolite need not reflect a change in all the metabolisms that contribute to the excretion of that metabolite - the change could have occurred in the peripheral, central, or in both metabolisms.

Karoum et al. (1976) reported that ethanol significantly increased rat brain MOPEG concentration while VMA was either unchanged (in normal rats) or also increased (in ethanol dependent rats). They concluded that no reversal of catecholamine metabolism from an oxidative to a reductive pathway, analogous to that produced by ethanol in the periphery, could be established in the brain.

As with noradrenaline, there appears to be no shift in central dopamine metabolism in response to ethanol. Several workers have demonstrated increased levels of the oxidized metabolites HVA and/or DOPAC in rat brain after ethanol (Karoum et al. (1976), Bustos and Roth (1976), Reggiani et al. (1980)). In previously abstinent alcoholics, there were no consistent increases or decreases of HVA levels in cerebro-spinal fluid after ethanol ingestion (Orenberg et al. (1976)). If there is any change in the pathway, it could occur peripherally, as Tank et al. (1976) demonstrated with rat liver slices.

It is generally acknowledged that if ethanol does induce a shift in brain serotonin metabolism it is not nearly as clear as that found peripherally (Noble and Tewari (1977)). That differences between peripheral and central serotonin metabolism exist was shown by Huff et al. (1971) who found that in both control and ethanol-treated rats the level of 5-HTPh was more than twice as high when $[^{14}C]$ - serotonin was given intravenously than when given intracranially. Arguments persist as to which of the two metabolites - 5-HIAA or 5-HTPh - is increased in response to ethanol in the CNS. Significant increases in brain 5-HIAA have been shown by Tabakoff and Boggan (1974) in mice and Fukumori et al. (1980) in rats. However,

Davis et al. (1969), found small but significant increases in the labelled products 5-HTPh and 5-HIAcetaldehyde after ethanol when labelled serotonin was injected into the lateral ventricle of rat brain. Other studies on rat brain, both in vitro (Eccleston et al. (1969)) and in vivo (Tyce et al. (1968)) have described little change in serotonin metabolism in response to ethanol.

From the above reports it is apparent that the shift in metabolism from oxidative to reductive pathways is a predominantly peripheral event, and does not occur to any comparable extent in the CNS. Therefore any changes in catecholamine or serotonin metabolite concentrations observed in urine as a consequence of ethanol can be assumed to be a peripheral metabolic shift, and not a central shift.

The fact that the effects of ethanol on peripheral and central catecholamine metabolism differ could explain the observation that urinary dopamine metabolites are not diverted away from oxidative pathways. It is possible that since dopamine is essentially a CNS neurotransmitter, a very major proportion of the dopamine metabolites found in urine is contributed by the central metabolism.

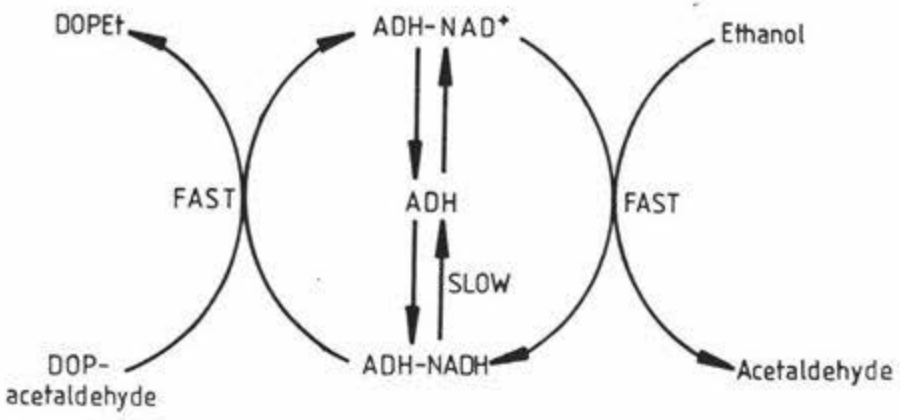
5.4.3 Theories to Explain Ethanol Induced Shifts in Catecholamine and Serotonin Metabolism

One of the first hypotheses presented to explain ethanol - induced shifts in catecholamine and serotonin metabolism was based on the fact that the oxidation of ethanol increases the hepatic $NADH/NAD^+$ ratio, therefore causing the cell to reduce aldehyde intermediates in preference to oxidation (Feldstein et al. (1967)). This hypothesis has since been disputed by Tank et al. (1976), who showed that increasing the NADH level by the addition of lactate failed to produce ethanol like alterations in the catecholamine metabolism of perfused rat liver.

In an alternative hypothesis, Walsh et al. (1970) suggested that the effect of ethanol occurs via its oxidation product, acetaldehyde, competitively inhibiting aldehyde dehydrogenase, the enzyme that catalyzes the formation of oxidized metabolites. They showed that ethanol alone only minimally altered the metabolism of $[^{14}C]$ - noradrenaline injected into rats, whereas acetaldehyde (injected intraperitoneally to give acetaldehyde levels at least 5 - 10 times higher than in the ethanol treated rats) produced

profound aberrations in noradrenaline metabolism. Inhibition of the oxidative pathway would cause an increased steady state concentration of biogenic aldehydes which, alternatively, would be reduced by aldehyde reductase. This mechanism appears to be operative mainly for noradrenaline (Wermuth and Münch (1979)) and would also explain the absence of a metabolic shift in the CNS since only a limited amount of alcohol dehydrogenase (ADH), but high levels of aldehyde dehydrogenase are present in brain extracts (Ris and von Wartburg (1973)). Consequently, acetaldehyde rarely reaches high levels in the CNS after ethanol ingestion (Stowell et al. (1979)).

A further mechanism, proposed to explain a shift in peripheral dopamine metabolism, has been postulated by Tank et al. (1976). Because the $NADPH/NADP^+$ ratio in liver is always high compared to the $NADH/NAD^+$ ratio (Krebs and Veech (1968)), the NADP - dependent aldehyde reductase responsible for the reduction of the biogenic aldehydes derived from the catecholamines and serotonin is most likely saturated with respect to its coenzyme. To increase the percentage of alcohol metabolite formed at a constant biogenic aldehyde level would require the NADH - dependent ADH system to contribute to the overall reduction. Since increasing NADH levels by the addition of lactate did not increase the levels of reduced products, it was suggested that ethanol mediates the reduction of aldehydes via the coupled oxidation - reduction shuttle between ethanol and the aldehyde which was first described by Gupta and Robinson (1966). For ADH, the slowest step in the overall conversion of ethanol to acetaldehyde is the release of NADH from the enzyme (Sund and Theorell (1963)). Thus, during the metabolism of ethanol, a sizeable steady - state concentration of ADH - NADH complex, which is capable of reducing biogenic aldehydes, is formed. Tank et al. proposed that the dopamine metabolite dihydroxyphenylacetaldehyde binds to the ADH - NADH complex faster than the complex breaks down. Hence the effect of ethanol was proposed to keep the ADH - NADH complex in the reduced form, not in its normal enzyme - NAD^+ oxidized form, so that it may bind and reduce biogenic aldehydes even under condition of high cellular $NAD^+/NADH$. This mechanism would also explain the failure of ethanol to induce changes in central catecholamine metabolism, again due to the low levels of brain ADH enzyme.



Mechanism proposed by Tank et al. (1976) for ethanol induced changes in dopamine metabolism.

None of these mechanisms, however, explains the observation by Davis et al. (1967b), and confirmed in this study, that ethanol increases the MOPEG/VMA ratio, but not the DOPEG/DOMA ratio.

5.4.4 The Effects of Diuresis on Catecholamine and Serotonin Metabolite Excretion

A significant diuretic response to ethanol only occurred in three out of six subjects investigated, which is in contrast to the long held view that ethanol elicits an increased urine volume (Beard and Knott (1971)). However, it is known that the diuretic response to ethanol varies widely between individuals and with different beverages (Haggard et al. (1941)). It was noted that two subjects (NC, PB) out of the three who failed to show a significant diuresis both ingested their alcohol dose without the use of diluent. Ogata et al. (1971) observed that diuresis does not persist when high blood alcohol levels are achieved and suggested that since subjects tend to drink considerable amounts of fluid other than ethanol when ingesting alcohol, they may be in positive water balance and therefore likely to produce higher urine volumes.

Only HVA showed a significantly increased excretion relative to creatinine during the water induced diuresis, the other metabolite levels were unchanged. Hence the lower levels of VMA and 5-HIAA observed in alcohol loaded subjects were, in fact, ethanol induced because the renal clearance of creatinine is not affected by ethanol (Ogata et al. (1971)). It is, however, possible that the increased

HVA excretion observed in some subjects in response to ethanol was diuretically induced, since the two subjects who showed the greatest elevation of HVA also exhibited the most extensive diuretic responses.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Methodology

The GCMS technique developed in this study was shown to be capable of accurately quantitating the low levels of acidic catecholamine and serotonin metabolites extracted from normal urine. It was appreciated that the technique requires further development before it can be applied to the analysis of alcoholic metabolites. Future work aimed at extending the precision of this method would require the utilisation of apparatus that was not available for this study.

Multiple ion detection would allow the simultaneous quantitation of all the acidic metabolites in a single chromatographic run, and would overcome the interference problems that prevented the application of the developed technique to the analysis of alcoholic metabolites. The use of MID would also permit the utilisation of deuterated internal standards, a highly precise method of quantitation.

The use of computerized data - separation techniques provides the analyst with the capability to measure tens or hundreds of components simultaneously, a technique described as 'metabolic profiling' (Gates et al. (1978)). Such a method could provide an accurate assessment of the overall change in an individuals metabolism in response to ethanol ingestion.

6.2 Alcohol Studies

The alcohol loading experiments, while not producing any significant original findings, did provide a clarification of the conflicting results previously reported in the literature. It was concluded that although this field of alcohol research has received considerable attention, there are several aspects that still require extensive investigation before a thorough understanding of the ways in which ethanol induces changes in metabolic pathways can be achieved.

The differences in the effects of ethanol on peripheral and central biogenic amine metabolism still requires further investigation. Since studies of the effects of ethanol on human CNS metabolism are restricted due to sample inaccessibility, several workers have

alternatively studied rat CNS metabolism. However, species differences make the extrapolation of these results to humans of questionable validity.

Correlations between ethanol induced changes in metabolism and behavioural parameters have received comparatively little attention. While not necessarily constituting a cause and effect relationship, the detection of metabolic changes may provide an indication of a subject's pharmacological sensitivity to ethanol. By detecting subjects who exhibit a high tolerance to the pharmacological effects of ethanol, a 'high risk of alcoholism' group could possibly be identified, and preventative measures taken. Through further investigation of ethanol/neurotransmitter interactions, it may eventually be possible to accurately assess the alcohol dependence of an individual through routine urine analysis, and to gain a greater understanding of the mechanism of alcohol addiction.

APPENDIX I MATERIALS

Materials:	Supplier:
DOPAC, HVA, DOMA, VMA, MOPEG, DOPEG, 5-HIAA, and 5-HTPh	Sigma Chemical Company
MOPEt	Calbiochem
Propyl Gallate, Rescorcinol	Sigma Chemical Company
β-Glucuronidase (Helix pomatia preparation with sulphatase activity)	Sigma Chemical Company
Homogentisic Acid (HGA)	Sigma Chemical Company
Creatinine	BDH Laboratory Chemicals
DEAE Sephadex A-25 anion exchange Resin	Pharmacia Fine Chemicals
Dowex AG 1x4, 100-200 mesh, chloride form resin	Riedel-de Haen AG, Seelze-Hannover, Germany
N,O-bis-TMS-trifluoroacetamide (BSTFA) (containing 1% TMCS as catalyst)	Pierce Chemical Company
Pyridine (silylation grade)	Pierce Chemical Company
Tri-Sil 'TBT'	Pierce Chemical Company
Silyl-8 (column conditioner)	Pierce Chemical Company
OV-1, OV-17 stationary phases	Varian Aerograph
XE-60, OV-101, OV-225 stationary phases	Supelco Inc.

DOPEt was synthesized from DOPAC by reducing in tetrahydrofuran with lithium aluminium hydride as described by Muskiet et al. (1978c).

All other chemicals and solvents were reagent grade or better and supplied by Sigma Chemical Company, BDH Laboratory Chemicals, Koch-Light Laboratories Ltd., or May and Baker Ltd.

APPENDIX II

MASS SPECTRA OF THE TMS DERIVATIVES OF THE METABOLITES INVESTIGATED IN THIS STUDY

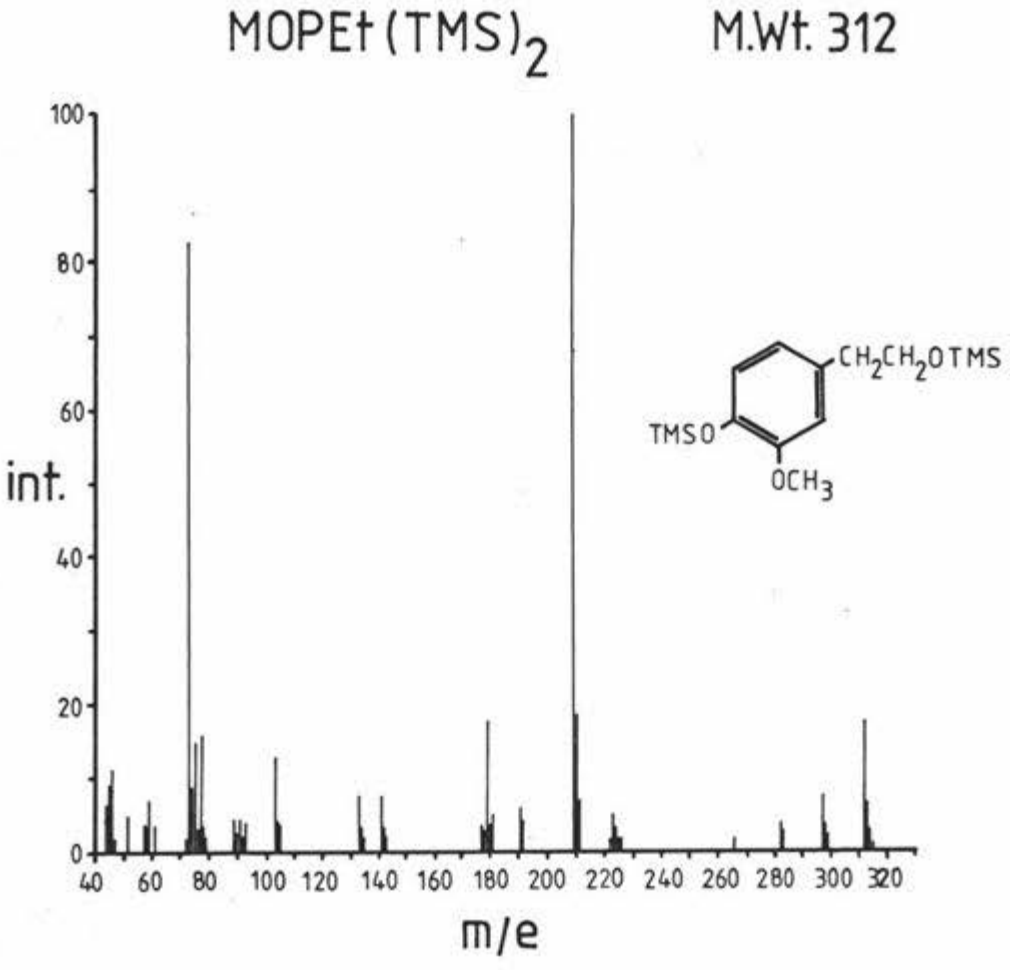
Index:

References: A - Markey et al. (1974)

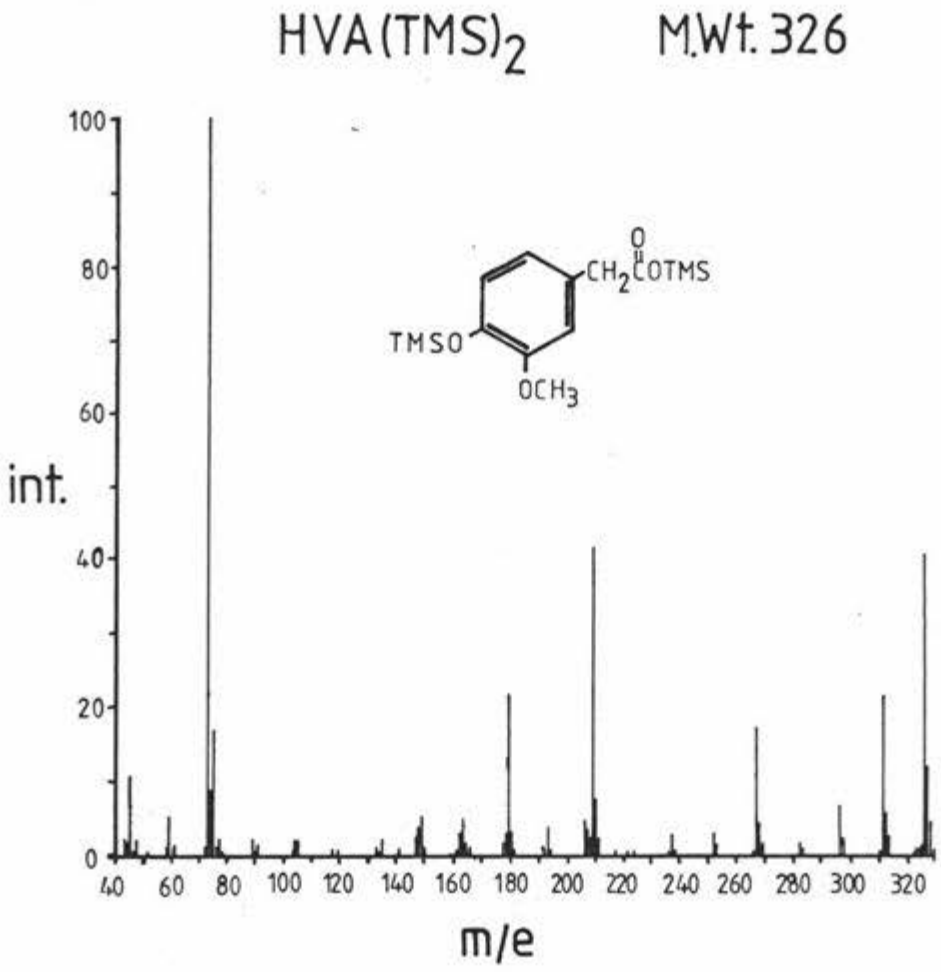
B - Waterbury and Pearce (1972)

C - this study

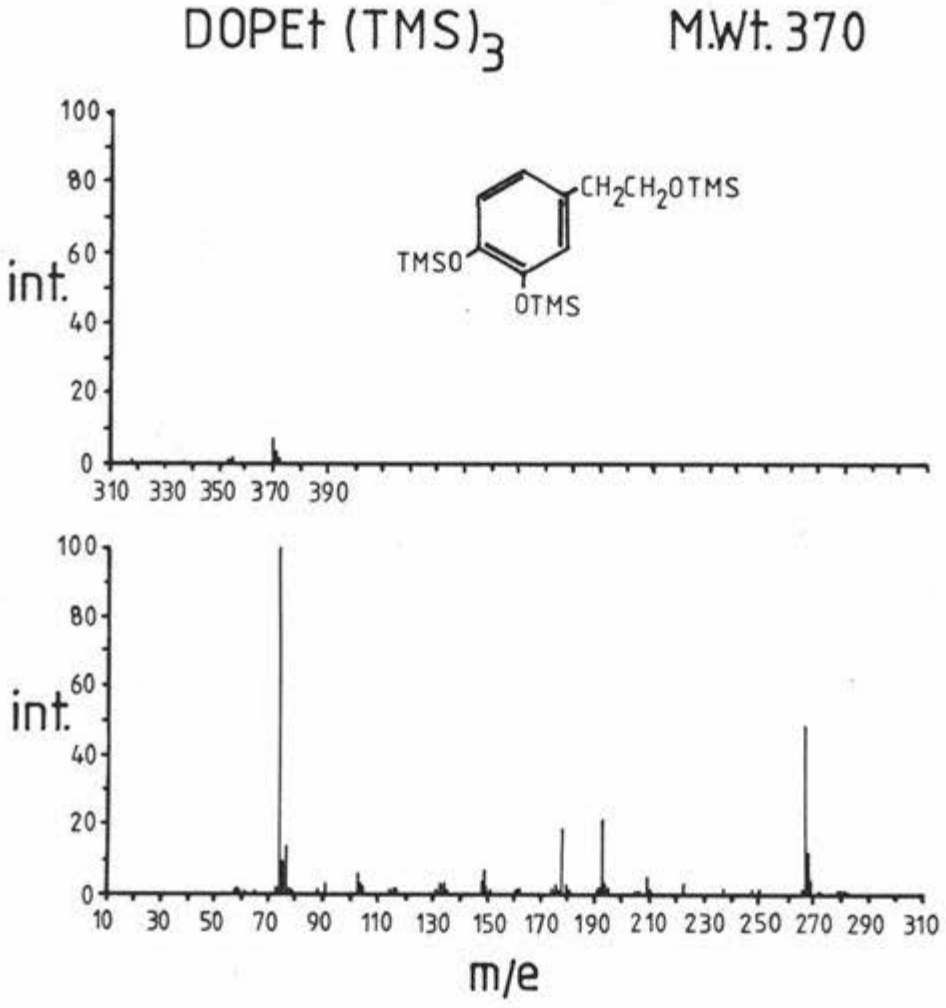
D - Hattox and Murphy (1978)



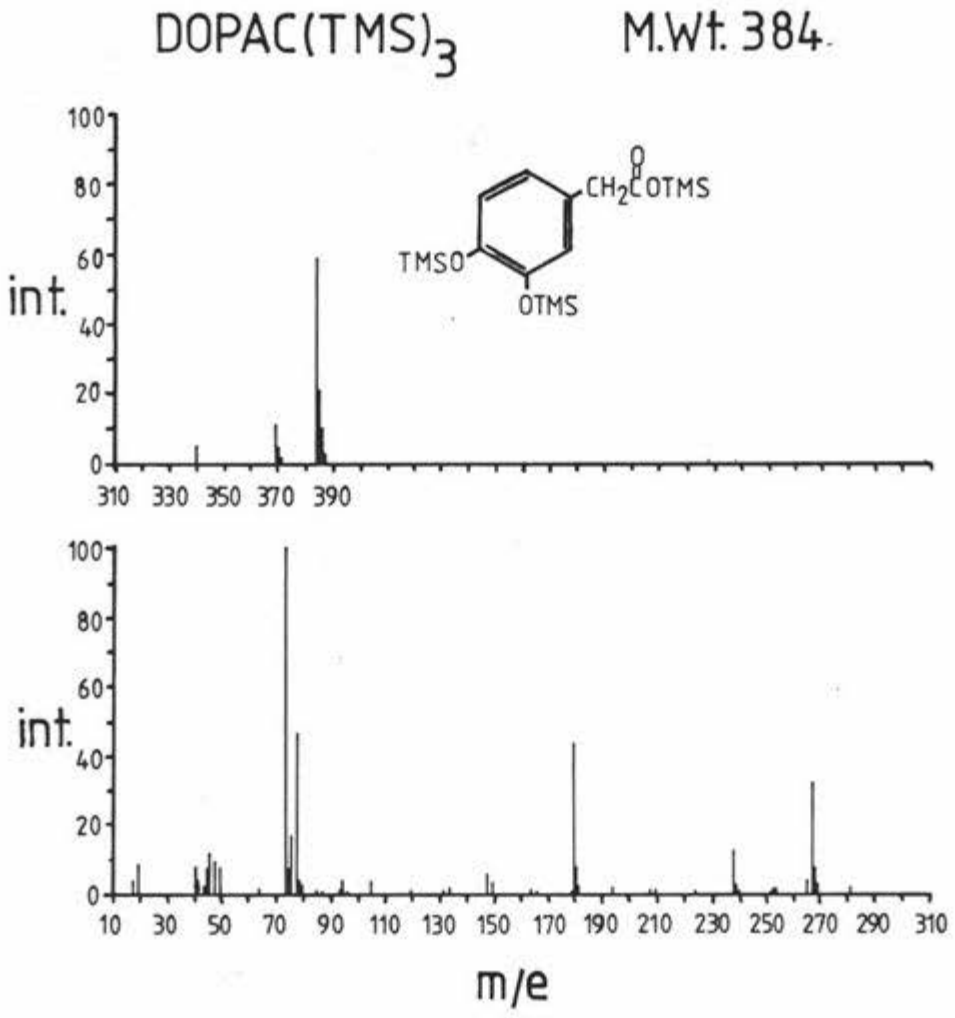
3-Methoxy-4-hydroxyphenylethanol-diTMS



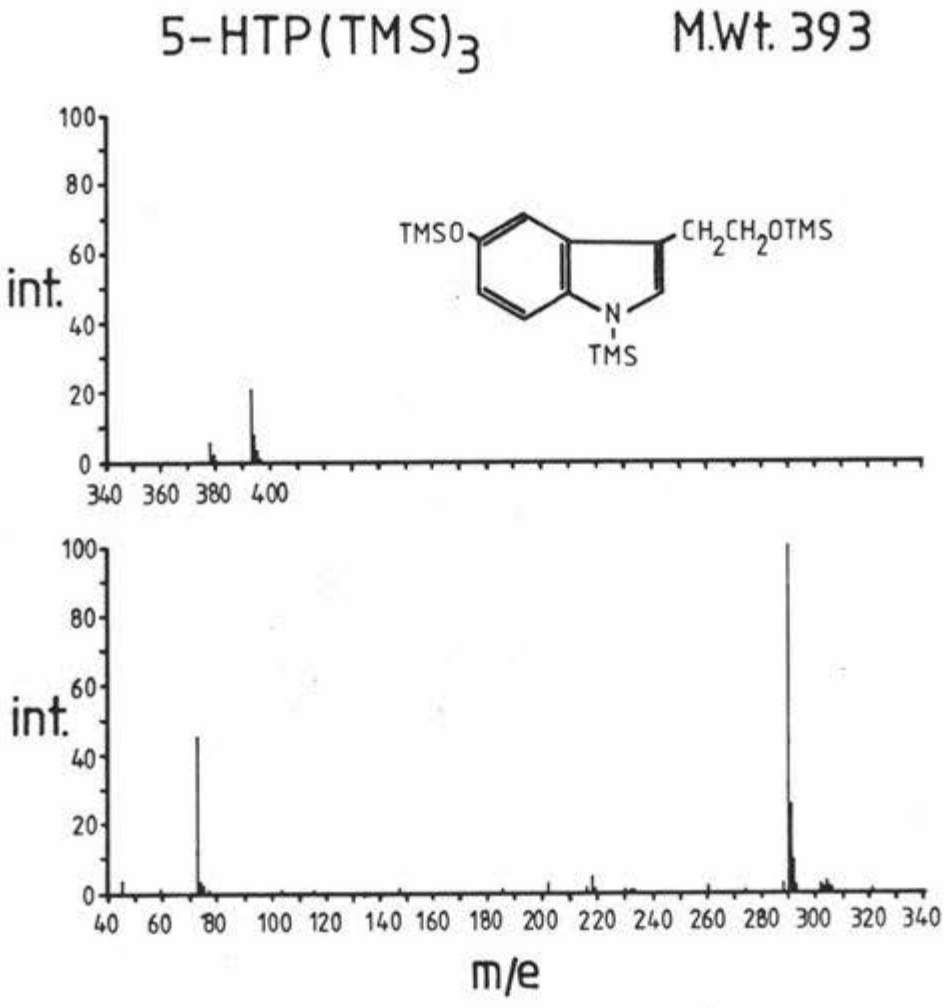
3-Methoxy-4-hydroxyphenylacetic Acid-diTMS



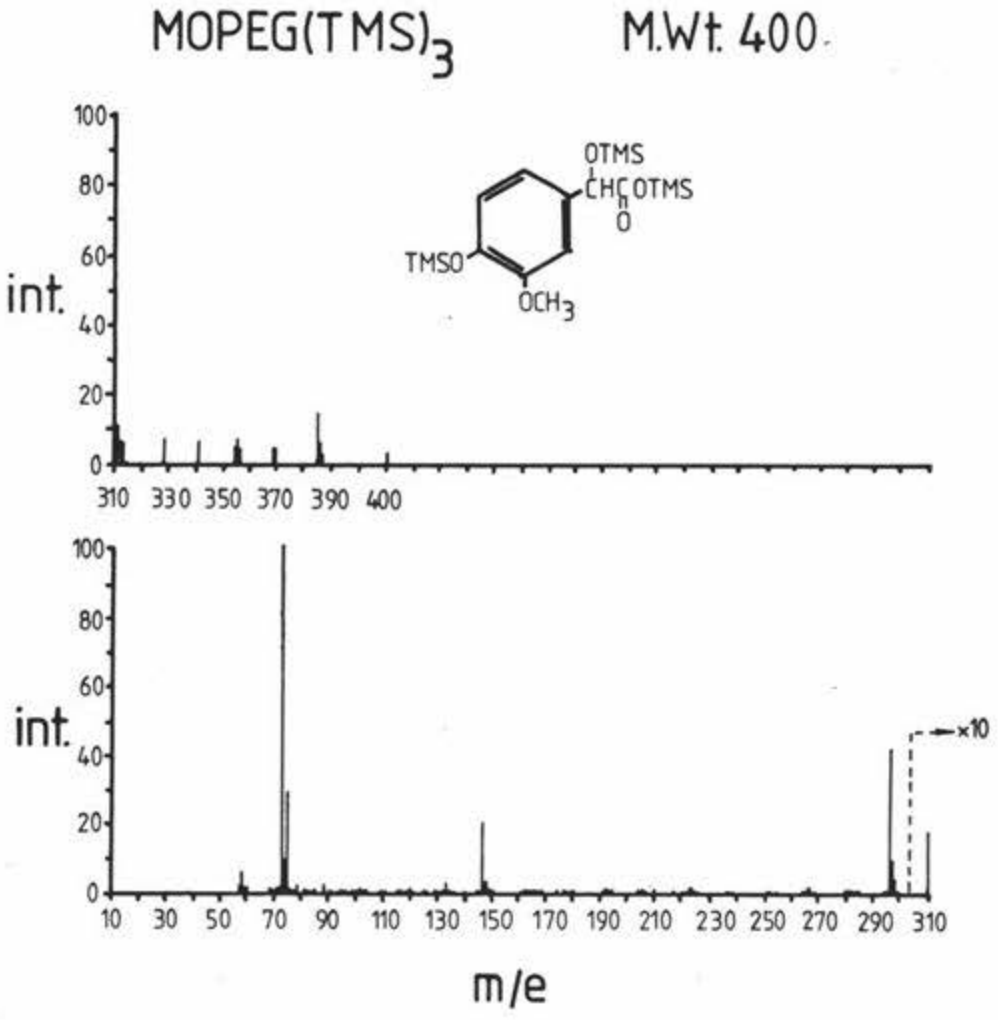
3,4-Dihydroxyphenylethanol-triTMS



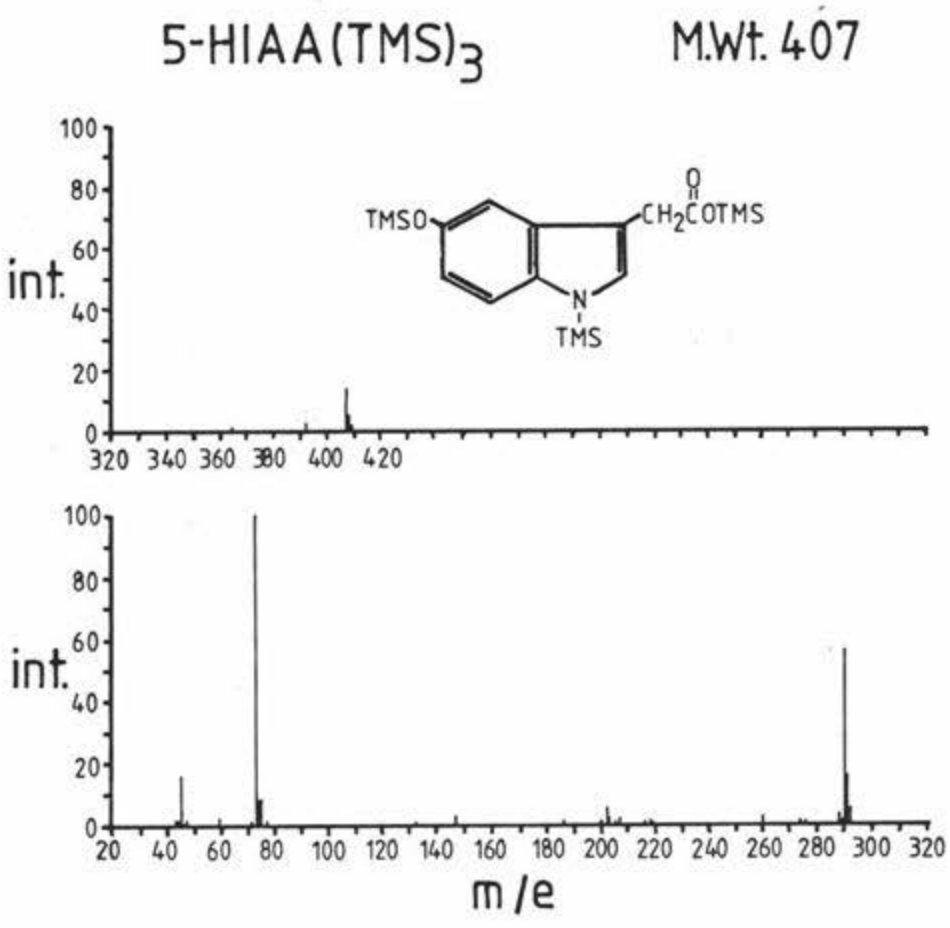
3,4-Dihydroxyphenylacetic Acid-triTMS



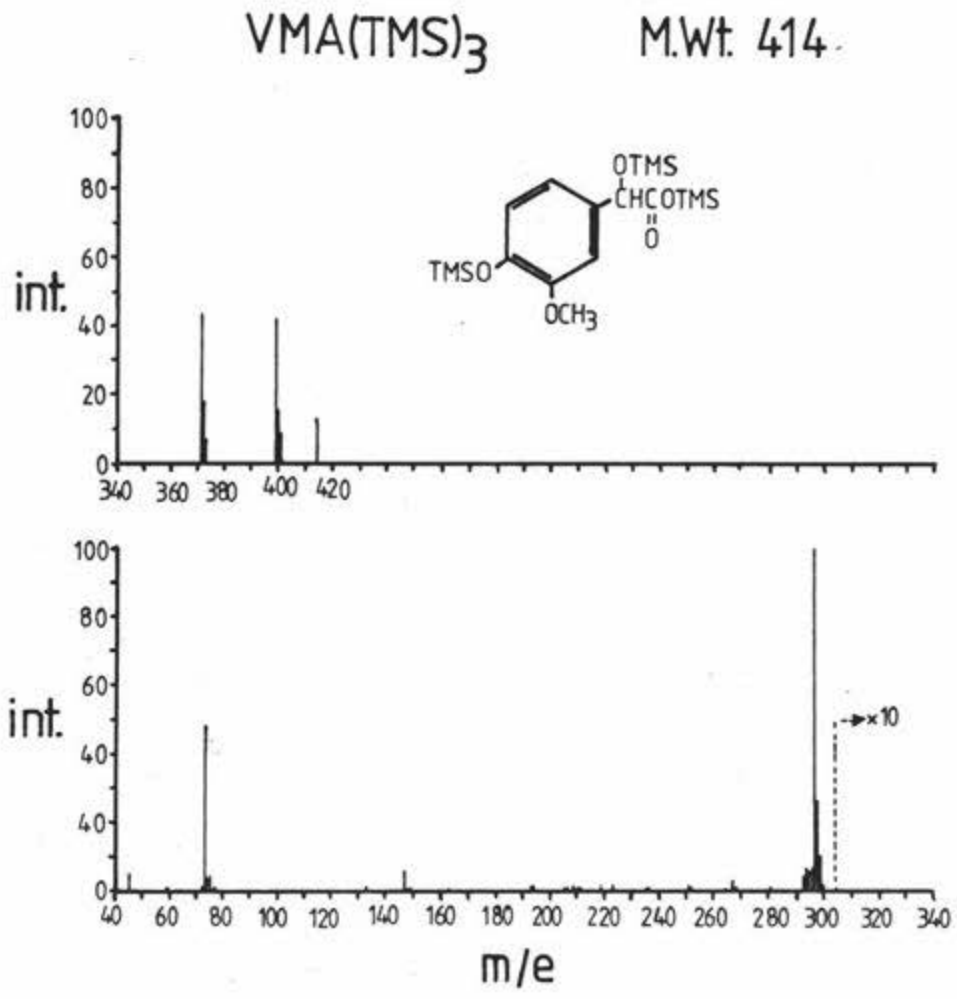
5-Hydroxytryptophol-triTMS



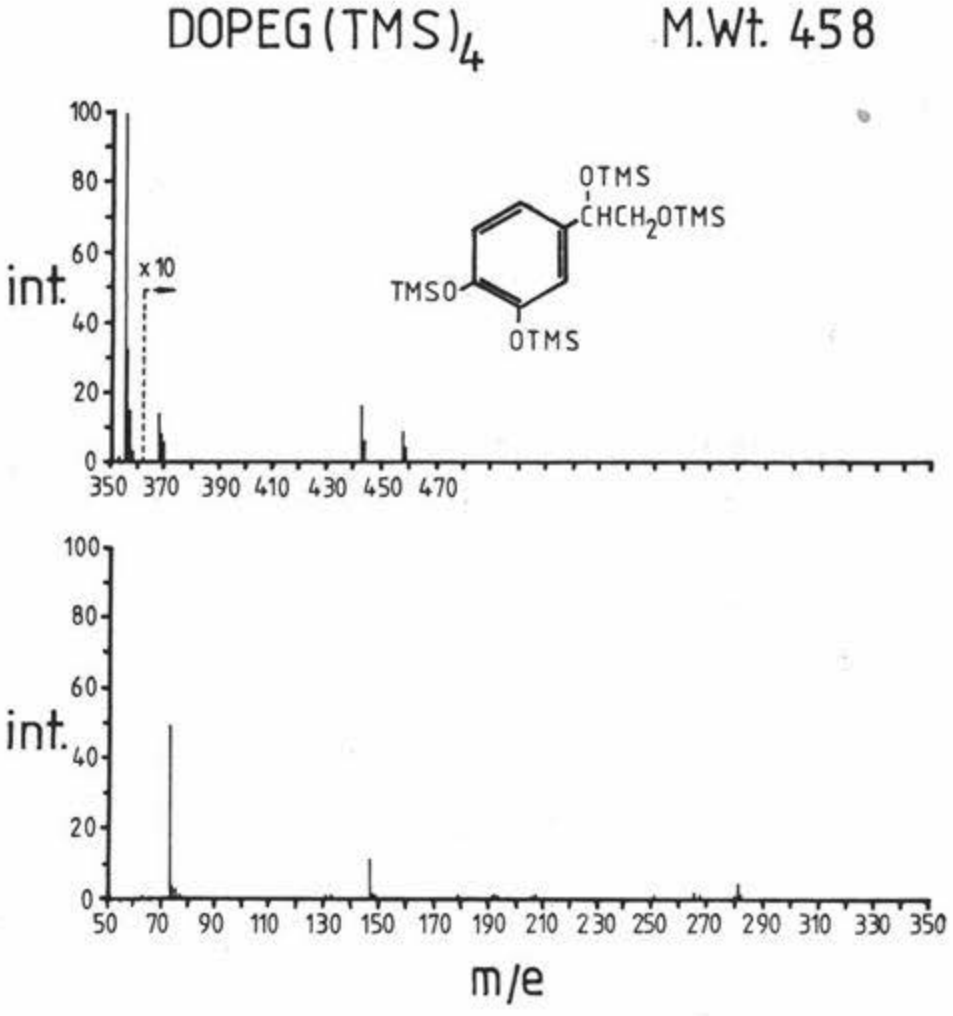
3-Methoxy-4-hydroxyphenylethyleneglycol-triTMS



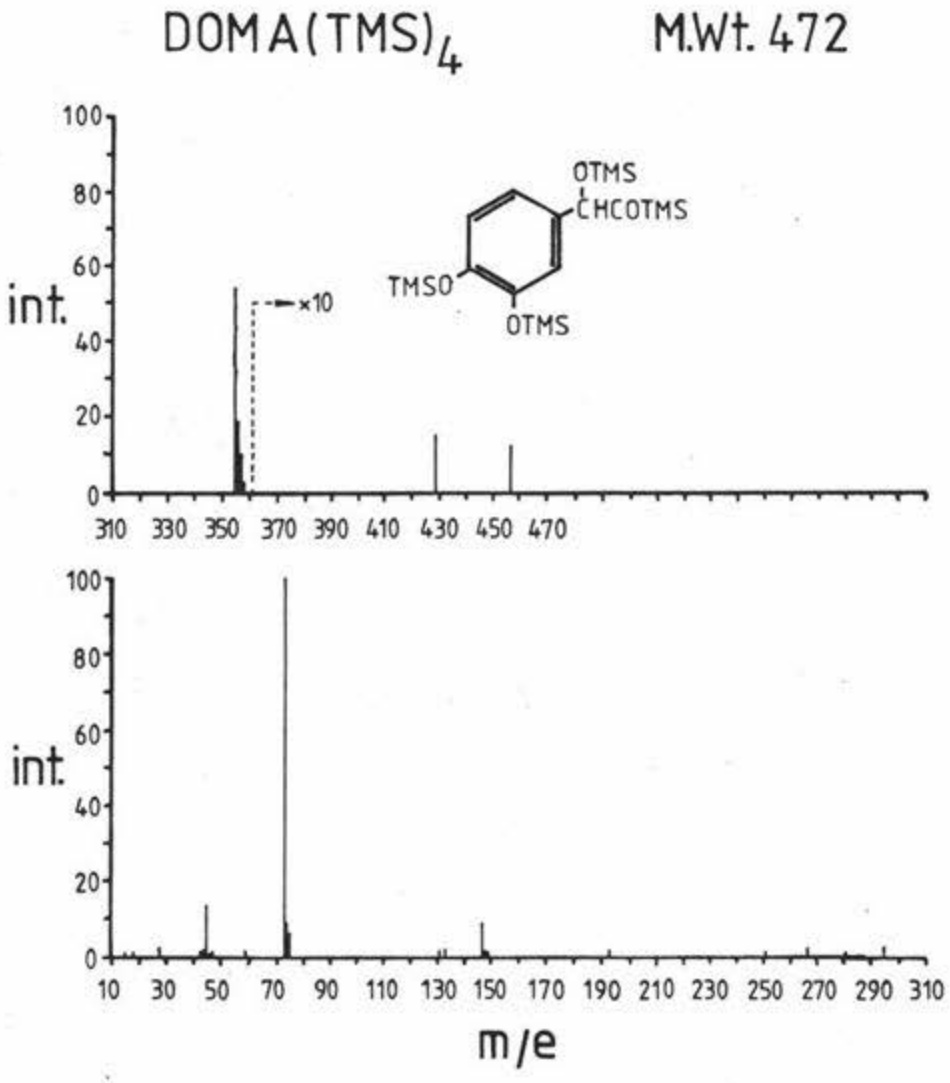
5-Hydroxyindoleacetic Acid-triTMS



3-Methoxy-4-hydroxymandelic Acid-triTMS



3,4-Dihydroxyphenylethyleneglycol-tetraTMS



3,4-Dihydroxymandelic Acid-tetraTMS

APPENDIX III ALCOHOL LOADING EXPERIMENTAL DATA

III(a) Blood Ethanol Concentrations During Alcohol Loading

NC		KA		KC		RG		AB		PB	
t	B.E.	t	B.E.	t	B.E.	t	B.E.	t	B.E.	t	B.E.
0	-	0	-	0	-	0	-	0	-	0	-
38	9.4	30	47.1	24	28	25	24.1	28	34.9	37	18.3
48	8.5	42	19.0	34	20	37	31.1	41	20.2	50	12.8
58	10.7	51	18.0	40	22	47	36.4	54	29.1	68	15.0
70	11.6	63	21.6	66	22	62	27.6	67	22.6	83	16.4
82	18.3	75	19.9	78	25	74	28.1	79	26.4	96	14.3
93	16.5	88	21.7	89	20	86	23.2	94	22.8	109	11.9
103	15.3	100	19.8	103	21	102	21.7	105	21.3	125	8.8
115	14.4	111	17.7	115	17	114	17.8	117	17.9	138	6.1
128	12.1	123	15.0	128	16	127	14.0	134	14.5	150	3.1
139	11.0	134	14.2	143	12	141	10.3	141	13.8	162	2.0
151	7.8	149	10.4	157	8	154	8.1	155	11.3	175	1.3
163	6.1	159	6.3	172	5	167	4.6	166	8.5	189	1.1
174	3.8	170	4.4	180	4	181	2.6	178	7.4		
187	2.7	180	3.2								

t=time after commencement of ethanol ingestion (mins)
B.E.=blood ethanol concentration (in mg%, 4.6 mg%=1.0 mM)

III(b) Diuresis Experiment, subject KC, urinary metabolite concentrations (μg metabolite mg^{-1} creatinine). t=midpoint of the time span over which the sample was collected.

t	DOPAC	HVA	DOMA	VMA	5-HIAA
-	1.11	3.61	0.26	2.16	2.59
15	1.30	4.93	0.63	2.82	2.18
45	1.41	6.03	0.63	2.91	2.55
75	1.35	5.28	0.76	2.63	2.60
105	1.53	5.25	0.76	3.13	2.51
135	1.49	5.29	0.90	2.93	2.61
165	1.55	5.01	1.00	3.32	1.88

III(c) Alcohol Loading, Urinary DOMA Levels (μg DOMA mg^{-1} creatinine)

NC		KA		KC		RG		AB		PB	
t	DOMA	t	DOMA	t	DOMA	t	DOMA	t	DOMA	t	DOMA
-	0.41	-	0.24	-	0.45	-	1.36	-	1.65	-	0.16
15	0.47	19	0.21	15	0.75	15	1.55	15	2.35	33	0.12
45	0.46	57	0.19	45	0.76	45	1.41	45	2.19	85	0.24
75	0.57	95	0.18	75	0.93	75	1.90	75	2.53	119	0.40
105	0.65	128	0.16	105	0.74	105	1.89	105	2.08	149	0.28
135	0.48	162	0.17	135	0.87	135	1.79	135	2.51	179	0.43
165	0.45			165	0.80	165	1.56	165	2.19		

III(d) Alcohol Loading, Urinary VMA Levels (μg VMA mg^{-1} creatinine)

NC		KA		KC		RG		AB		PB	
t	VMA	t	VMA	t	VMA	t	VMA	t	VMA	t	VMA
-	2.60	-	2.41	-	4.27	-	3.12	-	2.33	-	3.22
15	2.50	19	2.03	15	4.58	15	3.47	15	1.87	33	2.63
45	1.48	57	0.78	45	3.10	45	1.88	45	1.03	85	2.21
75	1.12	95	0.54	75	2.90	75	1.64	75	0.80	119	0.78
105	1.04	128	0.37	105	2.87	105	1.32	105	0.57	149	0.78
135	0.96	162	0.52	135	2.64	135	1.54	135	0.63	179	3.94
165	1.29			165	2.64	165	2.29	165	0.64		

III(e) Alcohol Loading, Urinary DOPAC Levels (μg DOPAC mg^{-1} creatinine)

NC		KA		KC		RG		AB	
t	DOPAC	t	DOPAC	t	DOPAC	t	DOPAC	t	DOPAC
-	2.36	-	4.05	-	3.10	-	0.75	-	4.03
15	2.44	19	3.66	15	3.34	15	1.05	15	4.36
45	2.48	57	3.95	45	3.92	45	1.09	45	4.04
75	2.33	95	3.78	75	3.64	75	1.19	75	4.01
105	2.31	128	2.80	105	3.61	105	1.21	105	3.14
135	2.20	162	2.88	135	3.75	135	1.15	135	3.54
165	2.64			165	3.49	165	1.32	135	3.32

Appendices III(c)-(e) t=midpoint of the time span over which the sample was collected.

III(f) Alcohol Loading, Urinary HVA Levels (µg HVA mg^{-1} creatinine)

NC		KA		KC		RG		AB	
t	HVA	t	HVA	t	HVA	t	HVA	t	HVA
-	3.83	-	4.72	-	6.78	-	2.70	-	3.44
15	4.64	19	3.60	15	10.20	15	4.38	15	4.44
45	3.79	57	3.60	45	9.22	45	5.76	45	4.07
75	3.55	95	3.51	75	8.20	75	5.79	75	3.58
105	4.92	128	2.75	105	8.66	105	5.90	105	2.38
135	3.83	162	3.16	135	7.86	135	5.79	105	3.70
165	6.67			165	8.04	165	6.75	165	3.52

III(g) Alcohol Loading, Urinary 5-HIAA Levels (µg 5-HIAA mg^{-1} creatinine)

NC		KC		RG		AB		PB	
t	5-HIAA	t	5-HIAA	t	5-HIAA	t	5-HIAA	t	5-HIAA
-	3.56	-	8.48	-	3.98	-	4.02	-	3.51
15	4.54	15	7.69	15	3.38	15	3.99	33	8.23
45	3.29	45	6.77	45	3.22	45	3.00	88	7.11
75	2.71	75	6.65	75	3.69	75	2.60	119	5.20
105	2.47	105	6.12	105	4.04	105	2.09	149	4.23
135	3.56	135	6.17	135	3.95	135	2.68	179	2.78
165	4.40	165	7.35	165	3.58	165	3.12		

III(h) Alcohol Loading, Alcoholic Metabolite Levels (µg Metab. mg^{-1} creatinine) subject PB.

t	DOPEG	MOPEG	5-HTPh
-	0.43	1.03	0.88
33	0.51	1.18	1.65
85	0.60	2.14	2.99
119	0.62	2.74	3.82
149	0.54	2.62	2.90
179	0.55	2.96	2.52

Appendices III(f)-(h) t=midpoint of time span over which the sample was collected.

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