STUDIES ON THE RENAL FORMATION AND ACTIONS OF 5-HYDROXYTRYPTAMINE USING ITS PRODRUGS IN MAN

by

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To

My wife Lisa and children, Matthew and Isabelle,

who have sustained me during this work.

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DECLARATION

This thesis has been composed by myself and the work presented in it is my own. Contributions and assistance by others are as indicated in the acknowledgements.

T.C. Li Kam Wa

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THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

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-egree PhD Date 17.11.95

-tle of Thesis Studies on the renal formation and actions of 5-hydroxytryptamine using its prodrugs in man

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The mammalian kidney, in addition to being a target for 5-hydroxytryptamine (5-HT), may itself be capable of locally producing and degrading 5-HT. In this thesis, I have employed the γ-glutamyl prodrug approach to targeting drugs to the kidney to explore the renal formation and actions of 5-HT in healthy human subjects.

I compared the effects of intravenous infusions of 5-hydroxy-L-tryptophan (L-5-HTP), the immediate precursor of 5-HT, and γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP), the glutamyl derivative of L-5-HTP. There were marked increases in the urinary excretion of 5-HT, without changes in circulating 5-HT levels measured in platelet-rich plasma, consistent with intrarenal synthesis of 5-HT. Both compounds reduced urinary sodium excretion in the absence of significant alterations in renal haemodynamics suggesting a predominantly tubular effect on sodium reabsorption. The urinary metabolite data and reduced extrarenal effects of glu-5-HTP, as evidenced by changes in growth hormone and aldosterone release, blood pressure and incidence of adverse effects, suggested that the glutamyl prodrug was relatively more selective for the kidney than L-5-HTP.

Carbidopa produced a 99% reduction in urinary excretion of 5-HT after glu-5-HTP and abolished its antinatriuretic effect. These observations indicated that glu-5-HTP, or its intermediate metabolite L-5-HTP, had no effect *per se* and required conversion to 5-HT to exert an effect on sodium excretion.

I compared the relative effectiveness of γ-L-glutamyl-L-tryptophan (glu-TRP), the glutamyl derivative of L-tryptophan, and glu-5-HTP as substrates for 5-HT synthesis in the kidney. Glu-TRP, unlike glu-5-HTP, failed to increase the urinary excretion of 5-HTP or 5-HT and to reduce sodium excretion suggesting an absence of effective renal synthesis of 5-HT following glu-TRP infusion.

I infused equimolar amounts of γ-L-glutamyl-L-dopa (gludopa) and glu-5-HTP in salt replete individuals to investigate whether the administration of one amine precursor affects the other amine synthesis and whether dopamine or 5-HT is preferentially generated. Relatively more dopamine than 5-HT was synthesised and the natriuretic effect of dopamine was more potent that the sodium retaining action of 5-HT *under the conditions of the study*. Comparison of the urinary metabolite data after the separate and concomitant infusion of the two precursor compounds provided no evidence of competitive inhibition of synthesis of either amine. There was an increase in urinary excretion of 5-HT after infusion of gludopa.

Frusemide produced the expected increase in urinary dopamine excretion but had no effect on 5-HT excretion in contrast to the increased 5-HT excretion reported in the rat. The absence of a rise in 5-HT excretion during the marked diuresis induced by frusemide can be cautiously taken as evidence that the increased 5-HT excretion after gludopa was unlikely to be due to a 'wash-out' of preformed 5-HT in the tubules by gludopa-induced diuresis.

Glu-5-HTP could be a valuable experimental tool for manipulating renal 5-HT production and exploring the renal formation and effects of 5-HT in man in health and disease.

CHAPTER ONE

INTRODUCTION

1.1 5-HYDROXYTRYPTAMINE

5-Hydroxytryptamine (5-HT; serotonin) is a naturally occurring indolealkylamine synthesised from the essential amino acid L-tryptophan. It possesses wide and varied biological activities and has been implicated in a number of physiological functions and pathological conditions.

1.1.1 History

For almost eighty years before 5-HT was identified as a distinct chemical substance, it has been known that a vasoconstrictor material appears in serum when blood is allowed to clot. This serum vasoconstrictor was given various names including 'vaso-constrictines', 'adrenalinähnliche Substanz', 'vasoconstrictor principle or substance', 'spätgift', ‘vaso-tonin’, 'thrombocytin' or 'thrombotonin' [1,2]. In 1948, Irvine Page's group at the Cleveland Clinic Foundation, U.S.A., isolated the active substance in pure crystalline form from beef serum and called it *serotonin* to denote its origin in serum and its ability to increase tone in blood vessels [3,4]. Shortly afterwards, the active moiety was tentatively identified as 5-HT [5]. In 1951, Hamlin and Fisher synthesised 5-HT and this was shown to be chemically and pharmacologically identical to the naturally occurring compound [6].

Independently, in the 1930s, Vittorio Erspamer and his co-workers in Italy started their investigation with the purpose of extracting, characterising and isolating the substance which imparts peculiar histochemical properties to the enterochromaffin (argentaffin) cells of the gastrointestinal mucosa [1]. Their experiments led them to discover the existence of a smooth muscle stimulant in the intestinal mucosa, and later in other tissues, which they termed *enteramine*. By the late 1940s, Erspamer has shown that it is present in many tissues of vertebrates and invertebrates and suggested that it is an indolealkylamine. In 1952, Erspamer and Asero isolated enteramine from the posterior salivary gland of the octopus (*Octopus*

vulgaris) and the skin of the Sicilian amphibian (*Discoglossus pictus*), and identified enteramine as 5-HT [1,7,8].

So by the early 1950s, both serotonin and enteramine were identified with 5-HT and with each other. 5-HT was subsequently found to come from many sources other than the blood or the gastrointestinal tract and to have a variety of effects. Neither serotonin nor enteramine is, therefore, an appropriate name and it has been suggested that these names should be abandoned in favour of 5-HT [9].

1.1.2 Chemistry

5-HT ($C_{10}H_{12}N_2O$) is the best known representative of the indolealkylamines, a group of natural and synthetic compounds which has as common basic structure an indole nucleus linked to an alkyl chain in position 3 of the heterocyclic ring (Figure 1-1). Its systematic chemical name is 3-(2-aminoethyl)-indol-5-ol and it has a molecular weight (MW) of 176.2. It does not possess any asymmetric carbon atoms and is, therefore, optically inactive. 5-HT is usually extracted or prepared as the creatinine sulphate salt. It is also available as the salts of other acids such as the hydrochloride, maleate and oxalate. It is easily oxidised in air, developing a brown-violet colour, particularly at alkaline pH.

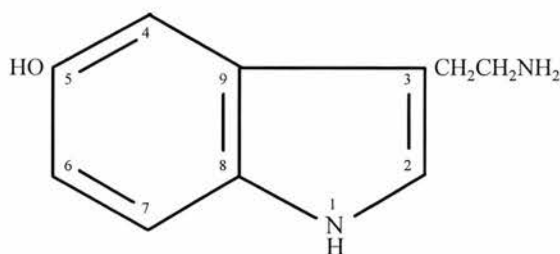


Figure 1-1. Structure of 5-hydroxytryptamine.

1.1.3 Source and Distribution

5-HT is widely distributed in nature occurring in vertebrates, in invertebrates such

as tunicates, molluscs, arthropods and coelenterates, and in a variety of plants. It is present in numerous stings and venoms including those of the common stinging nettle, wasp and scorpion. Some edible fruits, in particular walnuts, plantains, bananas and pineapples, contain significant amounts of 5-HT and their ingestion may lead to an increase in the urinary excretion of 5-hydroxy-3-indoleacetic acid (5-HIAA), the main excretory product of 5-HT metabolism [10].

5-HT occurs in highest concentrations in the gastrointestinal tract, platelets, pineal gland and brain in the body. About 90% of all the 5-HT in the adult human body is found in the enterochromaffin cells of the gastrointestinal tract, particularly in the duodenum (3.7 $\mu g\ g^{-1}$of tissue) and ileum (2.9 $\mu g\ g^{-1}$) [11,12]. 5-HT is synthesised *in situ* from L-tryptophan in these enterochromaffin cells and the amine is stored in large, electron dense neuro-secretin-like granules [13]. Such 5-HT containing enterochromaffin cells also appear to be present in other body tissues such as the pancreas, lung, thymus, thyroid and urogenital tract [14].

Most of the 5-HT in blood is present in a functionally inactive form, stored in electron dense 500-1500 Å granules in platelets at a concentration of about 5.2×10^5 molecules per platelet in man [15]. It is generally believed that platelets lack the enzymes required for the synthesis of 5-HT [16-18] but others have suggested that low level activities of the enzymes may be present [19]. The platelets derive their 5-HT content primarily from the plasma by means of an active uptake mechanism. Most of it is thought to originate in the enterochromaffin cells of the gastrointestinal mucosa during the passage of platelets through the intestinal blood vessels, where they encounter relatively high concentrations of 5-HT released into the circulation by the enterochromaffin cells [10]. This hypothesis is supported by the observation that there was more than 90% decrease of platelet 5-HT in a patient with an almost complete resection of the intestinal tract [20]. The absorption capacity of platelets for 5-HT is not saturated under normal conditions. In one study, the maximum uptake of 5-HT by human platelets was found to range from 180 to 960 ng per 10^8

platelets, the normal content being 10-90 ng per 10^8 platelets [21]. 5-HT which escapes the high affinity uptake system of the platelets is metabolised by monoamine oxidase type A (MAO-A), particularly in the liver, or taken up into endothelial cells, especially in the lungs, followed by rapid oxidative deamination of the amine by MAO-A. These processes lead to very low concentrations of 'free' 5-HT in plasma, with estimates ranging from 3 to 20 ng ml^{-1}[22], and will tend to limit any increase in the level of free 5-HT in the blood which may occur in the carcinoid syndrome [23]. Unambiguous data regarding the level of free 5-HT in plasma is difficult to obtain because of the well-known extreme fragility of platelets and it has even been suggested that there may be no free 5-HT in plasma, since any measured 5-HT may be derived from disintegrated platelets during sample collection and/or processing. However, if we accept that the 5-HT in platelets has its origin in the enterochromaffin cells of the gastrointestinal tract, some 5-HT, though perhaps only a very small amount, must circulate free in plasma since the exchange of 5-HT between platelets and enterochromaffin cells can only occur through the mediation of plasma. In addition, 5-HT released following damage or lysis of the platelets is of necessity set free and must enter the plasma for at least a short time. The 5-HT in platelets is more strongly bound or more slowly utilised than that in the enterochromaffin cells since the half-life is about 2.2-3.6 days in platelets compared to about 7-12 h in the gastrointestinal tract in man. It appears that the platelets only lose their 5-HT when disintegrating [10,24,25].

The brain synthesises its own 5-HT from L-tryptophan entering through the blood-brain barrier using the transport system for large neutral amino acids and is not dependent upon peripheral 5-HT synthesis [26-28]. 5-HT is formed and stored in the vesicles of the so-called tryptaminergic neurones in the central nervous system. High concentrations of 5-HT are found in the thalamus, hypothalamus, midbrain and pineal gland. The turnover rate of 5-HT in the central nervous system is high, the half-life of cerebral 5-HT being estimated as not longer than 10-20 min

[10,29]. There is some evidence that circulating 5-HT may enter the brain contrary to the generally held view that it does not penetrate the blood-brain barrier [30].

5-HT has been detected in a number of other tissues including the enteric plexuses, heart, lung, spleen, liver, kidney, thyroid and blood vessels [10,29].

1.1.4 Biochemical Pathways of 5-HT

1.1.4.1 Biosynthesis of 5-HT

The first step in the biosynthesis of 5-HT, represented schematically in Figure 1-2, is the hydroxylation at the C5 position of the indole ring of L-tryptophan to form 5-hydroxy-L-tryptophan (L-5-HTP). The L-5-HTP thus formed is decarboxylated to 5-HT by the enzyme aromatic L-amino acid decarboxylase [31].

L-tryptophan is a true essential amino acid and is not synthesised by mammalian cells. The dietary consumption of L-tryptophan is usually less than 1-2 g per day and normally only 1-2% of it is utilised for the daily synthesis of about 5-10 mg of 5-HT in man [24,32]. The intestinal tract is the most important site quantitatively for 5-HT synthesis. The conversion of L-tryptophan to L-5-HTP represents only a minor pathway in the overall mammalian metabolism of L-tryptophan. Most ingested L-tryptophan is metabolised via the kynurenine pathway [33] (Figure 1-3). The 5-hydroxylation pathway may, however, assume a major role in patients with malignant carcinoid tumours in whom as much as 60% of a daily intake of 500 mg of L-tryptophan may be converted to 5-hydroxyindoles, whereas only about 1% is metabolised by this route in normal individuals [34].

Tryptophan 5-hydroxylase

Tryptophan 5-hydroxylase [systematic name: L-tryptophan, tetrahydrobiopterin: oxygen oxidoreductase (5-hydroxylating); EC 1.14.16.4] catalyses the hydroxylation of L-tryptophan in the C5 position to form L-5-HTP. It is stereospecific for L-tryptophan and requires molecular oxygen and reduced

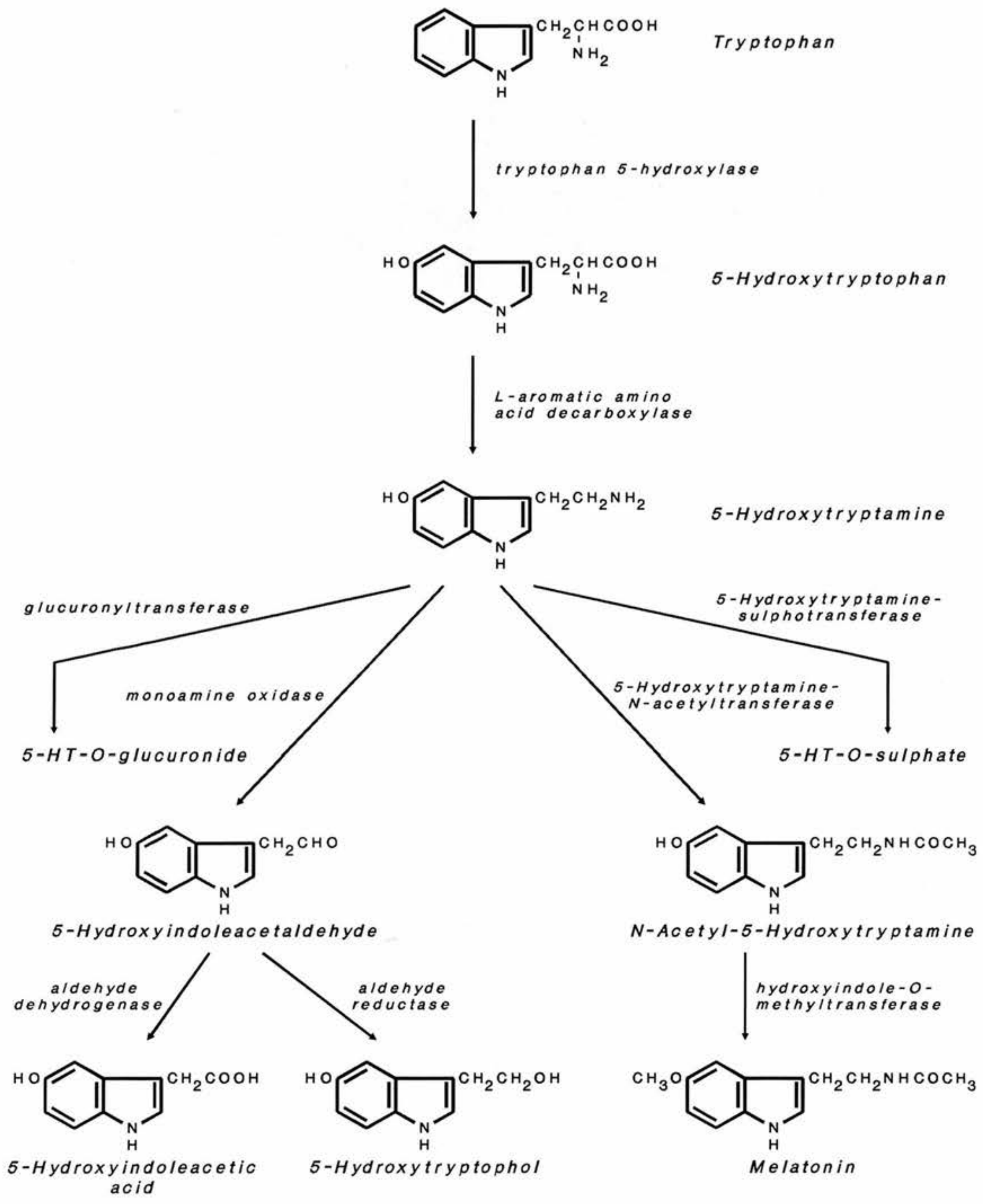


Figure 1-2. 5-HT metabolism.

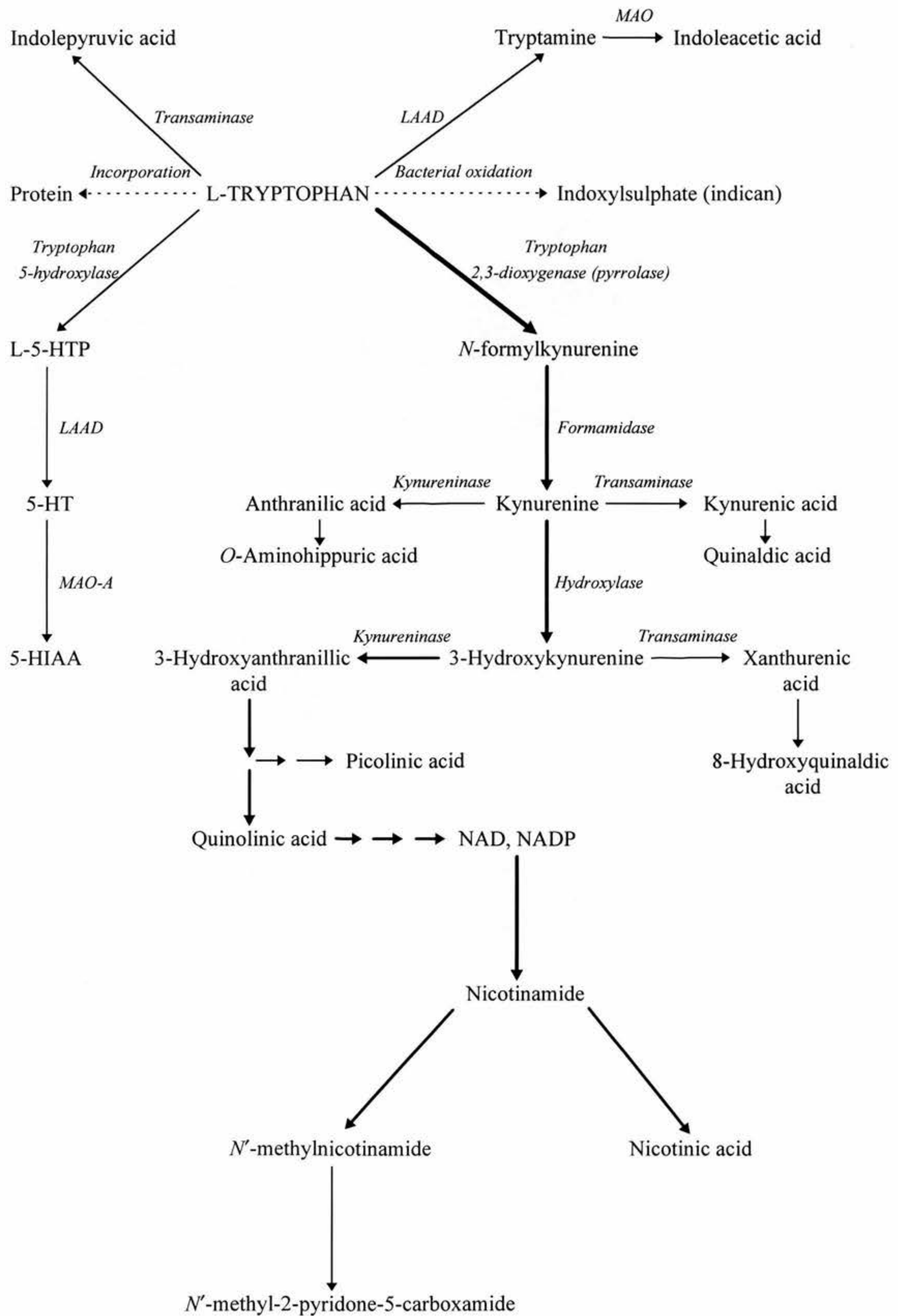
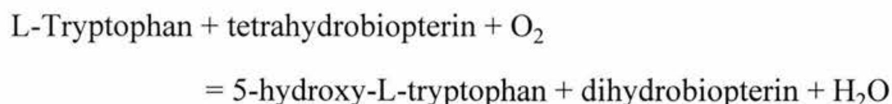


Figure 1-3. Major metabolic pathways of L-tryptophan degradation in man.

pteridine as cofactors. The reaction is represented by the equation:

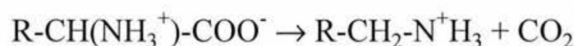


$$\text{L-Tryptophan} + \text{tetrahydrobiopterin} + O_2$$
$$= \text{5-hydroxy-L-tryptophan} + \text{dihydrobiopterin} + H_2O$$

This hydroxylation is the rate limiting step in the biosynthesis of 5-HT from L-tryptophan [31]. Under normal conditions, the enzyme is not fully saturated with the substrate and the rate of synthesis of 5-HT is dependent upon the availability of free L-tryptophan. Tryptophan 5-hydroxylase has been detected in the intestinal mucosa, brain, pineal gland, kidney, liver, mouse mast cells and carcinoid tumour cells. No tryptophan 5-hydroxylase activity was detected in platelets [18] but other investigations indicated the presence of low level of enzyme activity [19]. There was no tryptophan 5-hydroxylase activity in the heart, aorta or adrenal gland in the rat [35] but it was demonstrated to be present in the frog adrenal gland [36].

Aromatic L-amino acid decarboxylase

Aromatic L-amino acid decarboxylase (LAAD) [aromatic-L-amino-acid carboxy-lyase; EC 4.1.1.28], previously also known as hydroxytryptophan decarboxylase or dopa decarboxylase, catalyses the decarboxylation of L-5-HTP and L-dopa (L-3,4-dihydroxyphenylalanine) to 5-HT and dopamine (3,4-dihydroxyphenylethylamine) respectively [31]. It requires pyridoxal phosphate as a coenzyme. LAAD also catalyses the decarboxylation of the L-isomers of other aromatic amino acids including tyrosine to tyramine, phenylalanine to phenylethylamine, and tryptophan to tryptamine in different species. The enzyme prepared from the human kidney, however, appears to decarboxylate only L-dopa and L-5-HTP [37]. The reaction can be represented by the general formula:



$$\text{R-CH}(NH_3^+)\text{-COO}^- \rightarrow \text{R-CH}_2\text{-N}^+H_3 + CO_2$$

LAAD is widely distributed throughout mammalian tissues, occurring most abundantly in the kidney, liver, gastrointestinal tract, adrenal gland and brain [31,38,39]. Spleen, bone marrow and blood platelets possess little or no activity. Within the cell, the enzyme is localised mainly in the cytosol, although some

activity may be associated with particulate fractions. In LAAD deficiency, an inborn error of metabolism that affects both dopamine and 5-HT biosynthesis, there are increases in the concentrations of L-dopa and 5-HTP in urine, plasma and cerebrospinal fluid, together with low levels of catecholamines in plasma and 5-HT in whole blood [40].

There was, and still remains, some controversy as to the homogeneity of the decarboxylase(s) and whether one single or two separate decarboxylases catalyse the decarboxylation of L-dopa to dopamine and that of L-5-HTP to 5-HT [41]. It was originally considered that decarboxylation of L-dopa and L-5-HTP occurs under the influence of two distinct enzymes [16]. These were given separate code numbers by the Enzyme Commission and were known as dopa decarboxylase [EC 4.1.1.26] and 5-hydroxytryptophan decarboxylase [4.1.1.28] respectively. Later work, however, concluded that a single enzyme acts on both substrates [42-44] and that dopa decarboxylase and 5-HTP decarboxylase are one and the same enzyme which the IUPAC Commission on Biomedical Nomenclature in 1972 named as aromatic L-amino acid decarboxylase [EC 4.1.1.28]. There is still some doubt as to the strength of the evidence for a single enzyme capable of decarboxylating both substrates. The optimal pH and temperature conditions as well as the kinetic parameters are different for the decarboxylation of L-dopa to dopamine and that of L-5-HTP to 5-HT. These two activities exhibit different responses to changes in the levels of substrates, pyridoxal phosphate and the presence of reducing substances. There is also regional variation in the distribution of these two activities [39,45]. On the other hand, however, dopa and 5-HTP decarboxylase activities from a number of tissues of various species were immunologically indistinguishable and were inhibited to the same extent by a mono-specific antiserum to hog kidney decarboxylase supporting the concept of a 'single enzyme' hypothesis [44]. A monoclonal antibody directed against the aromatic LAAD from rat kidney also revealed that the enzymes from the striatum, adrenal medulla, pineal gland, liver

and kidney were indistinguishable with respect to immunological cross-reactivity and molecular size [46]. Furthermore, when the expression vector containing the full-length cDNA of human LAAD was transfected in COS cells, the expressed enzyme was found to decarboxylate both L-dopa and L-5-HTP [47]. Strong support, therefore, exists for the concept of a single aromatic LAAD but there may also be non-uniform local regulatory mechanisms governing the differential synthesis of catecholamines and indoleamines throughout mammalian tissues [41].

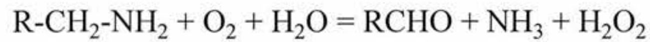
1.1.4.2 Metabolism of 5-HT

The principal metabolic pathway of 5-HT, endogenous or ingested, is oxidative deamination by MAO-A to form 5-hydroxyindoleacetaldehyde (Figure 1-2). This is promptly degraded, mainly by further oxidation by the enzyme aldehyde dehydrogenase to produce 5-HIAA, the major catabolic and excretory product of 5-HT metabolism. 5-Hydroxyindoleacetaldehyde can also be reduced to the alcohol, 5-hydroxytryptophol (5-HTOL), in a reaction catalysed by alcohol dehydrogenase. Under normal conditions, this pathway is less important than the oxidation to 5-HIAA. Other minor metabolic pathways for 5-HT include conjugation with sulphate or glucuronide, *N*-acetylation, *O*-methylation and *N*-methylation. 5-HIAA and 5-HTOL may also be conjugated or converted metabolically to the 5-methoxy derivatives. About 2 to 8 mg of 5-HIAA is excreted daily in the urine by the normal adult, along with much smaller amounts of other metabolites, as result of the metabolism of endogenous 5-HT [31].

Monoamine oxidase

Monoamine oxidase (MAO) [amine: oxygen oxidoreductase (deaminating) (flavin containing); EC 1.4.3.4] inactivates 5-HT to 5-hydroxyindoleacetaldehyde. It also catalyses the oxidative deamination of other biogenic amines, such as dopamine, adrenaline, noradrenaline, tyramine, phenylethylamine and tryptamine, to yield the corresponding aldehyde which is further converted enzymatically to the

corresponding acid or alcohol by aldehyde dehydrogenase or reductase respectively. The reaction catalysed by MAO is of the general form:



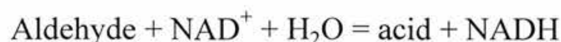
$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O = RCHO + NH_3 + H_2O_2$$

MAO is localised to the outer membrane of the mitochondrion. Its activity can be attributed to the effects of two structurally, immunologically and genetically distinct forms of MAO, designated type A and type B [31,48-50]. MAO-A is sensitive to inhibition by clorgyline and the B form is sensitive to inhibition by deprenyl. Both isoforms are widely distributed and are found in high concentrations in the liver, kidney, adrenal gland, heart, brain and intestines in man. MAO is absent (or present in very low concentrations) in blood plasma and erythrocytes, and human platelets contain MAO-B only. The human placenta is a rich source of MAO-A. MAO may play a protective role by oxidising amines in blood or preventing their entry into the circulation. MAO-A is localised both intra- and extraneuronally and prefers as substrates the hydroxylated amines such as 5-HT, noradrenaline and adrenaline. MAO-B, which is predominantly if not exclusively localised extraneuronally, preferentially deaminates non-hydroxylated amines such as phenylethylamine and benzylamine. Dopamine and tyramine are readily oxidised by both MAO-A and MAO-B. 5-HT may be metabolised to 5-HIAA by amine oxidases other than MAO-A [51]. It is of interest that patients suffering from Norrie disease with a deletion of the X-chromosomal region containing the MAO-A and MAO-B genes have essentially normal urinary levels of 5-HIAA [52]. Later studies showed that they possess normal plasma amine oxidase activity [53].

Aldehyde dehydrogenase

Aldehyde dehydrogenase [aldehyde: NAD^+ oxidoreductase; EC 1.2.1.3] is the enzyme responsible for the oxidation of 5-hydroxyindoleacetaldehyde to 5-HIAA. This enzyme is able to oxidise a variety of aliphatic and aromatic aldehydes to the corresponding carboxylic acids. Nicotinamide adenine dinucleotide (NAD^+) is a better cofactor than nicotinamide adenine dinucleotide phosphate ($NADP^+$), and the

reaction can be represented by the following equation:

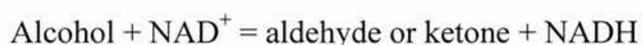


$$\text{Aldehyde} + NAD^+ + H_2O = \text{acid} + \text{NADH}$$

The enzyme is present in many body tissues including liver, adrenal gland, intestine, kidney, ovary, uterus, testis, seminal vesicles, bladder, adipose tissue, heart, lung, brain, spleen, skeletal muscle and brain, with the highest activity in liver and kidney [31].

Alcohol dehydrogenase

Alcohol dehydrogenase [alcohol: NAD^+ oxidoreductase, EC 1.1.1.1], also known as aldehyde reductase, catalyses the reduction of 5-hydroxyindoleacetaldehyde to 5-HTOL which is excreted in the urine, free or as a conjugate. The enzyme is capable of catalysing both the oxidation of alcohols to aldehydes and ketones, and the reduction of these carbonyl compounds to the corresponding alcohols. The coenzymes for these oxidation and reduction reactions are NAD^+ and NADH respectively. NADP+ and NADPH can also function as coenzymes.



$$\text{Alcohol} + NAD^+ = \text{aldehyde or ketone} + \text{NADH}$$

Alcohol dehydrogenase has a wide tissue distribution and is typically found in the soluble fractions of tissue homogenates. It is present in especially high concentrations in the liver and brain. Although under normal conditions, the reduction of 5-hydroxyindoleacetaldehyde to 5-HTOL is less important than its oxidation to 5-HIAA, 5-HTOL has been detected in peripheral tissues and brain. Ingestion of ethanol diverts 5-hydroxyindoleacetaldehyde from the predominantly oxidative route of 5-HIAA formation to the reductive pathway resulting in a decrease in urinary excretion of 5-HIAA and a corresponding increase in excretion of 5-HTOL. This has been attributed to an increase in $NADH/NAD^+$ ratio resulting from ethanol metabolism [31].

Sulphation and glucuronidation

Two conjugation reactions have been observed in the metabolism of 5-HT. One involves the transfer of sulphate from 3′-phosphoadenosine-5′-phosphosulphate to

5-HT to produce 5-HT-*O*-sulphate. This reaction is catalysed by the enzyme phenol sulphotransferase [3′-phosphoadenylylsulphate: phenol sulphotransferase; EC 2.8.2.1] which is present in many mammalian tissues including liver, kidney, lung, brain, gastrointestinal tract, heart and spleen. *O*-Sulphation of 5-HT results in a loss of biological activity; however, in many tissues, aryl sulphatase enzymes can hydrolyse 5-HT-*O*-sulphate and regenerate free 5-HT [31].

The other conjugation pathway involves conjugation of 5-HT with uridine diphosphoglucuronic acid (UDP glucuronic acid) to produce 5-HT-*O*-glucuronide in a reaction catalysed by UDP glucuronyltransferase [UDPglucuronate β-D-glucuronosyltransferase (accepto-unspecific); EC 2.4.1.17]. This enzyme is widely distributed in mammals, being found primarily in liver, kidney, gastrointestinal tract, epidermis and urinary tract. The liver has the highest activity and is the major site of glucuronidation of 5-HT but glucuronidation can occur elsewhere including within the kidney. This could have physiological importance as the kidney might be able to partially compensate in those states in which there is impaired hepatic glucuronidation. In the presence of MAO inhibitors, 5-HIAA excretion is decreased while excretion of 5-HT-*O*-glucuronide and 5-HT-*O*-sulphate rises thus compensating for the blocked pathway. Conversely, there is an increase in 5-HIAA excretion after administration of 5-HT when formation of the glucuronide is inhibited by sodium-*o*-aminobenzoate [31,54,55].

N-*acetylation,* O-*methylation,* N-*methylation*

N-acetylation of 5-HT is the first step in the biosynthesis of melatonin in the pineal gland (Figure 1-2). This involves the transfer of an acetyl group from acetyl-CoA to 5-HT producing *N*-acetyl-5-HT and is catalysed by arylamine acetyltransferase [acetyl-CoA: arylamine *N*-acetyltransferase; EC 2.3.1.5]. The activity of this enzyme regulates the diurnal rhythm of the synthesis of melatonin. *N*-acetylation of 5-HT has also been described in the liver and brain. *N*-acetyl-5-HT is converted to melatonin in the pineal gland via the *O*-methylation of the 5-hydroxyindole moiety.

This reaction is catalysed by hydroxyindole-*O*-methyltransferase [S-Adenosyl-L-methionine: *N*-acetylserotonin *O*-methyltransferase; EC 2.1.1.4] and uses S-adenosylmethionine as the methyl donor. 5-HT itself can be *O*-methylated in the brain resulting in the formation of 5-methoxytryptamine. *N*-methylation of 5-HT has also been reported in the brain and lung [31].

1.1.5 5-HT Receptors

The existence of pharmacologically distinct types of 5-HT receptors was first demonstrated by Gaddum and Picarelli in 1957 [56]. They identified two types of 5-HT receptors in the guinea pig ileum, the 'D' receptor mediating contraction by a direct action on smooth muscle which was blocked by *dibenzyline* (former name for phenoxybenzamine), and the 'M' receptor mediating depolarisation of cholinergic myenteric neurones which was blocked by *morphine*. In 1979, Peroutka and Snyder [57] classified the 5-HT receptors on the basis of radioligand-binding studies in brain tissue. The sites labelled by [^{3}H]5-HT were designated as 5-HT$_1$ receptors and those labelled by [^{3}H]spiperone were called 5-HT$_2$ receptors. In 1986, Bradley *et al.* [58] proposed that there are three main groups of 5-HT receptors, namely 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ receptors, each recognising different synthetic ligands that act as either agonists or antagonists. The 5-HT$_1$-like receptors were defined by their susceptibility to antagonism by methiothepin and/or methysergide, and potent agonism by 5-carboxamidotryptamine (5-CT). Their scheme integrated Gaddum and Picarelli's D (5-HT$_2$) and M (5-HT$_3$) classification based on functional studies with that of Peroutka and Snyder's 5-HT$_1$ and 5-HT$_2$ classification based on radioligand-binding studies (Figure 1-4). Since then, a large amount of information has been generated, particularly by the molecular biologists, and further receptors have been identified. These developments have prompted a review of the classification of 5-HT receptors [59-61].

The current classification (proposed in 1994) reflecting an international view

supported by the Serotonin Club Receptor Nomenclature Committee which reports to the International Union of Pharmacology Committee for Receptor Nomenclature is shown in Figure 1-4 and Table 1-1 [60,61]. It is based on three main criteria: operational data derived from quantification of the functional activities of selective agonists/antagonists and from radioligand-binding studies; the transduction mechanism to which the receptor is coupled; and the structural data of the receptor i.e. the nucleotide sequence of the receptor gene and the amino acids of the corresponding receptor protein. A receptor is only fully characterised if

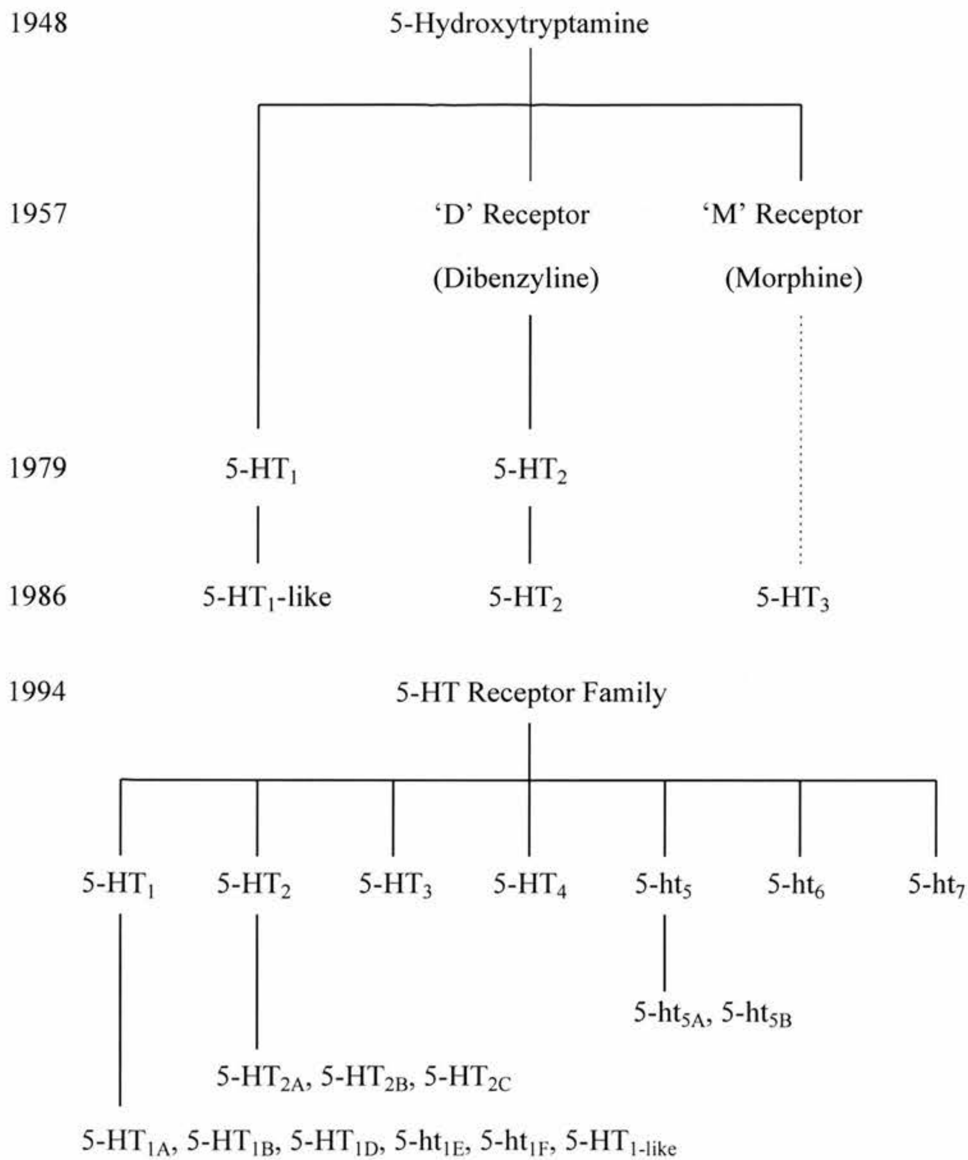


Figure 1-4. A history of the classification of 5-HT receptors.

Table 1-1. Serotonin receptor subtypes.

Receptor *Type*	*Subtype*	*Distribution*	*Effector pathways*	*Some responses and comments*
5-HT_1	5-HT_{1A}	Mainly CNS, limbic system (i.e. hippocampus, septum, amygdaloid), raphé nuclei, neocortex, hypothalamus, and substantia gelatinosa of spinal cord.	↓ cAMP, K^+ channel	Neuronal hyperpolarisation. Possible roles in regulation of emotion, mood and behaviour, modulation of activity of tryptaminergic neurones, regulation of functions of hypothalamus, proprioception, and integrative functions of neocortex. Reduction in heart rate and blood pressure.
	5-HT_{1B}	CNS, particularly in substantia nigra, basal ganglia, dorsal subiculum, superior colliculi, and some peripheral nerves. Found in rodents only.	↓ cAMP	Rodent equivalent of 5-HT_{1D_B} receptor. 5-HT autoreceptor in brain. Control release of other neurotransmitters such as acetylcholine and glutamate. Inhibition of noradrenaline release. May mediate behaviour and locomotion.
	5-HT_{1D}	Mainly CNS, particularly in substantia nigra, basal ganglia, superior colliculus, hippocampus, raphé, cortex. Cerebral blood vessels.	↓ cAMP	Contraction of some blood vessels. 5-HT autoreceptor. Modulate release of neurotransmitters such as acetylcholine and glutamate. Mediate the endothelium-dependent relaxation in pig coronary artery. May mediate behaviour and locomotion.
	5-ht_{1E}	Only CNS, generally in brain regions similar to those for 5-HT_{1D}	↓ cAMP	Functions not known
	5-ht_{1F}	Receptor mRNA identified in brain (hippocampus, dorsal raphé, striatum, cortex, thalamus, hypothalmus), mesentery, uterus.	↓ cAMP	Functions not known
	5-HT_1-like	Intracranial arteries, carotid arteriovenous anastomotic vessels. Some peripheral blood vessels contain 5-HT_1-like receptors either exclusively e.g. dog and rabbit saphenous vein, guinea pig iliac artery, and rabbit renal artery, or in addition to 5-HT_2 receptors e.g. human coronary artery.		Smooth muscle contraction, inhibition of noradrenaline release from sympathetic nerves, inhibition of 5-HT and glutamate release. Not definitively characterised but may be 5-HT_{1D}, 5-ht_{1F}, or other recombinant receptor.
5-HT_2	5-HT_{2A}	Vascular, bronchial, uterine, urinary, and gastrointestinal smooth muscles, platelets, CNS particularly in neocortex, claustrum, olfactory nuclei, basal ganglia.	↑ IP_3	Contraction of various smooth muscles, platelet aggregation, increased capillary permeability, amplify effects of other vasoconstrictors, some behavioural effects in rodents, neuroendocrine functions such as release of β-endorphin, corticosterone, luteinizing hormone in rats, prolactin in rhesus monkeys, adrenaline in dogs.

Table 1-1. *Continued.*

Receptor *Type*	*Subtype*	*Distribution*	*Effector pathways*	*Some responses and comments*
5-HT_2	5-HT_{2B}	? mainly peripheral. Receptor mRNA found in gastric fundus, gut, heart, kidney, lung, and to less extent in brain.	↑ IP_3	Rat stomach fundic muscle contraction
	5-HT_{2C}	Choroid plexus, globus pallidus, substantia nigra	↑ IP_3	Possible roles in locomotion, feeding, CSF production, adrenocorticotrophic hormone release, migraine, obsessive compulsive disorders, anxiety.
5-HT_3		Peripheral (autonomic, nociceptive sensory, and enteric) and central neurones (dorso-vagal complex, spinal trigeminal nucleus, nucleus tractus solitarius, area postrema, substantia gelatinosa, cortex, limbic system)	Na^+ & K^+ cation channel	Neuronal depolarisation. Involved in von Bezold-Jarisch-like reflex and other cardiovascular reflexes elicited by pulmonary and carotid body chemoreceptors, emesis associated with chemotherapy and radiotherapy, control of intestinal tone and secretion, modulation of visceral pain in gut, dermal pain & flare response, vasodilatation, possible roles in psychosis, anxiety, cognition, rewarding and withdrawal effects from drugs of abuse, and eating disorders.
5-HT_4		Gastrointestinal tract (myenteric neurones, smooth muscle, secretory cells), CNS (especially cortex, olfactory tubercles, nucleus accumbens, corpus striatum, globus pallidum, substantia nigra, colliculus, hippocampus), heart, urinary bladder.	↑ cAMP	Activation of acetylcholine release in gut, gastrokinetic action and peristaltic reflex, intestinal secretion, myocardial (atrial) stimulation. Possible roles in affective disorders, psychoses, motor co-ordination, arousal and visual perception, learning and memory. Steroid secretion from adrenocortical cells.
5-ht_5	5-ht_{5A} 5-ht_{5B}	5-ht_{5A} receptor mRNA found in cortex, hippocampus, habenula, olfactory bulb, cerebellum. 5-ht_{5B} receptor mRNA found in habenula, hippocampus.	Unknown	Functions in intact tissues not known
5-ht_6		Receptor mRNA localised in striatum, olfactory tubercle, cortex, hippocampus.	↑ cAMP	Functions in intact tissues not known
5-ht_7		Receptor mRNA localised in hypothalamus, thalamus, forebrain, cerebellum, heart & intestine	↑ cAMP	Functions in intact tissues not known

comprehensive information on its operational pharmacology, transduction mechanism and receptor structure is available. The present classification recognises the existence of at least three, but possibly up to seven, classes or groups of 5-HT receptors. They comprise the 5-HT_1, 5-HT_2 and 5-HT_3 classes, as well as the 'uncloned' 5-HT_4 receptor. The 5-ht_5, 5-ht_6 and 5-ht_7 receptor genes are cloned but the receptors have yet to be fully characterised operationally and transductionally in intact tissues, and as such their appellations are considered provisional. The 5-HT_1, 5-HT_2 and 5-ht_5 classes are further subdivided. Lower case letters are used to denote recombinant receptors to distinguish them from native receptors identified in whole tissues. The distribution and possible functional responses of these receptors are shown in Table 1-1 and examples of agonists and antagonists at the different receptors are given in Table 1-2. Relatively few agonists or antagonists interact selectively with only one type of 5-HT receptor.

5-HT_1 receptors occur mainly in the brain (Table 1-1). The 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E} and 5-ht_{1F} receptors have all been cloned and shown to share a high degree of homology (> 60% in the transmembrane domains) and to have intronless genes in the coding sequence region. They share a common transduction system in being negatively coupled to adenylate cyclase, presumably via a common or similar G-protein link, and their neuronal effects are predominantly inhibitory. The 5-HT_{1A} subtype may be important in the brain in relation to mood and behaviour. 5-HT_{1A} agonists, especially those considered as partial agonists, such as buspirone, gepirone, ipsapirone or tandospirone, have anxiolytic effects in animal models of anxiety, and buspirone, for instance, is used in the treatment of anxiety. A number of 5-HT_{1A} agonists, e.g. 8-OH-DPAT, flesinoxan, urapidil and 5-methyl-urapidil, reduce blood pressure and heart rate by activation of central 5-HT_{1A} receptors. The 5-HT_{1D} receptor subtype in the cerebral blood vessels is thought to be important in migraine and is the target for the anti-migraine drug, sumatriptan. Two human 5-HT_{1D} receptors have been demonstrated, designated 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$, the

Table 1-2. Examples of 5-HT agonists and antagonists (*see* Appendix I for glossary of drug names and abbreviations).

Receptor Type	*Subtype*	*Agonists*	*Antagonists*
5-HT_1	5-HT_{1A}	DP-5-CT, 5-CT, 5-Methyl-urapidil, 8-OH-DPAT, RU 24969, Flesinoxan, Ipsapirone, Buspirone, Gepirone, MDL 72832, Urapidil.	SDZ 216525, NAN 190, MDL 73005, 5-F-OH-DPAT, Cyanopindolol, Pindolol, Methiothepin, Metergoline, WAY 100135, BMY 7378, Spiperone, Propranolol.
	5-HT_{1B}	RU 24969, 5-CT, CP 93129, CGS 12066, Ergotamine	Cyanopindolol, Methiothepin, SDZ 21009, Pindolol, Metergoline.
	5-HT_{1D}	L 694247, 5-CT, Sumatriptan, Ergotamine.	GR 127935, Methiothepin, Metergoline
	5-ht_{1E}	5-HT	None (Methiothepin - weak)
	5-ht_{1F}	5-HT	None (Methiothepin - weak)
	5-HT_1-like	Sumatriptan, 5-CT	Methiothepin
5-HT_2	5-HT_{2A}	DOI, α-Methyl-5-HT, quipazine, MK 212, LSD (CNS)	Pirenperone, Ketanserin, Ritanserin, Cinanserin, Spiperone, Mesulergine, Metergoline, Mianserin, Cyproheptadine, LY 53857, ICI 169369, Pizotifen, LSD (peripheral), Methysergide.
	5-HT_{2B}	α-methyl-5-HT, DOI	SB 200646, LY 53857
	5-HT_{2C}	α-Methyl-5-HT, DOI, mCPP, TFMPP, MK 212, LSD	Metergoline, Mesulergine, Ritanserin, LY 53857, Methiothepin, Cyproheptadine, SB 200646, ICI 169369, Mianserin, Methysergide
5-HT_3		2-Methyl-5-HT, m-Chlorophenyl-biguanide, Phenylbiguanide	Tropisetron, Zacopride, Granisetron, MDL 72222, Ondansetron, Metoclopramide
5-HT_4		5-MeOT, α-methyl-5-HT, Benzamides (Cisapride, Renzapride, Zacopride, Metoclopramide), Benzimidazolones (BIMU1, BIMU8).	SB 204070, SB 207710, GR 113808, RS 23597190, SDZ 205557, DAU 6285, Tropisetron (weak)
5-ht_5		5-HT	Methiothepin
5-ht_6		5-HT	Methiothepin
5-ht_7		5-HT	Methiothepin

latter having very close homology to, and reflecting the distribution and function of, the 5-HT_{1B} receptor found in the rodent. The '5-HT_{1C} receptor', which does not act via cAMP, is now declared non-existent, having been assigned to the 5-HT_2 class and renamed as 5-HT_{2C} (*see* below). The 5-ht_{1E} and 5-ht_{1F} (previously 5-$HT_{1E\beta}$ or 5-HT_6) receptors are classified as 5-HT_1 receptor subtypes on the basis of their amino acid homology and their negative coupling to adenylate cyclase in cell lines. However, these receptors are operationally different from other 5-HT_1 receptors because 5-CT has little or no affinity or agonist activity at these sites. Similarly, methiothepin displays low affinity and, where tested, low antagonist potency at these receptors. The 5-HT_1-like appellation is now used to denote a group of 5-HT_1 receptor subtypes that have not yet been fully characterised and positively equated with any of the recognised 5-HT_1 subtypes. Some have been characterised solely using operational studies. They can be clearly distinguished from the 5-HT_{1A}-, 5-HT_{1B}- and 5-HT_{2C}- (previously 5-HT_{1C}) binding sites but it is not always easy to distinguish them from 5-HT_{1D}-binding sites particularly in peripheral tissues such as the blood vessels. There is a 5-HT_1-like sumatriptan-sensitive receptor that mediates vascular smooth muscle contraction and is negatively coupled to adenylate cyclase which has close similarities but is not identical to the 5-HT_{1D} receptor subtype in its pharmacological responses. There are no completely selective agonists or antagonists for 5-ht_{1E}, 5-ht_{1F}, and 5-HT_1-like receptors.

Three 5-HT_2 receptor subtypes are currently recognised, namely 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}, the 5-HT_{2A} subtype being functionally the most important (Table 1-1). Each has been cloned and shown to be a G-protein-linked single protein molecule of similar size and close homology. They are coupled to phospholipase C which catalyses phosphatidylinositol hydrolysis resulting in formation of diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3), a major calcium-releasing messenger. Their stimulation in vascular and other smooth muscles, therefore, results in an increase in intracellular Ca^{2+}. The operational characteristics of the three subtypes

are also very similar. The historical 5-HT_2 receptor ('classical' 5-HT_2 or Gaddum's D receptor) is now called 5-HT_{2A}. It is widely distributed in peripheral tissues and mediates the contraction of a variety of smooth muscles and the aggregation of platelets (Table 1-1). It is also found centrally and may mediate some of the behavioural effects of agents such as LSD. The cloned 5-HT_{2B} receptor (previously termed 5-HT_{2F}) is the receptor that mediates the contractile action of 5-HT in the rat isolated fundus. The 5-HT_{2C} receptor is a new appellation for the 5-HT_{1C} receptor. It has been reassigned as 5-HT_2 receptor subtype because of its close structural homology to 5-HT_2 receptor together with a shared second messenger transduction system and very similar operational characteristics. Knowledge of the functional role of 5-HT_{2C} receptors is limited by the lack of truly selective 5-HT_{2C} receptor agonists and antagonists. It would not be surprising if some of the effects attributed to 5-HT_{2A} receptor activation were indeed mediated by 5-HT_{2C} receptors or *vice versa* given the very close structural and pharmacological similarities between the 5-HT_{2A} and 5-HT_{2C} receptors.

The 5-HT_3 (Gaddum's M receptors) receptors are associated exclusively with peripheral and central neurones. They are structurally intrinsic to membrane cation channels, and do not involve any G-protein or second messengers in their transduction mechanism. They mediate the neuronal depolarising actions of 5-HT and exert a strong excitatory action. They are involved in many of the neuronal reflex effects of 5-HT in the periphery and have a variety of other possible functions (Table 1-1). The 5-HT_3 receptors in the gastrointestinal tract and brain stem are thought to be involved in the emesis reflex. Cytotoxic drugs and radiotherapy are believed to cause nausea and vomiting by stimulating the release of 5-HT from enterochromaffin cells of the intestine. The released 5-HT activates 5-HT_3 receptors on vagal afferent neurones and impulses are sent to the emesis centre in the brain stem. 5-HT_3 antagonists, such as ondansetron and granisetron, have been developed as antiemetic drugs especially for use in chemotherapy and radiotherapy

induced emesis and they exert their therapeutic effect by blocking $5\text{-}HT_3$ receptors in the peripheral vagal site and in the central emetic system. At high doses, the anti-emetic activity of metoclopramide is also mediated by $5\text{-}HT_3$ antagonism, rather than by dopamine D_2 receptor antagonism noted at low doses.

$5\text{-}HT_4$ receptors have been identified in a variety of tissues (Table 1-1), including the brain and the gastrointestinal tract. It is positively linked to adenylate cyclase. Its definitive characterisation awaits the cloning of the receptor gene. Two putative mouse and rat $5\text{-}ht_5$ receptor genes have been cloned and the recombinant receptors called $5\text{-}ht_{5A}$ and $5\text{-}ht_{5B}$. Their structural data suggest that they are quite distinct from other known mammalian 5-HT receptor types. Their function and transductional characteristics are unknown. Similarly, there are insufficient data for the full characterisation of the recently cloned $5\text{-}ht_6$ and $5\text{-}ht_7$ receptors which are linked positively to adenylate cyclase. In addition to the 5-HT receptors mentioned above, there are a number of functional receptors for 5-HT that do not fulfil the criteria for admission into any of the receptor types described and are 'orphans' of the present classification scheme. These include the 5-HT receptor mediating smooth muscle relaxation which is positively linked to adenylate cyclase (previously referred to as $5\text{-}HT_1$-like) and the 5-HT receptor on vascular endothelium mediating release of an endothelium derived relaxing factor which has been shown to be nitric oxide in some tissues [60].

1.1.6 Actions of 5-HT

Administration of 5-HT to an intact animal or isolated preparation produces a wide spectrum of responses that are complicated by the species, the physiological state, or the integrity of the preparation. The main actions of 5-HT are described below.

Cardiovascular System

Blood vessels. 5-HT can directly stimulate or relax vascular smooth muscles, act on vascular endothelial cells to release nitric oxide, and influence the release of

noradrenaline from sympathetic nerve terminals. The overall response in a vascular bed depends on the relative contribution by each of these responses.

5-HT usually produces contraction of most large blood vessels, both arteries and veins, and venules although their sensitivity varies greatly [60,62,63]. The pulmonary, splanchnic, renal and cerebral vascular beds are particularly affected. This effect is due to a direct action on vascular smooth muscles following activation of 5-HT_{2A} receptors. In some blood vessels, such as the cranial vessels, the 5-HT-induced vasoconstriction may be mediated by 5-HT_{1D} or 5-HT_1-like receptors. Low concentrations of 5-HT, with little or no direct contractile action, may also amplify the vasoconstrictor responses to other vasoactive agents such as angiotensin II and noradrenaline, an effect which is probably mediated by 5-HT_{2A} receptors.

5-HT can elicit vasodilatation via several mechanisms [60,62,64]. It has a direct relaxant effect on vascular smooth muscles in some blood vessels; it can stimulate endothelial cells to release nitric oxide; and it acts presynaptically at sympathetic nerve terminals to inhibit the release of noradrenaline in response to nerve stimulation, thus promoting a reduction in vascular tone. The 5-HT receptors at these three morphological sites have previously been thought to be, and classified as, of the '5-HT_1-like' type, but are 'orphans' of the present classification scheme of 5-HT receptors [60]. The 5-HT receptor mediating the direct vasorelaxant effect of 5-HT on vascular smooth muscle, for instance, is positively linked to adenylate cyclase, and it has been recommended that it should no longer be classified within the 5-HT_1 group of receptors which are all negatively coupled to adenylate cyclase. Similarly, the endothelial 5-HT receptor involved in the release of nitric oxide does not entirely match with any of the 5-HT receptor types in the present classification scheme although it shares some similarities with 5-HT_{1D} and the 5-HT_2 receptor class. It has been suggested that the vasorelaxant action of 5-HT may also be mediated by the release of other vasodilators such as prostacyclin. Infusion of 5-HT in low doses into the human brachial artery stimulates 5-HT_3 receptors to increase

forearm blood flow, but at high doses stimulates 5-HT_{2A} receptors to produce vasoconstriction [65,66].

In the microcirculation, the dilatation of arterioles, together with constriction of venules, produce an increase in capillary pressure and escape of fluid from the capillaries. In addition, 5-HT may have a direct effect on capillary permeability, an effect probably mediated by 5-HT_{2A} receptors [60].

Heart. 5-HT increases the rate and force of contraction of the isolated animal and human atria by stimulating 5-HT_4 receptors [60,67]. These receptors are not found in the human ventricular muscle. The cardio-stimulatory effect of 5-HT may also be partly caused by a 5-HT_3 receptor-mediated release of noradrenaline from the postganglionic cardiac sympathetic nerves or by a 5-HT_{2A} receptor-mediated release of catecholamines from the adrenal medulla. These indirect sympathomimetic effects are displayed by 5-HT at higher doses in contrast to its prejunctional inhibitory effect of noradrenaline release at sympathetic nerve terminals. The inotropic and chronotropic effects of 5-HT are of doubtful clinical significance and the effects of 5-HT on the heart *in situ* do not generally reflect a direct action on myocardial cells but are rather largely mediated indirectly through reflexes. Stimulation of 5-HT_3 receptors on the afferent vagal nerve endings in the heart, for instance, initiates a von Bezold-Jarisch-like reflex which is characterised by increased activity of the vagal efferent cholinergic neurones, leading to bradycardia and hypotension. 5-HT can also act on the carotid body chemoreceptors and the pulmonary J receptors to elicit cardiovascular reflexes, again mediated by 5-HT_3 receptors [60,67,68]. Intracoronary infusion of 5-HT dilates the normal coronary arteries but reduces coronary blood flow in patients with coronary atherosclerotic lesions presumably because the damaged endothelium is unable to synthesise or release adequate amounts of nitric oxide [69].

Blood pressure. The effect of 5-HT on blood pressure is variable and depends on the species, dose and route of administration, the initial level of blood pressure, and

the conditions under which the observations are made. A triphasic blood pressure response may be observed following intravenous 5-HT in conscious or anaesthetised animals. There is an initial brief profound fall in blood pressure caused by a decrease in heart rate and consequently cardiac output. This von Bezold-Jarisch-like reflex is initiated by stimulation of 5-HT_3 receptors on afferent cardiac vagal nerve endings leading to the activation of vagal efferent cholinergic neurones. The initial hypotensive phase is followed by a short-lived rise in blood pressure which is mainly due to direct vasoconstrictor effects mediated by 5-HT_{2A} receptors on the vasculature although, at least in the dog, adrenal release of catecholamines may also participate in this effect. The third phase is a longer-lasting hypotension attributed to vasodilatation in vessels supplying skeletal muscle. This depressor phase is not affected by selective 5-HT_2 or 5-HT_3 receptor antagonists and probably involves an action on the previously termed 5-HT_1-like receptors. A variety of blood pressure responses have been reported after intravenous administration of 5-HT in man including a triphasic blood pressure response as above, a biphasic response with a fall followed by a rise in blood pressure or *vice versa*, a pure pressor or depressor response, and no significant changes in blood pressure. 5-HT_{1A} receptor agonists, such as flesinoxan and urapidil, induce a centrally mediated decrease in blood pressure and heart rate by a combined inhibition of sympathetic tone and increase in vagal tone [60,63,67,70].

Platelets. 5-HT causes alteration in shape and reversible aggregation of human platelets by activating surface membrane 5-HT_{2A} receptors. This response is not normally followed by release of 5-HT stored in the platelets but, in the presence of low concentrations of other platelet proaggregators, 5-HT can produce maximal activation of platelets. 5-HT potentiates platelet aggregation induced by adenosine diphosphate (ADP), collagen and noradrenaline. It also enhances the secretion of α-granule constituents and liberation of arachidonate induced by collagen, and the secretion of dense granules induced by thrombin. During the platelet adhesion-

aggregation reaction at sites of vascular injury, 5-HT is released from the platelets along with ADP, thromboxane A_2 and other mediators. Acting in concert with thromboxane A_2, it can amplify the platelet aggregation reaction and speed clot formation. Since the vascular endothelial cells may be damaged at the site of injury, 5-HT can also act directly on vascular smooth muscles to cause vasoconstriction thereby aiding haemostasis [60,71,72].

Respiratory System

5-HT causes a marked contraction of the trachea in the guinea pig but not in the rat. It induces pulmonary vascular and airway constriction by stimulating 5-HT_{2A} receptors in different animal species. It does not produce pronounced broncho-constriction in asthmatic patients, and ketanserin has no effect on exercise-induced asthma suggesting that 5-HT is not an important mediator in human asthma [73]. Bronchoconstriction occurs in some patients with the carcinoid syndrome and is thought to be due to elevated levels of the amine [74] although bradykinin and substance P may also be implicated [23]. 5-HT may also cause hyperventilation as a result of stimulation of the carotid body arterial chemoreceptors or pulmonary J receptors, and 5-HT_3 receptors are involved in these responses [67,68].

Gastrointestinal Tract

5-HT causes contraction of gastrointestinal smooth muscles, increasing tone and motility thus facilitating peristalsis, and it has been proposed that it may play a role in the control of gastrointestinal motility. The 5-HT-induced contraction is partly due to a direct stimulant effect of 5-HT_{2A} receptors on the smooth muscle cells and partly due to an indirect excitatory effect of 5-HT mediated via 5-HT_3 receptors on ganglion cells located in the enteric nervous system causing the postganglionic neurones to release acetylcholine [73]. Activation of 5-HT_4 receptors also enhances the release of acetylcholine and there is evidence that 5-HT_4 receptor may also mediate intestinal secretions and the peristaltic reflex [60]. Increased pressure within the intestine may initiate the latter by releasing 5-HT from the

enterochromaffin cells. The intravenous administration of 5-HT, or its precursor, 5-HTP, produces intestinal colic and evacuation of the bowels in man [75,76], and diarrhoea is a prominent feature of the carcinoid syndrome [74]. 5-HT may also exert an inhibitory effect on the intestinal smooth muscle [73].

Genito-urinary System

The effect of 5-HT on ureteric contraction differs among species [55,77]. 5-HT was reported to cause contraction of the perfused *in situ* dog ureter with an intact blood supply but not in the isolated dog ureter. It contracted the ureter of the pig and the rabbit but had no effect on ureteric contraction in the cat. Studies also demonstrated that 5-HT has a stimulatory effect on the bladder in some animal species and man [70,73]. It produces an initial rapid contraction of the detrusor muscle of the bladder, possibly mediated by 5-HT_3 receptors, followed by a more prolonged tonic contraction, due to 5-HT_2 receptor-mediated direct stimulation of the smooth muscle of the bladder. 5-HT contracts the uterus by stimulating 5-HT_{2A} receptors and may also cause contraction of the vas deferens. The effects of 5-HT on renal haemodynamics, electrolytes and water excretion are covered later (*see* section 1.2).

Nervous System

Nerve endings. 5-HT may stimulate or inhibit neurones, depending on the site and type of receptor involved. It is a potent stimulant of nociceptive and itch nerve endings. It evokes pain when injected into the skin or applied to a blister base and is responsible for some of the symptoms caused by insect and plant stings. These responses are due to activation of 5-HT_3 receptors on sensory neurones leading to their depolarisation. Stimulation of 5-HT_3 receptors on afferent vagal nerve endings in the heart evokes the Bezold-Jarisch-like reflex. 5-HT may exert an inhibitory effect on the release of noradrenaline from adrenergic nerve terminals [60,67,68].

Central nervous system. Besides its role as a neurotransmitter, HT is thought to be implicated in a number of functions which include regulation of behaviour, mood and emotion, sleep, feeding, sensory pathways, and various autonomic and

endocrine functions (Table 1-1). These effects are mediated by different receptor types but in many instances the specific receptor involved is still unclear. Drugs that selectively inhibit reuptake of 5-HT, such as fluoxetine and paroxetine, are used as antidepressants, and buspirone, a 5-HT_{1A} receptor agonist, is effective in treating anxiety. The 5-HT_3 receptor antagonists, such as ondansetron, are also effective in animal models of anxiety [60].

Endocrine System

Anterior pituitary hormones. 5-HT stimulates prolactin secretion and appears to participate in the regulation of prolactin surges such as occur during suckling in lactating animals. The 5-hydroxytryptaminergic neurones involved seem to project from the dorsal raphé nucleus to medial basal hypothalamus. 5-HT induces the release of growth hormone and an increase in basal growth hormone secretion occurred after administration of the 5-HT precursors, tryptophan and 5-HTP, in man although this was not consistently observed in all studies. The data regarding the effects of 5-HT on thyroid stimulating hormone (TSH) release are conflicting. Most reports suggest an inhibitory role of 5-HT in the regulation of TSH but there may also be an opposite stimulatory system at a different site in the brain. Similarly, 5-HT has been reported to both stimulate and inhibit ACTH secretion but the majority of studies with 5-HT agonists and antagonists or 5-HTP favour a stimulatory action of 5-HT on ACTH secretion [78].

Vasopressin. 5-HT may stimulate the release of arginine vasopressin (AVP) since 5-HT precursors, 5-HT reuptake inhibitors such as fluoxetine [79], 5-HT releasing agents like *p*-chloroamphetamine [80,81], and 5-HT receptor agonists such as TFMPP, quipazine, MK 212, mCPP [80-84] all increase AVP concentrations in the rat. It is debatable whether this response is due to activation of 5-HT_{2A} or 5-HT_{2C} receptors since compounds claimed to be selective for 5-HT_{2A} receptor also show some affinity for 5-HT_{2C} receptor and *vice versa*. The available evidence suggests that AVP release is more likely to be mediated by 5-HT_{2C} than 5-HT_{2A} receptors

since MK 212, TFMPP, mCPP have a higher affinity for 5-HT_{2C} than for 5-HT_{2A} receptors [60,85], and the antagonist LY 53857 which blocks the AVP releasing effects of MK 212, quipazine and mCPP also has a higher affinity for 5-HT_{2C} than 5-HT_{2A} sites [81,83,84]. In addition, the 5-HT_2 agonist DOI that has a higher affinity for 5-HT_{2A} binding sites than MK 212, quipazine, TFMPP or mCPP, did not increase plasma AVP concentrations, and ketanserin, a 5-HT_2 receptor antagonist with selectivity for 5-HT_{2A} over 5-HT_{2C} receptor, did not antagonise the vasopressin response to mCPP [84]. The location of the neuroanatomical site(s) of the target 5-HT receptors is not known but anatomical lesions of 5-hydroxytryptaminergic pathways between the hindbrain and the hypothalamus prevented AVP release induced by 5-HT agonist or releasing agents in rats [81].

Renin. 5-HT has no effect on release of renin from the rat kidney *in vitro* [86]. Intramuscular or subcutaneous 5-HT increased plasma renin activity (PRA) *in vivo* in the rat [87,88]. An increase in renin secretion was also observed after infusion of 5-HT into the renal artery of the denervated kidney of the anaesthetised dog [89]. This response was suppressed by methysergide and ketanserin suggesting involvement of 5-HT_2 receptor. In contrast, an earlier study reported no increase in renin release after infusion of 5-HT into the renal artery of anaesthetised dog whose renal nerves were left intact [90] and no rise in PRA was observed after intravenous infusion of 5-HT in man [91]. Zimmermann and Ganong [92] showed that the 5-HT precursors, L-tryptophan and 5-HTP, which penetrate the blood-brain barrier to increase brain 5-HT, increased PRA without a concomitant increase in sympathetic output in the dog whose renal perfusion pressure was kept constant. This response was inhibited by the 5-HT antagonist metergoline. The rise in PRA was reduced or abolished when both peripheral and central LAAD activities were inhibited by high dose benserazide, but was not blocked, and was even possibly enhanced, when only peripheral LAAD activities were inhibited by carbidopa. Renal denervation blocked the increase in PRA produced by L-tryptophan. Their

observations suggest that the 5-HT precursors act on the central nervous system to produce an increase in renin secretion via the renal nerves. L-5-HTP also increased PRA in the rat [88,93,94], but unlike the situation in the dog, peripheral LAAD inhibition blocked the increase induced by L-5-HTP suggesting that L-5-HTP must be converted to 5-HT peripherally to increase PRA [88]. It is not known if changes in renal haemodynamics may have contributed to these results. Tryptophan also increased PRA in humans [95] but no increase was observed after administration of L-5-HTP [96,97]. The 5-HT_2 receptor agonists, quipazine, MK 212 and DOI, were shown to increase PRA in the rat [80,84,98,99]. These agents probably act on either 5-HT_{2A} or 5-HT_{2C} receptors within the central nervous system to increase renin secretion from the kidney [60,85]. Others have, however, suggested that some of the 5-HT agonists like quipazine increased renin release as a result of alterations in renal haemodynamics since quipazine reduced renal blood flow and its intracerebroventricular administration did not increase PRA in the rat [83].

Aldosterone. 5-HT is a potent stimulator of aldosterone secretion from the human and rat adrenal zona glomerulosa cells *in vitro* and this release is inhibited by methysergide and ketanserin indicating involvement of the 5-HT_2 class of receptors, possibly the 5-HT_{2A} receptor [96,100-102]. More recent *in vitro* experiments with frog and human adrenocortical cells, however, suggest that the stimulatory effect of 5-HT on aldosterone production is mediated by activation of 5-HT_4 receptor [103,104]. L-5-HTP has also been reported to increase aldosterone secretion *in vitro* [101] but this response was not observed by others [96]. Intravenous infusion of 5-HT increases plasma aldosterone without a concomitant increase in PRA or cortisol in man [91]. A similar response is observed after administration of zacopride, a 5-HT_4 receptor agonist, in subjects pretreated with dexamethasone suggesting involvement of 5-HT_4 receptor [104]. A rise in aldosterone secretion also occurs after administration of the 5-HT precursors, tryptophan [95] and L-5-HTP [96,97,105]. In subjects pretreated with dexamethasone, it was shown that the

aldosterone release induced by L-5-HTP is not mediated by changes in ACTH, PRA, potassium, or sodium concentrations [96,97]. This response is enhanced by peripheral LAAD inhibition and is thought to be mediated within the central nervous system but the mechanism or site of stimulation is unknown [96]. Ketanserin and methysergide are without effect on L-5-HTP-induced aldosterone secretion *in vivo* [105].

Other hormones. 5-HT may affect the secretion of other endocrine hormones including insulin, glucagon and progesterone. It may also modify exocrine secretory activity such as inhibition of gastric acid secretion induced by histamine or pentagastrin, and stimulation of pepsin, intestinal fluid or mucus secretion [106].

1.1.7 5-HT and Diseases

Carcinoid Syndrome

Carcinoid syndrome is a clinical state associated with malignant tumours of the enterochromaffin cells, arising most commonly in the small intestine. It typically occurs after hepatic metastasis is present, in part because of the liver's capacity to inactivate 5-HT or other substances released into the portal circulation. The rare carcinoid tumours that arise from extraportal sites, such as ovaries and thyroid gland, release their secretory products directly into the systemic circulation and can cause the carcinoid syndrome before metastasising. The clinical features include cutaneous flushing, facial telangiectasia, diarrhoea, abdominal pain, bronchospasm, fibrotic valvular lesions of the heart and peripheral oedema. The carcinoid tumours may synthesise and secrete, usually sporadically, large amounts of 5-HT. With massive tumours, 50% or more of dietary tryptophan may be diverted to 5-HT production in the tumour and niacin synthesis may be inadequate resulting in pellagra [23,74,107,108].

The whole blood and platelet concentrations of 5-HT are frequently raised in carcinoid syndrome. 5-HT levels in platelet-poor plasma have also been reported to

be elevated but these values may be unreliable since the platelet-poor fraction may contain mainly 5-HT from platelets disintegrated during centrifugation or from platelets aggregating during withdrawal of the sample [109-111]. The daily urinary excretion of 5-HIAA is raised to as much as 350 mg thus providing a reliable diagnostic test for the syndrome. Urinary 5-HT excretion is usually less than 1% of the urinary 5-HIAA level. Other metabolites that may be found in the urine in increased amounts include *N*-acetyl-5-HT, 5-HT-*O*-glucuronide, 5-HIAA-*O*-sulphate, indoleacetic acid, and 5-HTP [107,111].

Carcinoid syndrome has been regarded as a naturally occurring model of 5-HT excess and can provide useful information regarding the pharmacological responses of the human subject to large amounts of 5-HT. However, a variety of other biologically active substances, such as substance P, histamine, bradykinin and prostaglandins, are also secreted by the tumours, and the changes seen in carcinoid patients must therefore be interpreted with caution. 5-HT contributes to the development of diarrhoea, bronchoconstriction, oedema and fibrosis. Vasodilatation and flushing, on the other hand, may be primarily due to release of substance P and the formation of kinins [74]. The 5-HT_2 receptor antagonists methysergide, cyproheptadine, and ketanserin are effective in controlling diarrhoea and other gastrointestinal symptoms. They do not improve flushing although there is evidence from a few studies that ketanserin may be of benefit in some patients. The selective 5-HT_3 receptor antagonist, ondansetron, was reported to produce a dramatic improvement in diarrhoea, flushing, vomiting and appetite in a patient with the carcinoid syndrome [112]. A number of other treatments have been employed in patients with inoperable carcinoid tumours including *p*-chlorophenylalanine, an inhibitor of tryptophan 5-hydroxylase, to reduce 5-HT synthesis, and the use of somatostatin or one of its longer acting analogues which suppresses hormone secretion from various neuroendocrine, including carcinoid, cells [23,74,107,108].

Migraine

The cause of migraine is not well understood but a number of observations suggest that 5-HT is implicated in its pathogenesis. There is an increase in the urinary excretion of 5-HIAA following a migrainous attack. The blood and platelet concentrations of 5-HT fall during the attack as a result of depletion of platelet 5-HT. The 5-HT released from platelets and possibly other tissues may be responsible for triggering the vascular events, production of inflammatory mediators, and stimulation of nociceptive afferents that occur in migraine. Further evidence for a role of 5-HT in migraine is provided by the response to agents which interact with 5-HT. Agents that release 5-HT like reserpine or inhibit its uptake such as zimelidine may trigger migraine-like headaches. The 5-HT_2 receptor antagonists, such as methysergide, pizotifen and cyproheptadine, have been used for the prophylaxis of migraine. Their precise mechanism of action is unknown but it has been suggested that 5-$HT_{2A/2C}$ receptor activation is an initiating event in migraine, and their antagonism therefore prevents the onset of migraine. The β-adrenoceptor antagonist, propranolol, used in migraine prophylaxis also demonstrates 5-HT_1 and 5-HT_2 antagonist activities. Ergotamine and sumatriptan, which are used in the treatment of acute migraine attacks, have 5-HT_{1D} or 5-HT_1-like receptor agonist activity and the effectiveness of these drugs may be related to their vasoconstrictor effects on the cephalic blood vessels which become dilated during a migraine attack [113,114].

Hypertension

Several observations suggest that 5-HT, besides many other factors, may play a role in the development and/or maintenance of hypertension. The sensitivity of various vascular preparations to the vasoconstrictor effect of 5-HT is enhanced in animal models of hypertension compared with normotensive animals, and the contractile response to 5-HT in the mesenteric or renal vasculature is greater than those due to other vasoconstrictors such as noradrenaline or angiotensin II. In addition, there is a

delay in the development of tachyphylaxis to 5-HT in hypertensive compared with normotensive animals [115-117]. A reduction in platelet uptake of 5-HT, an increase in the aggregability of platelets and a reduced pulmonary clearance of 5-HT have been reported in human and animal hypertension [114,117,118]. These may contribute to increased availability of free 5-HT at the level of the resistance vessels where it may cause vasoconstriction by a direct action on the vascular smooth muscles or by potentiating the actions of other vasoactive agonists. In addition, impaired endothelial function, with decreased release of nitric oxide, may occur in hypertension and this may enhance the vasoconstrictor response to 5-HT [119]. The renal actions of 5-HT may also contribute to the pathogenesis of hypertension. 5-HT has been reported to decrease renal blood flow, glomerular filtration rate, sodium and water excretion (*see* section 1.2), and a sustained reduction in renal excretory capacity could contribute to the development and maintenance of hypertension [120]. In addition to the enhanced renal vascular response to 5-HT mentioned earlier, there is evidence that the urinary excretion of 5-HT in hypertensive subjects is higher than in normotensives and this may reflect overproduction of renal 5-HT [121]. This contention is supported by the finding that the renal content of 5-HT is higher in hypertensive than normotensive rats [122]. The observation that ketanserin, a 5-HT_2 receptor antagonist, is a hypotensive agent in several species including hypertensive humans has been taken as further proof that 5-HT receptors may participate in hypertension [117]. Central 5-HT_{1A} receptors may also be involved in the regulation of blood pressure since 5-HT_{1A} receptor agonists, such as 8-OH-DPAT, urapidil and flesinoxan, reduce blood pressure and heart rate in hypertensive animals and man. These effects appear to be due to a reduction in sympathetic outflow and an increase in cardiac vagal activity following activation of central 5-HT_{1A} receptors [67,123,124].

However, a number of observations equally argue against a role of 5-HT in hypertension. Experimental hypertension is associated with increased reactivity to a

number of vasoactive agents and not just to 5-HT. There is a marked variability in the responsiveness to 5-HT between different vascular preparations and some studies showed no selective increase in sensitivity to 5-HT in the isolated renal arteries or hindquarter vascular bed compared with normotensive control rats [124-126]. Consistent changes in circulating 5-HT levels have not been demonstrated in hypertension. While some studies have reported a reduction in platelet uptake and content of 5-HT [127-129], others have reported that platelet 5-HT levels are normal [118,127,130]. Some studies have demonstrated an increase in the circulating level of free 5-HT [118] but this is still rather low and may be subthreshold in producing vasoconstriction. In any case, it is unclear whether elevated plasma levels of 5-HT would cause hypertension, and it is worth noting that carcinoid syndrome is only rarely associated with hypertension [23,131]. Not all studies are in agreement regarding the effects of 5-HT on renal haemodynamics, sodium and water excretion (*see* section 1.2). It is also now accepted that the antihypertensive activity of ketanserin cannot be explained solely by its 5-HT_2 receptor blockade [67,124]. It possesses other properties including α_1-adrenoceptor blocking activity which may contribute to its hypotensive action, and other 5-HT_2 receptor antagonists, like ritanserin and LY 53587, which lack α_1-adrenoceptor antagonist activity do not lower blood pressure. It is also unclear whether derangement of central 5-HT function play a role in hypertension. Intracisternal administration of 5,6-dihydroxytryptamine, a depletor of central 5-HT, prevented or reversed the neurogenic hypertension induced by sino-aortic baroreceptor denervation in the rabbit, but depletion of central 5-HT failed to alter blood pressure in other experimental models of hypertension [132].

Other Pathological Processes

5-HT has been implicated as a possible mediator in a variety of other conditions besides those mentioned above, including depression, Raynaud's phenomenon, pulmonary hypertension, peripheral vascular disease, coronary spasm, pre-

eclampsia and eclampsia [114]. It is involved in the serotonin syndrome which consists of behavioural, neuromuscular and autonomic changes, typically resulting from combining 5-HT agents with MAO inhibitors, and is believed to arise from hyperstimulation of brainstem and spinal cord 5-HT_{1A} receptors [133]. The potential role of 5-HT in the pathophysiology of some renal conditions is discussed in section 1.2.7.

1.1.8 Drugs Affecting 5-HT Function

A summary listing of the major classes of drugs that influence 5-HT systems is shown in Table 1-3 [134]. A number of these drugs have been or are currently used as therapeutic agents. 5-HT *per se* has no clinical application as a drug but its precursors, L-tryptophan and L-5-HTP, have been used as antidepressants. *Para*-chlorophenylalanine (Fenclonine) is a potent inhibitor of tryptophan 5-hydroxylase and has been used to reduce 5-HT synthesis in patients with inoperable carcinoid tumours. Buspirone, a partial agonist at 5-HT_{1A} receptors, is licensed as a non-benzodiazepine anxiolytic. Sumatriptan, a 5-HT_{1D}/5-HT_1-like agonist, is effective in the treatment of acute migraine, and other 5-HT antagonists, such as methysergide and pizotifen, are used in its prophylaxis. The 5-HT_3 receptor antagonists, ondansetron and granisetron, are anti-emetic drugs used particularly for the treatment or prevention of nausea and vomiting associated with cancer chemotherapy or radiotherapy. Several compounds release 5-HT selectively from nerve terminals. The appetite suppressants fenfluramine and dexfenfluramine are such substances, but it is not clear whether this action is involved in their effect on appetite. Fenfluramine also reduces reuptake by blocking 5-HT transfer [135]. The selective serotonin reuptake inhibitors (SSRI), such as fluoxetine and paroxetine, specifically inhibit the reuptake of 5-HT by neurones and are effective antidepressants. Tricyclic antidepressants also impair neuronal reuptake of 5-HT. Some of the ‘recreational’ drugs interact with 5-HT. Lysergic acid diethylamide

(LSD), which among many actions is a partial agonist at brain 5-HT_2 receptors, is a hallucinogen [136]. Methylenedioxymethamphetamine (MDMA, "ecstasy"), releases 5-HT from nerve terminals. In large doses, it produces long-term 5-HT depletion and is possibly damaging to 5-HT neurones.

Table 1-3. Drugs affecting 5-HT function.

Site/locus of action	*Mechanism of action*	*Drug*
Synthesis	Precursors of 5-HT	L-tryptophan, L-5-HTP
	Inhibition of tryptophan 5-hydroxylase	*p*-Chlorophenylalanine, DL-6-Flurotryptophan, *p*-Chloroamphetamine
	Inhibition of LAAD	Carbidopa, Benserazide, α-Methyl-dopa
Storage	Inhibition of storage, release and depletion	Reserpine, Tetrabenazine
	Release of 5-HT	Fenfluramine, Dexfenfluramine, MDMA, *p*-Chloroamphetamine
Receptor interaction	Agonist	*See* Table 1-2
	Antagonist	*See* Table 1-2
Reuptake mechanism	Specific 5-HT reuptake inhibition	Fluvoxamine, Fluoxetine, Paroxetine, Sertraline
	Non-specific 5-HT reuptake inhibition	Amitriptyline, Imipramine, Chlorimipramine, Fenfluramine, Dexfenfluramine
Metabolism	Non-selective irreversible MAO inhibition	Iproniazid, Isocarboxazid, Phenelzine, Pargyline, Tranylcypromine
	Irreversible MAO-A inhibition	Clorgyline
	Reversible MAO-A inhibition	Meclobemide
	Irreversible MAO-B inhibition	Selegiline
	Reversible MAO-B inhibition	Lazabemide
Neurotoxin	Specific damage to 5-HT neurones	5,6-Dihydroxytryptamine, 5,7-Dihydroxytryptamine

1.2 5-HYDROXYTRYPTAMINE AND THE KIDNEY

Exogenous 5-HT may affect renal haemodynamics and function. These responses may, however, be heterogeneous and differ not only between species but also within animals of the same species depending on the experimental conditions. In addition, there is evidence that 5-HT may be produced locally within the kidney. The physiological role of 5-HT in the kidney and its pathological importance in renal disorders remain unclear.

1.2.1 Renal Metabolism of 5-HT

The pathways for 5-HT synthesis from L-tryptophan was outlined in Figure 1-2. L-Tryptophan is hydroxylated by tryptophan 5-hydroxylase to form L-5-HTP. This is followed by decarboxylation of L-5-HTP by aromatic LAAD to produce 5-HT. The latter is primarily inactivated by MAO-A to 5-hydroxyindoleacetaldehyde which is rapidly converted by aldehyde dehydrogenase to 5-HIAA, the major catabolic and excretory product of 5-HT metabolism [31].

Both the enzymes required for the synthesis of 5-HT from L-tryptophan are present in the mammalian kidney. Cooper and Melcer [137] first demonstrated the presence of tryptophan 5-hydroxylase activity in the rat and guinea pig kidney, and this was found to be 25% of the activity in the small intestine. The presence of tryptophan 5-hydroxylase activity in kidney homogenates of the rat has been confirmed by others, and the renal tryptophan 5-hydroxylase activity was reported to be equivalent to, or greater than, that found in the brain stem [35,138]. The tryptophan 5-hydroxylase activity in the rat kidney appeared to be localised in the cortex, specifically in a subfraction highly enriched in proximal tubules. There was practically no activity in the glomeruli and medulla. Surgical denervation or treatment with the neurotoxin 5,7-dihydroxytryptamine failed to alter enzymatic activity suggesting that renal tryptophan 5-hydroxylase is not localised in

5-hydroxytryptaminergic nerve terminals. *P*-Chlorophenylalanine, an inhibitor of tryptophan 5-hydroxylase, produced a marked and proportionate fall in both renal cortical tryptophan 5-hydroxylase activity and urinary 5-HT excretion in the absence of significant changes in blood 5-HT levels in the rat suggesting that L-tryptophan is the precursor for renal 5-HT. Sole *et al.* [35] also found significant tryptophan 5-hydroxylase activity in the three human cadaveric kidneys that they examined. The enzyme was more uniformly distributed across the kidney in man than in the rat, being present in both the cortex and medulla. No measurable tryptophan 5-hydroxylase activity was present in the small intrarenal arteries.

LAAD is present in high concentrations in the mammalian kidney [31]. The relative tissue activities of this enzyme were found to be liver 100, kidney 31, lung 15, heart 7, and brain 5 in the human adult [139]. Its ability to decarboxylate L-5-HTP was first described in dog and guinea pig renal tissue homogenates [16,140]. It is located primarily within the proximal tubular cells of the renal cortex [35,141-143] and its activity is higher in the proximal convoluted tubules than in the proximal straight tubules [142,143]. LAAD is also present in the glomeruli, renal medulla and within autonomic nerve cells. Its activity in the glomeruli is 16-fold lower than in the proximal tubules in the rat [35]. Highly purified glomeruli without arterioles or tubules obtained from rat kidney homogenates did not, however, produce dopamine from L-dopa suggesting the absence of significant LAAD activity in the glomeruli [141]. This contradicts an earlier report of synthesis of dopamine from L-dopa by isolated rat glomeruli [144].

The degrading enzyme MAO is also present abundantly in the mammalian kidney [31,49,145,146]. Histochemically, it was initially found to be localised almost exclusively in the renal cortex, particularly in the proximal tubules, with little activity in the medulla. No enzyme activity was demonstrable within the glomeruli of the guinea pig and cat kidney [145,146]. Later studies identified MAO in both the cortex and medulla of the cat and rat kidney [147,148]. Both isoforms

of MAO are present in the human kidney. Fernandes and Soares-da-Silva [148] found that MAO-A activity was similar in the cortex and medulla whereas MAO-B activity was higher in the cortex than in the medulla. They observed a similar pattern of distribution of MAO-A and MAO-B in the rat kidney. MAO-A, however, appeared to be the predominant form of MAO in the rat renal cortex in contrast to the human renal cortex which contained approximately equal amounts of the two forms of MAO. Lewinsohn *et al.* [49], on the other hand, showed that MAO-A was also the predominant form of the enzyme in the renal cortex of the human adult kidney whereas MAO-B activity was slightly higher than MAO-A activity in the foetal and neonate kidney. In contrast with that described for the human and rat kidney, MAO-B was found to be the predominant form of MAO in the renal cortex of the dog [149] and cat [147]. Selective inhibition of MAO-A by Ro 41-1049 in the rat produced a three-fold increase in urinary excretion of 5-HT and a 14-25% reduction in urinary 5-HIAA. It increased urinary dopamine by about 23% and reduced urinary DOPAC (3,4-dihydroxyphenylacetic acid; dopacetic acid) by 37-54%. Administration of the MAO-B inhibitor Ro 19-6327 did not change urinary excretion of 5-HT, 5-HIAA, dopamine and DOPAC. Urinary sodium or potassium excretion was unaffected by either inhibitor [150].

In addition to oxidative catabolism, the kidney has the capacity to metabolise and conjugate 5-HT to form sulphate and glucuronide metabolites [31,55]. 5-HT appears in the urine along with 5-HIAA and other metabolites. The daily urinary excretion of free 5-HT in normal human subjects was found to range from 31 to 296 μg with a mean value of 131 μg in one study [151].

1.2.2 Effect of Administration of 5-HT or its Precursors on Blood and Urinary Levels of 5-HT and its Metabolites

5-HT. The subcutaneous or intraperitoneal injection of 5-HT increased serum, plasma and platelet 5-HT levels in the rat [152]. 5-HT (1 mg kg^{-1} i.p.) produced an

eight-fold increase in urinary 5-HT excretion and a six-fold increase in 5-HIAA excretion in the rat [153]. About 0.6% of 5-HT administered intraperitoneally was excreted unchanged and 25-35% of the injected 5-HT was recovered as free and conjugated 5-HIAA in the rat [111]. Following intravenous 5-HT (1-10 mg kg^{-1}), 0.6-1.5% of the injected 5-HT was excreted as 5-HT and about 25% as 5-HIAA. The excretion of 5-HIAA was higher after subcutaneous than intravenous administration of 5-HT [154]. The urinary recovery of subcutaneously injected 5-HT as 5-HIAA decreased from 46% to 27% with increasing dose of 5-HT (1-15 mg kg^{-1}); this may be due to 5-HT-induced dose-dependent reductions in glomerular filtration [152]. Others reported that the urinary recovery of unchanged 5-HT was negligible after subcutaneous injection of 5-HT [155]. Intravenous administration of 5-HT increased whole blood and plasma 5-HT concentrations [156] and the urinary excretion of 5-HIAA in the dog but had little, if any, effect on the levels of free 5-HT in the urine [157]. About 25-40% of 5-HT injected intravenously was excreted as 5-HIAA [157,158]. In addition to increasing 5-HIAA excretion, 5-HT has been reported to enhance the excretion of glucuronide and sulphate conjugated metabolites of 5-HT, 5-HIAA and 5-HTOL in varying amounts depending on the animal species [111,159,160]. In rabbits and rats, 5-HT was excreted mainly as 5-HIAA and the glucuronides of 5-HT, 5-HTOL and 5-HIAA whereas in cats the corresponding *O*-sulphates were the major metabolites.

In man, the intravenous infusion of 5-HT (2.2-8.7 μg kg^{-1} min^{-1}) produced only a small increase in 5-HT level in platelet-rich plasma, approximating 2% of the infused amount [161]. The oral or intravenous administration of 5-HT produced no significant increase in urinary excretion of free 5-HT but a marked increase in the excretion of 5-HIAA and 5-HT conjugates [151,161,162]. The percentage of the 5-HT dose recovered in urine as 5-HIAA ranged between 65-92% after intravenous 5-HT [161,162] and 52-70% after oral 5-HT [151]. In the carcinoid syndrome, platelet 5-HT levels are usually elevated [110,111] but metabolites of 5-HT such as

5-HIAA generally increase many more times in the urine than does 5-HT itself [151]. Endogenous or exogenous 5-HT presented to the bloodstream may be either rapidly sequestered in platelets or efficiently metabolised in either the hepatic or pulmonary circulation. Very little free 5-HT may, therefore, be available for filtration at the renal glomerulus or secretion by the renal tubules and this may explain the failure to produce a substantial increase of 5-HT in the urine.

Tryptophan. The oral feeding of large amounts of L-tryptophan (14.7 or 24.7 mmol) in dogs produced a slight but variable increase in urinary 5-HIAA suggesting that hydroxylation of L-tryptophan may proceed at a maximal rate even at normal intakes of the amino acid [157]. Increased plasma levels of 5-HT and 5-HIAA levels were, however, observed after intraperitoneal injection of L-tryptophan (10-100 mg kg^{-1}) [163]. Oral administration of more than 50 mg kg^{-1} of L-tryptophan in rats produced an increase in urinary excretion of 5-HIAA and this was greater than after its subcutaneous or intraperitoneal administration [164]. The main site of hydroxylation of L-tryptophan is believed to be in the gastrointestinal mucosa [165] and this probably explains the greater increase in urinary 5-HIAA obtained after oral L-tryptophan. In contrast, D-tryptophan produced no increase in urinary 5-HIAA excretion [164]. Intravenous infusion of L-tryptophan (75 μg min^{-1}) produced no changes in plasma or urinary levels of 5-HTP or 5-HT in the rat [166].

Yuwiler and his colleagues [167] reported an increase in whole blood 5-HT after single oral doses of 50 and 100 mg kg^{-1} of L-tryptophan in normal man. They also observed an increase in 5-HT measured in platelet-poor plasma after 100 mg kg^{-1} load of L-tryptophan. Benedict and his co-workers [168] administered placebo or 1 g of L-tryptophan with the three main meals for 3 days. The mean total plasma tryptophan level 60-90 min after the last dose of L-tryptophan was 130 μmol l^{-1} compared to a value of 51 μmol l^{-1} after placebo but there was no increase in platelet 5-HT concentration. Similarly, Sole and his colleagues [35] found no changes in the concentrations of 5-HT in blood or platelet-poor plasma after 3 g of

L-tryptophan. There was, however, a five-fold increase in the urinary excretion of 5-HT over the 6 h period following administration of L-tryptophan which is indicative of renal synthesis of 5-HT. A rise in urinary excretion of 5-HT was reported after a single oral 2 g load of L-tryptophan [121,169] and an increase in 5-HIAA excretion after oral administration of 5 g of L-tryptophan [170]. Other investigators, however, reported that acute (25, 50 or 100 mg kg^{-1}) or chronic (50 mg kg^{-1} twice daily for 7 days) oral intake of L-tryptophan produced only small or negligible changes in urinary L-tryptophan and 5-HIAA excretion but increased urinary kynurenine and indoleacetic acid excretion [171-174].

5-HTP. Administration of DL-5-HTP increased plasma and whole blood 5-HT concentrations and the urinary excretion of 5-HTP, 5-HT and 5-HIAA in the dog [157,175]. Following its intravenous administration, 20% of the given dose was excreted as 5-HT and 30% as 5-HIAA, calculated on the basis that only the L-isomer was metabolised [157]. Large amounts of 5-HTP was detected in urine which was thought to be mostly due to unmetabolised D-isomer. Parenteral administration of DL-5-HTP in the rat increased blood concentrations and the renal content of 5-HT [153,155,162]. It also increased the urinary excretion of 5-HTP, 5-HT, 5-HIAA, and the glucuronide and sulphate conjugates of 5-HT and 5-HIAA [111,176]. Intravenous infusion of L-5-HTP produced marked increases in plasma levels of 5-HTP and urinary excretion of 5-HT and 5-HTP without significant increases in plasma 5-HT [166]. The elevation in urinary level of 5-HT after intraperitoneal DL-5-HTP was more marked than that after administration of 5-HT itself [155,176]. Similarly, Airaksinen and Uuspää [153] observed that intraperitoneal administration of 0.05-50 mg kg^{-1} of DL-5-HTP in rats produced a 10- to 5000-fold increase in urinary excretion of 5-HT representing 38.6% of the infused dose of L-5-HTP, whereas 5-HT (1 mg kg^{-1} i.p.) increased urinary 5-HT eight-fold and this represented only 0.6% of the given dose of 5-HT.

Davidson *et al.* [162] first reported that intravenous infusion of 50 mg of DL-5-

HTP over 1 h in two human subjects resulted in a marked increase in urinary excretion of 5-HT and 5-HIAA for 8 h. The percentages of the administered dose of 5-HTP represented by urinary 5-HT in the two subjects were 4 and 7% respectively. There was no detectable increase in urinary 5-HT following administration of 10 mg of 5-HT base itself. The average percentages of intravenously infused DL-5-HTP, 5-HT and 5-HIAA excreted as 5-HIAA over a 10 h period were 28%, 77% and 55% respectively. Brengelmann *et al.* [177] also reported the presence of large amounts of 5-HT and 5-HIAA in urine after intravenous injection of 25 mg of DL-5-HTP in healthy volunteers. In addition, they observed a significant increase of 5-HTP in urine which they presumed was, to a large extent, due to the unchanged D-isomer. Oates and Sjoerdsma [178] administered L- or DL-5-HTP intravenously over 90 to 150 min in 2 subjects with hypertension. About 70% of the dose of the L-isomer was recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA. The relative ratio of these three metabolites were approximately 0.15: 0.25: 0.60 respectively.

1.2.3 Evidence of 5-HT Synthesis in the Kidney

The origin of the 5-HT in normal urine is not entirely clear. A first possibility is that urinary 5-HT originates from plasma 5-HT either by glomerular filtration or tubular secretion. The second possibility is that at least part of the 5-HT in urine is contributed directly by kidney metabolism. Rodnight [169] reported that the 24 h urinary excretion of 5-HT in 12 normal human subjects ranged from 60 to 150 μg. In view of the very low level of free 5-HT in plasma, he suggested that either the renal clearance of 5-HT is high or that platelet-bound 5-HT is released into plasma at or near the sites of urine formation. He also raised the possibility that the 5-HT in the urine may in part be due to the renal synthesis of 5-HT.

The presence of tryptophan 5-hydroxylase and aromatic LAAD within the kidney suggests that the kidney may indeed be a site of 5-HT production. There is little doubt from both *in vitro* and *in vivo* studies that renal LAAD is able to

decarboxylate L-5-HTP to 5-HT. Conversion of L-5-HTP to 5-HT occurs readily in renal tissue homogenates from different mammalian species [16,39,42,140]. Histofluorescence studies using renal cortex slices showed that the synthesis of 5-HT from L-5-HTP occurred in the proximal convoluted tubules [142]. Incubation of isolated proximal convoluted tubules with increasing concentrations of L-5-HTP results in a dose-dependent formation of 5-HT [179,180]. When the rat kidneys were perfused *in situ*, addition of DL-5-HTP to the Locke's solution was followed by abundant appearance of 5-HT in the filtrate collected from the bladder and also in the perfusate from the inferior vena cava [153]. Similarly, Stier *et al.* [181] demonstrated that infusion of L-5-HTP into the renal artery of the isolated rat kidney resulted in the appearance of 5-HT in the urinary and venous effluents. The kidneys were perfused with Krebs-Henseleit solution to ensure that any direct intrarenal formation of 5-HT from L-5-HTP was independent of other tissues or blood elements. Infusion of the D-isomer, on the other hand, produced only a minimal increase in 5-HT output relative to that obtained after L-5-HTP consistent with stereospecific formation of 5-HT from its amino acid precursor L-5-HTP.

There is also indirect evidence from *in vivo* animal studies that the kidney is capable of 5-HT synthesis from L-5-HTP. A consistent observation in experimental animals is that the urinary excretion of 5-HT increases following parenteral administration of L-5-HTP or DL-5-HTP (*see* section 1.2.2). The rise in urinary 5-HT excretion was found to be much higher than after administration of 5-HT itself in the rat [153,155,176]. Although uptake of 5-HT by platelets or its extrarenal metabolism may partly account for the lower urinary 5-HT levels observed after administration of 5-HT [176], further evidence indicates that intrarenal formation of 5-HT from L-5-HTP may be largely responsible for the higher urinary levels of 5-HT that occurred after administration of 5-HTP. The renal content of 5-HT increased ten-fold within an hour of DL-5-HTP administration [153]. In addition, blood 5-HT concentration in the renal vein was

elevated and higher than that in the abdominal aorta. The blood 5-HT level was also higher in the renal vein than in the abdominal aorta even before DL-5-HTP injection. These data suggest that the kidney is able to convert 5-HTP to 5-HT which it transfers to the blood and urine, and that intrarenal synthesis of 5-HT also appears to take place physiologically [153]. Further support for *in vivo* formation of 5-HT from L-5-HTP by the rat kidney is provided by the work of Stier and Itskovitz [166]. They observed that urinary excretion of 5-HT increased markedly without concomitant increases in plasma 5-HT after infusion of L-5-HTP and that more than 20% of the administered L-5-HTP was recovered in the urine as 5-HT. In contrast, there were large increases in plasma levels of 5-HTP with only relatively small increases in urinary 5-HTP. Both *in vivo* and *in vitro* animal studies, therefore, show that the kidney can synthesise 5-HT from L-5-HTP.

Urinary excretion of 5-HT also increased markedly following intravenous administration of DL- or L-5-HTP in man whereas little increase in urinary 5-HT excretion occurred after administration of 5-HT [162; *see* section 1.2.2). These observations would be consistent with the proposition that 5-HT is also produced in the human kidney and that locally synthesised 5-HT is less accessible to the catabolising enzymes than infused 5-HT. However, plasma 5-HT was not measured in any of these studies and the contribution of extrarenal formation of 5-HT to the elevated urinary 5-HT excretion cannot therefore be assessed.

The evidence for intrarenal formation of 5-HT from its original precursor L-tryptophan is not so clear-cut. Homogenates prepared from kidneys of dogs and guinea pigs yielded 5-HT when incubated aerobically with DL-5-HTP but not with L-tryptophan [140]. Sole and his colleagues [35] demonstrated the accumulation of significant quantities of 5-HT in both the perfusate and urine of isolated rat kidneys perfused with a solution containing L-tryptophan, but free of 5-HTP, 5-HT and other amines, thus demonstrating the capacity of the whole kidney to synthesise 5-HT from L-tryptophan. On the other hand, Stier & Itskovitz [166] failed to

demonstrate increases in blood and urinary levels of 5-HTP or 5-HT during intravenous infusion of L-tryptophan in the rat *in vivo*. This contrasted with increases in urinary 5-HT excretion following infusion of L-5-HTP under the same experimental conditions. Their observations, which are at variance with data obtained in the isolated perfused kidneys, suggest that, for some reason, L-tryptophan transported by the kidney was not available to renal tryptophan 5-hydroxylase and that renal conversion of L-tryptophan to L-5-HTP and 5-HT was not very efficient *in vivo.* L-tryptophan loading increased urinary excretion of 5-HT *in vivo* in man (*see* section 1.2.2). This occurred without increases in 5-HT levels measured in blood or platelet-poor plasma [35] but other studies demonstrated an increase in 5-HT levels in whole blood and platelet-poor plasma after administration of L-tryptophan [167] making it difficult to assess the relative contribution of extra- and intrarenal synthesis of 5-HT to the increased urinary 5-HT excretion. Studies have, therefore, yielded inconsistent results regarding the renal formation of 5-HT from L-tryptophan. It is possible that 5-HT is formed from L-5-HTP but not L-tryptophan in the kidney. This would require the presence of L-5-HTP in the circulating blood. Plasma levels of L-5-HTP are, however, low *in vivo* but this is in part due to significant peripheral decarboxylation since carbidopa increases plasma 5-HTP levels several-fold in man [182,183].

1.2.4 Renal 5-HT Stores

The presence of 5-HT in the mammalian kidney was recognised soon after the amine was identified and characterised. There is, however, a wide variation in the reported levels of 5-HT in whole mammalian kidney tissue, from as low as 0.018 $\mu g\ g^{-1}$ tissue weight in the dog kidney up to 1.3 $\mu g\ g^{-1}$ in the mouse kidney [29]. Several factors other than species differences account for the disparity of 5-HT levels and widely differing values were reported within the same species. In many of the early studies, the chosen assay was unable to distinguish 5-HT from other

indolealkylamines or products converted to 5-HT during the extraction procedure. The methods of tissue preparation may also suffer from significant contamination by platelets or mast cells which contain significant amounts of 5-HT. Fischer and Aprison [184] reported that the mean levels of 5-HTP and 5-HT in the rat kidney were 4.9 μmol g^{-1} (22.3 ng g^{-1}) and 1.2 μmol g^{-1} (6.8 ng g^{-1}) tissue weight respectively. A more recent study using a highly specific assay for 5-HT combined with a careful flushing of the kidney and stripping of the renal capsule to minimise contamination by platelets or mast cells also demonstrated that the rat kidney contains relatively little 5-HT [35]. The 5-HT 'stores' in the medulla (21 ng g^{-1}) exceeded those in the cortex (7 ng g^{-1}) but they were uncertain whether the three-fold increase in medullary, as compared to cortical, 5-HT represents an actual heterogeneity in 5-HT distribution or a flushing artefact. The origin of 5-HT in the normal kidney is unclear. The blood platelets may be an important source of 5-HT in the kidney, especially in diseases characterised by intravascular coagulation, and there is also evidence that the kidney itself may be capable of synthesising 5-HT.

1.2.5 Renal Handling and Excretion of 5-HT, its Precursors and its Derivatives

Tryptophan. Tryptophan is partially bound to plasma proteins. It is filtered at the glomerulus and is both secreted and reabsorbed in the renal tubules [185-187]. Its stereoisomers are transported in a specific fashion in the mammalian kidney. L-Tryptophan was rapidly taken up by isolated rabbit renal tubules against a concentration gradient whereas its enantiomer, D-tryptophan, was not [185]. *In vivo* clearance and stop-flow experiments in the dog, and microperfusion studies in the rat revealed that L-tryptophan is both reabsorbed and secreted by the proximal tubule, with the reabsorptive process predominating [185,186]. The active proximal tubular reabsorption of L-tryptophan appears to operate via a stereospecific transport mechanism since D-tryptophan was reabsorbed to a minimal degree in the proximal tubule. The process also displays a high degree of structural specificity

since other tryptophan derivatives, like *N*-acetyl-L-tryptophan, DL-5-HTP and 5-HT, were not reabsorbed to any extent by the proximal tubule [185,186]. Microperfusion studies in the rat showed that the proximal tubular reabsorption of L-tryptophan exhibits saturation kinetics and a tubular transport maximum (Tm) for L-tryptophan occurred at a luminal (perfusate) concentration of 8 mM [186]. The reabsorptive process was competitively inhibited by L-phenylalanine suggesting that these amino acids share a common transport mechanism located in the proximal tubule [185,186]. It was unaffected by the metabolic inhibitors sodium azide and 2,4-dinitrophenol suggesting that the lumen-to-tubular cell transport mechanism for L-tryptophan does not require energy obtained through oxidative phosphorylation [185,186]. Probenecid, a potent blocker of the anionic secretory pathway, inhibited the tubular secretion of L-tryptophan showing that the latter is secreted in the proximal tubule by this pathway. It was without effect on the tubular reabsorption of L-tryptophan indicating that this process is not mediated by an organic acid transport mechanism [185].

D-tryptophan is handled by the kidney in a different fashion from L-tryptophan. In contradistinction to the bidirectional flux of L-tryptophan, D-tryptophan was primarily secreted and not reabsorbed in the proximal tubule to any significant degree [185-187]. Its proximal tubular secretion was probenecid sensitive and D-tryptophan, like L-tryptophan, is therefore secreted by an anionic secretory system [185,187]. Clearance studies showed that it undergoes net renal tubular reabsorption in the rat [187] in contrast to the observation of net tubular secretion in the dog [185] and chick [188]. Using micropuncture technique, D-tryptophan was found to be secreted in the proximal convoluted tubule but reabsorbed from a more distal segment of the nephron in the rat [187]. Its reabsorption was uninfluenced by luminal fluid pH [187]. D-tryptophan is apparently not utilised by humans to support nitrogen balance [189] and a considerable portion was found in the urine after its ingestion in man [190].

5-HTP. There is less information regarding the renal handling and transport of 5-HTP. Studies of uptake of [^{14}C]DL-5-HTP showed that 5-HTP was taken up by the kidneys and other tissues which have significant LAAD activity [191]. 5-HTP was shown to be taken up by the separated rat renal tubules by a saturable transport system [192]. Administration of L-5-HTP to rats *in vivo* increased plasma levels of 5-HTP but little appeared in the urine and the authors suggested that L-5-HTP may be efficiently reabsorbed by the kidney [166]. Microperfusion studies in the rat showed that DL-5-HTP, unlike L-tryptophan, was not reabsorbed by the proximal tubule [186], but it is possible that L-5-HTP is reabsorbed more distally.

5-HT. 5-HT is filtered at the glomerulus and secreted by the tubules. Clearance studies in the renal portal circulation of the chicken, and microperfusion and micropuncture studies in the rat showed that it is actively secreted by the proximal renal tubules in the rat and chicken kidney [187,193,194]. It is not reabsorbed anywhere along the mammalian nephron to any significant degree as would be expected since it is lipid insoluble [186,187,194]. The tubular secretion of 5-HT was inhibited by mepiperphenidol and quinine, both blockers of the organic cation transport system, suggesting that 5-HT is mainly secreted by the ordinary organic base transport system [194]. This is also supported by data showing that this process was inhibited by tolazoline, brom cresol green, and cyanine dye #863 which cause tubular inhibition of base transport [195]. Probenecid either had no effect [195] or only partially inhibited the secretion of 5-HT unlike its action on the transport of tryptophan or 5-HIAA [187,194]. Reserpine, despite its effect on platelets, did not inhibit the renal tubular secretion of 5-HT [193,195]. An early report suggested that 5-HT excretion was higher in acid urine in rat [176]. However, subsequent studies showed that the excretion of 5-HT is unaffected by urinary pH [187,194,195]. These data are consistent with the lipid insolubility of 5-HT and suggest that non-ionic diffusion mechanisms play no role in its excretion.

5-HIAA. 5-HIAA is excreted by the kidney by glomerular filtration and active

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tubular secretion [110,187,194,196]. Micropuncture studies in the rat demonstrated active secretion of 5-HIAA in both the proximal tubules and the more distal portions of the nephron. Microperfusion studies showed that it undergoes slight reabsorption from the proximal tubular fluid but this does not appear to be quantitatively important. Probenecid blocked the tubular secretion of 5-HIAA at all nephron levels implying that 5-HIAA, like tryptophan, is actively secreted in the renal tubules by the organic anionic transport system [187]. Similar observations were made in other species [194], and administration of probenecid to patients with the carcinoid syndrome was followed by a decrease in the urinary excretion of 5-HIAA and an increase in the plasma level of 5-HIAA [110,196].

The intrarenal site of this transport system has been established in the chicken. Probenecid completely blocked the active tubular secretion of intravenously infused labelled [^{14}C]5-HIAA in the chicken but had no effect on the excretion of [^{3}H]5-HIAA produced intracellularly within the kidney from simultaneously infused [^{3}H]5-HT [194]. Similarly, novobiocin, an inhibitor of *para*-aminohippuric acid (PAH) transport, completely blocked the excretion of intravenously infused [^{14}C]5-HIAA in the chicken, but this drug did not block the excretion of [^{3}H]5-HIAA produced intrarenally from intravenously administered [^{3}H]5-HT [197]. PAH is transported by the organic anion transport system and these observations, interpreted more broadly, indicate that novobiocin blocks the anionic secretory system that handles exogenously administered PAH or 5-HIAA thus providing further support for the probenecid data. Novobiocin had no effect on the transport of 5-HT or its metabolism to 5-HIAA. The absence of an effect of probenecid or novobiocin on the excretion of 5-HIAA produced within the renal tubular cell indicates that the probenecid- or novobiocin-sensitive anionic secretory step is located at the peritubular, and not the luminal, side of the cell [194,197].

There was no significant change in urinary 5-HIAA excretion with varying urinary pH in man [198]. Reabsorption of 5-HIAA was, however, observed to be

greater at pH 5 than at pH 7 in microperfusion experiments in the rat [187].

1.2.6 Effects of 5-HT on Renal Blood Flow and GFR

The effect of 5-HT on the renal circulation is variable depending on the animal species studied, as well as the dose and route of administration, with investigators reporting no change, an increase, a decrease, and even bi- or multiphasic changes in renal blood flow. The renal vasculature of the rat appears to be more sensitive than that of the rabbit, guinea pig or dog [55,70,199-201]. The injection of 5-HT directly into the renal artery of the isolated perfused rat kidney increased the renal vascular resistance and reduced renal blood flow even after denervation suggesting that its effect was not dependent on intact renal nerves [116,202]. This response was inhibited by ketanserin, a 5-HT_{2A} receptor antagonist [181,203]. 5-HT reduced renal blood flow and GFR *in vivo* in the rat independent of whether it was administered by the intravenous, subcutaneous or intraperitoneal route [55,70,204-206]. The vasoconstriction may be preceded by an initial vasodilatation [207]. The reduction in GFR in well-hydrated rats undergoing mannitol diuresis was independent of changes in systemic blood pressure [206]. 5-HT-induced renal ischaemia occurred primarily in the outer cortex and morphological lesions similar to renal cortical or tubular necrosis have been reported following acute and chronic dosing with 5-HT [205,206,208-210]. 5-HT, 5-CT (5-HT_1 receptor agonist) and 8-OH-DPAT (selective 5-HT_{1A} receptor agonist) caused vasodilatation in the isolated rat kidney preconstricted with noradrenaline and pretreated with 5-HT_2 and 5-HT_3 receptor antagonists. This response was antagonised by the selective 5-HT_{1A} receptor antagonist BMY 7378 and attenuated by the nitric oxide synthase inhibitor nitro-L-arginine (L-NNA) suggesting the existence of 5-HT_{1A}-mediated endothelium-dependent renal dilatation [211].

5-HT has a variable effect on the renal circulation of the dog. Most studies suggested that 5-HT administered intravenously in high doses, e.g. as a single

injection of 10-20 μg kg^{-1} [212] or as an infusion of 15-20 μg kg^{-1} min^{-1} [213], caused vasoconstriction resulting in an increase in renal vascular resistance and a reduction in renal blood flow and GFR [70,201]. This was associated with shunting of blood from the renal cortex to the medulla [212,214]. Similar observations were made after administration of 5-HT into the aorta [215,216] or directly into the renal artery [214,217-219], and the increase in renal vascular resistance could be reversed by the 5-HT receptor antagonist methysergide [215]. The effect of 5-HT on renal haemodynamics may be modulated by renal innervation and catecholamines [201]. The vasoconstrictor response to 5-HT was increased after denervation by cord section [220] and was converted to a vasodilator response when the renal sympathetic nerves were stimulated [218]. It has also been observed that the vasoconstrictor effect of 5-HT was blocked by the non-selective α-blocker phenoxybenzamine [219]; however, an earlier study showed that the increased renal vascular resistance was not inhibited by phentolamine, another non-selective α-blocker [217]. In contradistinction from the above studies, other investigators have reported that the intravenous infusion of 5-HT caused an increase in renal plasma flow with or without a reduction in GFR [221-223]. There are also a number of studies which showed no change in renal blood flow or GFR following infusion of 5-HT, particularly when the administered dose was less than 5 μg kg^{-1} min^{-1} [221,224] although similar observations were also made in some studies when the infusion rate was 5-10 μg kg^{-1} min^{-1} [213,222]. Others have also reported that no change in renal blood flow occurred after infusion of 5-HT into the renal artery [90]. Blackshear *et al.* [225], using an electromagnetic flowmeter to measure renal blood flow, observed that infusion of 5-HT into the renal artery of the anaesthetised dog produced an initial sharp decrease in blood flow followed by a gradual increase at 3 to 6 min. Prostaglandin synthetase inhibition by indomethacin abolished the vasodilator response, resulting in a sustained reduction in blood flow which was abolished by ketanserin, suggesting that the vasoconstrictor response was mediated

by an action on the $5\text{-}HT_{2A}$ receptor. The complex non-specific 5-HT receptor antagonist methysergide inhibited the increase in renal blood flow induced by ketanserin in indomethacin-treated dogs. It was suggested that the vasodilatation may be mediated by a $5\text{-}HT_1$ receptor. Neither ketanserin nor methysergide affected renal blood flow when administered in the absence of indomethacin, and none of these agents affected the initial transient vasoconstrictor response. Shoji *et al.* [226] also reported a biphasic response of renal blood flow to an infusion of 5-HT into the renal artery of the denervated dog kidney. They however observed that the initial transient vasoconstrictor phase was abolished by methysergide but not by ketanserin and that the subsequent vasodilatation was abolished by infusion of either ketanserin or methysergide. They suggested that 5-HT-induced vasoconstriction was mediated via a $5\text{-}HT_1$ receptor and vasodilatation was mediated by a $5\text{-}HT_2$ receptor. Their findings are not consistent with those of Blackshear *et al.* [225] but there were differences in experimental conditions between the two studies. More recently, Cambridge *et al.* [227] observed that 5-HT, sumatriptan ($5\text{-}HT_{1D}$ or $5\text{-}HT_1$-like agonist) and 5-CT reduced renal blood flow in dogs, which were pretreated with ketanserin and MDL 72222 to block $5\text{-}HT_2$ and $5\text{-}HT_3$ receptors respectively. The 5-CT-induced vasoconstriction was attenuated by the $5\text{-}HT_1$ receptor antagonist methiothepin. They suggested that vasoconstrictor responses to tryptamine analogues in the renal vasculature were mediated by $5\text{-}HT_{1D}$ or $5\text{-}HT_1$-like receptors. The renal vasoconstrictor responses to sumatriptan and 5-CT, but not that due to angiotensin II, were significantly augmented by the nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), suggesting the existence of endothelial $5\text{-}HT_1$-like receptors coupled to the endogenous vasodilator, nitric oxide, in the canine renal vasculature [228].

Administration of 5-HT subcutaneously [200], intravenously [205,229,230] or into the renal artery [231] reduced renal blood flow in the rabbit. Absence of an effect was, however, reported after injection of 5-HT into the aorta just above the

origins of the renal arteries [199]. The reduction in blood flow in the renal artery was antagonised by ketanserin indicating that it was mediated by 5-HT_{2A} receptor [230,231]. Dilatation of the renal resistance vascular bed was observed after administration of 5-CT, a 5-HT_1 receptor agonist, suggesting that 5-HT-induced vasodilatation may be mediated by a 5-HT_1 receptor [230]. Tadipatri *et al.* [232] concluded from their experiments with the rabbit isolated renal artery that 5-HT receptors mediating vasodilatation were not present in the smooth muscle or endothelium of the rabbit renal artery and may be predominantly located on arterioles. The receptor mediating 5-HT-induced vasoconstriction of the renal artery was not of 5-HT_2, 5-HT_3 or 5-HT_4 receptor type. It was also not of the 5-HT_{1A}, 5-HT_{2B} or 5-HT_{1D} receptor subtypes and it was felt that the pharmacological properties of this receptor most closely resemble those described for the heterogeneous 5-HT_1-like category.

The effect of 5-HT upon renal blood flow or GFR is even more difficult to evaluate in man. There are fewer studies and they have yielded contradictory results. Two normal human subjects given 5-HT intravenously at 10-20 $\mu g\ kg^{-1}\ min^{-1}$ showed a decrease in both renal plasma flow and GFR [233]. Bojs [161] also reported parallel reductions in renal plasma flow and GFR when 5-HT was infused at a rate of 4.3 $\mu g\ kg^{-1}\ min^{-1}$ in normal subjects. The changes were, however, small and only slightly exceeded the variations in renal plasma flow and GFR observed during the control periods. In addition, there were no significant changes in renal plasma flow and GFR compared to the control periods when the infusion rate of 5-HT was increased stepwise to 6.5 and 7.8 $\mu g\ kg^{-1}\ min^{-1}$ suggesting an adaptation of these responses to the infusion of 5-HT. Bojs concluded from his studies that 5-HT has a negligible effect on renal plasma flow and GFR in normal man. In hypertensive patients, the intravenous injection of a single 1 mg dose of 5-HT was reported to produce a 30% reduction in renal plasma flow rates for 15 to 45 min but GFR was not necessarily reduced [234]. In a further report, involving a larger

number of hypertensive subjects, Hollander and his colleagues [235] reported that the intravenous injection of 1 mg of 5-HT resulted in a mean reduction in renal plasma flow rates of 12% and GFR of 9%. These effects were unrelated to changes in systemic blood pressure. At variance with the above results, however, are those of other investigators who found no changes in renal plasma flow and GFR, or a reduction in GFR without any change in renal plasma flow [55,70]. The role of 5-HT in regulating renal blood flow in man therefore remains unclear given the inconsistent changes in renal haemodynamics that have been reported.

1.2.7 Effects of 5-HT on Electrolyte and Water Excretion

The most precise data regarding the effects of 5-HT on sodium and water excretion were obtained in the rat and dog, while less evidence is available in man. 5-HT, administered to rats intravenously, subcutaneously or intraperitoneally, reduced urinary flow rate, especially in well-hydrated animals [70,204,206,208,236-238]. The subcutaneous route was more effective than the intravenous or intraperitoneal route in producing this effect whereas 5-HT had no antidiuretic effect when given orally [70,154,204]. A simultaneous decrease in GFR and PAH clearance occurred in most instances and it has been suggested that 5-HT-induced antidiuresis is due primarily (and essentially) to a reduction in GFR brought about by constriction of the afferent glomerular blood vessels [204,206]. The percentage reduction in urine output was, however, greater than that in GFR and even more than that in renal plasma flow indicating that a higher percentage of the glomerular filtrate was reabsorbed by the tubules. In addition, a sustained antidiuresis occurred despite the prevention of a significant decrease in GFR by subcutaneously administered dibenamine [237]. These observations suggest the possibility of some direct or indirect effect of 5-HT on the tubular epithelium to increase water reabsorption separate from its effect on GFR. Others however argued that a satisfactory explanation of these observations may still be based on the theory that 5-HT-

induced redistribution of blood flow from the superficial cortex to the juxtamedullary area may allow the deep juxtamedullary nephrons with long loops of Henle to reabsorb more salt and water than the superficial cortical nephrons with short loops thus reducing urinary sodium and chloride excretion and urine output [70,239,240].

It has been suggested by some investigators that the antidiuretic effect produced by 5-HT could be partly due to the release of the antidiuretic hormone, AVP, perhaps induced by the pain stimulating effect of injected 5-HT [221,241]. However, an antidiuresis was also observed when 5-HT was administered subcutaneously at sufficiently low doses to avoid painful stimuli that might release AVP [237]. In addition, a reduction in chloride excretion accompanied the decrease in urine output after 5-HT administration in contrast to the antidiuresis induced by AVP which was accompanied by an increase in urinary chloride excretion [236]. Furthermore, the antidiuretic effect of 5-HT was observed in hypophysectomized rats [242], and drugs with 5-HT antagonist activities inhibited or diminished 5-HT-induced antidiuresis but had no effect upon the antidiuresis which followed release of endogenous AVP or administration of exogenous AVP [238,243]. These observations suggest that the antidiuretic effect of 5-HT is unlikely to be due to the release of endogenous AVP although the latter may contribute to antidiuresis in certain situations. 5-HT directly stimulates the adrenal gland to release aldosterone [100,244]. Aldosterone release does not, however, seem to account for the antidiuresis seen in the rat, since free water clearance should rise if the antidiuresis is primarily due to increased mineralocorticoid action whereas urine osmolality usually increases after 5-HT. Aldosterone secretion might account partially for the decrease in sodium excretion, but no firm data for potassium excretion in the rat are available to evaluate this possibility.

The dog, like the rat, is able to respond in certain experimental situations to 5-HT administration with a reduction in urinary volume. However, it requires higher

doses of 5-HT to exhibit this response. The minimum effective antidiuretic dose of 5-HT by single intravenous injection varies from 10 to 20 $\mu g\ kg^{-1}$ [212] and by intravenous infusion from 3 to 10 $\mu g\ kg^{-1}\ min^{-1}$ [222-224]. The subcutaneous and intramuscular routes are less effective [212,245]. The antidiuresis may be associated with reduction in GFR and/or renal plasma flow [212,214]. A number of studies, however, showed that 5-HT can reduce urinary volume in the absence of a change in GFR in the dog [213,222,245]. In one of these, 5-HT infused intravenously at 10 $\mu g\ kg^{-1}\ min^{-1}$ markedly decreased urine output in the absence of any changes in GFR or blood pressure and with only an insignificant increase in renal plasma flow [222]. There was an increase in the ratio of the concentration of inulin in the urine to that in plasma and a decrease in the ratio of urine volume to inulin clearance. These observations strongly suggest an increase in the tubular reabsorption of water. At higher infusion doses, a further decrease in urinary volume occurred which was now associated with a decrease in GFR and an increase in renal plasma flow. Very similar results have been reported by other investigators after either intravenous injection of 5-HT [245] or infusion of 5-HT [213] although in the latter study a decrease in renal plasma flow occurred at higher doses.

In contrast to the above observations, there are studies which failed to show a consistent antidiuresis with 5-HT [212,221]. In one study, 5-HT administered directly into the renal artery of either normal or denervated kidneys increased renal vascular resistance but did not affect urinary flow rate [217]. In another study, infusion of 5-HT into the renal artery of the denervated dog kidney increased urine flow and sodium excretion [226]. There was, however, a brief reduction followed by a more prolonged increase renal blood flow [226]. Pretreatment with ketanserin inhibited the increase in renal blood flow and changed the diuresis and natriuresis induced by 5-HT into antidiuresis and antinatriuresis, without any effect on GFR. Methysergide abolished the haemodynamic, diuretic and natriuretic responses to infusion of 5-HT. The authors suggested that 5-HT exerted its antidiuretic and

natriuretic actions via a 5-HT_1 receptor but that the renal haemodynamic changes induced by 5-HT may overcome these responses and produced the observed diuresis and natriuresis. It has also been suggested that the antidiuresis which may follow systemic administration of 5-HT does not result from a direct effect of 5-HT since it has frequently been noted that subcutaneous, intramuscular or intravenous 5-HT produced maximal antidiuresis when associated with manifestations of pain, respiratory distress, peripheral vascular changes or hypotension. Antidiuresis may, therefore, have been simply the result of hypotension or a reduction in perfusion pressure. Ureteric spasm has also been suggested as a possible cause of the antidiuresis [246]. As in the rat, antidiuresis did not appear to be mediated by release of AVP since the antidiuretic effect of 5-HT was not accompanied by any increase in chloride excretion and could also be demonstrated in hypophysectomized dogs [247].

The decrease in urinary volume that follows intravenous administration of 5-HT in the dog is usually accompanied by a decrease in the absolute and fractional excretion of sodium [213,223,224]. The reduction in sodium excretion may occur in the absence of renal haemodynamic changes and changes in filtered load of sodium at low doses of 5-HT. It may also occur when urinary flow is unaltered. The sodium-retaining effect of 5-HT was completely abolished after adrenalectomy raising the possibility that this action of 5-HT may be mediated through the adrenal gland. It is possible, however, that the increase in sodium excretion which commonly takes place after adrenalectomy was able to obscure the 5-HT effect quantitatively and the 5-HT-induced reduction in sodium excretion was just not measurable [248]. An effect produced during the first 30 min after 5-HT administration would seem too rapid for an effect on aldosterone synthesis or release and, in addition, there was no change in urinary potassium excretion. 5-HT had no effect on tubular reabsorption of electrolytes and water during mannitol diuresis in the dog [221]. The results in relation to the effect of 5-HT on urinary

potassium excretion is conflicting with some studies reporting a reduction [224] and others demonstrating no effect [213,221]. Similarly, a few studies suggested a reduction in chloride excretion [224] while others showed no effect [221].

In man, Hulet and Perera [249] demonstrated that the intravenous infusion of 1-2 mg of 5-HT base over one hour reduced sodium and chloride excretion but no consistent trends were noted in either urine or potassium output. Sinclair [76] showed that when doses of 4-8 mg of 5-HT were given by slow intravenous injection to normal subjects at the same time as a rapid intravenous infusion of 850 ml of isotonic dextrose (to induce maximal water diuresis), the duration of the diuretic response tended to be reduced. However, no antidiuretic effect was observed in the absence of water loading. Schneckloth *et al.* [233] observed a fall in urine and sodium output together with an increase in urinary potassium excretion in two normal human subjects given 5-HT intravenously at 10-20 $\mu g\ kg^{-1}\ min^{-1}$. Renal plasma flow and GFR also decreased. Bojs [161] found that decreased renal sodium excretion was the most prominent finding during intravenous infusion of 5-HT at a dose of 4.3-8.7 $\mu g\ kg^{-1}\ min^{-1}$ in normal man. This was independent of the effect on water handling or changes in systemic and renal haemodynamics. There was often a good dose response relationship and the average decrease in sodium excretion at the highest 5-HT dose was 63%. The reduction in sodium excretion was rapid in onset (within 10 min) and reversal (within 20 min), making it most unlikely that this response was mediated through the liberation of humoral agents. A reduction in sodium excretion was also observed in patients with bilateral adrenalectomy (for Cushing's disease) and in individuals with complete hypophysectomy indicating that adrenocortical or pituitary factors are unlikely to be involved in this response. Bojs's observations support the view that 5-HT-induced sodium retention was mainly a direct effect of 5-HT on tubular cells resulting in augmented tubular sodium reabsorption, although decreased tubular sodium load may sometimes be a contributing factor when a fall in GFR was observed. The fall

in chloride excretion and osmolar clearance during 5-HT infusion was similar to the decrease in sodium excretion suggesting that these changes may be secondary to the 5-HT effect on sodium excretion. Renal potassium excretion was not affected by infusion of 5-HT. The influence of 5-HT on water excretion was variable but antidiuresis was often observed when the initial urine flow was high, a situation consistent with low AVP activity. 5-HT had no antidiuretic effect when attempts were made to inhibit liberation of the antidiuretic hormone by rapid infusion of hypotonic glucose in water. It is therefore possible that a mechanism similar to release of AVP may operate during 5-HT infusion and be responsible for the antidiuresis. However, antidiuresis was also observed in a hypophysectomized patient with diabetes insipidus indicating that 5-HT does not act through liberation of AVP. The mechanism of the antidiuretic action of 5-HT remains unclear.

There are a few other studies in normal man, in addition to those quoted above, which demonstrated a reduction in urine output or sodium excretion after intravenous or intramuscular administration of 5-HT but a small number of experiments have also reported that 5-HT has little or no effect upon urinary volume. However, some of the negative results cannot be taken into consideration because of unsuitable experimental protocol such as determination of urine volume and composition only at the end of a 24 h observation period. These studies, published in non-English literature, are detailed in the reviews by Garattini and Valzelli [77], Erspamer [70] and Adler [55].

A reduction in urine output of about 40% was reported in hypertensive patients after an intravenous injection of 1 mg of 5-HT [235]. This occurred not only in those with side effects but also in those with no symptoms and the changes in urine volume were not consistently related to changes in systemic blood pressure or GFR. In those subjects with disturbing side effects (75% cases had mild to severe symptoms), emotional factors might have operated to cause an antidiuresis. There was a mean reduction in renal plasma flow rates of 12% and GFR of 9%, together

with a reduction in urinary excretion of sodium (17%) and potassium (14%). They argued that since these measurements generally changed in the same direction as urine flow, they might not have been due to actual reductions in haemodynamic function or electrolyte excretion but to inadequate washout of 'dead space' consequent to the reduction in urine flow. However, in some cases, the reductions in these functions appeared to be real, since they occurred without any reduction in urine flow. Periodic oliguria has also been reported in some cases of carcinoid syndrome but it is not known whether these episodes are associated with episodic increases in circulating 5-HT [76,109].

1.2.8 Effects of 5-HT Precursors on Renal Functions

Tryptophan. L-Tryptophan, in oral doses of 200 mg kg^{-1} or more, caused a dose-dependent reduction in urine output in well-hydrated rats. This may be a consequence of its conversion to 5-HT in the gastrointestinal mucosa (as well as intrarenally) since oral administration was more effective than subcutaneous or intraperitoneal administration in achieving this effect [164]. In contrast, D-tryptophan, in oral doses up to 1000 mg kg^{-1}, was ineffective. In another study, intraperitoneal injection of L-tryptophan (500 mg kg^{-1}) in rats produced a reduction in urinary sodium excretion and a slight increase in potassium elimination without changes in GFR and water excretion [250]. On the other hand, others have reported that intravenous infusion of L-tryptophan produced no significant changes in renal plasma flow, GFR, urine flow or sodium excretion in the rat [166]. Administration of L-tryptophan produced renovasoconstriction, a reduction in GFR and antinatriuresis in the rabbit concomitant with an increase in urinary 5-HT excretion [251]. Carbidopa abolished the changes in renal plasma flow, GFR and urinary 5-HT excretion induced by L-tryptophan and increased urinary sodium excretion. Ketanserin prevented the decreases in renal plasma flow, GFR and sodium excretion induced by L-tryptophan. These results suggest that antinatriuresis was mediated

by 5-HT_{2A} receptor in the kidney and that increased central 5-HT production induced a natriuresis. A single oral load of 2 g of L-tryptophan produced reductions in both renal plasma flow and GFR with no change in filtration fraction in man [121]. These changes were accompanied by reduced urine output and fractional excretion of potassium, and an increased fractional excretion of sodium. Plasma concentrations of vasopressin increased slightly after L-tryptophan suggesting that the antidiuresis may be partly mediated by release of vasopressin. There were no changes in blood pressure, PRA or plasma aldosterone concentrations. Reductions in renal plasma flow, GFR and urinary flow rates after administration of L-tryptophan in man have also been reported by others [252,253].

5-HTP. Infusion of L-5-HTP, but not D-5-HTP, increased renal vascular resistance in the isolated perfused rat kidneys. This response was inhibited by carbidopa and the 5-HT_{2A} receptor antagonist ketanserin [181]. 5-HTP, in parenteral (s.c. or i.p.) doses of 20 mg kg^{-1} or more, reduced urine output *in vivo* in well-hydrated rats probably secondary to its conversion to 5-HT within the kidney. The oral route was less effective than the subcutaneous or intraperitoneal route in achieving this effect. The antidiuretic effect of 5-HTP was at least 200 times less potent than that following 5-HT administration possibly because intrarenally produced 5-HT produced less vasoconstriction of the afferent glomerular arteries than exogenous 5-HT [155]. Intravenous infusion of L-5-HTP in rats reduced renal plasma flow, GFR, urine flow and sodium excretion without affecting mean arterial pressure [166]. These changes were reversed by carbidopa. Similarly, carbidopa was reported to reverse the reduced renal blood flow and increased renal vascular resistance induced by intravenous and intraarterial bolus injections of 5-HTP [254].

1.2.9 5-HT receptors in the Kidney

Little information is available on the nature, distribution and functional significance of 5-HT receptors in the kidney. 5-HT was shown to exert a dose-dependent

stimulatory effect on cAMP accumulation in isolated rat glomeruli [255]. This increase in cAMP was blocked by the 5-HT_2 antagonists methysergide, cinanserine [255] and ketanserin [256]. 5-HT elicited a smaller increase in cAMP levels in tubular fragments derived from diverse nephron segments in the renal cortex and had no effect on cAMP accumulation in tissue slices from the renal medulla or papilla [255]. Immunohistochemical and radioligand binding studies showed the presence of 5-HT_{1A} receptors on the basolateral surfaces of the medullary and cortical thick ascending limbs, distal convoluted tubules, connecting tubule cells, and principal cells of the initial collecting tubule in the human and rat kidney. There was an absence of immunostaining in the glomeruli, proximal tubules, thin limbs of Henle, collecting ducts, inner medulla and blood vessels. The specific localisation of 5-HT_{1A} receptors suggests that they may play a role in the regulation of salt and water transport in mammalian kidney [257]. Ligand autoradiography demonstrated 5-HT_{1B} receptors negatively coupled to adenylate cyclase in the outer stripe of the mouse, but not the rat, renal medulla [258]. 5-HT_1-like receptors were identified in the isolated perfused rat kidney which inhibited the release of noradrenaline induced by stimulation of the sympathetic nerves to the kidney [259]. 5-HT receptors have also been demonstrated in renal cell lines. 5-HT_{1B} receptors negatively coupled to adenylate cyclase are present in the opossum kidney (OK) cell line which retains several properties of the renal proximal tubule epithelium [260-262]. 5-HT_1 receptors that activate inward rectifier K^+ channels and inhibit cAMP accumulation were identified on Madin Darby Canine Kidney (MDCK) cells, a permanent cell line displaying many properties of the distal tubule [263,264]. 5-HT activates phospholipase C and protein kinase C, and stimulates mitogenesis in rat glomerular mesangial cells probably through 5-HT_2 receptors [265,266]. It has also been shown to be the major serum factor that induces phospholipase C-mediated hydrolysis of phosphoinositides in normal rat kidney (NRK) cells [267].

1.2.10 Potential Role of 5-HT in Renal Pathophysiology

As discussed previously, 5-HT may affect renal function and there is evidence that it may be synthesised within the kidney. The function of locally produced 5-HT is unclear but it has been speculated that 5-HT may act as a counterregulatory paracrine substance to dopamine in the regulation of renal haemodynamics, sodium and water excretion [35,268]. Its renal actions may contribute to the pathogenesis of hypertension (*see* section 1.1.7), and it has been suggested that 5-HT may also play a pathophysiological role in some renal conditions [55].

Glomerulonephritis. A variety of glomerulonephritides are characterised by increased capillary permeability and are mediated by immunological mechanisms. There is evidence that 5-HT or platelets may be involved in mediating this form of renal injury. 5-HT can increase the permeability of blood vessels to colloidal and macromolecular materials in various vascular beds including glomerular capillaries [269-271] and enhance the deposition of antigen-antibody immune complexes in blood vessels [272]. Furthermore, immune complexes can induce the aggregation of platelets and the liberation of 5-HT and other amines [273]. Released 5-HT may then act directly to increase the permeability of the vascular endothelium and allow penetration of the immune complexes into the subepithelial and mesangial areas where further damage may ensue. Studies in animal models of glomerulonephritis suggest that 5-HT may influence the deposition of immune complexes in glomeruli in the course of immune-mediated glomerulonephritis. Renal lesions in experimental acute and chronic serum sickness models of glomerulonephritis in the rabbit worsened after the administration of 5-HT. In contrast, treatment with the 5-HT receptor antagonist methysergide, the vasoamine depletor reserpine, the tryptophan 5-hydroxylase inhibitor *p*-chlorphenylalanine, or depletion of platelets by anti-platelet globulin reduced the severity of the glomerular lesions [272,274]. The 5-HT receptor antagonists, cyproheptadine and methysergide, have also been reported to reduce the deposition of immune complexes within the glomerulus and

proteinuria in autologous immune complex (Heymann) nephritis in the rat, a condition which is very similar to idiopathic membranous glomerulonephritis in man [275,276]. Proliferation of glomerular mesangial cells may be a feature of progressive glomerular diseases and 5-HT has been shown to stimulate proliferation of cultured mesangial cells [265]. Abnormal metabolism of 5-HT has been observed in patients with those forms of idiopathic glomerulonephritis that generally progress to renal failure, but not in those with more benign glomerular pathology [277,278]. These patients have low platelet 5-HT levels and elevated plasma 5-HT concentrations. These changes have been ascribed to *in vivo* activation of platelets by immune complexes (or other immunological mechanisms), and it been suggested that platelet activation and release of 5-HT may play a role in glomerulonephritis [277-279]. Antiplatelet drugs have also been tried in various forms of glomerular disease with variable efficacy [279].

There are however a number of observations which are in conflict with a proposed role of 5-HT in the pathogenesis of glomerulonephritis. In contrast to the above cited studies, several investigators have found that 5-HT antagonists and vasoactive amine depletors like reserpine did not influence the glomerular deposition of immune complexes or proteinuria in various models of experimentally induced immune complex glomerulonephritis [280-283]. 5-HT receptor antagonists also failed to inhibit the development of the morphological lesions and proteinuria in animal model of antiglomerular basement membrane antibody-induced nephritis [272,284]. In addition, many of the studies arguing for a role of 5-HT do not distinguish between a role for 5-HT, histamine, or other amines. The 5-HT receptor antagonists methysergide and cyproheptadine, for example, have other actions including inhibition of histamine release making it difficult to state conclusively that the reduction in immune complex deposition is solely attributable to their anti-5-HT action [272,285]. Moreover, the effect of 5-HT on glomerular capillary permeability is not firmly established. There is evidence that 5-HT increases the

leakage of macromolecular particles through venules rather than through the arteriolar or capillary wall [286]. Furthermore, 5-HT administered intravenously, directly into the renal artery, or into the kidney, failed to cause vascular leakage of circulating carbon particles into the renal parenchyma [287]. The role of 5-HT in modulating immune-mediated renal injury therefore remains unclear.

Renal failure. 5-HT decreases renal blood flow and can induce acute tubular or cortical necrosis, especially when given in large doses [205,208-210,288-290]. The reduced cortical blood flow with shunting of blood to the medullary region and the morphological lesions induced by 5-HT in the rat kidney show similarities to those described in acute renal failure in man or experimental animals [208,291-294]. In addition, conditions that predispose to acute renal failure in man potentiate the action of 5-HT upon the animal kidney. Thus, the severity of the renal lesions was reduced in saline-loaded rats but increased if the animals were sodium-deplete, sick or had previously been traumatised [290]. Furthermore, frusemide-induced diuresis, which is often employed to prevent acute tubular necrosis in patients, lowers renal 5-HT content in rat [295]. Counter to these results supporting an involvement of 5-HT in acute renal failure, are the observations that dogs which developed oliguria after burning of the skin showed a decrease, not an increase, in renal 5-HT content [296], and a thiazide diuretic, which was shown to lower 5-HT in the rat kidney [295], was without effect against 5-HT-induced renal lesions in this species [209]. In addition, very large pharmacological doses of 5-HT were used in experimental animals to produce renal failure and necrosis, and it is debatable whether comparable levels occur *in vivo* in the human kidney. Despite the circumstantial evidence mentioned earlier, it thus appears unlikely that 5-HT, functioning alone, participates in the production of acute renal failure in man. The possibility remains that it may act synergistically with other agents [55,209,288]. It is also conceivable that release of 5-HT intrarenally by damaged platelets could lead to high concentrations of 5-HT and a local pathological effect.

Abnormalities in 5-HT metabolism have been reported in experimental animals and man with chronic renal failure. The renal content and blood levels of 5-HT were found to be elevated in proportion to the extent of azotaemia in rats with experimentally-induced nephritis. There was also an inverse correlation between renal 5-HT content and urinary sodium excretion suggesting a possible role for 5-HT in sodium retention in this model of renal failure [297]. The uptake of 5-HT by human platelets was reduced in patients with renal failure of diverse aetiology [298] providing one possible mechanism for the reduced platelet and elevated plasma 5-HT levels observed in patients with severe uraemia [130,278,299]. Sebeková *et al.* [300], on the other hand, found that platelet 5-HT as well as plasma 5-HT levels were significantly higher in patients with decreased renal function than in controls. Urinary excretion of 5-HT was reduced, possibly reflecting decreased synthesis in the residual renal parenchyma while 5-HIAA excretion was normal. They suggested that the accumulation of 5-HT in the circulation could contribute to platelet hyperaggregation and/or consumption hypocoagulation, maintenance of hypertension, and the acceleration of atherosclerosis. The uptake of 5-HT by platelets *in vitro* has also been shown to vary inversely with pH [301] and acidosis occurs in renal failure. The activity of MAO enzyme has been reported to be increased in platelet-rich plasma in patients with chronic renal failure and reduced in the kidney of chronically uraemic rats, and it has been suggested that the increased enzyme activity in platelets may contribute to their reduced content of 5-HT [299]. Human platelets, however, contain MAO-B only and 5-HT is preferentially deaminated by MAO-A [50]. Urea also inhibited the MAO prepared from rabbit renal tissue homogenates *in vitro*, but the depressant action was less evident at higher concentrations [302]. The pathophysiological significance of disturbed 5-HT metabolism to the uraemic state is not known and it remains unclear whether the described abnormalities in platelet or plasma 5-HT levels contribute to, or result from, impaired renal and platelet function.

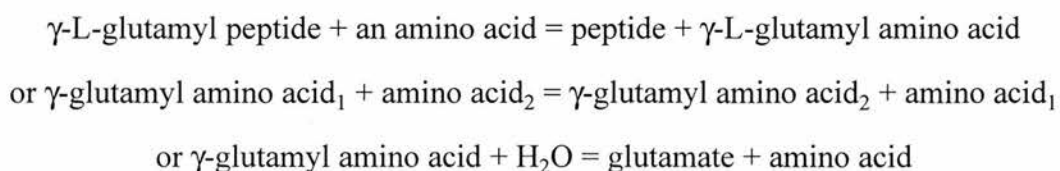
1.3. CONCEPT OF THE RENAL PRODRUG

The term *prodrug*, which was first used by Albert in 1958 [303], describes a pharmacologically inactive chemical derivative of a drug molecule that requires enzymatic or non-enzymatic transformation within the body in order to release the active drug and elicit a pharmacological effect [304]. Albert defined drug as 'any biologically active substance' [303]. A molecule with optimal structural configuration and physicochemical properties for evoking the desired therapeutic response at its target site does not necessarily possess the best molecular form and properties for its delivery to the site of ultimate action. Only a small fraction of the administered dose usually reaches the target area and, since most agents interact with non-target tissues as well, an inefficient delivery system may result in undesirable side effects. Prodrugs have been used to 'target' drugs to a particular organ or tissue with the hope that such site-specific drug delivery would optimise therapy and minimise toxicity. For this to be achieved, the prodrug must be readily transported to the site of action, where it must be selectively cleaved and transformed to the active drug relative to its conversion at other sites, and the active drug must be retained by the tissue at the target site [305]. Site-specific prodrug activation makes use of some specific property at the target site such as different pH or high activity of certain enzymes relative to non-target tissues.

Site-specific drug release exploiting the high activity of one, or more than one, enzyme at the target site to activate a prodrug has been used to produce selective delivery of the active parent drug to the kidney. This concept of a renal prodrug was first developed by Biel *et al.* [306]. They showed that the aminoacyl derivative of dopamine, *N*-L-isoleucyl-dopamine, produced a selective increase in renal blood flow with only minimal systemic haemodynamic effects in the dog and monkey. The basis of the relative selectivity was the conversion to dopamine by the enzyme aminoacylarylamidase [EC 3.4.11.14] which is abundant in the kidney. This

enzyme is, however, ubiquitous and others have reported elevated plasma levels of free dopamine and no real separation of the renal and systemic haemodynamic effects [307].

γ-Glutamyltransferase (γGT) [systematic name: (5-L-glutamyl)-peptide: amino-acid 5-glutamyltransferase; EC 2.3.2.2], also known as glutamyl transpeptidase, is an enzyme which is present in relatively high concentration in the kidney [308,309]. If the enzyme activity in the human kidney is given an arbitrary activity of 100, then the relative activities in other tissues would be: pancreas 8.3, liver 3.9, spleen 1.5, intestine 0.95, brain 0.5, lung 0.31, skeletal muscle 0.067, and heart 0.045 [308]. This membrane-bound enzyme is heavily concentrated in the brush border of the proximal tubules in animals [309-312] and man [310,313], and its activity is higher in the proximal straight tubules than in the proximal convoluted tubules [314-316]. It is orientated in the brush border membrane so as to react with substrates present in the extracellular milieu [317]. A small but significant portion of the total γGT activity in the kidney is present at the antiluminal border (basolateral side) of the proximal tubular cells [312], in association with the peritubular microvasculature [316,318,319] and/or basolateral membrane of the tubular cells [320]. Low activity of the enzyme has also been localised in the loop of Henle, distal tubule, collecting tubule and in the glomeruli [314,315,318]. γGT catalyses the transfer of the γ-glutamyl moiety from a peptide or amino acid to another peptide, amino acid or water, e.g.:



γ-L-glutamyl peptide + an amino acid = peptide + γ-L-glutamyl amino acid

or γ-glutamyl amino $acid_1$ + amino $acid_2$ = γ-glutamyl amino $acid_2$ + amino $acid_1$

or γ-glutamyl amino acid + H_2O = glutamate + amino acid

γGT is one of the key enzymes of the γ-glutamyl cycle, a metabolic pathway that accounts for the enzymatic synthesis and degradation of glutathione (γ-L-glutamyl-L-cysteinylglycine) [321-323]. It is capable of utilising both glutathione and glutamine as the natural γ-glutamyl donor. The high concentration of the enzyme at

the proximal tubule suggests that it may have an important physiological role in amino acid handling, glutathione transport and metabolism, and renal ammoniagenesis [316].

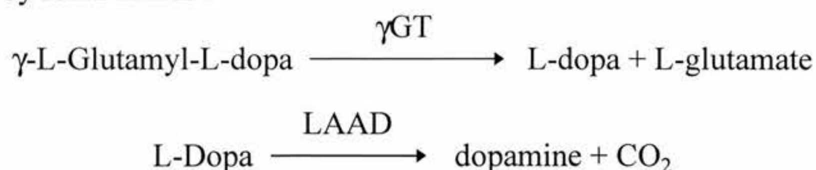
The presence of relatively high concentration of γGT in the proximal tubules suggests that γ-glutamyl derivatives of compounds containing an amino function may be selectively cleaved, thus releasing the active agent locally. Studies by Orlowski and Wilk [324,325] demonstrated that the kidney is indeed highly active in the uptake and metabolism of γ-glutamyl derivatives of amino acids and peptides. Intraperitoneal injection of the dipeptide γ-L-glutamyl-L-2-aminobutyrate in the mouse led to a marked accumulation in the kidney of free L-2-aminobutyrate together with glutamate, glutamine and aspartate derived from the γ-glutamyl moiety indicating that the renal uptake of the γ-glutamyl derivative was accompanied by hydrolysis of the peptide to its component amino acids by γGT. The level of L-2-aminobutyrate in the kidney was three times higher than that achieved after administration of an equimolar dose of free L-2-aminobutyrate. Similarly, administration of other γ-glutamyl derivatives, including glutathione, γ-L-glutamylglycylglycine, γ-L-glutamyl-L-phenylalanine and γ-L-glutamylmethionine, was followed by a marked increase in the concentration of aspartate, glutamate and glutamine in the kidney together with the amino acid(s) (cysteine, glycine, phenylalanine or methionine) constituting the other part of the molecule.

The enzyme γ-glutamylcyclotransferase [(5-L-glutamyl)-L-amino-acid 5-glutamyltransferase (cyclizing); EC 2.3.2.4] can also degrade γ-glutamyl amino acids producing the corresponding free amino acid and pyrrolidone carboxylate (5-oxo-L-proline) [323,326,327]. It shows little activity towards γ-glutamyl derivatives of aromatic and branched chain amino acids. It also requires the presence of an unsubstituted free carboxyl group in the amino acid participating in the γ-glutamyl linkage [326,328]. γ-L-Glutamyl-L-2-aminobutyrate, unlike glutathione and other γ-glutamyl dipeptides, is a substrate for this enzyme

[324,326,328]. An increase in the renal concentration of pyrrolidone carboxylate was observed after administration of γ-L-glutamyl-L-2-aminobutyrate suggesting that a significant amount of the γ-glutamyl peptide was degraded through the action of γ-glutamylcyclotransferase [324]. This reaction can only take place intracellularly since the enzyme is associated with the soluble cytoplasmic fraction of kidney proteins and this provides indirect evidence that glutamyl derivatives can enter the proximal tubular cells in an intact form. Injection of a tracer dose of γ-L-[^{14}C]glutamyl-L-2-aminobutyrate resulted in an accumulation of the isotope in kidney glutamate, glutamine and aspartate [325]. Although part of the dipeptide was degraded to its constituent amino acids, a significant proportion of γ-L-[^{14}C]glutamyl-L-2-aminobutyrate was directly incorporated into kidney ophthalmic acid (γ-L-glutamyl-L-2-aminobutyrylglycine), an analogue of glutathione, providing additional evidence that the γ-glutamyl derivative can enter into the kidney in an intact form.

The selective accumulation and metabolism of γ-glutamyl derivatives of amino acids and peptides in the kidney suggest that the γ-glutamyl group can provide a convenient carrier for directing compounds containing an amino group into kidney metabolism [324]. These observations prompted Wilk and co-workers [329] to explore the possibility of using γ-glutamyl derivatives of pharmacologically active agents as kidney-specific prodrugs. They postulated that the active agent would be released from its inactive γ-glutamyl derivative within the kidney by renal γGT thus yielding organ specificity and absence of systemic effects. They tested the possibility of using the γ-glutamyl derivative of L-dopa as a kidney-specific dopamine prodrug. They demonstrated that intraperitoneal administration of γ-L-glutamyl-L-3,4-dihydroxyphenylalanine (γ-L-glutamyl-L-dopa; gludopa) to mice led to a selective generation of dopamine in the kidney via the sequential actions of two enzymes which are highly active in the kidney. γGT catalysed the cleavage of the γ-glutamyl linkage, and the resulting L-dopa was decarboxylated to

dopamine by renal LAAD.



$$\gamma\text{-L-Glutamyl-L-dopa} \xrightarrow{\gamma GT} \text{L-dopa} + \text{L-glutamate}$$

$$\text{L-Dopa} \xrightarrow{\text{LAAD}} \text{dopamine} + CO_2$$

It is likely that L-dopa was released from the prodrug in the proximal tubules, and possibly the loop of Henle, which are the main sites of γGT location. The presence of γGT on both the luminal and antiluminal membranes suggests that uptake of gludopa into proximal tubular cells can occur after filtration from the tubular lumen and/or from the renal circulation. The enzyme may function both as carrier and hydrolytic unit so that free L-dopa is liberated into the proximal tubular cells. L-dopa will then be available for conversion to dopamine under the action of LAAD which is also present in high concentrations in proximal tubular cells [143]. Tissues other than the kidney showed only low levels of dopamine, and it is noteworthy that the second highest concentration of dopamine was found in the duodenum-pancreas consistent with the previous finding that the pancreas is the second richest site of γGT activity [308]. The dopamine level in the kidney after gludopa was almost five-fold greater than that produced by an equimolar dose of L-dopa. The administration of L-dopa also resulted in a more uniform distribution of dopamine among the organs examined. Thus, the ratio of dopamine concentration in the kidney to that in the heart was 25.8 after gludopa but was only 4.3 after L-dopa. Dopamine, selectively generated in the kidney after administration of gludopa, increased renal plasma flow while minimally affecting non-target sites as was demonstrated by no change in systemic blood pressure. The minimum dose required to produce renal vasodilatation was also shown to be lower for gludopa than L-dopa. It was suggested that γ-glutamyl derivatives of certain drugs may be useful as kidney specific prodrugs [329].

Further evidence that gludopa is a kidney-specific prodrug in the rat was provided by Cummings *et al.* [330] who showed that the concentration of dopamine in the kidney was 31-fold higher than that in the liver after intravenous

administration of gludopa. Wang *et al.* [331] showed that urinary dopamine increased 1200-fold and 7800-fold, without significant changes in plasma dopamine, after gludopa administration at doses of 25 $\mu g\ kg^{-1}\ min^{-1}$ and 100 $\mu g\ kg^{-1}\ min^{-1}$ respectively in the conscious rabbit. Gludopa elicited significant increases in urine flow, urinary sodium excretion and renal blood flow, and these renal effects occurred without alterations in blood pressure, heart rate and hindlimb blood flow. In contrast, however, Drieman *et al.* [332] reported that gludopa increased blood flow in the hindlimb and mesentery to the same extent as in the renal vascular bed in the anaesthetised rat.

Barthelmebs *et al.* [333] showed that the addition of gludopa to the perfusate in the isolated perfused rat kidney led to release of dopamine both into urine and perfusate. Dopamine released into the renal venous effluent could be considered as released from the basolateral side of the proximal tubular cells whereas dopamine released into urine could be considered as a release from the luminal side. In their experiments with filtering and non-filtering isolated kidneys, they demonstrated that glomerular filtration and access to γGT on the brush border membrane of the proximal tubular cells were required for maximal conversion rate of gludopa to dopamine and that gludopa was metabolised with a greater yield via the luminal approach to the enzymatic system. Inhibition of γGT by AT-125 (L-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid) inhibited the synthesis of dopamine by 80% and reduced gludopa-induced renal vasodilatation indicating that γGT intervened in the metabolism and vascular action of gludopa. γGT, rather than LAAD, was also shown to be the first enzymatic pathway in the synthesis of dopamine from gludopa since γ-glutamyl dopamine did not accumulate in the presence of AT-125. The study by Cummings *et al.* [330], however, showed that gludopamine was detected in urine in the rat, but not in man, after administration of gludopa suggesting that gludopa could act as a substrate for LAAD in this species.

Worth and colleagues [334] infused gludopa in normal volunteers at doses of

12.5 and 100 μg kg^{-1} min^{-1} for 2 h and showed that it increased renal plasma flow with a small increase in GFR and a reduction in filtration fraction, and was natriuretic. At the higher dose, there was a small reduction in diastolic blood pressure and a small, non-significant, increase in pulse rate. However, at the low dose, it was devoid of effects on systemic haemodynamics suggesting that the renal dopaminergic effects resulted from a local action of gludopa. The effectiveness of gludopa as a renal prodrug with relative renal specificity was demonstrated by the 280-fold and 2500-fold increases in urine dopamine excretion at low and high doses, whereas plasma dopamine rose to only 4 and 25 times the baseline levels respectively. Filtered dopamine contributed only 0.04% and 0.1% respectively to urinary free dopamine excretion during low and high dose gludopa infusion indicating that urinary dopamine was largely derived from renal transformation of L-dopa. The increase in plasma dopamine during low and high doses was believed to be caused either by a 'spill-over' from the kidney or extrarenal transformation of gludopa to dopamine. Studies with this relatively renally selective dopaminergic prodrug proved extremely valuable in understanding the dopamine paracrine system in the kidney in man [334-342].

Kyncl *et al.* [307] investigated the potential of the γ-L-glutamyl derivative of dopamine as a renal dopamine prodrug in the dog. γ-L-Glutamyl dopamine differs from γ-L-glutamyl-L-dopa in requiring only γGT for its activation. Oral administration of this compound produced significant levels of the intact amide in the plasma with almost no elevation in plasma levels of free dopamine and dopamine sulphate. There were, however, marked increases in urinary dopamine and dopamine sulphate. In contrast, an equimolar oral dose of dopamine increased the plasma concentrations of free and conjugated dopamine but most of the dopamine was excreted as dopamine sulphate and urinary excretion of dopamine was almost thirty-fold lower than that produced after administration of the glutamyl derivative. The biologically inactive γ-L-glutamyl dopamine appeared to circulate

in the blood as a depot from which dopamine was slowly released on passage through the kidney. The selective generation of the active dopamine in the kidney caused a sustained increase in renal blood flow. The dopamine was then rapidly metabolised and excreted into the urine without re-entering the systemic circulation in a high enough concentration to produce adrenergic stimulation of the heart or affect systemic blood pressure. Wilk *et al.* [343] compared the renal and cardiovascular effects of γ-L-glutamyl derivatives of L-dopa and dopamine, and found that gludopa was a more potent renal vasodilator and elicited a less pronounced pressor response than γ-L-glutamyl dopamine. The greater renal selectivity and activity of gludopa may be related to a greater affinity of this compound for γGT [344] and/or the involvement of a second enzyme (LAAD) [333].

Orlowski *et al.* [345] attempted to extend the prodrug approach to achieve kidney specificity for other drugs. They studied whether γ-L-glutamyl and various *N*-acyl-γ-L-glutamyl derivatives of sulphamethoxazole could be used as kidney-selective prodrugs of sulphamethoxazole. Intraperitoneal administration of γ-L-glutamyl sulphamethoxazole led to some preferential accumulation of the sulphonamide in the mouse kidney but the concentration was no greater than that achieved after administration of the free sulphamethoxazole and relatively high concentrations of sulphamethoxazole were also found in other tissues. The lack of kidney selectivity was apparently due to the rapid cleavage of the γ-glutamyl derivatives even in tissues with a low γGT activity. High kidney-selective accumulation of sulphamethoxazole was observed after incorporating an *N*-acetyl, *N*-butyryl or *N*-chloroacetyl group on the γ-glutamyl prodrug. It was thought that acylase [*N*-acyl-L-amino-acid aminohydrolase; EC 3.5.1.14], which is present in high concentrations in the kidney, hydrolysed the acyl group, and that this was followed by removal of the γ-glutamyl group by γGT thus releasing sulphamethoxazole. The renal selectivity of these *N*-acyl-γ-glutamyl prodrugs was attributed to the combined

action of these two enzymatic processes.

The above explanation for the renal selectivity of *N*-acyl-γ-glutamyl prodrugs was disputed by Drieman and co-workers [346,347]. They argued that the localisation of the active site of γGT on the extracellular side of the brush border and basolateral membranes [317] makes it unlikely that conversion of the prodrug by this enzyme can generate high intracellular concentrations of the active drug. The cytosolic enzyme γ-glutamylcyclotransferase, another enzyme of the γ-glutamyl cycle, is also able to hydrolyse γ-glutamyl compounds [323,326,327], and they suggested that the combination of this enzyme, rather than γGT, with acylase is responsible for the intracellular conversion of *N*-acyl-γ-glutamyl prodrugs. Drieman and colleagues also suggested that an active transport or carrier system is required to get the prodrugs into the proximal tubular cells since anionic compounds do not diffuse easily across biological membranes. They studied the processes underlying the renal selectivity of the vasodilator prodrug CGP 22979 (*N*-acetyl-L-glutamic acid-*N*-[N^2-(5-*n*-butyl-2-pyridyl)hydrazide]), the *N*-acetyl-γ-L-glutamyl derivative of the hydralazine-like vasodilator CGP 18137 (5-*n*-butyl,2-hydrazinopyridine) in the rat. They found that inhibition of γGT by AT-125 did not lower the kidney concentrations of CGP 18137 suggesting that γGT was not the enzyme involved in the conversion of CGP 22979 to CGP 18137 or the renal accumulation of CGP 18137. γGT may be partly responsible for the renal haemodynamic responses to CGP 22979 since AT-125 caused a decrease in the renal response to the prodrug. The transport processes involved in the renal selectivity of CGP 22979 were investigated by pretreating the animals with the anion transport inhibitor probenecid, the γ-glutamyl transport inhibitor buthionine sulphoximine, and glutathione which has been shown to reduce renal uptake of γ-glutamyl cysteine. All three compounds reduced the renal accumulation of CGP 18137 produced by CGP 22979 and attenuated the renal response to the prodrug. These results showed that carrier-mediated transport of the prodrug into the renal

tubular cells, followed by its intracellular conversion to the active drug, were the main determinants of the kidney selectivity of this prodrug. The authors suggested that CGP 22979 is partly taken up by the proximal tubular cells through the basolateral membrane, possibly via a sodium-dependent system or by the anion transport system. Once it is intracellular, CGP 22979 is converted to CGP 18137 by γ-glutamylcyclotransferase with or without prior deacetylation by acylase. Drieman *et al.* [347] also found that the renal accumulation of sulphamethoxazole after *N*-acetyl-γ-L-glutamyl sulphamethoxazole was inhibited by probenecid and buthionine but was unaffected by AT-125. These results again support their hypothesis than an active or facilitated transport of the prodrug followed by intracellular conversion are responsible for the kidney selective delivery of sulphamethoxazole. Like Orlowski *et al.* [345], they found that γ-L-glutamyl sulphamethoxazole lacked renal selectivity. They argued that sulphamethoxazole is a polar drug and does not easily cross biological membrane. Sulphamethoxazole resulting from hydrolysis of filtered γ-glutamyl sulphamethoxazole by γGT at the brush border membrane will be excreted into the urine while sulphamethoxazole released by γGT at the basolateral membrane will enter the blood stream rather than into the tubular cells. The latter is equivalent to an intraarterial infusion of sulphamethoxazole and results in no selectivity for the kidney because of the low extraction rate of sulphamethoxazole in the kidney. They suggested that the difference in renal selectivity between γ-glutamyl and *N*-acetyl-γ-L-glutamyl sulphamethoxazole lies in the site of conversion to the active drug; extracellularly by γGT for γ-L-glutamyl sulphamethoxazole and intracellularly in the case of *N*-acetyl-γ-L-glutamyl sulphamethoxazole.

A number of other γ-L-glutamyl or *N*-acyl-γ-L-glutamyl compounds have been designed and tested for as kidney selective prodrugs including γ-L-glutamyl-XAC (xanthine amine congener) as a kidney-selective adenosine receptor antagonist [348], and SC-47792 (*N*-acetyl-γ-glutamyl fusaric acid hydrazide) as a dopamine-β-

hydroxylase inhibitor [349]. These studies and those cited previously suggest that the γ-L-glutamyl or *N*-acyl-γ-L-glutamyl prodrug derivatives of a variety of drugs may be of benefit if it is a desired objective to restrict the drug action to the kidney or urinary tract. The activation of these prodrugs appears to be quite different. *N*-Acetyl-γ-L-glutamyl prodrug is delivered to the kidney via the plasma. A part is filtered by glomerular filtration and excreted into the urine or possibly taken up via the brush border membrane. Another part can be taken up via the basolateral membrane. Once intracellularly, prodrug is converted to drug by γ-glutamylcyclo-transferase and acylase. The active drug can be retained by kidney cells, metabolised, excreted into the urine or into the blood. The *N*-acetyl-γ-glutamyl prodrugs may, therefore, be particularly useful for the intracellular delivery of drugs with an intracellular site of action in the kidney. γ-L-Glutamyl compounds, on the other hand, are converted extracellularly by γGT, either at the basolateral side or at the luminal side of the renal tubular cells. This implies that they are not suited for intracellular delivery of an active drug, and the only application can be for drugs acting in the renal tubular lumen unless other features are present, such as is the case with γ-L-glutamyl-L-dopa where the enzyme LAAD is also involved in its activation [329].

A prerequisite for the applicability of a γ-glutamyl derivative of a given drug as a kidney selective prodrug is that it can function as a substrate for γGT. γ-Glutamyl amide bonds are not invariably cleaved by γGT [350]. The γ-glutamyl derivatives of adamantanine, 4-aminobutyric acid, L-thiazolidine-4-carboxylic acid, 1-amino-cyclopentanecarboxylic acid, for instance, were not readily hydrolyzed by γGT [351,352]. Similarly, not all *N*-acetyl-γ-L-glutamyl compounds were found to be kidney selective prodrugs. Drieman *et al.* [353] utilised an *in vitro* system to screen seven prodrugs for their potential renal selectivity. They found that the *N*-acetyl-γ-L-glutamyl derivatives of *para*-nitroaniline, aminophenyl acetic acid, sulphamethoxazole, sulphadimethoxine, propranolol and metoprolol were

accumulated in rat kidney slices by a probenecid-sensitive carrier. The derivatives of *para*-nitroaniline, aminophenyl acetic acid and sulphamethoxazole were also accumulated via a buthionine sulphoxine-sensitive process. The difference in transport inhibition by probenecid and buthionine sulphoxine suggests that at least two carriers are involved in the transport of the prodrugs. The accumulation of the *N*-acetyl-γ-L-glutamyl 4′-aminoantipyrine was not affected by either probenecid or buthionine sulphoxine. Unlike all other prodrugs, those of 4′-aminoantipyrine and propranolol were not, or only to a very limited extent, converted to the parent compounds by kidney homogenates. The derivatives of *para*-nitroaniline and aminophenyl acetic acid were selectively accumulated by the kidney *in vivo* whereas *N*-acetyl-γ-L-glutamyl propranolol was not. The authors found that the main factors determining the selectivity of *N*-acetyl-γ-L-glutamyl prodrugs are: the transport into the kidney, the conversion rate, the residence time of the prodrug in the kidney, and the presence or absence of competition for uptake and conversation by other tissues such as liver. *N*-acetyl-γ-L-glutamyl-4′-aminowarfarin was also found to lack renal selectivity [354]. The γ-L-glutamyl or *N*-acyl-γ-L-glutamyl prodrug approach therefore offers the possibility of delivering drugs selectively to the kidney but is not universally applicable.

1.4 OBJECTIVES OF THESIS

The data regarding the effects of 5-HT on urinary sodium and water excretion in man are at variance. Most studies suggested that it reduced both urine output and sodium excretion. These responses were inconsistently related to changes in the renal circulation, and it is unclear whether they were due to changes in renal haemodynamics or increased tubular reabsorption (*see* sections 1.2.6 and 1.2.7).

The studies in man largely focused on the pharmacological reactions to intravenous administration of 5-HT. Exogenous 5-HT reaching the kidney first comes into contact with the arterioles and only subsequently with the capillaries and other structures. It may reduce blood flow through the afferent glomerular arterioles as a result of its vasoconstrictor action and this may obscure any tubular effect. It is also difficult to evaluate from systemic infusion studies whether the observed effects of 5-HT on the kidney are solely due to its direct effects on the renal vasculature or tubules since 5-HT has a wide spectrum of actions in the body (*see* section 1.1.6). The renal responses to infusion of 5-HT may be largely modified by and/or possibly be due to the confounding influences produced by effects in other tissues by the amine. Thus, in addition to affecting systemic blood pressure, 5-HT may stimulate the release of aldosterone by a direct action on the adrenal cortex or it may release renin as result of alterations in systemic or renal haemodynamics. These extrarenal effects of 5-HT may secondarily alter renal functions and need to be taken into consideration when interpreting the observed renal effects of 5-HT. The dose of 5-HT that can be given intravenously in man is also limited by unpleasant adverse effects such as chest tightness, flushing, heaviness and tingling in the extremities, thrombosis or pain along the course of the infused vein, cough, dyspnoea, headache, abdominal pain, nausea, increased bowel or bladder activity [76,161,235,355]. These symptoms may interfere with the assessment of the antidiuretic activity of 5-HT in man since any painful or unpleasant stimulus may

produce a powerful antidiuresis [356,357].

Investigations of the renal effects of 5-HT in the intact organism may also be limited by the rapid uptake of 5-HT into platelets or its conversion to inactive metabolites in the pulmonary and hepatic circulation. 5-HT was found to be a more effective antidiuretic when the injection was made into the cubital vein rather than into the malleolar vein in the dog because of the shorter path, and hence time taken, to reach the kidney. In addition, if blood was first withdrawn into the syringe containing 5-HT and immediately reinjected, no antidiuresis was observed because the amount of free 5-HT was reduced [358]. It is thus conceivable that the kidney may indeed be highly sensitive to 5-HT but that, following its intravenous administration, it is so rapidly removed from the circulation that it may not reach the renal tissues in an adequate concentration to produce effects, and increasing the dose to achieve an effective renal concentration may merely increase the confounding influences resulting from its extrarenal effects. Studies in animals may be designed to provide information regarding the local renal effects of 5-HT but one needs to be careful in applying the findings obtained in animals to man because of the differences in the pattern of distribution, metabolism and actions of 5-HT between species (*see* section 1.2).

In considering the actions of 5-HT on the kidney, it is also important to distinguish between the effects of the extrarenal 5-HT delivered via the renal arterial blood and the effects of 5-HT generated *in situ* in renal tissue since there is evidence that the kidney is capable of producing and degrading 5-HT (*see* section 1.2.3). Plasma levels of free 5-HT are relatively low except in certain pathological conditions and the 5-HT content in renal tissue is due to either active extraction of 5-HT from perfusing blood and/or due to intrarenal synthesis. It can therefore be argued that in studies in which 5-HT is infused into the systemic circulation or even into renal artery, the observed effects are more likely to be pharmacological and not similar to the physiological or pathophysiological situations *in vivo*.

Administration of 5-HT precursors which are selectively converted to 5-HT within the kidney may allow the renal effects of 5-HT to be assessed separately from any systemic effects caused by intravenous infusion of 5-HT. Intrarenal synthesis of 5-HT has been demonstrated in rats given L-5-HTP [166,181,268] but administration of DL- or L-5-HTP is associated with adverse gastrointestinal and other effects in man [162,359-362], and this may limit the dose of 5-HTP than can be given. Furthermore, unlike 5-HT, L-5-HTP readily crosses the blood-brain barrier enhancing synthesis of 5-HT in the brain [156,175,362,363] and central 5-HT may stimulate the release of renin, aldosterone, cortisol and vasopressin making it difficult to interpret the renal effects of locally produced 5-HT (*see* section 1.1.6).

The γ-glutamyl prodrug approach to targeting drugs to the kidneys offers the possibility of delivering drugs selectively to the kidney with little or no systemic effects (*see* section 1.3). Gludopa (γ-L-glutamyl-L-dopa), the glutamyl derivative of L-dopa, is a renal dopamine prodrug which is sequentially converted in the kidney by γGT to L-dopa and decarboxylated by LAAD to dopamine. This prodrug has allowed the renal effects of dopamine to be explored in man [334-342]. The glutamyl derivative of L-5-HTP, γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP), may similarly be converted intrarenally to 5-HTP and then decarboxylated to 5-HT. This compound would allow, in theory, the manipulation of renal 5-HT production and permit the *in vivo* renal effects of 5-HT in man to be assessed with less interference from the effects on other systems produced by administration of 5-HT or L-5-HTP. The glutamyl derivative of L-tryptophan, γ-L-glutamyl-L-tryptophan (glu-TRP), may be another renally selective 5-HT precursor. The γ-glutamyl bond may be cleaved to release L-tryptophan which could then be sequentially hydroxylated by tryptophan 5-hydroxylase in the kidney to L-5-HTP and then further metabolised by decarboxylation to 5-HT.

The objectives of this thesis were:

1. To investigate the potential of γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP) as a renally selective 5-HT precursor and compare its effects with those of 5-hydroxy-L-tryptophan (L-5-HTP).

2. To investigate if the effects observed with glu-5-HTP are due to its intrarenal transformation to 5-HT.

3. To investigate whether γ-L-glutamyl-L-tryptophan (glu-TRP) increases renal 5-HT production.

4. To investigate any possible interaction between 5-HT and dopamine delivered to the kidney by glu-5-HTP and gludopa respectively.

5. To investigate whether frusemide-induced diuresis, which has been shown to increase urinary dopamine excretion, affects 5-HT excretion.

CHAPTER TWO

METHODOLOGY

2.1 Subjects Studied

The studies reported in this thesis were performed in male volunteers, aged 18 to 45 years, recruited by advertisements in the local press. They were all in good health as judged by medical history, physical examination, 12-lead electrocardiogram, urinalysis, full blood count, serum electrolytes, creatinine and liver function tests. They also underwent screening tests, after counselling, for the presence of hepatitis B surface antigen, antibody to hepatitis B core antigen, and antibody to human immunodeficiency virus. They were within 15% of their ideal body weight. None were on regular drug treatment and they avoided medication of any kind for at least 2 weeks before the start of a study and until its completion. Individuals who smoked more than 15 cigarettes daily were excluded from the studies.

2.2 Ethical Considerations

The studies were approved by the Lothian Research Ethics Committee, Lothian Health Board, Edinburgh, and written informed consent was obtained from all the volunteers prior to their taking part.

2.3 Drugs and other Chemicals

γ-L-Glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP; $C_{16}H_{19}N_3O_6$; FW 349.3). This dipeptide was supplied by Aalto Bio Reagents Ltd (Rathfarnham, Dublin, Republic of Ireland). The peptide content of the preparation, as determined by the nitrogen content, was 95%, the balance being counter ions (e.g. acetate salts) and water carried over from freeze-drying. The peptide purity, determined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), was greater than 99%. A stock solution of glu-5-HTP (5 mg ml^{-1}) was made up, sterile and pyrogen free, in 0.9% w/v sodium chloride (NaCl) solution by the Pharmacy Department at Edinburgh Royal Infirmary and stored in glass ampoules at -40° C.

γ-L-Glutamyl-L-tryptophan (glu-TRP; $C_{16}H_{19}N_3O_5$; FW 333.3). Glu-TRP was purchased from Sigma Chemical Co. Ltd (Poole, Dorset, U.K.). The preparation contained 97% pure peptide. The peptide purity was greater than 97% as determined by TLC. Glu-TRP was prepared for use in 0.9% NaCl by the Pharmacy Department at Edinburgh Royal Infirmary and aliquots of the solution (5 mg ml^{-1}) were stored in glass vials at -40° C.

γ-L-Glutamyl-L-3,4-dihydroxyphenylalanine (gludopa; $C_{14}H_{18}N_2O_7$; FW 326.3). Gludopa was supplied by Aalto Bio Reagents Ltd (Dublin, Republic of Ireland). Its peptide content was 95% and the peptide purity, determined by TLC and HPLC, was greater than 99%. It was prepared in 0.9% NaCl by the Pharmacy Department at Edinburgh Royal Infirmary and stored in vials (10 mg ml^{-1}) at -40° C.

5-Hydroxy-L-tryptophan (L-5-HTP; $C_{11}H_{12}N_2O_3$; FW 220.2). L-5-HTP was purchased from Sigma Chemical Co. Ltd (Poole, U.K.). It was dissolved in 0.9% NaCl and packed in ampoules (10 mg ml^{-1}) by the Pharmacy Department at Edinburgh Royal Infirmary. These were stored at -40° C.

Carbidopa. This was obtained from Merck Sharp and Dohme Ltd (Hoddesdon, Herts, U.K.). Capsules containing 50 mg of carbidopa and matched lactose placebo capsules were made up by the Pharmacy Department at Edinburgh Royal Infirmary.

Frusemide (Lasix®). Ampoules of this drug (10 mg ml^{-1}) were obtained from Hoechst UK Ltd (Hounslow, Middlesex, U.K.).

Polyfructosan (Inutest®). Ampoules of polyfructosan (5 g in 20 ml) were purchased from Laevosan-Gesellschaft (Linz, Austria).

Para-*aminohippurate sodium.* Vials of *para*-aminohippurate sodium (PAH; 2 g in 10 ml) were obtained from Merck Sharp & Dohme Ltd (Hoddesdon, U.K.).

Slow sodium. Slow sodium tablets (NaCl 600 mg; ≈10 mmol each of Na^+ and Cl^-) were purchased from CIBA Laboratories (Horsham, West Sussex, U.K.).

Sodium chloride (saline; 0.9% w/v) solution. 500 and 1000 ml packs of 0.9% NaCl (9 g l^{-1}; ≈150 mmol each of Na^+ and Cl^- per litre) were obtained from Baxter

Healthcare Ltd (Thetford, Norfolk, U.K.); 10 and 20 ml vials (Mini-Plasco) were obtained from B. Braun Medical Ltd (Aylesbury, Bucks, U.K.).

2.4 Experimental Protocol

All the studies were of a randomised, placebo-controlled (except for the study described in Chapter 5), cross-over study design in which each subject received all test medications. The study days were usually separated by at least 7 days to ensure that there was little chance of carry-over effects of treatments and to allow for re-equilibration of salt balance. The basic experimental protocol, except for the study reported in Chapter 9, is outlined in Figure 2-1. Specific details of each study are given in the individual experimental chapters.

The subjects refrained from alcohol for 24 h, abstained from xanthine-containing drinks (tea, coffee, chocolate, coke) from 18.00 h, and fasted from 22.00 h the evening before each study day. Smoking was prohibited during the study days. For some of the studies, the subjects were given general dietary advice to avoid large changes in their salt intake in an attempt to reduce the variability in their salt status between study days. The help of Ms. E. Sloan, dietitian, in compiling the dietary regimes is gratefully acknowledged.

The subjects arrived at the clinical investigation unit at about 08.00 h on each study day, having drunk 250 or 500 ml of tap water one hour previously. An intravenous cannula (18 G Venflon®, BOC Ohmeda AB, Helsingborg, Sweden) was inserted into a large vein in each forearm, one for administration of infusions and the other in the contralateral arm for blood sampling. In the studies in which ERPF and GFR were estimated, priming doses of PAH (0.5 g) and polyfructosan (Inutest; 3.5 g) were added to 0.9% NaCl solution to make up a total volume of 40 ml and infused over 8 min using a Perfusor VI infusion pump (B. Braun, Melsungen AG, Germany). This was followed by a maintenance infusion of PAH (3.75 g l^{-1}) and polyfructosan (4.5 g l^{-1}) in 0.9% NaCl solution at a constant rate of 5 ml min^{-1} using

a volumetric infusion pump (IMED 960; IMED Ltd, Abingdon, Oxon, U.K.) throughout the experiment. In other studies, saline alone was administered at a rate of 10 ml kg^{-1} h^{-1} (Chapter 3) or 5 ml min^{-1} (Chapters 5 and 7). After a three hour run-in period, the subjects received the test infusion(s) (glu-5-HTP, glu-TRP, gludopa or L-5-HTP, each made up to a total volume of 30 ml with saline) at a rate of 0.5 ml min^{-1} for one hour. Placebo was 30 ml of saline alone. These infusions were administered by Braun Perfusor VI infusion pumps. In addition to the saline given intravenously, the subjects drank 150 or 200 ml of water half-hourly (except for the study described in Chapter 3). These measures were used to promote an adequate urine flow and to facilitate complete bladder emptying. The subjects remained supine or semi-recumbent throughout each experimental day except when they stood up to micturate. Objective recordings of blood pressure and pulse were made at regular intervals using a Dinamap oscillometric semi-automated recorder (Critikon, Inc., Tampa, FL, U.S.A.) with the subject in the supine position. Mean arterial blood pressure (MAP) was calculated as the sum of diastolic blood pressure plus one third of the pulse pressure.

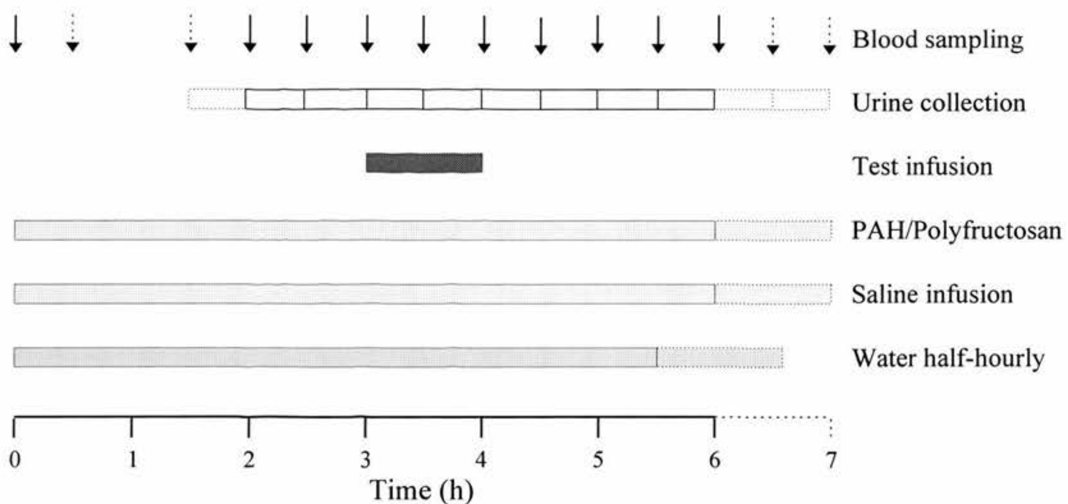


Figure 2-1. Outline of experimental protocol.

Blood sampling. Blood samples were always taken before the subject rose to pass urine. They were collected into lithium heparin tubes (10 ml) for measurement of

plasma sodium, potassium, PAH, polyfructosan, glu-TRP and tryptophan; and into plain tubes (5 ml) for serum growth hormone. Plasma and serum were separated after centrifugation at 1500 *g* and stored at -40° C. Venous blood samples (5 ml) for determination of PRA were taken after the subjects had been supine for at least 20 min [364,365]. They were transferred into chilled glass tubes containing 100 μl of a 5% w/v ethylenediaminetetra-acetic acid (EDTA) disodium salt as anticoagulant and kept on ice to minimise angiotensin I production in the samples. These were immediately centrifuged for 20 min at 1500 *g* and 4° C to minimise the cryoactivation of prorenin [366]. For the same reason, the supernatant plasma was promptly withdrawn and stored at -40° C until assayed. These plasma samples were also used for the measurement of plasma aldosterone. Venous blood samples for measurement of 5-HT and 5-HTP in platelet-rich plasma (PRP) were gently withdrawn via a 16 G Venflon cannula into a 10-ml plastic syringe without using a tourniquet. Each blood sample (9 ml) was dispensed into a plain polystyrene plastic tube containing 1 ml of an acid-citrate-dextrose anticoagulant mixture which consisted of citric acid (8 g l^{-1}), trisodium citrate (22 g l^{-1}) and glucose (20 g l^{-1}) [367]. After gentle mixing by tube inversion, the citrated whole blood was centrifuged at low speed (120 *g*) for 20 min at room temperature. The upper two-third of the supernatant (i.e. PRP) was carefully removed with a disposable transfer pipette and stored at -40° C in a sealed polystyrene tube until analysis.

Urine collection. Accurately timed urine collections of about 30 min duration were made before, during and after the test infusions. Urine volumes were recorded and aliquots stored in sterile 25-ml Universal containers at -40° C for determination of sodium, potassium, PAH, polyfructosan, 5-HTP, glu-TRP and tryptophan. Urine samples (20 ml) for measurement of 5-HT, 5-HIAA, dopa, dopamine and DOPAC were transferred into Universal containers containing 0.2 ml of 5 M hydrochloric acid (HCl) as an antioxidant [368]. The pH of each sample was measured to ensure that it was less than 3 and further HCl was added if required.

2.5 Analytical Methods

2.5.1 Measurement of Urinary Dopa, Dopamine and DOPAC

L-Dopa, dopamine, DOPAC and the internal standard epinine were adsorbed from urine onto active aluminium oxide at pH 8.5. This procedure is based on the original use of aluminium oxide as an extractant by Anton and Sayre [369,370]. Following washing of the oxide with deionised water, the metabolites were eluted with 2% perchloric acid and measured by HPLC with electrochemical detection.

Reagents

EDTA (0.5 M). This was prepared by dissolving 186 g of EDTA disodium salt ($Na_2EDTA.2H_2O$) in 1 litre of water.

Tris buffer (pH 11.0, 0.5 M). 12.114 g of tris(hydroxymethyl)aminomethane was dissolved in 200 ml of water.

Perchloric acid (2%). 16.67 ml of 60% perchloric acid was made up to 500 ml volume with water.

HCl (0.01 M and 1 M). These were obtained by diluting 5 M HCl with water.

Aluminium oxide active acidic, Brockmann grade 1. Aluminium oxide (500 g) was activated by heating at 200° C for 2 h after which it was stored at 100° C.

Stock L-dopa, dopamine and DOPAC standards (1 mg ml $^{-1}$). These stock solutions were obtained by dissolving L-dopa, dopamine hydrochloride and DOPAC in 1 M HCl and stored in aliquots at -40° C.

Working L-dopa standards (40, 80, 120, 160 and 200 ng ml $^{-1}$). The stock L-dopa standard was diluted 1:50 with water and the resulting solution further diluted 1:100 with 0.01 M HCl to produce a concentration of 200 ng ml $^{-1}$ of L-dopa. L-dopa was then prepared over a concentration range of 40, 80, 120, 160 and 200 ng ml $^{-1}$ in 20 ml volumes by adding 16, 12, 8, 4 and 0 ml of water to 4, 8, 12, 16 and 20 ml of the 200 ng ml $^{-1}$ solution of L-dopa respectively.

Combined working dopamine and DOPAC standards (40, 80, 120, 160 and 200

ng ml $^{-1}$***).*** The stock dopamine and DOPAC standards were each diluted 1:25 with water and the resulting solutions further diluted 1:100 with 0.01 M HCl to produce a concentration of 400 ng ml^{-1} of dopamine and DOPAC. 50 ml aliquots of these solutions were mixed together to produce a solution containing 200 ng ml^{-1} of dopamine and DOPAC. Dopamine and DOPAC were then prepared over a concentration range of 40, 80, 120, 160 and 200 ng ml^{-1} in 20 ml volumes by adding 16, 12, 8, 4 and 0 ml of water to 4, 8, 12, 16 and 20 ml of the 200 ng ml^{-1} dopamine/DOPAC solution respectively.

Stock epinine internal standard (1 mg ml $^{-1}$***).*** 50 mg of epinine hydrochloride was dissolved in 50 ml of 1 M HCl and stored in 1.1 ml aliquots at -40° C.

Working epinine internal standard (2 µg ml $^{-1}$***).*** The stock internal standard was diluted 1:10 with water and the resulting solution further diluted 1:50 with 0.01 of 1 M HCl to produce a final epinine concentration of 2 µg ml^{-1}.

Mobile phase used for HPLC. The HPLC solvent (pH 5.2) consisted of citric acid monohydrate (5.75 g), sodium acetate trihydrate (6.80 g), sodium hydroxide (2.40 g), 1-octanesulphonic acid sodium salt (0.10 g), acetic acid (1.05 ml) and EDTA disodium salt (0.10 g) made up to 1 litre with deionised water. Methanol 4% (v/v) was added. The solvent was filtered through a 0.22 µm membrane (Durapore, Division of Millipore, Milford, MA, U.S.A.) and degassed using helium for 3 min before use. The 1-octanesulphonic acid was incorporated as an ion-pairing reagent.

Sources of reagents. EDTA disodium salt, tris(hydroxymethyl)aminomethane, perchloric acid 60%, 5 M HCl solution, citric acid monohydrate, sodium acetate trihydrate, sodium hydroxide pellets, 1-octanesulphonic acid sodium salt, acetic acid (glacial), methanol (all chemicals being of analytical reagent grade) and aluminium oxide active acidic (Brockmann grade 1) were obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.). L-Dopa (L-3,4-dihydroxyphenyl-alanine; L-3-hydroxytyrosine; FW 197.2), dopamine hydrochloride (3,4-dihydroxyphenethylamine; 3-hydroxytyramine; FW 189.6), DOPAC (3,4-

dihydroxyphenylacetic acid; FW 168.1), and epinine hydrochloride (*N*-methyl-dopamine, deoxyepinephrine) were obtained from Sigma Chemical Co. Ltd (Poole, U.K.) Deionised water (Elgastat Spectrum Water Purification System, The Elga Group, Bucks, U.K.) was used to make up the solutions and dilutions for all the assays described in this thesis unless stated otherwise.

Method

Aliquots (0.5 g) of activated aluminium oxide were weighed into sufficient 10-ml tubes to cover the number of samples and standards to be extracted. The alumina aliquots were washed with 5 ml of 0.5 M EDTA for 10 min at room temperature on a Rollamixer (Denley Spiramix, Sussex, U.K.). The tubes were centrifuged and the supernatants were aspirated to waste.

5 ml aliquots of test urine samples (1:200 dilution with water may be required for urine collected after infusion of gludopa) or standard solutions (40, 80, 120, 160 and 200 ng ml^{-1}) of L-dopa, dopamine and DOPAC were added to the washed alumina followed by 0.5 ml of the internal standard (epinine, 2 $\mu g\ ml^{-1}$). The pH of all the urine and standard samples was raised to 8.5, the optimum for adsorption of the metabolites to alumina, by dropwise addition of 0.5 M Tris buffer. The change in pH was measured with a PHM 61 Laboratory pH meter with GK2321C combined electrode (Radiometer Ltd, Crawley, West Sussex, U.K.). The tubes were 'Rollamixed' for 40 min at room temperature to allow adsorption of L-dopa, dopamine, DOPAC and epinine. They were centrifuged at 1000 *g* for 5 min to deposit the alumina and the supernatants were removed by suction. The alumina deposits were washed twice with water to maximise removal of unadsorbed metabolites, each time siphoning off the supernatants after centrifugation. After the final wash, the supernatants were withdrawn as completely as possible and 2 ml of 2% perchloric acid was added to each of the alumina deposits. The tubes were shaken vigorously for 10 min at room temperature using a mechanical shaker to facilitate elution of L-dopa, dopamine, DOPAC and epinine into the acid phase.

They were then centrifuged at 1000 *g* for 5 min and the supernatants filtered through 0.2 μm Porex filters (Porex Medical, Fairburn, GA, U.S.A.). The filtered eluates were then ready for quantitation of dopa, dopamine and DOPAC by HPLC and electrochemical detection.

Analytical system

Each assay run consisted of test samples, standards and a quality control sample. These samples were loaded onto an automated sample processor (Waters Intelligent Sample Processor WISP 710B, Waters, Division of Millipore, Milford, MA, U.S.A.) and the instrument was programmed to inject 20 μl aliquots of the sample eluates, prepared as outlined above, at 15 min intervals into the mobile phase delivered at a flow-rate of 1 ml min^{-1} by a Beckman Altex solvent metering pump (Model 110A, Altex Scientific Inc., Berkeley, CA, U.S.A.). The solvent passed through a Waters 2 μm metal frit filter and a precolumn (Waters Guard-Pak precolumn insert Novo-Pak C_{18}) before reaching the reversed-phase chromatography analytical column. This was a Waters Nova-Pak C_{18} Radial-Pak cartridge, 100 x 5 mm (i.d.), packed with 4 μm spherical C_{18} bonded silica-based materials, and the cartridge was housed in a Waters RCM (Radial Compression Module) 8 x 10 cartridge holder.

The effluent was fed to a Waters M460 electrochemical (EC) detector which contained a 30 μl flow cell and a glassy carbon working electrode with a silver/silver chloride (Ag/AgCl) reference electrode. The potential of the working electrode was set at +0.8 V *versus* the Ag/AgCl reference electrode since this was found to give the optimum conditions for the detection of dopa, dopamine and DOPAC when the instrument was set in the oxidative mode with background current of approximately 0.5 mA. This potential forces the oxidation of *ortho*-dihydroxyl groups to yield the corresponding *ortho*-quinone. The electrons generated in this reaction pass into the electrode and the resulting current is measured as each oxidizable analyte passes the electrode. The amplitude of the

current is thus proportional to the quantity of metabolite passing through the cell. The signals from the EC detector were analysed by a computing integrator (Shimadzu C-R3A Chromatopac, Shimadzu Corporation, Kyoto, Japan) which indicated retention time of each peak detected and also calculated the area under each peak. A representative chromatogram obtained from a urine sample collected after infusion of gludopa is shown in Figure 2-2.

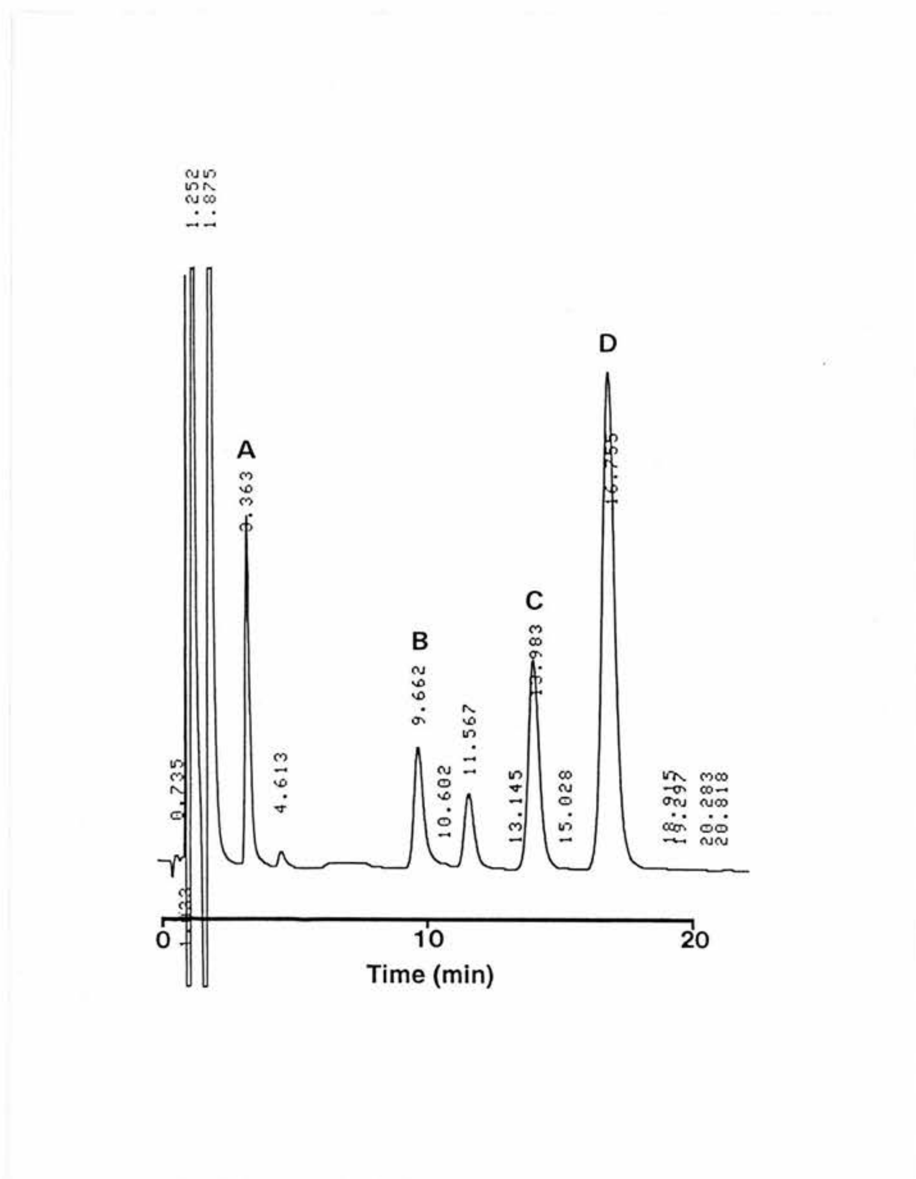


Figure 2-2. Chromatogram obtained from a urine sample collected after infusion of gludopa. The peaks are: A = dopa; B = DOPAC; C = dopamine; D = epinine (internal standard).

The dopa, dopamine, DOPAC and internal standard (epinine) peaks were identified by their relative retention times. The integrated area of each standard dopa, dopamine and DOPAC peak was divided by the area of the internal standard in the same sample. Standard plots of the ratios of peak areas *versus* the corresponding 40, 80, 120, 160 and 200 ng ml^{-1} concentrations of the standards were constructed to verify linearity of the relationship and goodness of fit for each metabolite. Linear regression analysis was then performed on the ratios against the standard concentrations, and the concentrations of dopa, dopamine and DOPAC in the test and quality control samples were derived from the corresponding regression line by computation. Urinary dopa, dopamine and DOPAC concentrations were converted and expressed in nmol l^{-1}. The intra- and inter-assay coefficients of variation were 2.9% and 3.7% for dopa; 1.0 % and 2.2 % for dopamine; 2.5 % and 2.5 % for DOPAC. The values of the lower limit of detection of the assays were 25, 26 and 30 nmol l^{-1} for dopa, dopamine and DOPAC respectively.

2.5.2 Measurement of Urinary 5-HT

5-HT in urine was measured by an 'in-house' modification of the method described by Jouve *et al.* [371]. It was selectively adsorbed onto Amberlite CG-50, a weakly acidic cation exchange resin, and eluted into ammonium acetate prior to its measurement by HPLC and spectrofluorometric detection. This eliminated the significant quantities of other fluorescent amines in urine such as tyrosine, phenylalanine, tryptophan and dopamine. *N*ω-methyl-5-hydroxytryptamine (*N*-methyl-5-HT) oxalate was used as the internal standard.

Reagents

Amberlite CG-50 (weakly acidic cationic exchanger, carboxylic acid active group, 100-200 wet mesh, H^+ ionic form). This cation exchange resin was washed and equilibrated with 0.1 M HCl prior to storage as follows: 250 g of amberlite was sprinkled into 2 litres of water and dispersed by magnetic stirring. This was then

transferred to a 5-litre Buchner clarifying funnel and the water was allowed to pass through the resin to waste. The resin was washed sequentially with 5 litres of 4 M HCl followed by 5 litres of water; 5 litres of 2 M NaOH followed by 5 litres of water; and 5 litres of 4 M HCl followed by 5 litres of water. The resin was finally equilibrated with 5-10 litres of 0.1 M HCl. It was removed from the Buchner funnel into a 5-litre beaker and 0.1 M HCl added so that the resin sediment occupied approximately 20% of the total volume. The final suspension was mixed vigorously to maintain homogeneity, dispensed into 1-litre glass bottles, and stored at 4° C.

Phosphate buffer (0.2 M, pH 8.0). The buffer was prepared by mixing 53 ml of 0.2 M sodium dihydrogen phosphate solution with 947 ml of 0.2 M disodium hydrogen phosphate solution. The 0.2 M sodium dihydrogen phosphate solution was made up by dissolving 1.87 g of sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) in 60 ml of water, and the 0.2 M disodium hydrogen phosphate was obtained by dissolving 28.39 g disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) in 1 litre of water.

Ammonium acetate (0.05 M, pH 5.0). 3.85 g of ammonium acetate was dissolved in 1 litre of water and the pH adjusted to 5.0 by addition of acetic acid.

Ammonium acetate (3 M, pH 5.0). 231 g of ammonium acetate was dissolved in 1 litre of water and the pH adjusted to 5.0 by addition of acetic acid.

Sodium hydroxide (1 M and 2 M). The 2 M solution was prepared by dissolving 80 g of sodium hydroxide (NaOH) pellets per litre of water. This was diluted with an equal volume of water to give 1 M NaOH solution.

HCl (4 M, 1 M and 0.1 M). These were prepared by diluting 5 M HCl with water.

Stock 5-HT standard (1 mg ml $^{-1}$). 20 mg of 5-hydroxytryptamine hydrochloride was dissolved in 20 ml of 1 M HCl and stored in 1.1 ml aliquots at -40° C.

Working 5-HT standards (50, 100, 150, 200 and 250 ng ml $^{-1}$). The stock 5-HT standard solution was diluted 1:40 with water and the resulting solution further diluted 1:100 to produce a solution containing 250 ng ml^{-1} of 5-HT. 5-HT

hydrochloride was then prepared over a concentration range of 50, 100, 150, 200 and 250 ng ml^{-1} in 10 ml volumes by adding 8, 6, 4, 2 and 0 ml of water to 2, 4, 6, 8 and 10 ml of the 250 ng ml^{-1} 5-HT solution respectively.

Stock* N*-methyl-5-HT internal standard (100 μg ml* $^{-1}$*). 10 mg of *N*ω-methyl-5-hydroxytryptamine oxalate was dissolved in 100 ml of 1 M HCl and stored in aliquots of 1.1 ml at -40° C.

Working* N*-methyl-5-HT internal standard (4 μg ml* $^{-1}$*). This was prepared by diluting the stock solution 1:25 with water.

Mobile phase used for HPLC. 4 litres of 0.1 M sodium acetate was prepared by adding 54.43 g of sodium acetate trihydrate to 4 litres of water. 5 litres of 0.1 M citric acid was prepared by dissolving 105 g of citric acid monohydrate powder in 5 litres of water. The 0.1 M citric acid was added to the 0.1 M sodium acetate solution until the pH was 3.5. Methanol 4% (v/v) was added. The solvent was filtered through a 0.22 μm Durapore membrane and degassed using helium for 3 min prior to use.

Sources of reagents. 5 M HCl solution, sodium hydroxide pellets, sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate anhydrous, ammonium acetate, acetic acid, sodium acetate trihydrate, citric acid monohydrate and methanol (all chemicals being of 'analaR' grade) were obtained from BDH Laboratory Supplies (Poole, U.K.). Amberlite CG-50, 5-hydroxy-tryptamine hydrochloride and *N*ω-methyl-5-hydroxytryptamine oxalate were obtained from Sigma Chemical Co. Ltd (Poole, U.K.).

Method

Sufficient Poly-Prep columns (4 cm x 0.8 cm i.d., with a 10-ml integral reservoir) (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, U.K.) were set out to receive the test urine samples and standard solutions of 5-HT. 3 ml of washed Amberlite CG-50 resin, which was kept dispersed in 0.1 M HCl acid by magnetic stirring, was added to each column and the acid was allowed to elute to waste. Phosphate buffer

(10 ml) was added to each column and allowed to elute to waste. The columns were then ready to receive the standard and test urine samples.

Aliquots (2 ml) of test urine samples (diluted as necessary) and standard solutions of 5-HT were transferred into a series of 5-ml tubes. The pH of each urine sample was raised to pH 8.0 by dropwise addition of 1 M NaOH. To each tube were then added 0.2 ml of the phosphate buffer and 0.2 ml of the internal standard (*N*-methyl-5-HT, 4 $\mu g\ ml^{-1}$). All the urine and standard samples were pipetted onto the prepared Bio-Rad columns and the effluents allowed to run to waste. The columns were sequentially washed with 5 ml of 0.05 M ammonium acetate, followed by 0.5 ml of 3 M ammonium acetate, each time allowing the effluents to run to waste. The columns were then inserted into 10-ml tubes and 3 ml of 3 M ammonium acetate was added to each column. The eluates were collected and vortex-mixed prior to analysis by HPLC with fluorometric detection.

Analytical system

HPLC was performed with a Beckman Altex solvent metering pump (Model 110A) delivering the mobile phase at 1 ml min^{-1}, an automatic sample injection module (Waters 712 WISP) programmed to inject 20 µl aliquots of samples at 20 min intervals, and a Waters Nova-Pak C_{18} Radial-Pak cartridge, 100 x 5 mm (i.d.), packed with 4 µm spherical C_{18} bonded silica-based materials and held under radial compression in a Waters RCM 8 x 10 cartridge holder. The column packing was protected by an inline Waters 2 µm metal frit filter and by a precolumn (Waters Guard-Pak precolumn insert Novo-Pak C_{18}). The eluate from the analytical column passed through the flow cell of a Perkin-Elmer LS-5 luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, Bucks, U.K.). The excitation wavelength was set at 284 nm and the emission wavelength at 314 nm. The excitation slit was 15 mm and the emission slit was 20 mm. The signals produced were fed to a Shimadzu C-R3A computing integrator. A representative chromatogram obtained from a urine sample collected after infusion of glu-5-HTP is shown in Figure 2-3.

The integrated area of the 5-HT peak obtained from each standard 5-HT solution and test urine sample was divided by the area of the internal standard in the same sample. The concentrations of the unknown test and quality control samples were calculated as described in section 2.5.1. The lower limit of detection of the assay for 5-HT was 45 nmol l^{-1}, and the intra- and inter-assay coefficients of variation were 5.6% and 11.6% respectively.

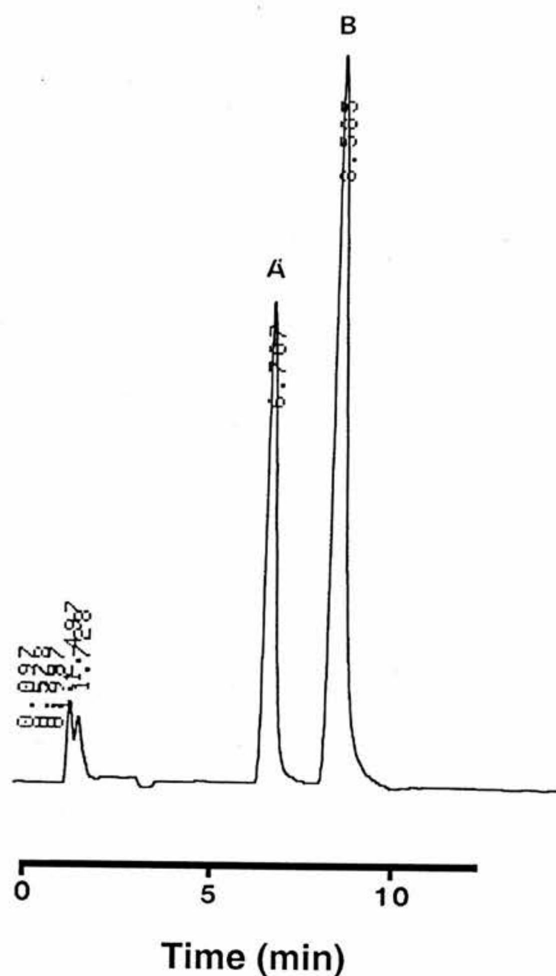


Figure 2-3. Chromatogram obtained from a urine sample collected after infusion of glu-5-HTP. The peaks are: A = 5-HT; B = *N*-methyl-5-HT (internal standard).

2.5.3 Measurement of Urinary 5-HTP

5-HTP in urine was measured by HPLC with spectrofluorometric detection. This was performed after simple dilution of the urine sample without the need for prior extraction because of the very high urinary 5-HTP concentrations following infusion of glu-5-HTP or L-5-HTP.

Reagents

HCl (1 M). 5 M HCl was diluted 1:5 with water.

Stock L-5-HTP standard (1 mg ml $^{-1}$). 20 mg of L-5-HTP was dissolved in 20 ml of 1 M HCl. It was stored in 1.1 ml aliquots at -40° C.

Working L-5-HTP standards (20, 40, 60, 80 and 100 ng ml $^{-1}$). The stock L-5-HTP standard was diluted 1:100 with water and the resulting solution further diluted 1:100 to produce a solution containing 100 ng ml $^{-1}$ of L-5-HTP. L-5-HTP was then prepared over a concentration range of 20, 40, 60, 80 and 100 ng ml $^{-1}$ in 2.0 ml volumes by adding 1.6, 1.2, 0.8, 0.4 and 0 ml of water to 0.4, 0.8, 1.2, 1.6 and 2.0 ml of the 100 ng ml $^{-1}$ L-5-HTP solution respectively.

Working epinine internal standard (10 μg ml $^{-1}$). The working internal standard was obtained as described in section 2.5.1.

Mobile phase used for HPLC. 4 litres of 0.1 M sodium acetate was prepared by adding 54.4 g of sodium acetate trihydrate to 4 litres of water. The pH was adjusted to pH 3.5 by addition of 0.1 M citric acid (105 g of citric acid monohydrate powder to 5 litres of water). 1-Octanesulphonic acid (100 mg l $^{-1}$) and methanol (4%) was added, and the mixture degassed using helium prior to use.

Sources of reagents. These were as described in sections 2.3, 2.5.1 and 2.5.2.

Method

0.1 ml of the working epinine internal standard (10 μg ml $^{-1}$) was added to 2 ml aliquots of test urine samples and standard L-5-HTP solutions (20 ,40, 60, 80 and 100 ng ml $^{-1}$). The urine samples obtained after infusion of glu-5-HTP or L-5-HTP

were diluted 1:250 and 1:500 with water prior to addition of epinine. 5-HTP was then measured by HPLC with spectrofluorometric detection using the same system as that described for measurement of 5-HT (*see* section 2.5.2). The fluorescence measurements were taken at 284 nm for excitation and 355 nm for emission. A representative chromatogram obtained from a urine sample collected after infusion of glu-5-HTP is shown in Figure 2-4. The limit of detection of the assay was 45 nmol l^{-1}, and the intra- and inter-assay coefficients of variation were 5.5% and 6.5% respectively.

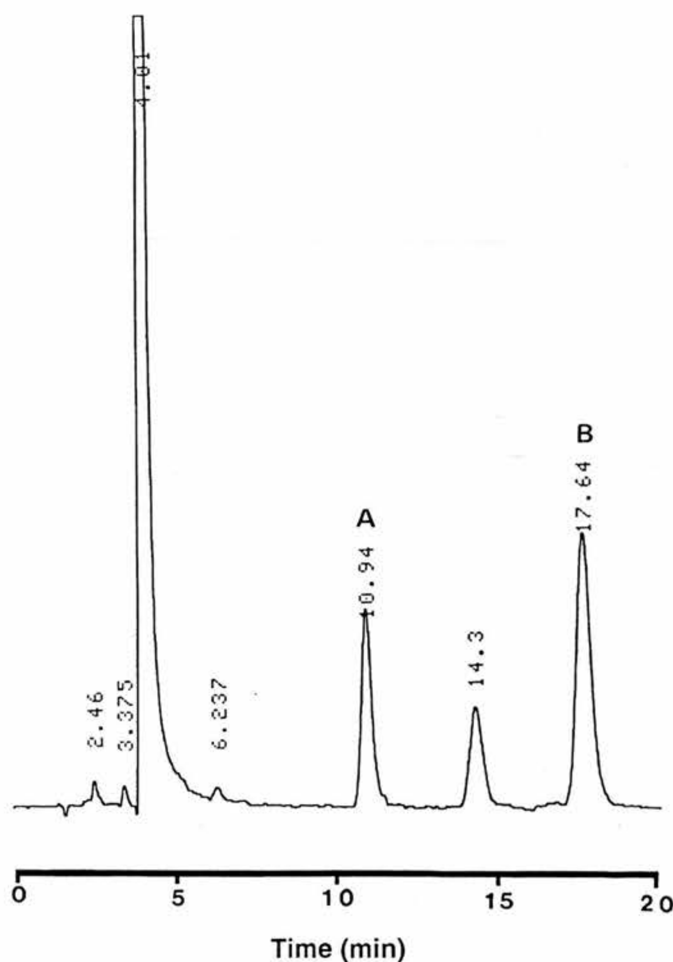


Figure 2-4. Chromatogram obtained from a urine sample collected after infusion of glu-5-HTP. The peaks are: A = 5-HTP; B = epinine (internal standard).

2.5.4 Measurement of 5-HTP and 5-HT in Platelet-Rich Plasma

5-HTP and 5-HT in PRP were assayed, after deproteinisation with perchloric acid containing L-cysteine, by HPLC with electrochemical detection using *N*-methyl-5-HT as the internal standard. These assays were kindly performed by Ms. N.J.T. Burns and Dr. B.C. Williams of the Department of Medicine, Western General Hospital, Edinburgh.

Reagents

Perchloric acid (15%) containing L-cysteine (2 mM, 242.4 mg l^{-1}).

Perchloric acid (1.5%) containing L-cysteine (0.2 mM).

Stock L-5-HTP, 5-HT and* N*-methyl-5-HT standards (10 μM). These stock solutions were prepared by dissolving L-5-HTP (2.20 mg l^{-1}), 5-hydroxytryptamine creatinine sulphate (3.87 mg l^{-1}) and *N*ω-methyl-5-hydroxytryptamine oxalate (2.80 mg l^{-1}) in a solution of perchloric acid (1.5%) and cysteine (0.2 mM).

Combined working L-5-HTP, 5-HT and* N*-methyl-5-HT standards (1 μM each). The stock solutions were diluted with 1.5% perchloric acid containing 0.2 mM L-cysteine to produce the combined working standards.

Mobile phase used for HPLC. The HPLC solvent (pH 4.8) contained the following: 0.1 M phosphate buffer (15.6 g l^{-1} of $NaH_2PO_4.2H_2O$), 1 mM of EDTA (372 mg l^{-1} of $Na_2EDTA.2H_2O$), 25 mg l^{-1} of 1-octanesulphonic acid and 5% v/v methanol. The solution was dissolved in double distilled water, filtered under vacuum using 0.45 μm filter and degassed at room temperature before use.

Sources of reagents. L-5-HTP, 5-hydroxytryptamine creatinine sulphate, Nω-methyl-5-hydroxytryptamine oxalate, L-cysteine free base and EDTA disodium salt were obtained from Sigma Chemical Co. Ltd (Poole, U.K.); sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) from BDH Laboratory Supplies (Poole, U.K.); 1-octanesulphonic acid sodium salt from FSA laboratory supplies (Fisons Scientific Apparatus, Loughborough, Leicester, U.K.); methanol and double distilled water, both HPLC grade, from Rathburn Chemicals Ltd (Walkerburn,

Peeble, U.K.); and perchloric acid (72%) from May and Baker Laboratory Chemicals (Dagenham, Essex, U.K.).

Method

Samples of PRP were thawed at room temperature and 400 μl aliquots removed. Each aliquot was spiked with 50 μl of 10 μM *N*-methyl-5-HT (internal standard) and the protein was precipitated by addition of 50 μl of a solution of 15% perchloric acid containing 2 mM L-cysteine. The samples were vortexed, left at 4° C for 15 min to allow complete precipitation of protein, and then centrifuged at 10,000 *g* and 4° C for 15 min. The supernatants were removed and stored in polypropylene tubes at 4° C, for not more than 24 h, until assayed for 5-HTP and 5-HT by HPLC and electrochemical detection.

Analytical system

The HPLC system consisted of a Waters 510 pump, an U6K injector, a Waters 150 x 3.9 (i.d.) mm μBondapak C_{18} reversed-phase analytical column (Waters Associates, Division of Millipore, Milford, MA, U.S.A.), a BAS LC-4A EC detector with a single glassy carbon electrode (Bioanalytical Systems, West Lafayette, NJ, U.S.A.) and a Waters Data Module. Before injecting the samples, the instrument was equilibrated by injection of 25 μl of the standard mixture containing 1 μM of L-5-HTP, 5-HT and the internal standard *N*-methyl-5-HT thus obtaining an elution profile. The standard mixture was then injected repeatedly at intervals throughout the day to check the consistency of retention time for each compound. A 25 μl extract from each sample was injected for HPLC measurement of all relevant compounds. The flow rate of the mobile phase was 2 ml min^{-1}. The detector potential was set at +0.6 V with 10 nA full scale deflection. Total elution time for all compounds was 40 min. The heights of the peaks in the standard or test samples were divided by the peak height of the internal standard in the same sample. The concentrations of 5-HTP and 5-HT in the test samples were derived by comparing the ratios of peak heights obtained in the test samples with

corresponding values obtained with the standard mixture containing 1 μM of each compound. The intra- and inter-assay coefficients of variation were: 0.6% and 5.6% respectively for L-5-HTP; 1.2% and 5.7% respectively for 5-HT.

2.5.5 Measurement of Urinary 5-HIAA

Urinary 5-HIAA was measured using the modified colorimetric method described by Goldenberg [372]. 5-HIAA, other phenolic acids and drug metabolites are extracted into diethyl ether from acidified urine. Sodium chloride is added to saturation to promote quantitative transfer of 5-HIAA into the ether phase. The 5-HIAA is then back-extracted into a phosphate buffer (0.1 M, pH 7.0) and reacted with 1-nitroso-2-naphthol in the presence of nitrous acid at 37° C to form a violet colour [373]. Phosphate buffer at pH 7 is chosen for efficient extraction of 5-HIAA since this improves with increasing pH reaching a maximum at about pH 6.8 and 5 HIAA becomes progressively more unstable at higher pH values [374]. In addition, urinary phenols lacking an acid group are not ionised at pH 7 and remain behind in the ether layer, thereby providing a relatively 'clean' 5-HIAA extract. An intense blue chromophore is formed upon subsequent addition of 2-mercaptoethanol and the intensity of the colour is proportional to the 5-HIAA concentration. Extraneous colours, caused by reactive phenols and indoleacetic acid, are removed by treatment with the mercaptoethanol and extraction into ethyl acetate. The absorbance maximum of the remaining blue solution occurs at 645 nm. Beer's law is not obeyed through a suitable range of 5-HIAA concentrations at this wavelength and a more useful linear range is obtained at 590 nm.

Reagents

HCl (1 M). This was prepared as described in section 2.5.3.

Phosphate buffer (0.1 M, pH 7.0). 5.18 g of potassium dihydrogen orthophosphate (KH_2PO_4) and 8.80 g of disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)

were dissolved in water and made up to a final volume of 1 litre.

1-Nitroso-2-naphthol (11.5 mM; 2 g l^{-1}). 200 mg of 1-nitroso-2-naphthol was dissolved in ethanol to a final volume of 100 ml and stored in a dark bottle at 4° C.

Sodium nitrite solution (0.36 M; 25 g l^{-1}). This was prepared by dissolving 2.5 g of sodium nitrite in water to make up a final volume of 100 ml.

Nitrous acid (0.014 M). This was prepared immediately before use by adding 1 M HCl to 1 ml of sodium nitrite solution (25 g l^{-1}) to make up a final volume of 25 ml.

2-Mercaptoethanol (3.58 M). This was prepared in a fume hood by diluting 25 ml of 2-mercaptoethanol (14.3 M) with water to a final volume of 100 ml. It was stored in the hood in a dark-brown bottle at room temperature.

Thiourea (0.013 M, 1 g l^{-1}). 1 g of thiourea was dissolved in water and the final volume adjusted to 1 litre.

Stock 5-HIAA standard (250 mg l^{-1}). 25 mg of 5-HIAA was dissolved in 100 ml of 1 M HCl, and stored in a dark-brown glass bottle at 4° C.

Working 5-HIAA standards (2, 6, 8 and 10 mg l^{-1}). This was prepared by diluting 4 ml of the stock standard to 100 ml with aqueous thiourea (1 g l^{-1}). The preparation is stable for 2 weeks when stored at 4° C in an amber-coloured bottle. 5-HIAA was then prepared over a concentration range of 2, 4, 6, 8 and 10 mg l^{-1} in 5 ml volumes by adding 4, 3, 2, 1 and 0 ml of thiourea to 1, 2, 3, 4 and 5 ml of the working 5-HIAA standard (10 mg l^{-1}) respectively.

Diethyl ether.

Sodium chloride dry powder.

Ethyl acetate (0.9 g ml^{-1}).

Sources of reagents. Potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate anhydrous, 5 M HCl, sodium nitrite, diethyl ether, sodium chloride, ethyl acetate and ethanol, all of analytical reagent grade, were obtained from BDH Laboratory Supplies (Poole, U.K.). 1-Nitroso-2-naphthol, 2-mercaptoethanol, thiourea and 5-HIAA were purchased from Sigma Chemical Co. Ltd, U.K.

Method

Sodium chloride (≈ 1.6 g), 2 ml of 1 M HCl and 10 ml of diethyl ether were added to 2 ml of deionised water (blank), test urine and standard samples in 25-ml glass stoppered Quickfit tubes (Mackay & Lynn, Edinburgh, U.K.). The tubes were capped, shaken mechanically in a horizontal position for 5 min at room temperature, and then centrifuged at 500 *g* for 3 min. 8 ml aliquots of the upper ether phase were transferred to a set of 10-ml Quickfit tubes and 1.6 ml of phosphate buffer was added. After shaking for 5 min at room temperature and centrifugation at 500 *g* for 3 min, the upper ether layer was aspirated to waste. 0.8 ml of the lower aqueous layer was transferred to a third set of tubes (care being taken not to carry over any ether), and 0.2 ml of 1-nitroso-2-naphthol reagent and 0.4 ml of the nitrous acid solution were added to each tube. All tubes were vortex-mixed and incubated in a water bath at 37° C for 5 min. 80 μl of 2-mercaptoethanol solution was then added. The tubes were vortexed and placed in a boiling bath for a further 5 min. At the end of the incubation period, 2 ml of ethyl acetate was added. Following vortex-mixing, the tubes were centrifuged at 500 *g* to separate the layers. The upper ethyl acetate layers were aspirated off and discarded. The lower aqueous layers were transferred to cuvettes and the absorbance of the standards and unknowns read against the water blank at 590 nm in an automatic recording spectrophotometer (Philips PU8720, Philips (Pye-Unicam) Ltd, Cambridge, U.K.). Standard responses were constructed automatically and the unknown responses read from these. A quality control sample of urine was incorporated into each run and any sample with a 5-HIAA level greater than 10 mg l^{-1} (52 μmol l^{-1}) was re-assayed after dilution of the urine. The lower limit of detection of the assay for 5-HIAA was 5.2 μmol l^{-1}. The intra- and inter-coefficients of variation were 4.5% and 7.3% respectively.

2.5.6 Measurement of Glu-TRP in Plasma and Urine

Glu-TRP was assayed both in plasma, after deproteinisation with perchloric acid,

and in urine by HPLC and spectrofluorometric detection.

Reagents

Glu-TRP standards (250, 500, 750 and 1000 ng ml $^{-1}$). Glu-TRP solution (5 mg ml^{-1}) was diluted 1:100 and the resulting solution further diluted 1:50 with water to produce a solution containing 1 μg ml^{-1} of glu-TRP. Glu-TRP was then prepared over a concentration range of 250, 500, 750 and 1000 ng ml^{-1} in 2 ml volumes by adding 1.5, 1.0, 0.5 and 0 ml of water to 0.5, 1.0, 1.5 and 2 ml of the 1 μg ml^{-1} glu-TRP solution respectively.

Mobile phase used for HPLC. The solvent consisted of 0.1 M sodium acetate (13.6 g l^{-1} of sodium acetate trihydrate) whose pH was adjusted to 3.3 by addition of 0.1 M citric acid (21 g l^{-1} of citric acid monohydrate). 1-Octanesulphonic acid (100 mg l^{-1}) and methanol (10%) were added and the mixture was degassed using helium.

Sources of reagents. These were as described in sections 2.3, 2.5.1 and 2.5.2..

Method

0.5 ml of 6% perchloric acid was added to 0.5 ml aliquots of test plasma samples. The mixtures were vortexed for 30 s and left to stand for 5 min at room temperature to precipitate the proteins. The supernatants were removed after centrifugation at 1500 *g* for 10 min. Glu-TRP was then measured by HPLC with spectrofluorometric detection at excitation and emission wavelengths of 280 and 336 nm respectively using the same system as that used for measurement of 5-HT (*see* section 2.5.2). The solvent stream was delivered at 1 ml min^{-1} and 20 μl aliquots of the prepared samples were injected into it at 20 min intervals. A standard plot of the ratios of peak areas *versus* the corresponding 250, 500, 750 and 1000 ng ml^{-1} concentrations of the standards was constructed to verify linearity of the relationship and goodness of fit. Linear regression analysis was performed on the peak areas against the standard concentrations, and the concentrations of glu-TRP were derived from the regression line by computation. A representative chromatogram obtained from plasma after infusion of glu-TRP is shown in Figure 2-5. All the plasma samples

after infusion of glu-TRP were assayed during one batch run and the intra-assay coefficient of variation was 4.1%. The lower limit of detection of the assay was 50 nmol l^{-1}. Glu-TRP was undetectable in urine using the present assay.

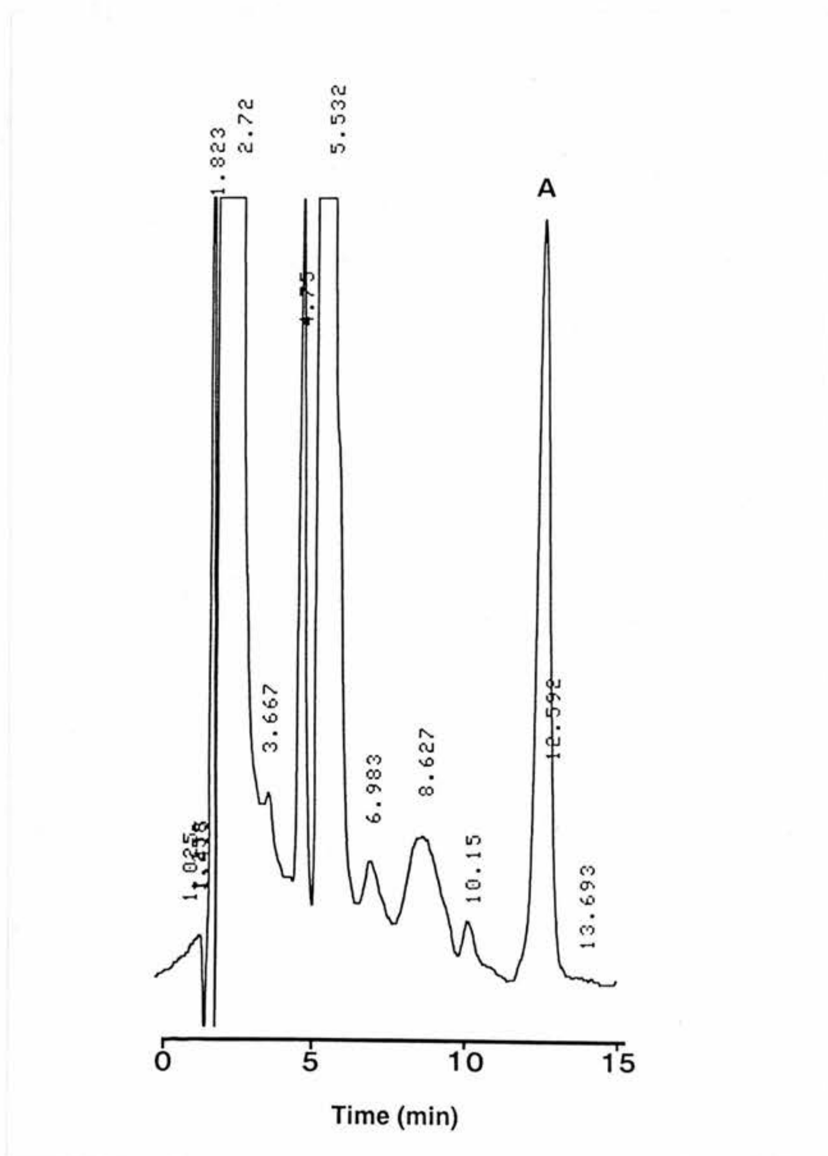


Figure 2-5. Chromatogram obtained from a plasma sample collected after infusion of glu-TRP. Peak A = glu-TRP.

2.5.7 Measurement of Tryptophan in Plasma and Urine

Tryptophan was assayed both in plasma (after deproteinisation with perchloric acid) and in urine by HPLC and spectrofluorometric detection using *O*-methyl-L-tyrosine as the internal standard.

Reagents

HCl (0.1 M and 1 M). These were prepared by diluting 5 M HCl with water.

Stock L-tryptophan standard (1 mg ml^{-1}). 20 mg of L-tryptophan was dissolved in 20 ml of 1 M HCl and stored in 1.1 ml aliquots at -40° C.

Working L-tryptophan standards (40, 80, 120, 160 and 200 ng ml^{-1}). The stock L-tryptophan standard was diluted 1:100 and the resulting solution further diluted 1:50 with 0.1 M HCl to produce a solution containing 200 ng ml^{-1} of L-tryptophan. L-tryptophan was then prepared over a concentration range of 40, 80, 120, 160 and 200 ng ml^{-1} in 1 ml volumes by adding 0.8, 0.6, 0.4, 0.2 and 0 ml of water to 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the 200 ng ml^{-1} L-tryptophan solution respectively.

Stock O-methyl-L-tyrosine internal standard (1 mg ml^{-1}). 50 mg of *O*-methyl-L-tyrosine was dissolved in 50 ml of 1 M HCl and stored in 1.1 ml aliquots at -40° C.

Working O-methyl-L-tyrosine internal standard (20 μg ml^{-1}). The stock internal standard was diluted 1:50 with water to produce a final concentration of 20 μg ml^{-1}.

Mobile phase used for HPLC. The solvent consisted of 0.1 M sodium acetate (13.6 g l^{-1} of sodium acetate trihydrate) whose pH was adjusted to pH 3.3 by addition of 0.1 M citric acid (21 g l^{-1} of citric acid monohydrate). Methanol (10%) was added and the mixture was degassed using helium prior to use.

Sources of reagents. These were as described in section 4.5.2. L-tryptophan ($C_{11}H_{12}N_2O_2$, FW 204.2) and *O*-methyl-L-tyrosine free base (4-methoxy-L-phenylalanine) were purchased from Sigma Chemical Co. Ltd (Poole, U.K.).

Method

0.5 ml of 6% perchloric acid was added to 0.5 ml aliquots of test plasma samples. The mixtures were vortexed for 30 s and left to stand for 5 min at room temperature to precipitate the proteins. After centrifugation at 1500 *g* for 10 min, 0.1 ml of the supernatants were removed and added to 4.9 ml of water (i.e. diluted 1:50). Urine samples were diluted 1:50 with water. 0.1 ml of the internal standard (20 μg ml^{-1}), was then added to 1 ml aliquots of the diluted protein-free plasma supernatants,

diluted urine samples and standard solutions of L-tryptophan (40, 80, 120, 160 and 200 ng ml^{-1}). Tryptophan was then measured by HPLC with spectrofluorometric detection at excitation and emission wavelengths of 280 and 336 nm respectively using the same system as that used for measurement of 5-HT (*see* section 2.5.2). The solvent stream was delivered at 1 ml min^{-1} and 40 µl aliquots of the prepared samples were injected into it at 15 min intervals. The intra- and inter-assay coefficients of variation were 3.8% and 4.6% respectively. A representative chromatogram obtained from a urine sample collected after infusion of glu-TRP is shown in Figure 2-6.

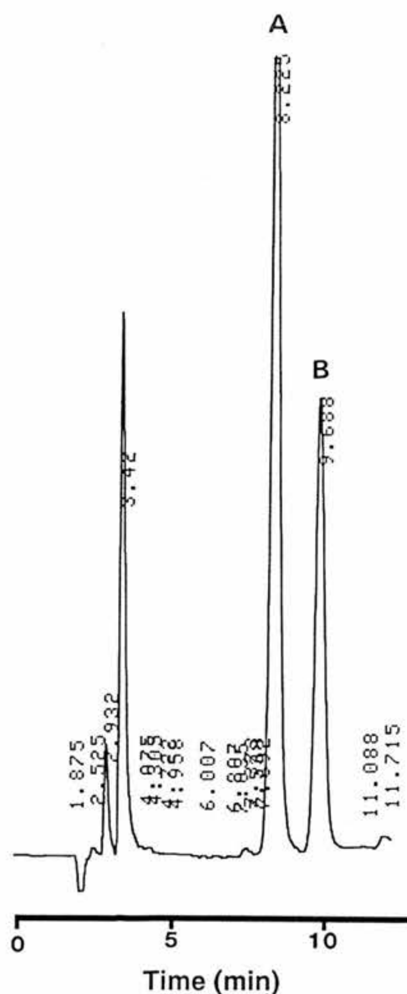


Figure 2-6. Chromatogram obtained from a urine sample collected after infusion of glu-TRP. The peaks are: A = *O*-methyl-L-tyrosine (internal standard); B = tryptophan.

2.5.8 Measurement of Polyfructosan in Plasma and Urine

Polyfructosan (Inutest) was measured using a modification of the method of Bacon and Bell [375]. Inulin, after hydrolysis with concentrated HCl at 80° C, generates a red colour when reacted with resorcinol. Ferric chloride ($FeCl_3$) was added to stabilise the colour. The intensity of the red colour is proportional to the inulin concentration and was estimated spectrophotometrically by absorbance at 480 nm.

Reagents

HCl/FeCl$_3$ solution. 7.5 mg of $FeCl_3$ was dissolved in 1 litre of concentrated HCl.

Resorcinol reagent (1.5 g l^{-1}). Resorcinol was dissolved in ethanol.

Perchloric acid (3% and 6%). Perchloric acid (60%) was diluted 1:10 and 1:20 with water to produce 6% and 3% perchloric acid respectively.

Stock Inutest standard (250 mg ml^{-1}). This was obtained from the manufacturer.

Working Inutest standards (100, 200, 300, 400 and 500 μg ml^{-1}). The stock Inutest standard was diluted 1:500 to produce a concentration of 500 μg ml^{-1} of Inutest. Inutest was then prepared over a concentration range of 100, 200, 300, 400 and 500 μg ml^{-1} in 0.5 ml volumes by adding 0.4, 0.3, 0.2, 0.1 and 0 ml of water to 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the 500 μg ml^{-1} Inutest solution.

Sources of reagents. Ferric chloride anhydrous and resorcinol (1,3-benzenediol) were obtained from Sigma Chemical Co. Ltd (Poole, U.K.). The other reagents were obtained as described in sections 2.3, 2.5.1 and 2.5.5.

Method

0.5 ml of 6% perchloric acid was added to 0.5 ml aliquots of test plasma samples. The mixtures were vortexed for 30 s and left to stand at room temperature for 5 min to precipitate the proteins. After centrifugation at 1500 *g* for 10 min, 0.5 ml of the supernatants were transferred to glass stoppered Quickfit tubes. 0.5 ml of 6% perchloric acid was also added to 0.5 ml of the standard solutions of Inutest (100, 200, 300, 400 and 500 μg ml^{-1}), and 0.5 ml aliquots of the resulting solutions

transferred to Quickfit tubes. Urine samples were diluted 1:20 with 3% perchloric acid by adding 0.1 ml of urine to 1.9 ml of 3% perchloric acid, and 0.5 ml aliquots of the diluted urine samples transferred to Quickfit tubes. 3 ml of resorcinol reagent and 3 ml of $HCl/FeCl_3$ solution were then added to each tube containing standard Inutest solutions, plasma supernatants or diluted urine samples. The tubes were vortex-mixed and incubated in a water bath at 80° C for 40 min. At the end of this period, the tubes were removed, cooled in tap water, and the extinction read against the blank at 480 nm in a spectrophotometer (Philips PU8720). The standard response was constructed automatically and plotted together with the test results of the unknown samples on a Philips S200 GP printer/plotter. The red colour developed is stable for 1 h and all samples were read within that time. The intra- and inter-assay coefficients of variation were 3.4% and 7.2% respectively for plasma samples, and 3.8% and 7.7% respectively for those from urine.

2.5.9 Measurement of *Para*-Aminohippuric acid in Plasma and Urine

PAH was assayed in deproteinised plasma and in urine by HPLC and spectrofluorometric detection using 3,4-dihydroxybenzylamine (DHBA) as the internal standard.

Reagents

Perchloric acid (6%). 60% perchloric acid was diluted 1:10 with water.

Stock PAH standard (1 mg ml^{-1}). This was prepared by diluting PAH solution (200 mg ml^{-1}) 1:200 with 1 M HCl and 1.1 ml aliquots frozen at -40° C.

Working urine PAH standards (125, 250, 500, 750 and 1000 ng ml^{-1}). The stock PAH standard was diluted 1:1000 with water to give a solution containing 1 μg ml^{-1} of PAH. PAH was then prepared over a concentration range of 125, 250, 500, 750 and 1000 ng ml^{-1} in 2 ml volumes by adding 1.75, 1.5, 1.0, 0.5 and 0 ml of water to 0.25, 0.5, 1.0, 1.5 and 2.0 ml of the 1 μg ml^{-1} PAH solution respectively.

Working plasma PAH standards (8, 16, 24, 32 and 40 μg ml^{-1}). The stock PAH standard was diluted 1:25 with PAH-free pooled plasma to produce a plasma

solution containing 40 μg ml^{-1} of PAH. Standard plasma PAH concentrations of 8, 16, 24, 32 and 40 μg ml^{-1} in 0.5 ml volumes were then prepared by adding 0.4, 0.3, 0.2, 0.1 and 0 ml of pooled normal plasma to 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the 40 μg ml^{-1} plasma PAH solution respectively.

Stock DHBA internal standard (1 mg ml^{-1}). 50 mg of 3,4-dihydroxybenzylamine hydrobromide was dissolved in 50 ml of 1 M HCl. It was stored in 1.1 ml aliquots (urine internal standard) and 3 ml aliquots (plasma internal standard) at -40° C.

Working urine DHBA internal standard (40 μg ml^{-1}). The stock DHBA standard solution was diluted 1:25 with water.

Working plasma DHBA internal standard (800 μg ml^{-1}). This was obtained by adding 2 parts of water to 8 parts of the stock plasma DHBA solution.

Mobile phase used for HPLC. The HPLC solvent is similar to that used for measurement of urinary L-5-HTP (*see* section 2.5.3) and consisted of 0.1 M citrate acetate buffer (pH 3.5), 100 mg l^{-1} 1-octanesulphonic acid and 4% v/v methanol.

Sources of reagents. 3,4-Dihydroxybenzylamine hydrobromide was obtained from Sigma Chemicals Co. Ltd (Poole, U.K.). The sources of other reagents were given in sections 2.3 and 2.5.1.

Method

0.1 ml of the plasma DHBA internal standard (800 μg ml^{-1}) was added to 0.5 ml aliquots of the test plasma samples and standard plasma PAH solutions (8, 16, 24, 32 and 40 μg ml^{-1}), followed by 0.5 ml of 6% perchloric acid. The mixtures were vortexed for 30 s, left to stand for 5 min at room temperature, and centrifuged at 1000 *g* for 10 min to deposit the proteins. The supernatants were removed and diluted 1:40 with water. Urine samples were diluted 1:4000 by adding 19.9 ml of water to 0.1 ml of urine and diluting 0.1 ml of the resulting solution with 1.9 ml of water. 0.1 ml of the urine DHBA internal standard (40 μg ml^{-1}) was then added to 2 ml aliquots of the diluted urine and the range of urine PAH standards.

Standard and test samples were measured by HPLC with fluorometric detection

using the same system as that used for measurement of urinary 5-HT (*see* section 2.5.2). The mobile phase was delivered at 1 ml min^{-1}, the injection volume was set at 20 μl, and fluorometric detection was performed with excitation and emission wavelengths set at 280 and 360 nm respectively. A representative chromatogram obtained from a plasma sample is shown in Figure 2-7. The intra- and inter-assay coefficients of variation were 2.8% and 4.0% respectively for plasma PAH, and 3.8% and 7.7% respectively for urinary PAH.

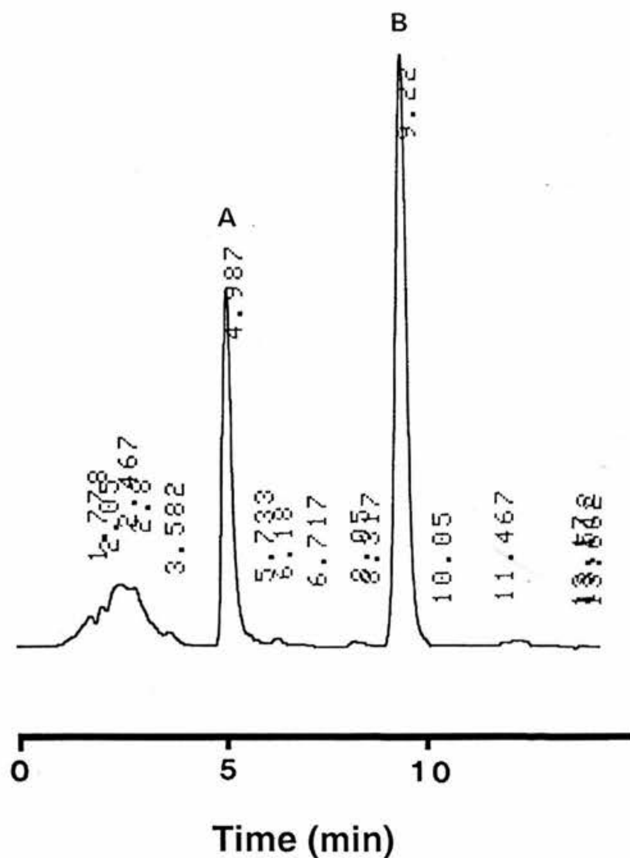


Figure 2-7. Chromatogram obtained from a plasma sample. The peaks are: A = PAH; B = 3,4-dihydroxybenzylamine (DHBA; internal standard).

2.5.10 Measurement of Plasma Renin Activity

Renin is a proteolytic enzyme produced by the juxtaglomerular cells of the kidney. In man, it cleaves on the Leu^{10}-Val^{11} peptide bond of angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-R), an α_2 macroglobulin synthesised in the liver, to produce the decapeptide angiotensin I (ANG I; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). Angiotensin converting enzyme (ACE) cleaves two additional amino acids (His-Leu) at the carboxyl end of ANG I to produce the biologically active octapeptide angiotensin II (ANG II; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). ANG II is rapidly broken down to smaller peptides including angiotensin III (ANG III; Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) by angiotensinases.

Incubation of plasma at 37° C in the presence of powerful inhibitors of ACE and angiotensinases allows the generation of ANG I but prevents its further degradation. EDTA and 8-hydroxyquinoline inhibit ACE and angiotensinase A, both of which are Ca^{2+} dependent; phenylmethylsulphonyl fluoride inhibits angiotensinase B; and dimercaprol, a metal chelator, is used to augment the action of EDTA and 8-hydroxyquinoline. The reaction is arrested after one hour by sudden cooling to 4° C. Generated ANG I is measured by radioimmunoassay (RIA) and PRA is expressed in ng of ANG I produced per ml of plasma per hour. The RIA is based on the competition of radioiodinated ANG I ($[^{125}I]$ANG I) and unlabelled ANG I for binding to the limited amount of antibody binding sites in a fixed amount of antiserum. As the quantity of ANG I in plasma or standard samples increases, the ability of the antibody to bind $[^{125}I]$ANG I decreases. Our 'in-house' method used for determination of PRA is based on that developed by Haber *et al.* [364].

Plasma samples were stored at -40 °C and assayed on ice (4° C) since prorenin can be activated to renin by exposure to temperatures between -5° C and +4° C [366]. Lysozyme is present in the buffer systems to prevent bacterial proliferation as they are a potent source of peptidases.

Reagents

Phosphate buffer (1 M, pH 5.3). 15.6 g of sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) was dissolved in 75 ml of water and the pH raised to 5.3 by addition of 1 M NaOH (40 g l^{-1}). The final volume was adjusted to 100 ml with water and 200 mg of EDTA disodium salt added.

Tris acetate buffer (0.1 M, pH 7.4). 6.05 g of tris(hydroxymethyl)aminomethane was dissolved in 480 ml of water. The pH was adjusted to 7.4 with acetic acid and the solution made up to a final volume of 500 ml with water. It was stored at 4° C.

Tris acetate buffer (0.1 M, pH 7.4) containing 0.5% BSA. This was prepared by dissolving 2 g of bovine serum albumin (BSA) in 500 ml of Tris acetate buffer.

Tris acetate buffer (0.1 M, pH 7.4) containing 0.5% BSA and 0.1% lysozyme chloride. This was obtained by dissolving 200 mg of lysozyme chloride in 200 ml of the above Tris acetate buffer containing 0.5% BSA for 1 h at room temperature.

Tris acetate buffer (0.1 M, pH 7.4) containing 0.25% BSA. Tris acetate buffer containing 0.5% BSA was diluted with an equal volume of Tris acetate buffer.

Dextran-coated charcoal. Dextran (100 mg) was dissolved in 50 ml of Tris acetate buffer (pH 7.4) kept on ice. When the dextran was in solution (≈ 5 min), 900 mg of charcoal was sprinkled into the solution and mixed by magnetic stirring for 45 min.

Phenylmethylsulphonyl fluoride 5% in ethanol. This was prepared by dissolving 100 mg of phenylmethylsulphonyl fluoride in 2 ml of ethanol. It was stored in a glass vessel at 4° C and renewed 3 monthly.

Dimercaprol (1 M). This was prepared by adding 0.2 ml of dimercaprol (1.25 g ml^{-1}) to 1.8 ml of ethanol. It was stored in glass at 4° C and renewed monthly.

8-Hydroxyquinoline. A saturated solution of 8-hydroxyquinoline free base in ethanol was prepared. It was stored at 4° C and renewed 3 monthly.

Protease inhibitor mix. The inhibitor mix consisted of phenylmethylsulphonyl fluoride 5% in ethanol (2 parts), dimercaprol 1 M solution in ethanol (1 part) and 8-hydroxyquinoline saturated solution in ethanol (one part).

Angiotensin I. ANG I (9 μg) was dissolved in 1 ml of water and 0.1 ml aliquots containing 900 ng of ANG I transferred to separate tubes. Each 900 ng aliquot was diluted to 20 ml with Tris acetate buffer containing 0.5% BSA and stored in 1 ml volumes (45 ng ml^{-1}) at -40° C. 45 ng of ANG I was further diluted with Tris acetate buffer containing 0.25% BSA to give a final volume of 25 ml. This working standard ANG I solution (180 pg 100 μl^{-1}) was stored in 0.8 ml aliquots at -40° C. It was double diluted using Tris acetate buffer containing 0.25% BSA to give a range of ANG I concentrations of 180, 90, 45, 22.5, 11.3 and 5.6 pg 100 μl^{-1}.

[^{125}I]angiotensin I. ANG I labelled with iodine^{125} ([^{125}I]ANG I; 1.25 μCi) was dissolved in 2 ml of Tris acetate buffer containing 0.25% BSA, followed by a 1:200 dilution to give the requisite amount of counts of 1500-2000 per 200 μl per minute.

Antibody to angiotensin I. This was prepared for use as a 1:20,000 dilution in Tris acetate buffer containing 0.25% BSA to give 50% binding to [^{125}I]ANG I.

Sources of reagents. Sodium dihydrogen orthophosphate dihydrate, sodium hydroxide pellets, EDTA disodium salt, tris(hydroxymethyl)aminomethane, acetic acid 'glacial', ethanol (all chemicals being of analytical reagent grade) and charcoal ('Norit GSX') were obtained from BDH Laboratory Supplies (Poole, U.K.). Bovine serum albumin (RIA grade, fraction V powder, 96-99% albumin), lysozyme chloride, dextran (average MW 77,800), phenylmethylsulphonyl fluoride, dimercaprol (2,3-dimercaptopropanol; BAL; British anti-lewisite; 1.25 g ml^{-1}), and 8-hydroxyquinoline (free base) were purchased from Sigma Chemical Co. Ltd (Poole, U.K.). Standard ANG I (9 μg per ampoule) was obtained from the National Institute of Biological Standards and Control (Potters Bar, Herts, U.K.). [^{125}I]ANG I (1.25 μCi per ampoule) was supplied by CIS UK Ltd (High Wycombe, Bucks, U.K.). Antibody to ANG I was raised in rabbit and kindly provided by the late Dr. Frank Goodwin (London Hospital Medical College, U.K.).

Method

Plasma samples were allowed to thaw at room temperature and kept at 4° C on ice.

The phosphate buffer (40 μl) was added to duplicate 200 μl aliquots of plasma samples followed by the inhibitor mix (10 μl). After thorough mixing, the samples were incubated at 37° C for exactly 1 h in a shaking water bath to allow generation of ANG I by the action of renin on angiotensinogen. The samples were then placed on an ice-tray and 1 ml of water kept at 4° C was added to arrest the generation of ANG I. Plasma dilution at this stage was 1:6.25.

To 100 μl volumes of these plasma samples or standard solutions of unlabelled ANG I (5.6, 11.3, 22.5, 45, 90 and 180 pg 100 μl^{-1}) were added 400 μl of Tris acetate buffer containing 0.5% BSA and 0.1% lysozyme chloride, 200 μl of [^{125}I] ANG I, and 200 μl of antibody. 200 μl of plasma and 300 μl of Tris acetate buffer containing 0.5% BSA and 0.1% lysozyme chloride were used if PRA was expected to be low. Test plasma samples and standards were prepared in duplicate. In addition, tubes were prepared containing only 200 μl of [^{125}I]ANG I to give total counts; antibody, [^{125}I]ANG I and buffer to assess the efficiency of binding of [^{125}I]ANG I to antibody and to provide the zero point on the standard curve; and two extra tubes contained only buffer and [^{125}I]ANG I to assess the efficiency of charcoal to adsorb free [^{125}I]ANG I and as a background check for how much non-angiotensin antibody was present. All the tubes were covered and incubated at 4° C for 24 h to allow any binding of labelled and unlabelled ANG I to the antibody to reach equilibrium. Dextran-coated charcoal (0.5 ml) was then added to each tube, except for the 'total count' tubes. Free ANG I will bind to dextran-coated charcoal whereas the antibody-bound ANG I will not. After mixing and standing for 5 min at 4° C, all tubes were centrifuged at 1500 *g* and 4° C for 15 min. The supernatants containing antibody-bound ANG I were carefully decanted into a fresh set of tubes.

The radioactivity of the antigen/antibody complex was counted for 1 min using an automated gamma counter (LKB-Wallac 1275 Mini Gamma, Selsdon, Surrey, U.K.). The 'antibody-bound counts' of the samples were expressed as a percentage of total counts. A standard curve can be obtained by plotting these values against

the standard ANG I concentrations and the concentrations of test samples could be determined from the curve. This was performed automatically through a RIA spline incorporated with the gamma counter. Results of paired test samples were averaged within the spline and the concentrations, derived from the standard plot, were printed on a FACIT 4510 serial matrix printer (LKB-Wallac). The values in pg 100 μl^{-1} were multiplied by a factor of 6.25 to take account of the plasma dilution and by a factor of 0.01 to express PRA as ng of ANG I generated per ml of plasma per h. The lower detection limit of the assay was 0.35 ng ANG I ml^{-1} h^{-1}, and the intra- and inter-assay coefficients of variation were 5.2% and 8.6% respectively.

2.5.11 Measurement of Plasma Aldosterone

Plasma aldosterone was measured by a single antibody solid-phase RIA without an extraction step using a commercially available kit ('Coat-A-Count Aldosterone', Diagnostics Products Corporation, Los Angeles, CA, U.S.A.).

All samples were analysed in duplicate. Aliquots (200 μl) of undiluted test plasma samples or standard concentrations of aldosterone (0, 25, 50, 100, 200, 600 and 1200 pg ml^{-1}) were added to polypropylene tubes coated with antibody to aldosterone, followed by 1 ml of radioactive [^{125}I]aldosterone. Controls for non-specific binding and total counts were incorporated. After gentle mixing, the tubes were incubated at 37° C for 3 h during which aldosterone in plasma or standard samples competed with [^{125}I]aldosterone for antibody binding sites. The supernatants were then decanted and the radioactivity of the antigen/antibody complexes retained in the tubes was measured using an automated gamma counter (LKB-Wallac 1282 Compugamma) which incorporated a RIA spline. A standard curve was automatically constructed and the sample concentration derived from it as described in section 2.5.10 for measurement of PRA. The lower detection limit of the assay was 10 pg ml^{-1}, and the intra- and inter-assay coefficients of variation were 5.0% and 7.0% respectively.

2.5.12 Measurement of Serum Growth Hormone

Serum human growth hormone (hGH) was measured by an immunoradiometric assay (IRMA) using a commercially available kit (ELSA-HGH, CIS UK Ltd, High Wycombe, U.K.). The IRMA is based on two mouse monoclonal anti-hGH antibodies raised against sterically remote sites on the hGH molecule. The first non-labelled antibody is bound to a polystyrene fin located in the tube. The hGH in standard or serum samples is allowed to bind to this antibody. Following addition of the second ^{125}I-radiolabelled anti-hGH antibody, the hGH is 'sandwiched' between the two types of antibodies. The amount of bound radio-labelled antibody is directly proportional to the concentration of hGH.

50 μl aliquots of test serum and standard concentrations of hGH (0, 0.2, 2, 10, 25 and 50 ng ml^{-1}) were added to the tubes provided, followed by 300 μl of radiolabelled antibody against hGH. Each tube was incubated at room temperature for 2 h, decanted and then washed three times with 3 ml of 'Tween 20' to remove any unbound label. The radioactivity was measured using a gamma counter (LKB-Wallac 1275 Mini Gamma). The concentrations of hGH in the test samples were determined from the standard plot constructed using the values obtained with standard concentrations of hGH. The intra- and inter-assay coefficients of variation of the assay were 2.8% and 4.0% respectively.

2.5.13 Measurement of Sodium and Potassium in Plasma and Urine

Sodium and potassium were measured by ion-selective electrode potentiometry using a dedicated analyser (Radiometer KNA-1 sodium-potassium analyser, Crawley, West Sussex, U.K.). This instrument measures sodium and potassium simultaneously by using K^+ and Na^+ ion-selective electrodes with a potassium chloride reference source. An automatic two point calibration for both electrolytes was initially performed using standard electrolyte concentrations, setting sodium measurements between 40 and 152 mmol l^{-1}, and potassium measurements between

4 and 80 mmol l^{-1}. Quality control plasma and urine samples containing low and high levels of the electrolytes were analysed to ensure that the results were within the stated ranges before running the test plasma and urine samples. The urine samples were diluted 1:3 before analysis with a Radiometer diluent as this helped to stabilise pH and ionic strength. The initial calibration was repeated after every 5 samples. Results in mmol l^{-1} were printed automatically by a Radiometer PRS 12 printer. The intra- and inter-assay coefficients of variation were 1.0% and 1.1% respectively for both sodium and potassium.

2.6 Calculation of Renal Clearance

Clearance is defined as the virtual volume of plasma from which a given substance is completely removed per unit time [376-378]. Renal clearance is derived by dividing total urinary excretion by the plasma concentration during the collection interval, i.e. $C = UV/P$, where C is the clearance in units of ml of plasma cleared of the substance per minute, U and P are the concentrations of the substance in urine and plasma respectively, and V is the urine flow rate in ml min^{-1}. An alternative formula is $C = A_e/AUC$, where A_e is the amount of the substance excreted in the urine and AUC is the area under the plasma concentration *versus* time curve during the clearance period. If a substance is not affected by the renal tubules, the amount found in urine will equal the amount filtered by the glomerulus per minute. Inulin, a polymer of fructose derived from plant tubers (MW $\approx$ 5,200), is not excreted or reabsorbed by the tubules and is cleared from plasma exclusively by glomerular filtration. Its renal clearance or that of the related substance polyfructosan (a polymer of fructose derived from algae; MW $\approx$ 3,000) is used as a measure of GFR. The clearance of a substance completely reabsorbed by tubules, e.g. glucose, is zero. In general, substances having clearances less than that of inulin undergo filtration and net tubular reabsorption, and those with clearances greater than that of inulin undergo filtration and net tubular secretion. PAH is almost completely extracted

from blood in one passage through the kidney and its clearance is used as a measure of renal plasma flow. The proportion of PAH in renal arterial blood excreted during a single passage through the kidney (extraction ratio) varies between species, and is between 90 and 95% in normal humans. The incomplete extraction is mainly due to shunting of blood through the inert perirenal fat and capsule, and recirculation of this small proportion of PAH makes the measured clearance a slight underestimate of the true renal plasma flow. This systematic source of error is acknowledged by using the term *effective* or *estimated* renal plasma flow (ERPF).

Effective renal plasma flow. ERPF was estimated by calculating the renal clearance of PAH (C_{PAH}) and the value was adjusted to a body surface area of 1.73 m^2. The body surface area for an individual was calculated using the formula given by Dubois and Dubois [379], $A = W^{0.425} \times H^{0.725} \times 71.84/10000$ where A is surface area in m^2, W is weight in kg, and H is height in cm.

Glomerular filtration rate. GFR was estimated by calculating the renal clearance of polyfructosan (Inutest) (C_{In}) and corrected to a body surface area of 1.73 m^2.

Filtration fraction (FF). FF was calculated as $(C_{In}/C_{PAH}) \times 100\%$.

Fractional clearance. The fractional clearance of a substance was defined as (Clearance/GFR) x100%. Thus, the fractional excretion of sodium, FE_{Na}, was calculated as renal clearance of sodium divided by GFR (C_{In}) and expressed as a percentage. FE_{Na} represents the percentage of the filtered sodium which is excreted in the urine and allows for changes in the filtered load of sodium.

2.7 Statistical Analysis

Descriptive statistics. Mean and standard deviation (SD) values were used to summarise the data unless otherwise stated. The latter usually follow a 'normal' or 'Gaussian' distribution and the normality of the data can be checked using the Shapiro-Wilk W test. Coefficient of variation is calculated by (SD/mean) x 100%.

Student's paired t test. This test was used where the data consist of two

measurements made on each of a number of subjects either before and after treatment or receiving each of two experimental conditions. The 95% confidence interval of the difference between the means (95% CI_{diff}) indicates the range of possible values within which the true difference is likely to lie. The P value was corrected for multiple comparisons by the Bonferroni method and the associated 99% CI_{diff} values quoted where appropriate. Non-parametrically distributed data were log-transformed before analysis.

Two-way analysis of variance. Two-way analysis of variance (ANOVA) was performed to identify simultaneously the component of total variance due to subjects and treatments. It was used in preference to Student's paired t test where a comparison between more than two treatments or time points of the same treatment was to be made at least in the preliminary analysis.

Repeated Measures ANOVA. The variables measured serially on the different experimental days were analysed by three-way repeated measures ANOVA giving the effects of subjects, type of infusion (treatment), time, and the interaction of treatment and time. This procedure allows for the non-independence of successive data points in experiments involving repeated measurements on a subject on one occasion, or on more than one occasion after different treatments. When inequality of variances between data sets was detected, the number of degrees of freedom for the F-ratio was adjusted using the Huynh-Feldt epsilon [380]. If there was a significant overall treatment or treatment x time interaction difference between the types of infusion, follow-up paired comparison between the different infusion regimens were made to determine where the differences lie, with correction of the P value by the Bonferroni method to allow for multiple comparisons and protect against a type 1 error.

All the statistical analyses in this thesis were performed using SPSS/PC+ 5.0 statistical software package (SPSS Inc, Chicago, IL, U.S.A.), and effects were considered to be statistically significant when P values were less than 0.05.

CHAPTER THREE

A PRELIMINARY COMPARISON OF 5-HYDROXY-L-TRYPTOPHAN AND γ-L-GLUTAMYL-5-HYDROXY-L-TRYPTOPHAN

3.1 Introduction

Parenteral administration of 5-HT has been employed to investigate the renal actions of 5-HT in man. Variable results have been reported but most studies have suggested that 5-HT produces a fall in renal blood flow, GFR, urine output and sodium excretion [55,70,77; *see* sections 1.2.6 and 1.2.7). 5-HT, however, has a wide spectrum of actions in the body and the renal responses to infusion of pharmacological doses of 5-HT may be largely modified by, or may possibly be due to, confounding influences produced by effects in other tissues arising from an increase in circulating amine. In addition, peripherally administered 5-HT may be taken up avidly into platelets and/or efficiently metabolised in the pulmonary and hepatic circulation [1,35,176]; it may, therefore, not reach the kidney in concentrations adequate to produce demonstrable effects.

Administration of 5-HT precursors which are selectively converted to 5-HT within the kidney may allow the renal actions of 5-HT to be assessed separately from any systemic effects resulting from intravenous infusion of 5-HT. It has been demonstrated in rats that 5-HT is formed intrarenally from its immediate precursor L-5-HTP, under the action of renal LAAD, and that the amine reduces renal blood flow, urine output and sodium excretion [153,166,181,254,268]. Administration of L-5-HTP is, however, associated with adverse gastrointestinal and other effects in man and this may limit the dose of L-5-HTP that can be given [162,359-362]. Furthermore, unlike 5-HT, it readily crosses the blood-brain barrier enhancing synthesis of 5-HT in the brain [156,175,362,363], and may exert central effects.

The γ-glutamyl prodrug approach to targeting drugs to the kidney offers the possibility of delivering drugs selectively to the kidney with little or no systemic effects [324,325,329; *see* section 1.3). Previous work has shown that gludopa, the glutamyl derivative of L-dopa, is a renal dopamine prodrug which is sequentially converted in the kidney by γGT to L-dopa and decarboxylated by LAAD to dopamine (Figure 3-1) [329-331,333,334,341]. This prodrug has allowed the renal

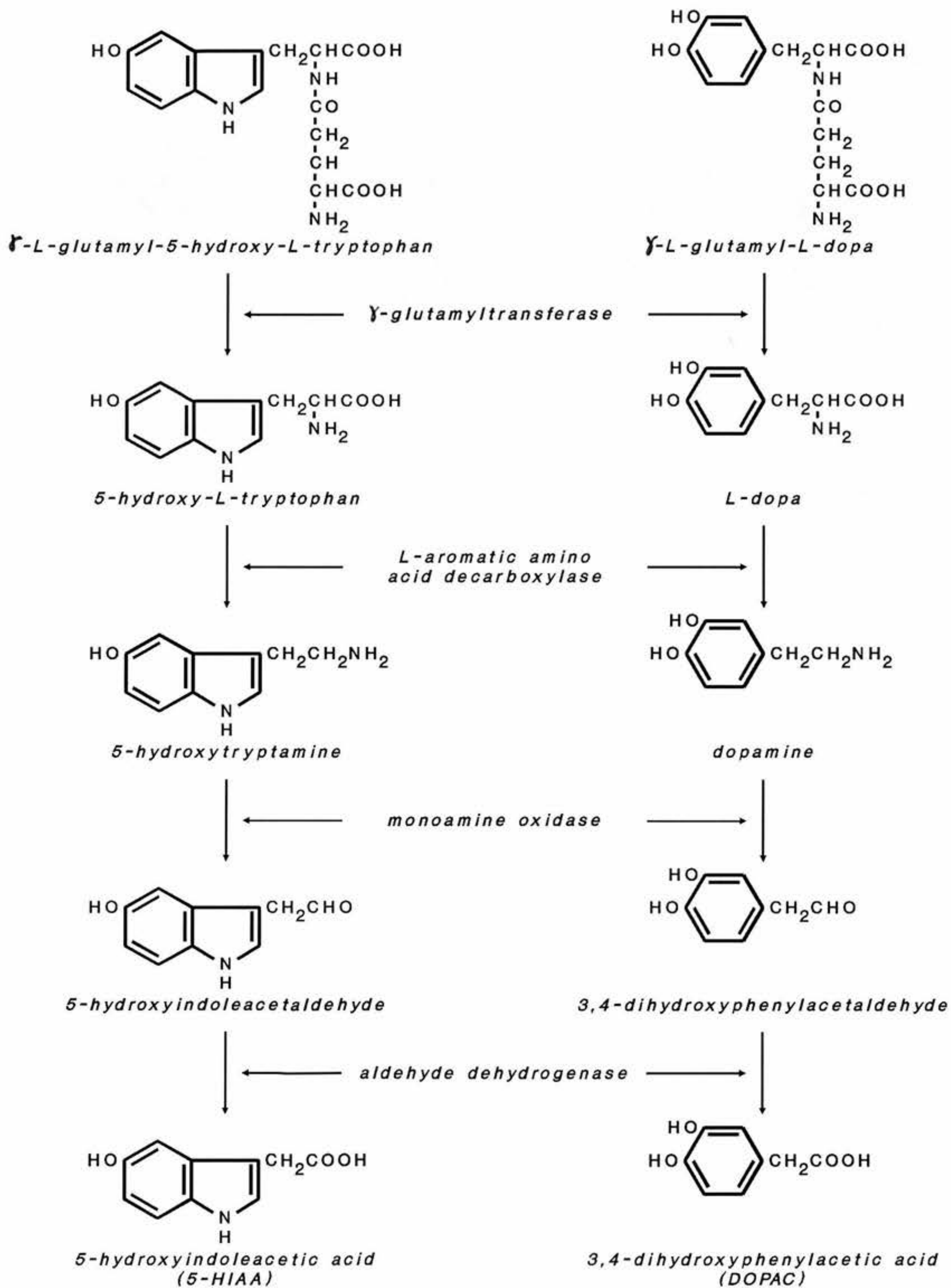


Figure 3-1. Possible metabolism of glu-5-HTP. Comparison with gludopa.

effects of dopamine to be explored *in vivo* in man [334-342]. In an analogous way, γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP), the glutamyl derivative of L-5-HTP, may be a putative 5-HT renal prodrug, which is converted intrarenally by γGT to L-5-HTP and then decarboxylated by LAAD to 5-HT (Figure 3-1). Our hypothesis was that glu-5-HTP may be a more renally selective 5-HT prodrug than L-5-HTP and that it may enable the renal actions of 5-HT to be assessed with less confounding extrarenal effects.

This small preliminary study compared the effects of intravenous infusions of equimolar amounts of glu-5-HTP and L-5-HTP with infusion of saline (placebo) in normal man. We were interested in observing changes in urinary excretion of 5-HTP, 5-HT, 5-HIAA, sodium, PRA and plasma aldosterone concentrations, as well as the tolerability of the two compounds.

3.2 Methods

Five healthy male volunteers, aged 22 to 32 years (mean 28 years) and weighing 61.5 to 99.5 kg (mean 71.3 kg), took part in this randomised, placebo-controlled, cross-over study. They each attended on 3 separate days, at least 1 week apart. They refrained from alcohol for 24 h, abstained from xanthine-containing drinks from 18.00 h, and fasted from 22.00 h the evening before each study day. They continued with their usual diet. They arrived at the clinical investigation unit at about 08.15 h having drunk 250 ml of tap water 1 h previously. They received intravenous 0.9% NaCl at a rate of 10 ml kg^{-1} h^{-1} for 6 h to increase urinary sodium excretion. After a 3 h run-in period (time = 3 h), an equimolar amount (45 nmol kg^{-1} min^{-1}) of L-5-HTP (10 μg kg^{-1} min^{-1}) or glu-5-HTP (16.6 μg kg^{-1} min^{-1}) in 0.9% NaCl was administered intravenously for 1 h. Placebo was saline alone at 0.5 ml min^{-1}. The subjects remained supine or semi-recumbent throughout the study except when standing up to pass urine. Accurately timed consecutive urine collections of about 30 min duration were made during the last half hour of the run-

in phase (period 2.5-3.0 h), during the administration of the test infusions (period 3.0-3.5 h and period 3.5-4.0 h), and for 2 h after the completion of the infusions (period 4.0-4.5 h to period 5.5-6.0 h). Urine volumes were recorded and aliquots removed and stored at -40°C for analysis of sodium, 5-HTP, 5-HT and 5-HIAA. Venous blood samples for estimation of PRA and aldosterone were taken at 2, 3, 3.5, 4, 5 and 6 h. Pulse rate and blood pressure were measured using a Dinamap semi-automated recorder with the subject in a supine position. Recordings were made half-hourly during the first 3 h, every 5 min during the test infusion, every 15 min for the next hour, and half-hourly afterwards. The average values for each 30 min period were calculated and used in the analysis. PRA, plasma aldosterone concentrations, and urinary sodium, 5-HTP, 5-HT and 5-HIAA concentrations were measured as described in section 2.5. Glu-5-HTP and L-5-HTP were prepared for use in 0.9% saline with 0.5% human albumin as a carrier. Blood was taken before and after the study for measurements of renal and liver functions (creatinine, electrolytes, bilirubin, alanine transaminase, γGT, alkaline phosphatase) and haematological indices (Hb, differential leucocyte count, platelets).

Results are given as means ± SD. Differences in the cumulative urinary metabolite data following L-5-HTP and glu-5-HTP were compared by Student's t test for paired observations and the 95% CI_{diff} of the means quoted. The urinary sodium, pulse rate, blood pressure and aldosterone data between the 3 treatment days were compared by repeated measures ANOVA for overall statistical significance. Values of $P < 0.05$ were considered statistically significant.

3.3 Results

Urinary excretion rates of 5-HTP, 5-HT and 5-HIAA for each 30 min period are shown in Table 3-1 and Figure 3-2. 5-HTP was not detectable in the urine during infusion with saline alone. Marked increases in urine 5-HTP occurred after infusion of both L-5-HTP and glu-5-HTP. The 5-HTP excretion was higher for all collection periods after infusion of glu-5-HTP when compared with L-5-HTP, and the 3-6 h cumulative urinary excretion of 5-HTP after administration of glu-5-HTP was 2.9 times that after glu-5-HTP infusion ($P < 0.001$) (Table 3-2). The mean urinary excretion rate of 5-HT was ≤ 0.4 nmol min^{-1} during the baseline period (2.5-3.0 h) on the 3 experimental days and did not change during infusion of placebo. It increased to a peak value of 293 ± 32 nmol min^{-1} after administration of L-5-HTP and 215 ± 17 nmol min^{-1} following infusion of glu-5-HTP. The 3-6 h cumulative 5-HT excretion after L-5-HTP infusion was not significantly different from that after glu-5-HTP (95% CI_{diff}: −1.9 to 7.2 µmol). Urinary 5-HIAA excretion increased after both compounds when compared with placebo infusion. The 3-6 h cumulative excretion of 5-HIAA after L-5-HTP infusion was greater than that after glu-5-HTP ($P < 0.05$). During the 3-6 h collection period, 41 ± 2% (on a molar basis) of the infused dose of L-5-HTP and 52 ± 8% of administered glu-5-HTP were recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA (Table 3-3).

The urinary sodium excretion rates prior to infusion of placebo, L-5-HTP and glu-5-HTP were 275 ± 115, 210 ± 68 and 280 ± 105 µmol min^{-1} respectively (Table 3-1). The post-infusion sodium excretion rates are presented in Figure 3-3 as changes from baseline (period 2.5-3.0 h) to allow for the lower baseline values on the L-5-HTP infusion day. Urinary sodium excretion increased progressively throughout all clearance periods in response to infusion of saline on the placebo day. Glu-5-HTP significantly attenuated the increase in sodium excretion when compared with placebo ($P < 0.001$) whereas L-5-HTP caused a reduction in mean urinary sodium output which was not statistically significant. The 3-6 h cumulative

natriuresis values after infusion of placebo, L-5-HTP and glu-5-HTP were 65.0 ± 19.1, 47.4 ± 16.9 and 46.5 ± 16.1 mmol respectively. The corresponding changes in cumulative sodium excretion, relative to the baseline excretion rates, on the 3 experimental days were +15.5 ± 6.5, +9.7 ± 14.1 and −3.8 ± 6.6 mmol. Cumulative natriuresis was reduced by 5.7 ± 11.9 mmol (95% CI_{diff}: −9.0 to 20.5 mmol; $P = 0.3$) after L-5-HTP infusion and by 19.3 ± 6.2 mmol (95% CI_{diff}: 11.5 to 27.0 mmol; $P < 0.005$) after glu-5-HTP when compared with placebo infusion.

Plasma aldosterone was significantly increased by L-5-HTP infusion ($P < 0.05$) but not after glu-5-HTP (Figure 3-4). PRA was undetectable (< 0.35 ng ANG I $ml^{-1} h^{-1}$) in two subjects on all three study days. In the other three subjects, PRA declined from 1.00 ± 0.49 ng ANG I $ml^{-1} h^{-1}$ at baseline to 0.68 ± 0.28 ng ANG I $ml^{-1} h^{-1}$ at the end of placebo infusion and 0.61 ± 0.22 ng ANG I $ml^{-1} h^{-1}$ one hour later. Equivalent values were 1.21 ± 0.34, 0.91 ± 0.34 and 0.70 ± 0.34 ng ANG I $ml^{-1} h^{-1}$ on the L-5-HTP day, and 1.16 ± 0.20, 0.66 ± 0.22 and 0.48 ± 0.19 ng ANG I $ml^{-1} h^{-1}$ on the glu-5-HTP day. Statistical analysis was deemed inappropriate due to the small number of subjects with detectable PRA values.

Two subjects complained of nausea at the end of L-5-HTP infusion but none had any adverse reactions with glu-5-HTP. There were no significant changes in pulse rate or blood pressure throughout the study (Table 3-4). No significant changes in measurements of liver and renal functions or haematological indices and no untoward effects were observed at 1 week after the end of the study.

Table 3-1. Mean (SD) urinary excretion rates of 5-HTP, 5-HT, 5-HIAA and sodium for each 30 min period before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo, L-5-HTP and glu-5-HTP (n = 5). A value of 0 is entered when 5-HTP was below the limit of detection of the assay.

	Time (h)						
	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
5-HTP excretion (nmol min^{-1})							
Placebo	0	0	0	0	0	0	0
L-5-HTP	0	129.4	185.8	113.2	75.6	38.3	29.9
		(42.5)	(59.0)	(28.1)	(37.9)	(12.3)	(11.9)
Glu-5-HTP	0	240.5	512.4	396.6	241.8	144.3	95.0
		(37.9)	(65.6)	(86.4)	(16.2)	(8.1)	(11.0)
5-HT excretion (nmol min^{-1})							
Placebo	0.4	0.4	0.4	0.4	0.4	0.3	0.3
	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)
L-5-HTP	0.3	200.3	293.2	184.9	119.3	60.8	42.4
	(0.1)	(89.3)	(31.9)	(23.5)	(63.5)	(27.0)	(22.3)
Glu-5-HTP	0.4	68.3	196.4	215.3	150.3	96.5	65.6
	(0.2)	(23.6)	(68.9)	(17.4)	(22.4)	(11.9)	(13.4)
5-HIAA excretion (nmol min^{-1})							
Placebo	17.8	18.0	18.4	18.8	16.8	15.9	15.8
	(5.2)	(4.4)	(6.9)	(5.0)	(5.3)	(6.2)	(6.0)
L-5-HTP	14.2	149.2	295.1	299.4	248.5	159.0	118.5
	(4.9)	(62.8)	(103.9)	(45.8)	(57.2)	(30.3)	(41.3)
Glu-5-HTP	17.8	81.1	167.8	208.2	182.2	154.3	122.4
	(6.6)	(62.3)	(42.2)	(37.5)	(28.2)	(31.5)	(16.5)
Sodium excretion (μmol min^{-1})							
Placebo	275.1	310.7	332.8	357.1	365.8	381.5	413.5
	(115.4)	(123.2)	(119.8)	(107.0)	(105.1)	(85.2)	(111.1)
L-5-HTP	209.6	219.2	230.9	235.1	251.5	289.4	302.0
	(67.5)	(71.7)	(69.9)	(79.2)	(99.6)	(127.7)	(134.8)
Glu-5-HTP	279.6	296.3	253.4	238.6	232.7	270.0	275.4
	(104.5)	(105.5)	(62.9)	(55.9)	(66.6)	(133.4)	(147.7)

Table 3-2. The 3-6 h cumulative excretion values (μmol) of 5-HTP, 5-HT and 5-HIAA after administration of placebo, L-5-HTP and glu-5-HTP. Values shown are means ± SD (n = 5).

	Infusion			*L-5-HTP vs glu-5-HTP*
	Placebo	L-5-HTP	Glu-5-HTP	95% CI_{diff}; P value
5-HTP	0	17.1 ± 5.1	50.0 ± 4.4	27.9 to 38.0; P < 0.001
5-HT	0.06 ± 0.01	26.9 ± 6.2	24.2 ± 3.1	−1.9 to 7.2; P = 0.19
5-HIAA	3.1 ± 0.8	38.1 ± 8.8	27.6 ± 3.2	1.4 to 19.7; P < 0.05

Table 3-3. Percentage urinary recoveries (%) of infused L-5-HTP and glu-5-HTP as 5-HTP, 5-HT and 5-HIAA. Values shown are means ± SD (n = 5).

	Infusion		*L-5-HTP vs glu-5-HTP*
	L-5-HTP	Glu-5-HTP	95% CI_{diff}; P value
5-HTP	8.8 ± 1.6	26.5 ± 4.9	12.1 to 23.3; $P < 0.001$
5-HT	13.9 ± 0.6	12.6 ± 1.3	-0.5 to 2.9; $P = 0.12$
5-HIAA	18.1 ± 2.9	12.9 ± 2.2	1.5 to 9.0; $P < 0.05$
Total	40.8 ± 1.9	52.0 ± 7.6	1.6 to 20.9; $P < 0.05$

Table 3-4. Mean (SD) pulse rate and blood pressure values before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo, L-5-HTP and glu-5-HTP (n = 5).

				Time (h)			
	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
Placebo infusion							
Pulse rate	55.2	54.5	54.8	53.5	55.1	54.4	55.0
(beats min^{-1})	(9.1)	(8.4)	(8.0)	(8.9)	(9.2)	(9.6)	(9.9)
Systolic blood	117.8	116.4	117.1	116.1	115.1	120.8	122.4
pressure (mmHg)	(14.7)	(11.6)	(12.6)	(11.4)	(10.9)	(13.1)	(16.5)
Diastolic blood	71.0	68.9	69.2	70.0	70.9	71.2	73.4
pressure (mmHg)	(12.0)	(9.9)	(11.6)	(11.1)	(11.9)	(11.1)	(13.4)
L-5-HTP infusion							
Pulse rate	53.4	53.1	54.4	53.4	52.0	52.6	53.2
(beats min^{-1})	(6.7)	(6.5)	(9.0)	(7.5)	(6.4)	(7.2)	(8.7)
Systolic blood	114.2	110.7	115.8	116.3	116.2	116.4	116.2
pressure (mmHg)	(5.8)	(7.3)	(8.4)	(10.5)	(8.4)	(6.4)	(6.4)
Diastolic blood	67.8	68.6	69.4	70.0	69.0	68.4	72.4
pressure (mmHg)	(8.6)	(7.8)	(7.8)	(8.2)	(7.3)	(6.5)	(7.7)
Glu-5-HTP infusion							
Pulse rate	53.0	52.3	52.6	53.5	52.2	52.4	51.6
(beats min^{-1})	(10.0)	(7.7)	(7.3)	(9.8)	(7.8)	(7.0)	(7.1)
Systolic blood	116.2	114.1	113.9	112.9	114.3	116.8	119.0
pressure (mmHg)	(12.1)	(11.9)	(10.6)	(8.7)	(9.4)	(10.6)	(14.3)
Diastolic blood	69.4	66.4	70.2	65.3	70.6	70.0	71.2
pressure (mmHg)	(12.2)	(6.5)	(7.2)	(8.0)	(9.4)	(9.3)	(8.8)

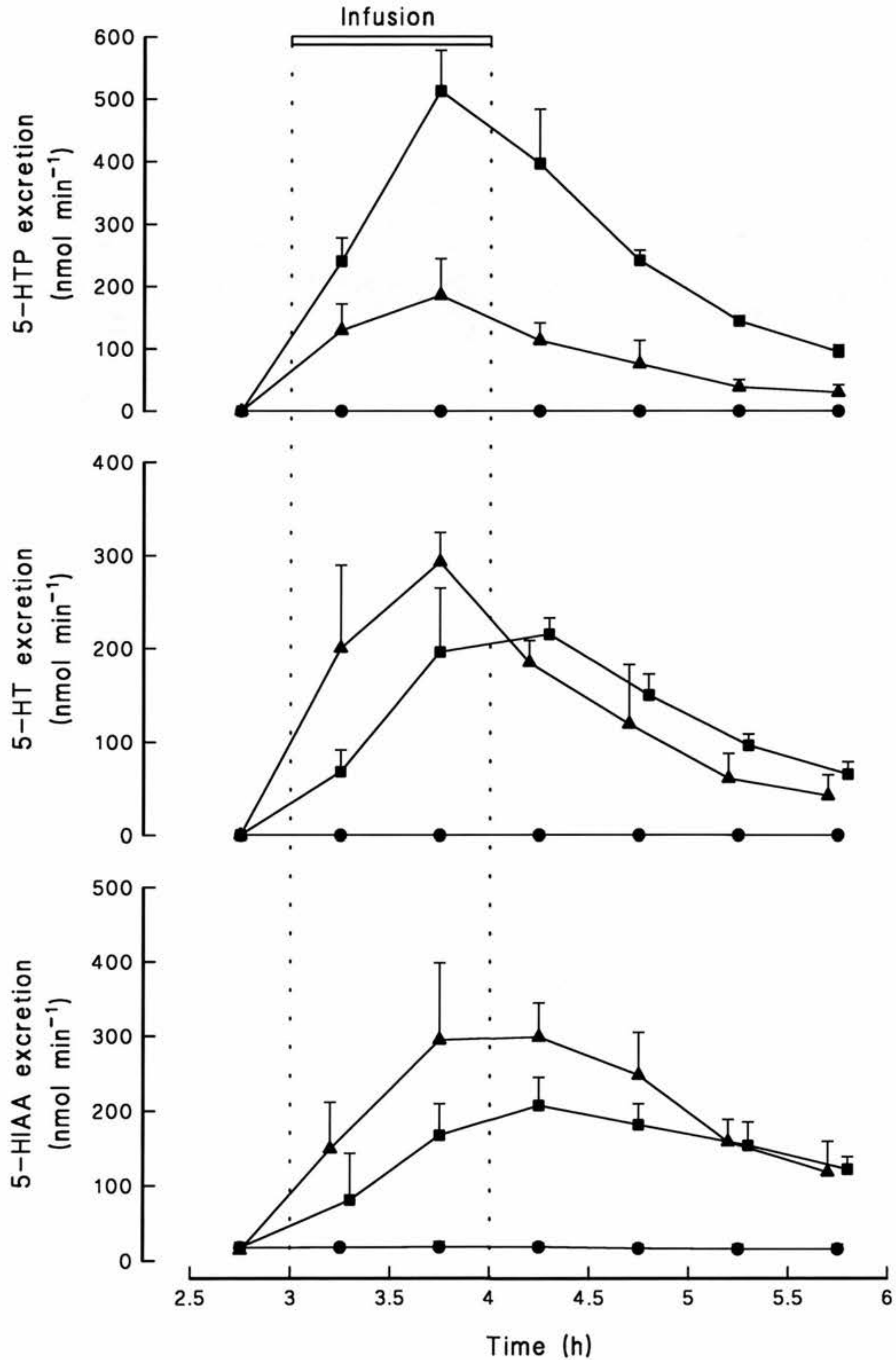


Figure 3-2. Urinary excretion rates of 5-HTP, 5-HT and 5-HIAA before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 5).

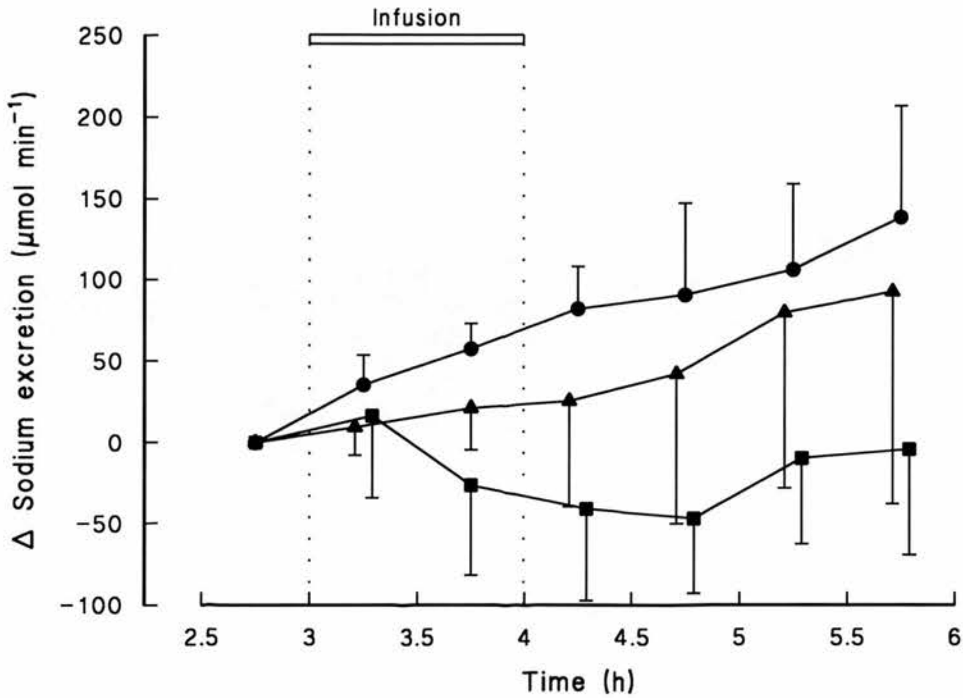


Figure 3-3. Changes (Δ) in urinary excretion of sodium from baseline (period 2.5-3.0 h) following infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 5).

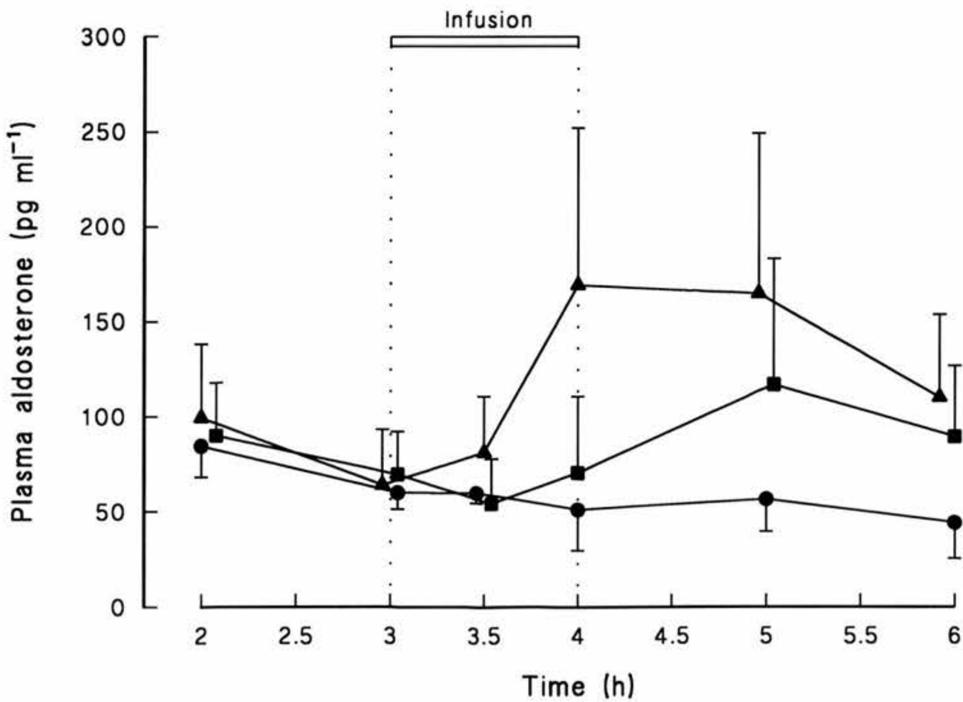


Figure 3-4. Plasma aldosterone concentration following infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 5).

3.4 Discussion

Urinary excretion of 5-HT increased markedly during and after infusion of L-5-HTP and glu-5-HTP. This finding suggests that 5-HT was synthesised in the human kidney from both putative 5-HT renal prodrugs since extrarenal production of 5-HT cannot account completely for the high urinary levels of 5-HT. 5-HT produced extrarenally and presented to the blood stream would be rapidly deaminated by MAO-A in the pulmonary and hepatic circulation or sequestered into platelets leaving little free 5-HT available for filtration at the renal glomerulus or secretion by the tubules [1,35,176]. The theory that circulating 5-HT would be rapidly destroyed is supported by previous observations that intravenous administration of 5-HT resulted in only a small increase in blood 5-HT concentration (equivalent to approximately 2% of the infused amount) and had virtually no effect on the level of free 5-HT in urine [161,162]. Moreover in the carcinoid syndrome, where there is an increase in circulating 5-HT, urinary 5-HT excretion is usually less than 1% of 5-HIAA excretion [107].

Urine 5-HTP levels were higher after the administration of glu-5-HTP than with L-5-HTP. This indicates that the amount of L-5-HTP delivered to the kidney for renal decarboxylation was greater following the infusion of glu-5-HTP and is in keeping with it being relatively more selective for the kidney than L-5-HTP. Cumulative urinary 5-HT excretion was not significantly different between L-5-HTP and glu-5-HTP infusions. Urinary 5-HIAA excretion was higher after infusion of L-5-HTP than with glu-5-HTP. These urinary metabolite data would be consistent with the greater extrarenal metabolism of L-5-HTP than glu-5-HTP and with the hypothesis that glu-5-HTP was less accessible to extrarenal catabolic enzymes. About 41% of the infused L-5-HTP was recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA during the three hour collection period in this study whereas the recovery of the three measured metabolites averaged 52% after glu-5-HTP. The quantitative difference indicates that the two compounds are handled

differently in the body and is consistent with greater delivery of the glutamyl compound to the kidney. The lower recovery value with L-5-HTP may be due to uptake of L-5-HTP or its metabolites into other body compartments such as the brain or circulating platelets. It is also possible that more of the L-5-HTP than glu-5-HTP was converted to products which were not measured in this study (e.g. sulphate and glucuronide conjugates).

Glu-5-HTP significantly attenuated urinary excretion of sodium. Mean urinary excretion of sodium after L-5-HTP infusion was lower than that after placebo infusion but the difference was not statistically significant. The failure to demonstrate a significant antinatriuresis with L-5-HTP in this study may, however, be due to a type 2 error because of the small number of subjects investigated. The greater antinatriuretic effect produced by the glutamyl derivative suggests that 5-HT generated intrarenally, following administration of this dipeptide, is more effective despite the similar increments in mean cumulative 5-HT excretion produced by L-5-HTP and glu-5-HTP. The retention of sodium, presumably mediated by local intrarenal synthesis 5-HT, may be a consequence of a reduction in renal blood flow, a fall in GFR or an increased tubular reabsorption of sodium. These questions are addressed in the next study (*see* Chapter 4).

L-5-HTP increased plasma aldosterone levels without any concomitant rise in PRA in keeping with previous studies in man that the release of aldosterone by L-5-HTP is independent of activation of the renin-angiotensin system [96,97]. This effect on aldosterone may be due to direct stimulation of the adrenal cortex [100-104] and/or may be mediated by central 5-HT pathways as L-5-HTP can cross the blood-brain barrier [96,97]. L-5-HTP also stimulates the release of other hormones such as prolactin, ACTH and growth hormone, providing further evidence that it can act centrally [78,361,381]. In contrast, glu-5-HTP caused a small, non-significant and delayed increase in aldosterone level in keeping with its effects being largely restricted to the kidney. The delayed rise in aldosterone concentration

following glu-5-HTP may be due to the conversion of the glutamyl compound to L-5-HTP or 5-HT in the kidney followed by recirculation of L-5-HTP or 5-HT. It is also possible that there may be some γGT [382] and LAAD activity in the adrenal gland [31,39] and 5-HT produced locally could stimulate aldosterone release. Release of aldosterone is unlikely to be responsible for the observed antinatriuresis after glu-5-HTP as the glu-5-HTP induced rise in aldosterone occurred later than the reduction in sodium output. In addition, no significant retention of sodium occurred after L-5-HTP infusion despite the higher plasma aldosterone concentrations. L-5-HTP has been reported to increase PRA in the dog and rat [88,92-94] but no increase was observed after its administration in human subjects [96,97]. Suppression of renin, in the present study, often to levels not detectable by our assay system, probably resulted from the sodium chloride infusion given to our subjects. Both renin and aldosterone release are suppressed by oral [364,383,384] and intravenous [385-390] sodium chloride loading. Expansion of extracellular fluid volume by saline infusion produces greater suppression of renin and aldosterone than equivalent expansion with non-saline containing solutions such as dextran or glucose [390]. There is some evidence that the chloride ion may perhaps be more important than sodium in mediating the renin response to saline infusion, given that sodium bicarbonate and potassium bicarbonate failed to inhibit renin release in the rat, in contrast to sodium and potassium chloride [391].

Two out of the five subjects complained of nausea at the end of L-5-HTP infusion. This and other gastrointestinal symptoms such as abdominal pain, vomiting and diarrhoea which have been reported by others following L-5-HTP administration are probably mediated by enteric or central 5-HT_3 receptors [162,359-362]. The dose of L-5-HTP used in our study was chosen in an attempt to minimise adverse gastrointestinal and cardiovascular effects. Glu-5-HTP, in equimolar amount, caused no adverse effects, providing further evidence that it is more renally selective and has less systemic toxicity. There were no significant

changes in pulse rate or blood pressure with either compound.

In conclusion, the results of this study indicate that 5-HT can be generated intrarenally in man and that it is antinatriuretic. Glu-5-HTP appears to be more selective for the kidney than 5-HTP and causes less systemic effects. This glutamyl compound may allow the renal formation and effects of 5-HT to be evaluated in man with little confounding extrarenal effects.

CHAPTER FOUR

A COMPARISON OF THE RENAL AND NEUROENDOCRINE EFFECTS OF 5-HYDROXY-L-TRYPTOPHAN AND γ-L-GLUTAMYL-5-HYDROXY-L-TRYPTOPHAN

4.1 Introduction

We demonstrated in the previous small study, involving 5 healthy volunteers, that both L-5-HTP and its glutamyl derivative, glu-5-HTP, produced marked increases in the urinary excretion of 5-HT consistent with intrarenal synthesis of 5-HT (*see* Chapter 3). Glu-5-HTP was significantly antinatriuretic whereas L-5-HTP caused a reduction in mean urinary sodium output which was not statistically significant. Our data also suggested that the glutamyl compound has greater renal selectivity than L-5-HTP. The present more extensive study, using a modified protocol, was designed to investigate further the effects of intravenous infusions of equimolar amounts of glu-5-HTP and L-5-HTP in normal man. In this study, we measured several additional parameters. Effective renal plasma flow (ERPF), glomerular filtration rate (GFR) and fractional excretion of sodium (FE_{Na}) were estimated in order to elucidate the antinatriuretic actions of these 5-HT precursors. Urine dopamine excretion was assessed since LAAD, the enzyme converting L-5-HTP to 5-HT, is also involved in the synthesis of dopamine from L-dopa [31,37,41-44]. In addition, we measured serum growth hormone concentration as a possible indicator of the central effect of these two 5-HT precursors since previous studies demonstrated that L-5-HTP causes release of growth hormone [361,381,392-396].

4.2 Methods

Nine healthy male volunteers, aged 21-30 years (mean 25.7 years) and weighing 63.4 to 96.9 kg (mean 71.2 kg), were each studied on 3 separate occasions (5-14 days between study days) in this randomised, placebo-controlled, cross-over study. They refrained from alcohol for 24 h, abstained from xanthine-containing drinks from 18.00 h, and fasted from 22.00 h the evening before each study day. They were maintained on their normal diet but were advised to consume the same diet in the 24 h before they attended. They arrived at the clinical investigation unit at about 08.00 h on each study day, having drunk 500 ml of tap water 1 h previously.

Priming doses of PAH (0.5 g) and polyfructosan (Inutest; 3.5 g), added to 0.9% NaCl to make up a total volume of 40 ml, were infused over 8 min using a Braun Perfusor VI infusion pump. This was followed by a maintenance infusion of PAH (3.75 g l^{-1}) and polyfructosan (4.5 g l^{-1}) in saline at a constant rate of 5 ml min^{-1} using an IMED volumetric infusion pump throughout the experiment to allow estimation of ERPF and GFR. After a 3 h run-in period, an intravenous infusion of an equimolar amount (45 nmol kg^{-1} min^{-1}) of L-5-HTP (10 μg kg^{-1} min^{-1}) or glu-5-HTP (16.6 μg kg^{-1} min^{-1}) in saline made up to a total volume of 30 ml was administered at 0.5 ml min^{-1} for 1 h. Placebo was 30 ml of saline alone. These were administered in a single blind, randomised order. In addition to the saline administered intravenously, the subjects drank 150 ml water half-hourly. These measures were used to promote an adequate natriuresis and diuresis. The subjects remained semi-recumbent or supine throughout the experiment except when standing up to micturate.

Accurately timed consecutive urine collections of about 30 min duration were made during the last hour of the run-in phase (period 2.0-2.5 h and period 2.5-3.0 h), during the administration of the test infusions (period 3.0-3.5 h and period 3.5-4.0 h) and for 2 h afterwards (period 4.0-4.5 h to period 5.5-6.0 h). Urine volumes were recorded and aliquots stored at -40°C for analysis of sodium, potassium, PAH, polyfructosan, 5-HTP, 5-HT, 5-HIAA, dopamine and DOPAC. Urine samples for 5-HT, 5-HIAA, dopamine and DOPAC were acidified ($pH < 3$) with 5 M HCl to prevent their oxidation. Venous blood samples were taken at 0, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 h for measurement of plasma electrolytes, PAH and polyfructosan; at 2, 3, 3.5, 4, 5 and 6 h for determination of PRA and plasma aldosterone; and at 3, 4, 4.5 and 5 h for measurement of serum growth hormone. Objective recordings of blood pressure and pulse rate were made in duplicate, with the subject in the supine position, using a Dinamap semi-automated recorder every half hour during the experiment except during and for one hour after the test

infusions when they were measured every 15 min. The mean values for each half hour period were calculated and used in the subsequent statistical analysis. Plasma aldosterone, PRA, serum growth hormone, plasma and urinary sodium, potassium, PAH and polyfructosan, and urinary 5-HTP, 5-HT, 5-HIAA, dopamine and DOPAC were measured as described in section 2.5. Blood was taken before and after the study for measurements of renal and liver functions (creatinine, electrolytes, bilirubin, aspartate transaminase, γGT, alkaline phosphatase) and haematological indices (Hb, differential leucocyte count, platelets).

Renal clearance was calculated using the standard formula UV/P, where U is the urine concentration, V is the urine flow rate and P is the mean of the plasma levels at the beginning and end of each clearance period (*see* section 2.6). ERPF and GFR were estimated by calculating the renal clearances of PAH (C_{PAH}) and polyfructosan (C_{In}) respectively. The filtration fraction (FF) was calculated as (C_{In}/C_{PAH}) x 100%. FE_{Na} was calculated as renal clearance of sodium divided by GFR (C_{In}) and expressed as a percentage.

Results are given as means ± SD. The data between the 3 experimental days were analysed by repeated measures ANOVA for overall statistical significance. Two-way ANOVA was employed to identify any differences between treatments at an individual time point. Differences in the cumulative urinary metabolite data following L-5-HTP and glu-5-HTP were analysed by Student's t test for paired observations. The growth hormone values were log transformed before analysis because of the marked skewing of the data. Values of $P < 0.05$ were considered statistically significant.

4.3 Results

Figure 4-1 shows the urinary excretion rates of 5-HTP, 5-HT and 5-HIAA for each 30 min period. The exact values for each collection period are given in Table 4-1. 5-HTP, which was undetectable in the urine during infusion of saline alone, increased markedly after administration of both L-5-HTP and glu-5-HTP. Urinary excretion of 5-HTP after infusion of glu-5-HTP was higher than that following L-5-HTP for all collection periods. Cumulative 5-HTP excretion over the 3 h following the start of the glu-5-HTP infusion was 3.2 times that after L-5-HTP infusion ($P < 0.001$) (Table 4-2). The mean urinary excretion rates of 5-HT prior to infusion of placebo, L-5-HTP and glu-5-HTP were similar at 0.5 nmol min^{-1}. There was no significant change in 5-HT excretion after the infusion of placebo. 5-HT excretion rose to a peak value of 349 ± 117 nmol min^{-1} during administration of L-5-HTP and 301 ± 44 nmol min^{-1} after glu-5-HTP. The 3-6 h cumulative urinary 5-HT excretion after L-5-HTP infusion was not significantly different from that following glu-5-HTP infusion. 5-HIAA was not detectable in the urine before administration of the test infusions. Marked increases in urinary 5-HIAA occurred after administration of L-5-HTP and glu-5-HTP. The 3-6 h cumulative 5-HIAA excretion values were higher following administration of L-5-HTP than after glu-5-HTP ($P < 0.01$). During the 3-6 h collection period, $47 \pm 5\%$ of infused L-5-HTP and $66 \pm 7\%$ of glu-5-HTP were recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA (Table 4-3). Urinary dopamine and DOPAC excretion rates were unaffected by L-5-HTP or glu-5-HTP when compared with placebo (Table 4-1).

The absolute and fractional excretion of sodium increased progressively on the placebo day in response to infusion of saline (Table 4-1 and Figure 4-2). Both L-5-HTP ($P < 0.01$) and glu-5-HTP ($P < 0.005$) significantly attenuated the increase in sodium excretion when compared with placebo. FE_{Na} was likewise reduced by L-5-HTP ($P < 0.025$) and glu-5-HTP ($P < 0.005$). The sodium excretion following L-5-HTP infusion was not significantly different from that following glu-5-HTP. The

3-6 h cumulative sodium excretion values after placebo, L-5-HTP and glu-5-HTP administration were 65.3 ± 16.5, 51.1 ± 12.5, and 47.0 ± 13.3 mmol respectively. The corresponding changes in cumulative sodium excretion, relative to the baseline excretion rates, on the 3 experimental days were +18.0 ± 16.9, −10.0 ± 11.6 and −2.0 ± 15.5 mmol. Cumulative natriuresis was reduced by 28.0 ± 24.8 mmol (95% CI_{diff}: 8.9 to 47.0 mmol; $P < 0.01$) after L-5-HTP infusion and by 19.9 ± 13.9 mmol (95% CI_{diff}: 9.3 to 30.6 mmol; $P < 0.005$) after glu-5-HTP when compared with placebo infusion. Urinary excretion of potassium increased progressively on the placebo day from 62.0 ± 35.5 $\mu mol\ min^{-1}$ during the 2.0-2.5 h period to 95.0 ± 32.0 $\mu mol\ min^{-1}$ during the 5.5-6.0 h period ($P < 0.01$). Neither L-5-HTP nor glu-5-HTP affected urinary potassium excretion when compared with placebo infusion (Figure 4-3 and Table 4-2). L-5-HTP, but not glu-5-HTP, significantly reduced urine output ($P < 0.01$) when compared with placebo (Figure 4-4 and Table 4-2). The ERPF, GFR and FF values were not significantly different between the 3 study days, but there was a trend towards a fall in ERPF and GFR after infusion of L-5-HTP (Figure 4-5 and Table 4-5).

Serial PRA and plasma aldosterone concentrations are shown in Figure 4-6. Both L-5-HTP ($P < 0.001$) and glu-5-HTP ($P < 0.05$) significantly increased plasma aldosterone when compared with placebo. The increase after glu-5-HTP was, however, smaller ($P < 0.005$) and delayed when compared with L-5-HTP. PRA was low at baseline and did not increase during infusion of either compound. Compared with placebo, there was a significant increase in serum growth hormone concentrations half an hour after the end of L-5-HTP infusion ($P < 0.05$) (Figure 4-6). There was a large intersubject variability in the growth hormone response to L-5-HTP. Although there was a significant response for the group *in toto*, a rise in growth hormone concentration of > 5 $ng\ ml^{-1}$ was present in only 4 subjects, all of whom experienced nausea. Infusion of glu-5-HTP caused no significant changes in growth hormone levels.

L-5-HTP, but not glu-5-HTP, significantly increased diastolic blood pressure when compared with placebo ($P < 0.05$) (Figure 4-7). There were no significant differences between treatments in systolic blood pressure ($P = 0.08$) or pulse rate. Six out of the nine subjects complained of nausea at the end of L-5-HTP infusion but only one experienced this effect with glu-5-HTP. No other adverse effect was noted.

No significant changes in haematological indices, renal and liver functions, and no untoward effects were present at 1 week after the end of the study.

Table 4-1. Mean (SD) urinary excretion rates of 5-HTP, 5-HT, 5-HIAA, dopamine, DOPAC and sodium for each 30 min period before (period 2.0-2.5 h and period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo, L-5-HTP and glu-5-HTP (n = 9). A value of 0 is entered when the measured variable was below the limit of detection of the assay employed.

				Time (h)				
	2.0-2.5	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
5-HTP excretion (nmol min^{-1})								
Placebo	0	0	0	0	0	0	0	0
L-5-HTP	0	0	137.1	235.1	128.0	75.0	48.9	35.1
			(32.6)	(57.4)	(33.7)	(21.3)	(10.3)	(6.8)
Glu-5-HTP	0	0	284.9	672.4	579.9	310.0	218.7	160.9
			(102.1)	(296.7)	(88.9)	(52.2)	(81.1)	(108.0)
5-HT excretion (nmol min^{-1})								
Placebo	0.4	0.5	0.4	0.5	0.4	0.6	0.4	0.6
	(0.2)	(0.2)	(0.2)	(0.1)	(0.1)	(0.3)	(0.2)	(0.2)
L-5-HTP	0.5	0.5	181.3	349.0	202.1	129.9	73.5	50.0
	(0.1)	(0.2)	(52.2)	(117.2)	(67.0)	(38.4)	(21.8)	(15.9)
Glu-5-HTP	0.6	0.5	98.8	300.2	300.7	186.2	133.9	93.9
	(0.2)	(0.3)	(48.9)	(145.7)	(44.2)	(61.2)	(77.7)	(84.3)
5-HIAA excretion (nmol min^{-1})								
Placebo	0	0	0	0	0	0	0	0
L-5-HTP	0	0	139.0	316.9	297.6	226.2	167.8	126.7
			(49.0)	(76.9)	(94.6)	(40.8)	(26.7)	(23.5)
Glu-5-HTP	0	0	69.6	186.4	218.1	173.2	157.8	137.9
			(40.7)	(135.0)	(75.9)	(50.0)	(38.6)	(76.7)
Dopamine excretion (nmol min^{-1})								
Placebo	1.6	1.6	1.7	1.6	1.5	1.8	1.5	2.0
	(0.5)	(0.6)	(0.6)	(0.5)	(0.5)	(0.9)	(0.6)	(1.1)
L-5-HTP	1.7	1.7	1.7	1.5	1.5	1.5	1.6	1.6
	(0.4)	(0.6)	(0.5)	(0.4)	(0.6)	(0.4)	(0.5)	(0.4)
Glu-5-HTP	1.7	1.6	1.7	1.8	2.0	1.8	1.7	2.0
	(0.6)	(0.5)	(0.7)	(0.7)	(0.8)	(0.8)	(0.6)	(1.4)
DOPAC excretion (nmol min^{-1})								
Placebo	3.2	3.5	3.3	3.2	3.1	3.6	3.1	3.6
	(0.8)	(1.3)	(0.8)	(0.9)	(0.8)	(1.6)	(1.2)	(1.4)
L-5-HTP	3.3	3.4	3.1	2.6	2.6	3.0	3.3	3.3
	(0.9)	(1.2)	(0.7)	(0.6)	(0.9)	(0.7)	(1.2)	(1.0)
Glu-5-HTP	3.4	3.3	3.5	3.7	3.7	3.6	3.3	4.4
	(2.2)	(1.4)	(1.7)	(2.0)	(2.4)	(2.5)	(2.0)	(4.5)

Table 4-2. The 3-6 h cumulative excretion values (μmol) of 5-HTP, 5-HT and 5-HIAA after administration of placebo, L-5-HTP and glu-5-HTP. Values shown are means ± SD (n = 9).

		Infusion		*L-5-HTP vs glu-5-HTP*
	Placebo	L-5-HTP	Glu-5-HTP	95% CI_{diff}; P value
5-HTP	0	21.0 ± 4.8	66.9 ± 14..7	37.5 to 54.4; $P < 0.001$
5-HT	0.09 ± 0.03	31.4 ± 8.7	33.4 ± 9.4	-3.9 to 7.9; P = 0.5
5-HIAA	0	38.8 ± 6.6	28.0 ± 5.8	3.6 to 17.9; $P < 0.01$

Table 4-3. Percentage urinary recoveries (%) of infused L-5-HTP and glu-5-HTP as 5-HTP, 5-HT and 5-HIAA. Values shown are means ± SD (n = 9).

	Infusion		*L-5-HTP vs glu-5-HTP*
	L-5-HTP	Glu-5-HTP	95% CI_{diff}; P value
5-HTP	10.7 ± 1.0	34.6 ± 4.5	20.6 to 27.1; P < 0.001
5-HT	16.0 ± 2.7	17.1 ± 3.0	-2.1 to 4.5; P = 0.4
5-HIAA	20.4 ± 4.9	14.6 ± 3.1	1.8 to 9.8; P < 0.01
Total	47.1 ± 5.4	66.3 ± 7.3	11.7 to 26.7; P < 0.001

Table 4-4. Mean (SD) urinary excretion rates of sodium and potassium, FE_{Na}, and urine output before (period 2.0-2.5 h and period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo, L-5-HTP and glu-5-HTP (n = 9).

	Time (h)							
	2.0-2.5	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
Sodium excretion (μmol min^{-1})								
Placebo	254.1	263.0	296.1	298.6	320.9	415.1	369.1	475.5
	(140.0)	(129.7)	(96.8)	(116.1)	(85.9)	(146.2)	(137.6)	(195.9)
L-5-HTP	290.7	339.6	298.4	228.7	239.4	285.0	324.5	361.4
	(89.4)	(119.7)	(81.2)	(72.0)	(77.1)	(74.2)	(93.9)	(109.5)
Glu-5-HTP	257.0	271.7	276.2	235.2	238.6	236.0	263.5	313.0
	(144.6)	(134.1)	(83.4)	(85.0)	(71.2)	(82.0)	(88.7)	(117.3)
FE_{Na} (%)								
Placebo	1.6	1.8	1.9	2.0	2.3	2.6	2.9	3.0
	(0.7)	(0.7)	(0.5)	(0.6)	(0.8)	(0.6)	(0.8)	(0.6)
L-5-HTP	2.1	2.3	2.2	1.8	2.0	2.1	2.5	2.6
	(0.6)	(0.7)	(0.6)	(0.6)	(0.6)	(0.6)	(1.0)	(1.0)
Glu-5-HTP	1.9	2.0	1.9	1.7	1.6	1.8	1.9	2.1
	(1.2)	(0.7)	(0.5)	(0.4)	(0.4)	(0.5)	(0.6)	(0.6)
Potassium excretion (μmol min^{-1})								
Placebo	62.0	64.5	70.9	69.8	76.6	93.3	76.7	95.0
	(35.5)	(33.0)	(29.7)	(28.5)	(25.0)	(31.7)	(22.6)	(32.0)
L-5-HTP	62.5	75.9	74.4	69.9	66.0	82.3	95.5	95.0
	(18.3)	(25.7)	(14.9)	(17.2)	(15.9)	(14.4)	(25.3)	(22.8)
Glu-5-HTP	65.0	72.5	79.2	75.6	83.4	81.0	86.6	91.3
	(19.0)	(23.9)	(24.6)	(24.1)	(26.6)	(26.1)	(27.1)	(38.5)
Urine flow (ml min^{-1})								
Placebo	7.9	6.5	7.6	6.8	6.8	7.8	6.3	7.8
	(2.6)	(1.9)	(1.4)	(0.4)	(1.6)	(3.3)	(2.3)	(3.8)
L-5-HTP	8.0	8.3	8.6	3.4	2.2	4.1	7.8	9.1
	(2.9)	(2.1)	(1.8)	(1.3)	(0.9)	(2.4)	(2.8)	(3.5)
Glu-5-HTP	6.1	7.4	8.0	6.5	5.7	5.2	6.4	7.3
	(3.4)	(2.9)	(2.1)	(2.4)	(2.0)	(1.8)	(1.8)	(3.0)

Table 4-5. Mean (SD) ERPF, GFR and FF values before (period 2.0-2.5 h and period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo, L-5-HTP and glu-5-HTP (n = 9).

	Time (h)							
	2.0-2.5	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
ERPF (ml min^{-1} 1.73 m^{-2})								
Placebo	556.9	535.0	561.8	528.3	557.9	541.8	506.3	563.5
	(108.1)	(125.9)	(104.0)	(128.7)	(99.6)	(116.0)	(117.8)	(107.5)
L-5-HTP	541.3	566.4	550.4	538.1	472.8	522.2	508.8	544.3
	(124.4)	(120.0)	(118.5)	(134.7)	(99.3)	(96.3)	(85.7)	(110.4)
Glu-5-HTP	499.6	480.1	545.9	494.5	508.0	513.3	509.2	576.1
	(110.4)	(89.2)	(75.0)	(33.2)	(95.0)	(120.0)	(76.6)	(145.1)
GFR (ml min^{-1} 1.73 m^{-2})								
Placebo	100.1	104.0	106.2	101.5	107.0	101.6	95.5	102.2
	(19.1)	(20.5)	(18.1)	(20.0)	(22.2)	(15.7)	(19.9)	(14.6)
L-5-HTP	97.4	97.1	93.4	88.7	83.7	90.8	94.3	98.0
	(14.0)	(8.7)	(12.5)	(11.9)	(16.2)	(19.0)	(10.5)	(12.2)
Glu-5-HTP	93.0	89.1	98.1	92.2	100.1	93.0	98.9	97.5
	(12.7)	(14.1)	(23.2)	(15.4)	(14.7)	(13.8)	(17.6)	(18.0)
FF (%)								
Placebo	18.0	19.7	19.2	19.5	19.3	19.2	19.5	18.7
	(1.4)	(2.4)	(3.1)	(2.4)	(3.1)	(3.0)	(4.5)	(4.0)
L-5-HTP	18.8	17.6	17.5	17.1	18.3	17.7	18.8	18.5
	(4.7)	(3.0)	(3.6)	(3.4)	(5.0)	(4.2)	(3.1)	(3.7)
Glu-5-HTP	19.3	18.9	18.3	18.7	20.2	19.0	20.0	17.9
	(4.5)	(3.6)	(5.1)	(3.4)	(4.6)	(5.0)	(5.2)	(5.2)

Table 4-6. Mean (SD) pulse rate and blood pressure values before (period 2.0-2.5 h and period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo, L-5-HTP and glu-5-HTP (n = 9).

	Time (h)							
	2.0-2.5	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
Placebo infusion								
Pulse rate	60.0	59.6	58.7	58.5	59.1	59.5	60.1	59.3
(beats min^{-1})	(8.4)	(8.0)	(5.8)	(6.7)	(6.3)	(7.1)	(7.5)	(7.2)
Systolic blood	117.4	116.3	115.4	115.2	115.7	118.2	120.1	121.0
pressure (mmHg)	(10.2)	(8.9)	(8.1)	(8.5)	(7.0)	(9.3)	(8.7)	(7.4)
Diastolic blood	64.1	64.8	62.0	62.2	62.4	64.3	65.8	66.3
pressure (mmHg)	(10.4)	(10.1)	(8.8)	(8.7)	(8.3)	(10.9)	(9.6)	(10.6)
L-5-HTP infusion								
Pulse rate	58.1	58.1	59.5	61.0	57.9	57.1	56.4	59.0
(beats min^{-1})	(8.8)	(8.2)	(7.8)	(9.9)	(8.4)	(6.6)	(7.7)	(6.6)
Systolic blood	115.3	116.8	115.8	120.6	120.8	118.3	118.8	121.2
pressure (mmHg)	(9.5)	(12.0)	(9.0)	(10.2)	(10.2)	(11.4)	(11.1)	(11.7)
Diastolic blood	63.2	63.7	65.3	68.8	69.8	67.5	63.1	67.3
pressure (mmHg)	(9.9)	(8.6)	(10.3)	(10.1)	(10.1)	(10.3)	(10.1)	(9.6)
Glu-5-HTP infusion								
Pulse rate	61.9	58.0	57.6	57.3	58.1	59.6	60.1	58.8
(beats min^{-1})	(10.3)	(8.4)	(8.6)	(9.0)	(8.5)	(5.9)	(8.1)	(6.8)
Systolic blood	118.9	118.9	114.6	115.4	118.1	119.4	122.9	120.3
pressure (mmHg)	(9.7)	(8.9)	(9.4)	(11.3)	(10.6)	(10.5)	(11.1)	(9.8)
Diastolic blood	63.6	62.9	61.9	62.1	63.2	63.9	64.6	63.8
pressure (mmHg)	(9.9)	(12.2)	(11.1)	(11.6)	(10.7)	(10.4)	(14.1)	(11.0)

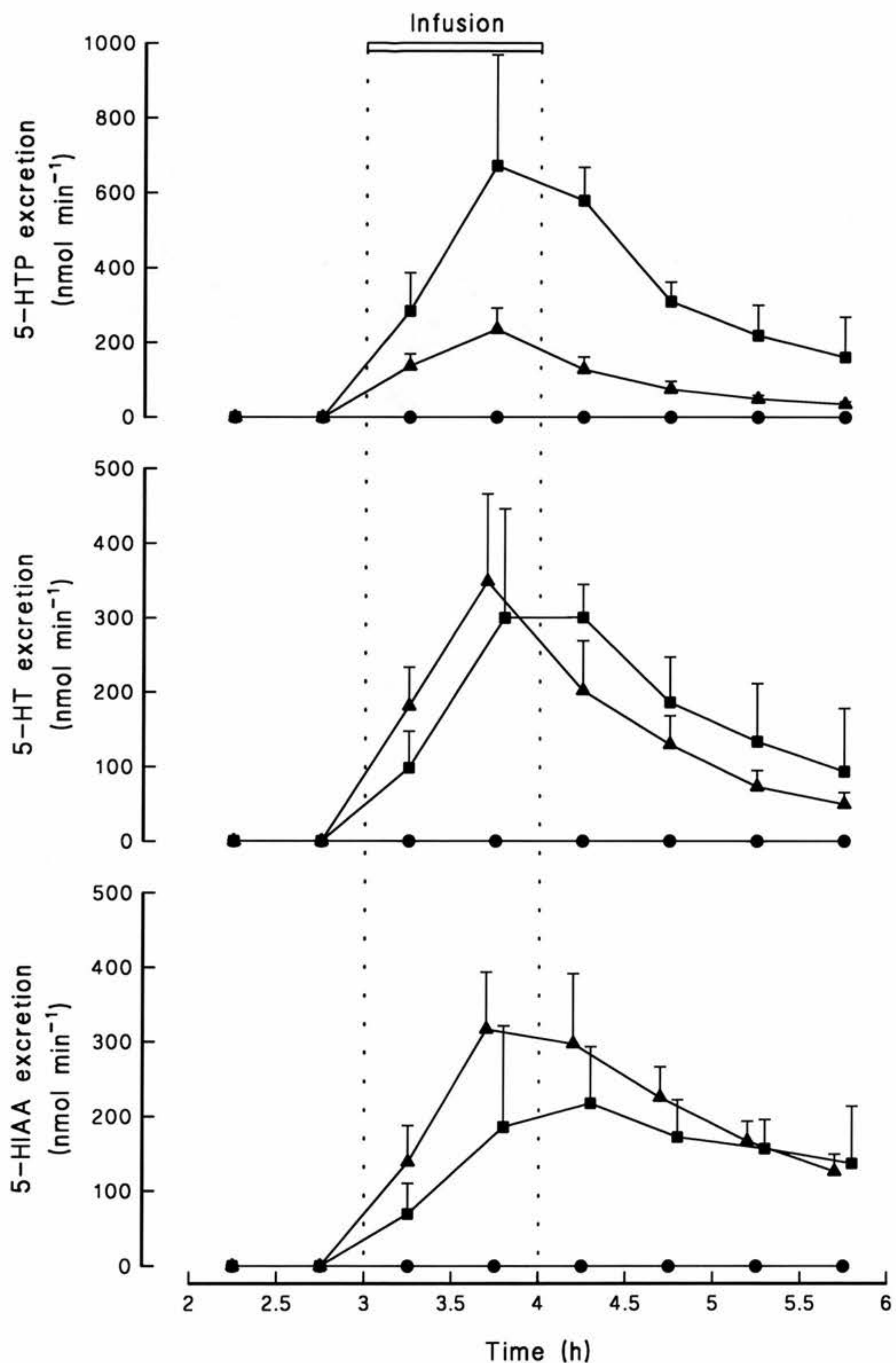


Figure 4-1. Urinary excretion rates of 5-HTP, 5-HT and 5-HIAA before (period 2.0-2.5 h and period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 9).

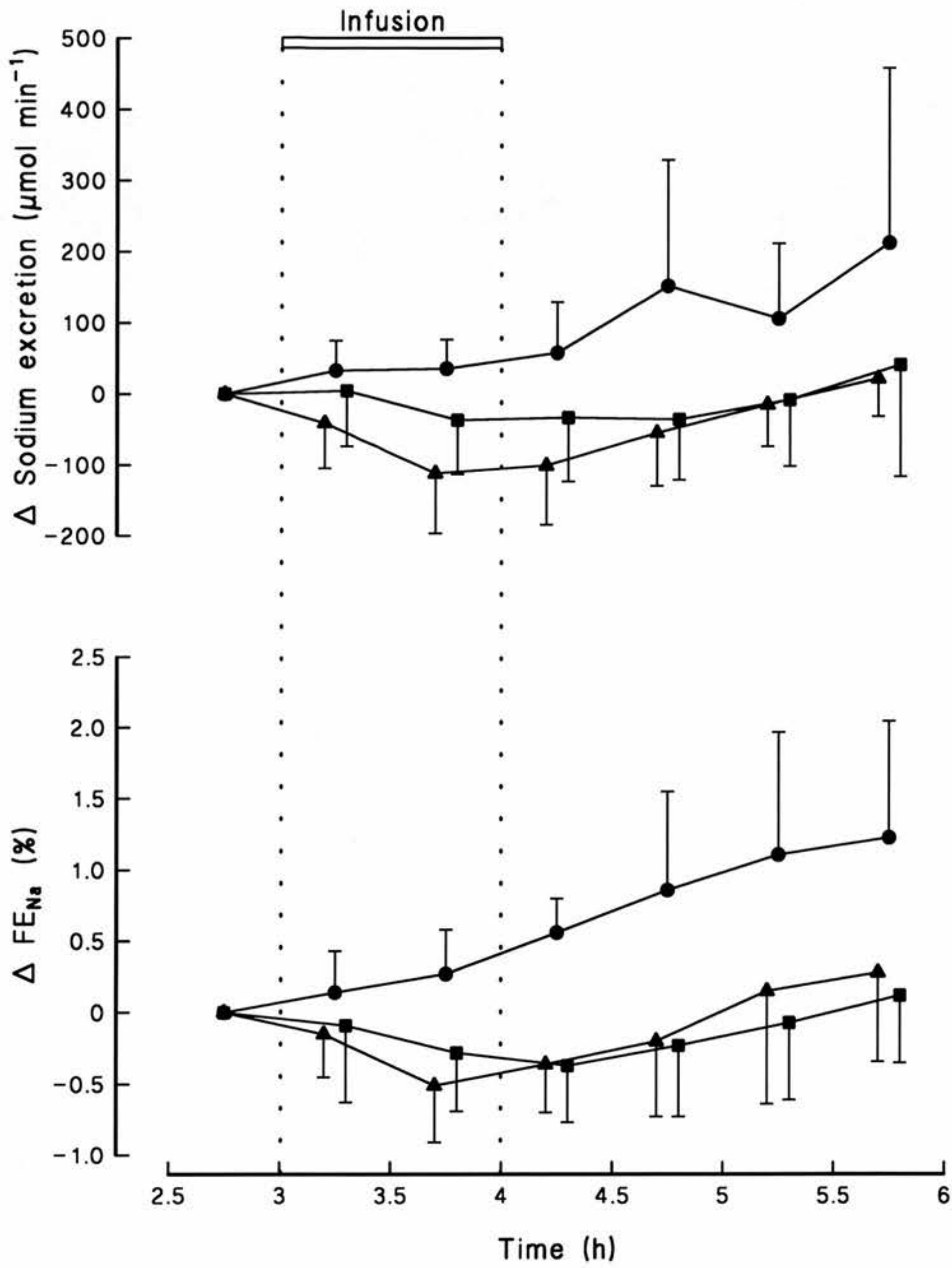


Figure 4-2. Changes (Δ) in urinary excretion of sodium and FE_{Na} from baseline (period 2.5-3.0 h) following infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 9).

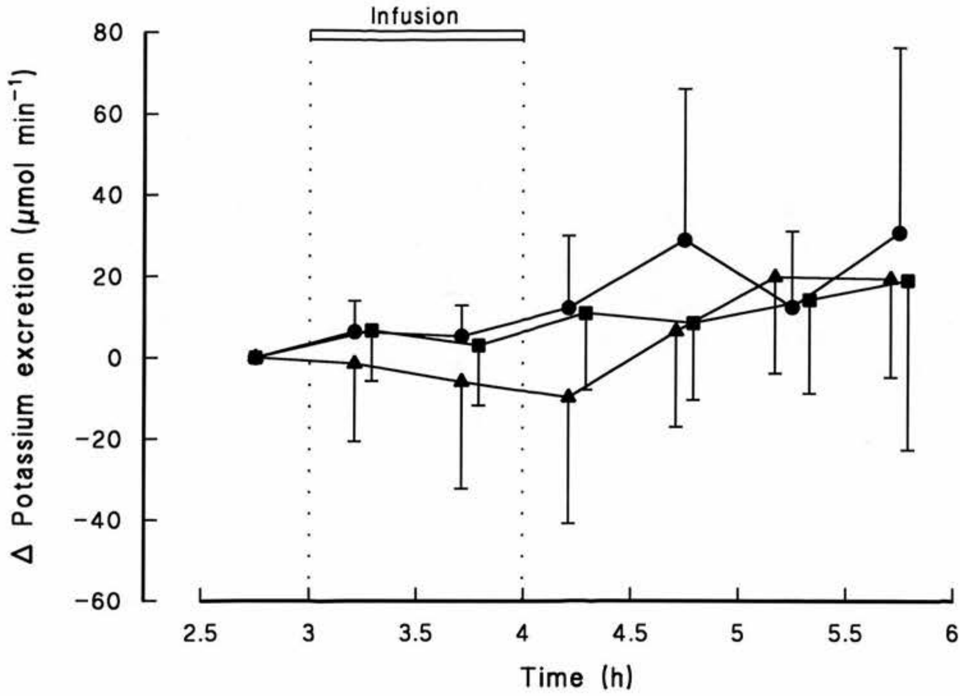


Figure 4-3. Changes (Δ) in urinary excretion of potassium from baseline (period 2.5-3.0 h) following infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 9).

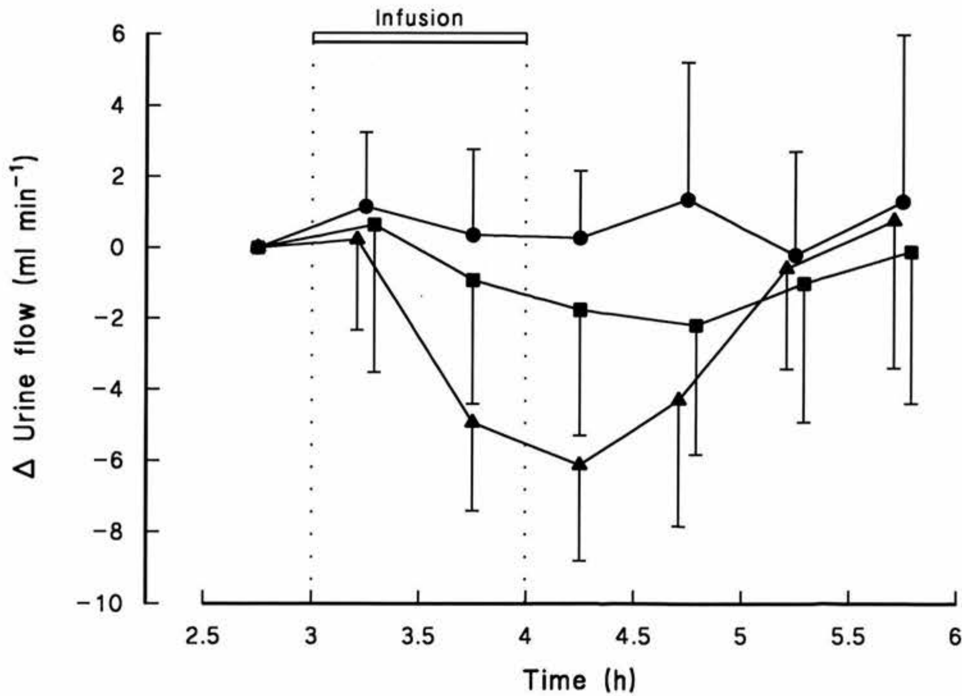


Figure 4-4. Changes (Δ) in urine output from baseline (period 2.5-3.0 h) following infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 9).

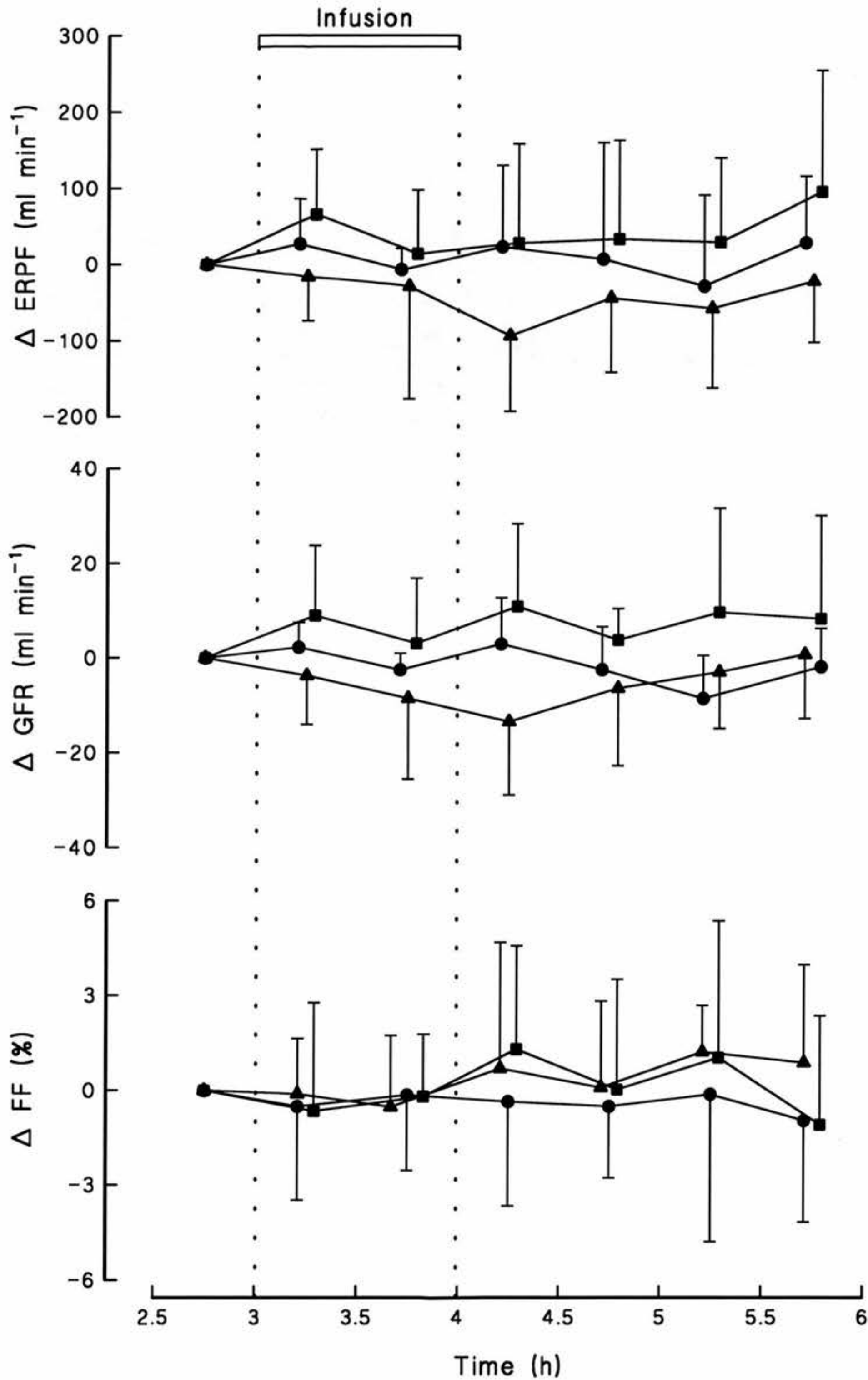


Figure 4-5. Changes (Δ) in ERPF, GFR and FF from baseline (period 2.5-3.0 h) following infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 9).

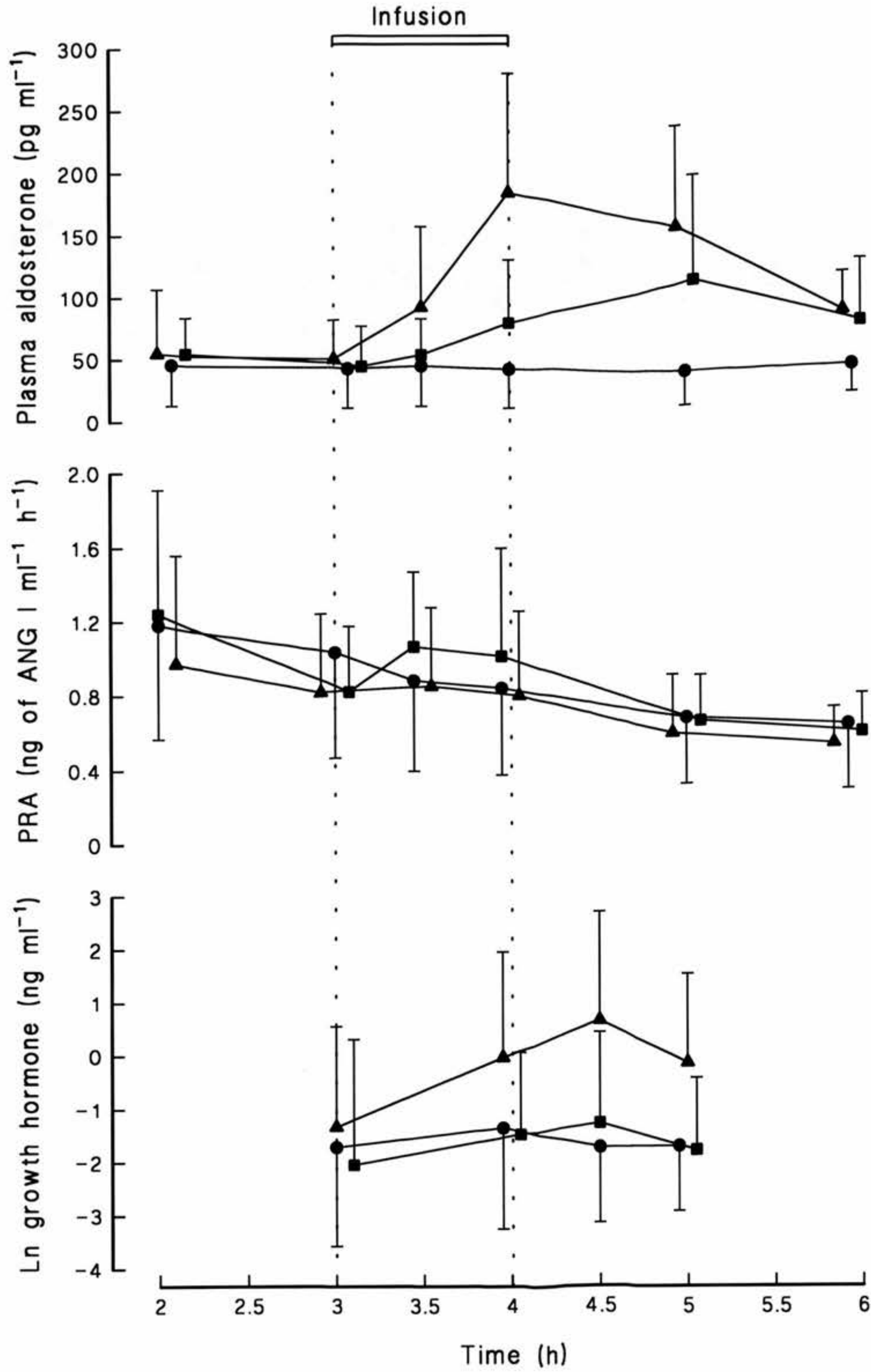


Figure 4-6. Plasma aldosterone, PRA and serum growth hormone before, during and after placebo (●), L-5-HTP (▲) and glu-5-HTP (■). The $\log_e$ (ln) transformed growth hormone values are plotted. Values shown are means ± SD (n = 9).

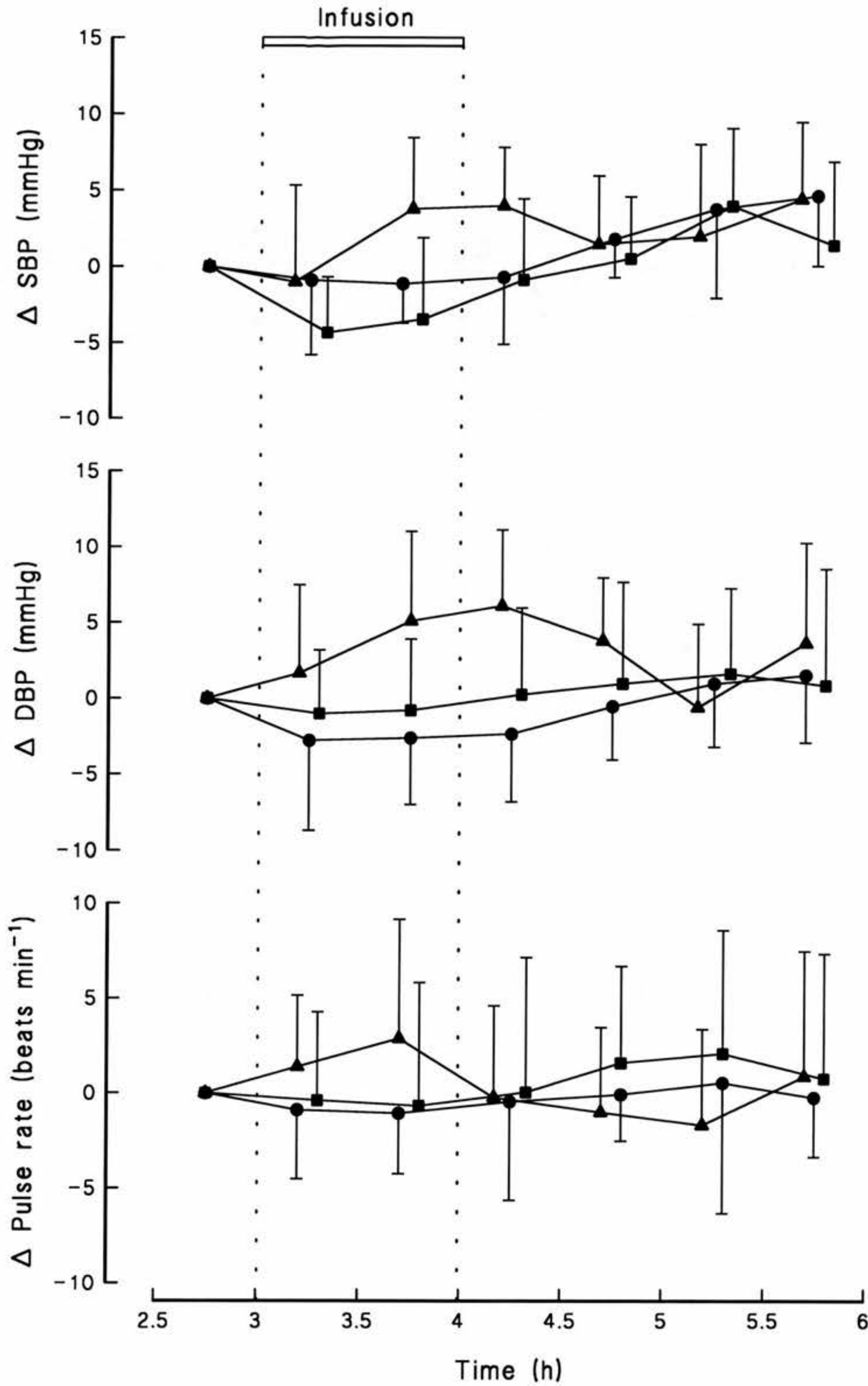


Figure 4-7. Changes (Δ) in systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse rate after infusion of placebo (●), L-5-HTP (▲) and glu-5-HTP (■). Values shown are means ± SD (n = 9).

4.4 Discussion

The present study confirms our previous observations that both L-5-HTP and glu-5-HTP markedly increase urinary 5-HT excretion. As was discussed in Chapter 3, such high urinary levels of 5-HT can only be achieved by generation of 5-HT within the kidney. Measurements of 5-HTP and 5-HT in the renal artery and vein, in addition to urinary measurements, will be required to further support the conclusion that 5-HT is synthesised intrarenally from these 5-HT precursors. It is difficult to perform these investigations in healthy volunteers because of ethical considerations. However, strong evidence to support the intrarenal formation of 5-HT from L-5-HTP is available from experiments with the isolated perfused kidney in the rat [181]. L-5-HTP is converted to 5-HT by renal LAAD. Glu-5-HTP is presumably converted to 5-HT via the sequential catalytic actions of renal γGT and LAAD analogous to the conversion of gludopa to dopamine [329,333,334,341]. Since both γGT [309-320] and LAAD [35,141-143] are concentrated mainly in the proximal renal tubules, our results would seem to indicate renal tubular formation of 5-HT with its subsequent urinary excretion.

The cumulative urinary excretion of 5-HTP after administration of glu-5-HTP was three times that after L-5-HTP infusion suggesting that the amount of L-5-HTP available intrarenally for decarboxylation to 5-HT was greater following infusion of glu-5-HTP. This would be consistent with glu-5-HTP being relatively more selective for the kidney than L-5-HTP whereas relatively more of L-5-HTP may be metabolised extrarenally. There were, however, no differences between the cumulative 5-HT excretion values after L-5-HTP and glu-5-HTP. On a molar basis, 16% of the administered dose of L-5-HTP and 17% of glu-5-HTP were recovered in the urine as 5-HT. The cumulative urinary outputs, measured as the sum of 5-HTP, 5-HT and 5-HIAA excreted during the 3-6 h collection period, were 47% and 66% of the infused dose after L-5-HTP and glu-5-HTP respectively. The higher recovery with glu-5-HTP again suggests greater delivery of the glutamyl compound to the

kidney.

5-HT and dopamine are synthesised from their respective immediate precursors, L-5-HTP and L-dopa, by a common enzyme, aromatic LAAD [31,37,41-44]. In addition, these biogenic amines are both metabolised by MAO to 5-HIAA and DOPAC respectively [31,48,397]. It is, therefore, possible that administration of L-5-HTP may alter the renal formation and/or excretion of dopamine or DOPAC. Indeed, because 5-HT and dopamine could have opposite effects on renal haemodynamics and function, it has been postulated that these biogenic amines may be generated within the kidney under physiological conditions as reciprocal regulators of renal haemodynamics and of salt and water excretion [35,268]. We did not, however, observe any effect of L-5-HTP or glu-5-HTP on the urinary excretion of dopamine or its major metabolite DOPAC in our study. The reason for this is unknown but it would be of interest to infuse equimolar amounts of gludopa and glu-5-HTP to investigate which prodrug is the preferred substrate for the renal enzymes and what is the overall pharmacological effect (*see* Chapter 8).

Both L-5-HTP and glu-5-HTP significantly reduced urinary sodium excretion and fractional excretion of sodium in the present study. In our previous study, a statistically significant antinatriuretic effect was observed following administration of glu-5-HTP, but not after 5-HTP (*see* Chapter 3). The mean urine excretion of sodium after L-5-HTP was, however, lower than that following placebo. A type 2 error probably explains the failure to demonstrate a significant antinatriuresis after L-5-HTP because only five subjects were studied. The reduction in urine excretion of sodium in the present study occurred without significant alterations in ERPF, GFR or FF suggesting that the antinatriuresis is mediated by a direct tubular action of 5-HT formed within renal tubular cells by LAAD. There was a suggestion of a fall in ERPF and GFR after L-5-HTP infusion in our study but the changes were inconsistent and not statistically significant. It is possible that an infusion of L-5-HTP for longer than one hour could produce a significant effect on renal

haemodynamics. However, the absence of an effect on ERPF and GFR, particularly by glu-5-HTP, may not be surprising given the likely tubular site of production of 5-HT. The lack of effect by L-5-HTP on renal haemodynamics in this study is in contrast with studies which demonstrated decreases in ERPF and GFR in rats treated with L-5-HTP [166,254,268]. It was suggested that glomerular LAAD, though of low activity, formed 5-HT within the glomerulus, or that an intrarenal transport system of 5-HT from a tubular site of formation to the blood vessels or glomerulus could explain the observed haemodynamic effects [268].

There was a progressive increase in urinary potassium excretion during infusion of saline on the placebo day. A mild kaliuresis with salt loading has been reported by other workers [383,386,398] but a reduction in urinary potassium excretion has also been observed [385]. Potassium excretion is dependent partly on the rate of sodium excretion as well as the level of aldosterone secretion [383]. It was demonstrated that significantly more potassium was excreted by normal subjects when salt loading follows a period of salt restriction [398] even though the rate of sodium excretion was lower in this situation presumably because of the higher level of aldosterone. L-5-HTP and glu-5-HTP infusion had no effect on urinary excretion of potassium when compared with placebo.

A significant reduction in urine flow rate was observed after L-5-HTP but not after glu-5-HTP. There is evidence from animal studies that 5-HT acts within the central nervous system to increase plasma vasopressin concentration [80,82,84]. The antidiuresis induced by L-5-HTP may therefore be due release of vasopressin subsequent to an increase in brain 5-HT, since L-5-HTP readily crosses the blood-brain barrier [156,175,362,363], whereas glu-5-HTP did not produce this effect because its conversion to 5-HT occurs mainly in the kidney. An alternative explanation is that the vasopressin release is due to the nausea produced by L-5-HTP since nausea is a potent stimulus to secretion of vasopressin in man [357,399-401].

There was an early and significant increase in plasma aldosterone concentrations without a concomitant increase in PRA following administration of L-5-HTP. This is in agreement with observations by other investigators that the release of aldosterone by 5-HTP does not depend on the activation of the renin-angiotensin system in man [96,97]. 5-HT has been shown to exert a direct stimulatory action on adrenocortical cells through activation of 5-HT_4 receptors in animals and man [100,101,103,104]. L-5-HTP may be converted to 5-HT by LAAD present in the adrenal gland [31,36,39] and the release of aldosterone by L-5-HTP may, therefore, be due to an effect of locally synthesised 5-HT on the adrenal cortex. Alternatively, the L-5-HTP-evoked aldosterone secretion may be mediated by central 5-HT pathways [96,97]. Glu-5-HTP caused a smaller and delayed rise in aldosterone level consistent with its effects being largely confined to the kidney. The delayed rise in aldosterone concentration may be due to the renal formation of L-5-HTP from glu-5-HTP and its subsequent recirculation. We cannot also exclude the possibility that glu-5-HTP may be converted to L-5-HTP or 5-HT within the adrenal gland. Aldosterone acts mainly at the cortical collecting tubule where it causes a decrease in the excretion of sodium and an increase in excretion of potassium [402-404]. Water passively follows the aldosterone-mediated transport of sodium. Following its intravenous infusion in man, there was a delay of 2 h before the effect of aldosterone on the excretion of the electrolytes became apparent in one study and the antinatriuresis and kaliuresis lasted 4-6 h after stopping the infusion [402]. Another study reported a latent period of 20-60 min after intravenous infusion of aldosterone before there was any demonstrable reduction in sodium chloride excretion [405]. The peak effect occurred at 2 to 4 h, and the depressed sodium chloride excretion lasted for as long as 6 to 8 h after the administration of aldosterone. In the present study, the time course of the changes in sodium excretion and the lack of effect on potassium excretion would, therefore, suggest that the antinatriuretic response to L-5-HTP or glu-5-HTP infusion did not depend

on the known action of aldosterone at the distal nephron [404].

Growth hormone concentrations were significantly increased after infusion of L-5-HTP. This is presumably mediated by the conversion of L-5-HTP to 5-HT in the central nervous system and is in agreement with studies by other workers which demonstrated a rise in growth hormone following administration of L-5-HTP [361,381,392-396] or L-tryptophan, the first substrate for 5-HT production [396,406-411]. However, discordant results have been obtained by other investigators [406,412,413] and the role of 5-HT in the regulation of growth hormone release remains unclear. It is possible that nausea may have been a factor in the release of growth hormone secretion in our study since all four subjects who experienced this side effect showed an increase in growth hormone concentration of more than 5 ng ml^{-1}. Emesis may increase plasma concentrations of growth hormone [399]. On the other hand, an increase in growth hormone has been reported in studies where only a minority of the subjects felt nauseated [361,381,392,393,395,406,407]. Glu-5-HTP had no effect on growth hormone release in keeping with its conversion to 5-HT being limited largely to the kidney.

Six of the nine subjects experienced nausea at the end of L-5-HTP infusion. This is a well-recognised adverse effect associated with DL- or L-5-HTP administration [162,359-362]. Glu-5-HTP, in equimolar amount, produced this symptom in only one subject. In addition, glu-5-HTP did not affect blood pressure whereas L-5-HTP caused a significant increase in diastolic blood pressure. This action of L-5-HTP is contrary to the depressor effect observed in most animal species [414]. However, hypertension was observed in children with Down's syndrome given L-5-HTP [415]. The low incidence of adverse effects and the lack of effect on blood pressure provide further evidence that glu-5-HTP is more renally selective and has less systemic toxicity than L-5-HTP.

In conclusion, the results of this study confirm that 5-HT can be generated intrarenally from both 5-HT prodrugs in man. When 5-HT is generated, it produces

an effect on tubular sodium reabsorption, independent of changes in ERPF or GFR, resulting in sodium retention. The urinary metabolite data and reduced extrarenal effects, as evidenced by changes in growth hormone and aldosterone release, blood pressure and adverse effects, supports the hypothesis that glu-5-HTP is relatively more selective for the kidney than 5-HTP. This relative selectivity is probably due to the avid uptake and metabolism of glutamyl compounds by the kidney [324,329]. Whatever the mechanism involved, the use of glu-5-HTP should allow the effects of increased renal synthesis of 5-HT to be studied with less confounding extrarenal effects.

CHAPTER FIVE

BLOOD AND URINE 5-HYDROXYTRYPTOPHAN AND 5-HYDROXYTRYPTAMINE LEVELS AFTER ADMINISTRATION OF 5-HYDROXY-L-TRYPTOPHAN AND γ-L-GLUTAMYL-5-HYDROXY-L-TRYPTOPHAN

5.1 Introduction

The mammalian kidney contains all the major enzymes required for the synthesis of 5-HT from L-tryptophan, as well as those necessary for its degradation, suggesting that it might have the capacity to synthesise and metabolise 5-HT locally (*see* sections 2.2.1 and 2.2.3). Studies in rats demonstrated that 5-HT can indeed be synthesised intrarenally and it has been suggested that urinary excretion of 5-HT reflects renal production of 5-HT [35,166,181,268]. We administered L-5-HTP and its glutamyl derivative, glu-5-HTP, to healthy men in the previous two studies and observed marked increases in the urinary excretion of 5-HT after both compounds (*see* Chapters 3 and 4). We argued that such high urinary levels cannot be accounted for solely by extrarenal production of 5-HT and can only be achieved by generation of 5-HT within the kidney where it would be less accessible to the effects of systemic degradative enzymes. The contribution of 5-HT produced extrarenally to the elevated urinary 5-HT excretion cannot, however, be fully assessed without knowledge of changes in 5-HT levels in the systemic circulation (and ideally those in the renal artery) over the course of the experiment.

In the present study, we have estimated 5-HTP and 5-HT concentrations in platelet-rich plasma (PRP), in addition to urinary 5-HTP and 5-HT excretion, after infusion of equimolar amounts of both 5-HT precursors to further investigate our proposition that the large increments in 5-HT excretion were principally due to 5-HT generated within the kidney. We chose to estimate the level of 5-HT in PRP rather than platelet-poor (or -free) plasma because the well-known fragility of platelets makes it difficult to ensure consistently that the 5-HT measured is not derived from platelets disintegrating during sample collection and/or processing. 5-HT was measured in PRP in preference to whole blood because processing of whole blood for measurement of 5-HT leads inevitably to disruption of red blood cells with concomitant release of oxyhaemoglobin which results in oxidation of 5-HT [416].

5.2 Methods

Six healthy male volunteers (age range 22-35 years) were studied on 2 separate days, at least 1 week apart, in this randomised cross-over study. They refrained from alcohol for 24 h, abstained from xanthine-containing drinks from 18.00 h, and fasted from 22.00 h the evening before each study day. They were maintained on their normal diet. The subjects attended the clinical investigation unit at 08.00 h after drinking 500 ml of water 1 h previously. They received intravenous 0.9% NaCl at 5 ml min^{-1} and drank 150 ml of water half-hourly over the next 6 h. They emptied their bladders at time 2.5 h and serial urine collections of 30 min duration were made thereafter. Half an hour later (time = 3 h), an equimolar dose (45 nmol kg^{-1} min^{-1}) of L-5-HTP (10 μg kg^{-1} min^{-1}) or glu-5-HTP (16.6 μg kg^{-1} min^{-1}) was infused intravenously for 60 min. Venous blood samples were withdrawn gently, without using a tourniquet to avoid platelet aggregation, via a 16 G Venflon cannula before and every 30 min for 3 h after the start of the infusion. The blood sample (9 ml) was dispensed into an acid-citrate-dextrose anticoagulant (1 ml) consisting of citric acid (8 g l^{-1}), trisodium citrate (22 g l^{-1}) and glucose (20 g l^{-1}) [367,417]. Following centrifugation at 120 *g* for 20 min at room temperature, the upper two thirds of the supernatant (i.e. PRP) was harvested. An aliquot was removed for platelet counting which was carried out on an electronic particle Coulter Counter (Coulter Electronics, Inc., Hialeah, FL, U.S.A.) in the Department of Haematology, Edinburgh Royal Infirmary, Edinburgh. The remaining PRP was stored at -40°C in sealed polystyrene tubes until analysis for 5-HTP and 5-HT. The volume of each urine collection was measured and aliquots stored at -40°C for analysis of 5-HTP and 5-HT. The urine samples were acidified with 5 M HCl to prevent their oxidation. 5-HTP and 5-HT in PRP and in urine were assayed as described in section 2.5. 'Platelet 5-HT' concentration, expressed in pmol of 5-HT per 10^8 platelets, was calculated by dividing the concentration of 5-HT in PRP by the concentration of platelets [417].

Results are expressed as means ± SD. The area under the plasma concentration *versus* time curve (AUC) was calculated using the trapezoidal rule. The apparent renal clearance of 5-HTP was estimated by dividing the amount of 5-HTP excreted in urine by the corresponding area under the concentration *vs* time curve [376]. The data on the 2 experimental days were compared by Student's paired t test and 95% CI_{diff} between means quoted where appropriate. Repeated measures ANOVA was used to identify any differences in the blood 5-HT levels between the two study days. Differences were considered statistically significant when the P value was less than 0.05.

5.3 Results

The 5-HTP concentrations in PRP and urinary excretion rates of 5-HTP on the 2 study days are shown in Figure 5-1. 5-HTP was undetectable in baseline PRP and urine samples. The peak concentration (C_{max}) of 5-HTP in PRP was 1365 ± 302 nmol l^{-1} and the AUC(3-6 h) was 1763 ± 250 nmol l^{-1} h after the administration of L-5-HTP. The corresponding values after glu-5-HTP infusion were lower at 471 ± 95 nmol l^{-1} (95% CI_{diff}: 539 to 1249 nmol l^{-1}; $P < 0.005$) and 934 ± 185 nmol l^{-1} h (95% CI_{diff}: 464 to 1193 nmol l^{-1}; $P < 0.005$). The 3-6 h cumulative urinary 5-HTP excretion after glu-5-HTP (44.0 ± 8.6 μmol) was 2.5 times that occurring after L-5-HTP infusion (17.6 ± 2.1 μmol; 95% CI_{diff}: 18.5 to 34.4 μmol; $P < 0.001$). The apparent renal clearance of 5-HTP during the 3-4 h period was higher after glu-5-HTP (1357 ± 348 ml min^{-1}) than after L-5-HTP (246 ± 56 ml min^{-1}; 95% CI_{diff}: 734 to 1487 ml min^{-1}; $P < 0.001$).

PRP 5-HT concentration was 812 ± 218 nmol l^{-1} before administration of L-5-HTP and 769 ± 140 nmol l^{-1} before glu-5-HTP and did not change significantly after administration of either compound (Figure 5-2). Expressing the 5-HT measurements relative to the number of platelets in the PRP, as pmol per 10^8 platelets, to reflect 'platelet 5-HT' concentrations [417] did not alter this conclusion.

There were, however, huge increases in urinary 5-HT excretion. Mean urinary excretion rate of 5-HT, which was < 0.7 nmol min^{-1} before dosing, rose to a peak value of 412 ± 92 nmol min^{-1} at the end of L-5-HTP infusion and 303 ± 29 nmol min^{-1} after the administration of glu-5-HTP. The mean urinary excretion rates of 5-HT after administration of L-5-HTP were greater than those seen after glu-5-HTP during the infusion of these compounds but less during the post-infusion phase. As a result, the 3-6 h cumulative 5-HT excretion values after L-5-HTP and glu-5-HTP were not significantly different at 37.4 ± 4.6 μmol and 32.0 ± 4.5 μmol respectively (95% CI_{diff}: -0.6 to 11.5 μmol).

Two subjects complained of nausea, and one of these two vomited, at the end of L-5-HTP infusion. There were no ill-effects following glu-5-HTP infusion.

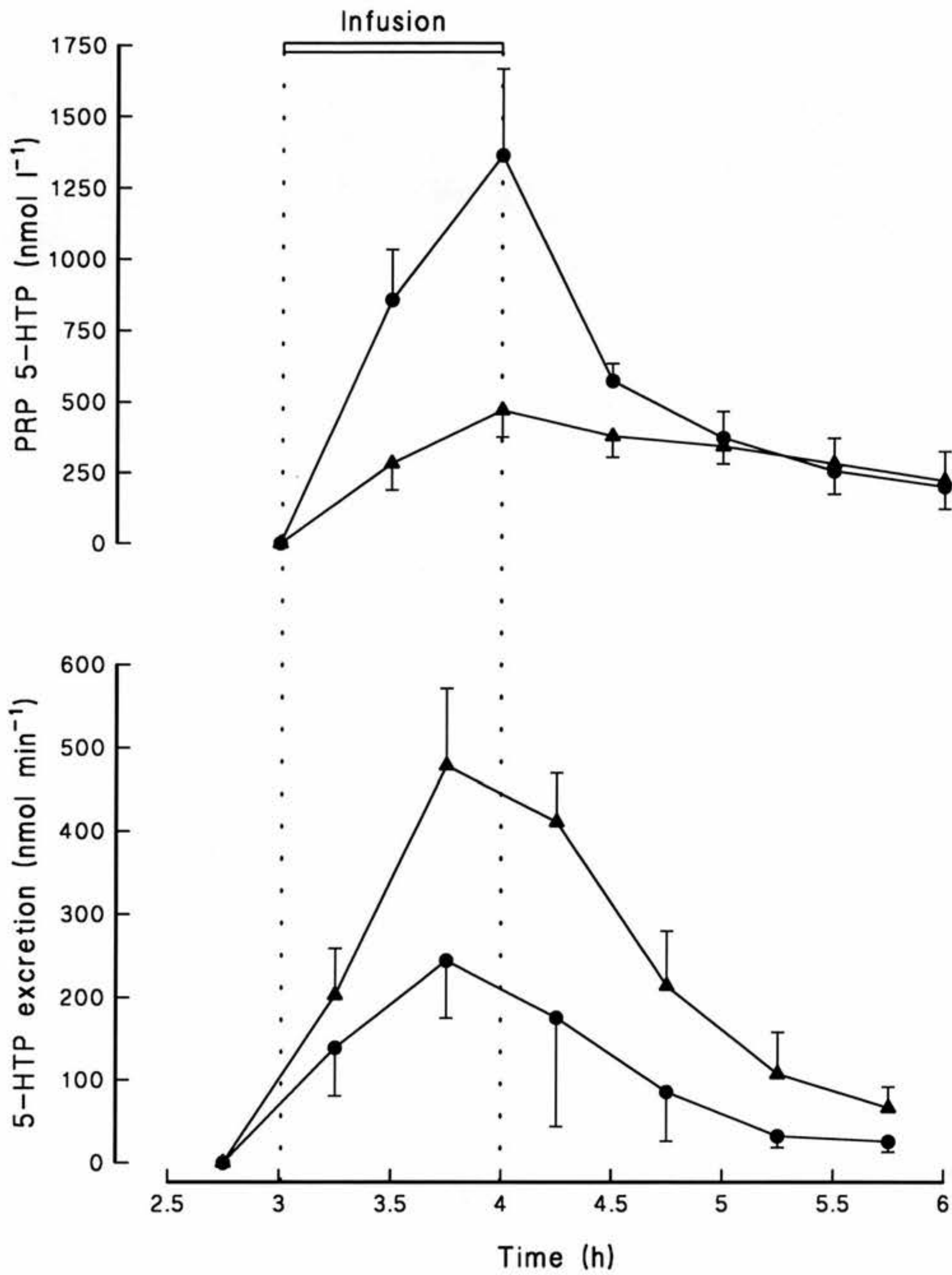


Figure 5-1. 5-HTP concentrations in PRP and urinary excretion rates of 5-HTP before, during and after infusion of L-5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SD (n = 6).

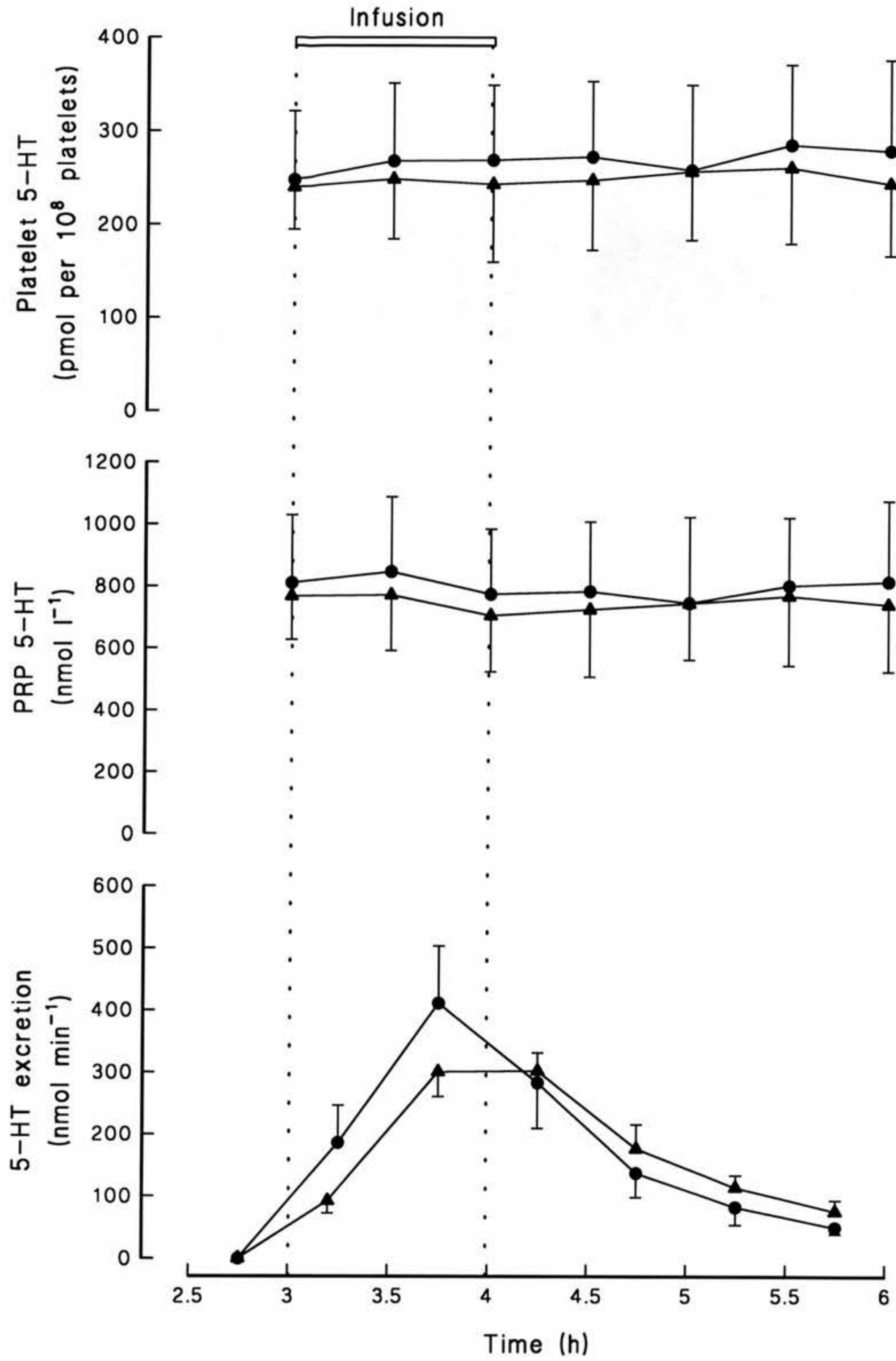


Figure 5-2. Platelet 5-HT concentrations, PRP 5-HT concentrations and urinary excretion rates of 5-HT before, during and after infusion of L-5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SD (n = 6).

5.4 Discussion

The platelets normally concentrate 5-HT released into blood from other tissues [15] and *in vitro* studies have shown that, under normal conditions, the storage capacity of platelets is not saturated [21,418]. The level of 5-HT in platelet (expressed as pmol per 10^8 platelets) or in PRP (nmol l^{-1} PRP) could, therefore, be used as an indicator of circulating 5-HT. These two measurements are highly correlated and it has been suggested that estimation of 5-HT in PRP is an equally accurate and reliable index of variation in blood 5-HT level when compared with platelet 5-HT concentration [417]. In the present study, we have demonstrated that the marked increases in urinary 5-HT excretion after both L-5-HTP and glu-5-HTP occurred without concomitant increases in platelet or PRP 5-HT concentrations. Similarly, intravenous infusion of L-5-HTP in the rat was reported to produce a large increase in urinary 5-HT excretion without affecting plasma 5-HT levels [166]. The present findings provide additional evidence that urine 5-HT, after infusion of both 5-HT precursors, was largely derived from intrarenal synthesis of 5-HT as we would have expected an increase in circulating 5-HT levels, if 5-HT produced extrarenally contributed significantly to the increased 5-HT excretion in urine. Although a placebo day was not included in this study, we had shown previously that the saline infusion and water loading employed in the protocol do not affect urinary 5-HTP or 5-HT excretion (*see* Chapter 4).

5-HTP, unlike 5-HT, does not appear to be concentrated in platelets and it has been reported that the concentrations of 5-HTP in PRP after administration of L-5-HTP were not significantly different from those measured in platelet-poor plasma [419]. In the present study, 5-HTP in PRP increased as expected after administration of L-5-HTP but an increase, although of a smaller magnitude, was also observed after glu-5-HTP. This suggests that glu-5-HTP metabolism was not completely confined to the kidney and that the delivery of 5-HTP via its glutamyl derivative was, therefore, only relatively selective for the kidney. This finding

would not be surprising since the enzyme γGT required for the conversion of glu-5-HTP to L-5-HTP is also present in significant concentrations at sites other than the kidney. Its concentration in the kidney is, however, considerably greater than elsewhere [308,309] and it can be expected that L-5-HTP will be predominantly released within this organ. This is supported by the observations that the peak level of 5-HTP and the AUC of its concentration-time curve were lower than those observed after administration of L-5-HTP. The amount of 5-HTP in urine after glu-5-HTP was, however, higher than that seen after L-5-HTP. It was greater than can be accounted for by the total clearance of 5-HTP from the plasma perfusing the kidney providing strong support for the intrarenal synthesis of L-5-HTP from glu-5-HTP and this is reflected by the high apparent renal clearance value of 5-HTP after infusion of glu-5-HTP. L-5-HTP produced intrarenally from glu-5-HTP may be excreted into the urine, converted to 5-HT or 5-HIAA, or reabsorbed into the circulation. Recirculation of L-5-HTP produced in the kidney, in addition to extrarenal transformation of glu-5-HTP to 5-HTP, could both contribute to the increased levels of circulating 5-HTP following the administration of glu-5-HTP.

L-5-HTP is decarboxylated to 5-HT by aromatic LAAD. This enzyme is widely distributed in the body with high activity in the kidney and liver [31,38,39,139]. Exogenous L-5-HTP, or L-5-HTP formed from glu-5-HTP, may, therefore, be converted to 5-HT extrarenally. The absence of an increase in circulating 5-HT, particularly after administration of L-5-HTP, suggests either that the assay we have used was unable to detect any increase in 5-HT levels or that 5-HT produced extrarenally was rapidly metabolised and cleared from the circulation [1,35,176]. The rapid uptake of L-5-HTP into other body compartments such as the brain may also be a factor [175].

Gastrointestinal side effects observed after administration of L-5-HTP appear to be related to the plasma 5-HTP concentrations [360,420,421]. In the present study, two subjects developed nausea at the end of L-5-HTP infusion at the time when

peak circulating levels of 5-HTP occurred. In contrast, peak circulating 5-HTP levels were much lower after infusion of the glutamyl compound and no adverse effects were observed.

The present study therefore provided further evidence that 5-HT is synthesised intrarenally after administration of both L-5-HTP and glu-5-HTP in man. In addition, the data supported the proposition that the glutamyl compound is better tolerated and that it exhibits greater renal selectivity (*see* Chapters 3 and 4).

CHAPTER SIX

THE ANTINATRIURETIC ACTION OF γ-L-GLUTAMYL-5-HYDROXY-L-TRYPTOPHAN IS DEPENDENT ON ITS DECARBOXYLATION TO 5-HYDROXYTRYPTAMINE

6.1 Introduction

Carbidopa (MK 486; L-α-methyl-α-hydrazino-β-(3,4-dihydroxyphenyl)-propionic acid, also known as L-α-hydrazino-3,4-dihydroxy-α-methylhydrocinnamic acid) inhibits the enzyme LAAD. It does not penetrate the blood-brain barrier even at high dosage, in contrast to benserazide (Ro 4-4602; N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)-hydrazine), another LAAD inhibitor [38,422-424]. Administration of carbidopa therefore results in selective extracerebral LAAD inhibition. The drug is about 50% absorbed after oral administration in human subjects. The plasma level reaches a maximum value within 4 h after dosing and its plasma half-life is approximately 2 h [425]. Its effectiveness as a decarboxylase inhibitor can, however, persist for a longer period possibly as a result of formation and accumulation of metabolites also capable of inhibiting the decarboxylase enzyme or because of its retention in tissues [426]. Plasma levels of endogenous 5-HTP may rise from less than 1 ng ml^{-1} to 5 ng ml^{-1} after administration of carbidopa [183]. Pretreatment with carbidopa caused about a 5 to 15-fold increase in the plasma concentration of 5-HTP obtained after oral administration of 100, 200 mg or 2 mg kg^{-1} of L-5-HTP [182,427] and reduced or prevented the accumulation of 5-HT in plasma or serum [427-429]. Carbidopa is usually prescribed in combination with L-dopa for the treatment of Parkinson's disease to inhibit the peripheral metabolism of L-dopa and enhance the ability of the ingested L-dopa to cross the blood-brain barrier for central production of dopamine by LAAD [430]. As a result, a lower dose of L-dopa is required for therapeutic effects and this reduces the incidence of peripheral adverse reactions.

In the previous studies, we demonstrated marked increases in the urinary excretion of 5-HT, without concomitant changes in 5-HT concentrations in platelet-rich plasma, after infusion of glu-5-HTP in keeping with intrarenal synthesis of 5-HT (*see* Chapters 3 to 5). Glu-5-HTP is presumably converted to L-5-HTP by γGT and then decarboxylated by LAAD to 5-HT. This is analogous to the

conversion of gludopa to L-dopa by γGT and then to dopamine by LAAD (*see* section 1.3). Glu-5-HTP was found to be relatively more selective for the kidney than L-5-HTP. It reduced urinary sodium excretion without significant alterations in renal haemodynamics. This antinatriuresis probably resulted from an effect of intrarenally generated 5-HT on the renal tubular reabsorption of sodium although we could not exclude the possibility of an effect of the intact dipeptide or of the intermediate metabolite L-5-HTP.

The present study was designed to investigate whether the extracerebral LAAD inhibitor, carbidopa, blocks the formation of 5-HT from glu-5-HTP and interferes with the actions of glu-5-HTP in normal volunteers.

6.2 Methods

Eight healthy male volunteers, aged 18-39 years (mean 28 years) and weighing 59.9-80.9 kg (mean 69.0 kg), took part in this randomised, single-blind, placebo-controlled, within-subject cross-over study. Each subject attended on 4 separate occasions, at least 1 week apart. They refrained from alcohol for 24 h, abstained from caffeine-containing beverages from 18.00 h, and fasted from 22.00 h the evening before each of the study days. They arrived at the clinical investigation unit at about 08.00 h on each study day, having drunk 500 ml of tap water 1 h previously. The subjects received an intravenous loading dose of 0.5 g of PAH and 3.5 g of polyfructosan (Inutest) at the start of the study (time = 0 h) followed by a maintenance infusion of PAH (3.75 g l^{-1}) and polyfructosan (4.5 g l^{-1}) in 0.9% NaCl at a rate of 5 ml min^{-1} for the next 6 h. They emptied their bladders at 1.5 h and accurately timed consecutive urine collections of about 30 min duration were made thereafter until the end of the study. Two hours after the start of the study (time = 2 h), the subjects took either 100 mg of carbidopa or placebo orally. This was followed 1 h later (time = 3 h) by a 60-min infusion of glu-5-HTP, made up to 30 ml with 0.9% saline, at a rate of 16.6 μg kg^{-1} min^{-1} or placebo (saline alone at 0.5 ml

min^{-1}). Each subject therefore received the following 4 combinations in a randomised sequence: placebo + placebo; placebo + glu-5-HTP; carbidopa + placebo; and carbidopa + glu-5-HTP. The subjects remained semi-recumbent or supine throughout the experiment except when standing to pass urine and drank 150 ml of water every 30 min to promote an adequate diuresis. Blood pressure and pulse rate were measured in duplicate, with the subject in the supine position, by a semi-automated Dinamap recorder every half hour during the study.

Venous blood samples were collected at 30 min intervals for measurement of plasma sodium, PAH and polyfructosan. Blood for determination of PRA and plasma aldosterone were collected at 0.5, 2, 3, 3.5, 4, 5 and 6 h. The volume of each urine collection was measured and aliquots stored at -40°C for analysis of sodium, potassium, PAH, polyfructosan, 5-HTP, 5-HT, 5-HIAA and dopamine. Urine samples for 5-HT, 5-HIAA and dopamine were acidified (pH < 3.0) with 5 M HCl to prevent their oxidation. The measurements of plasma aldosterone and PRA; urinary potassium, PAH, 5-HTP, 5-HT, 5-HIAA and dopamine; plasma and urinary sodium were described in section 2.5.

ERPF and GFR were estimated from the renal clearances of PAH (C_{PAH}) and polyfructosan (C_{In}) respectively using the standard formula UV/P, where U is the urine concentration, V is the urine flow rate and P is the mean of the plasma levels at the beginning and end of each clearance period. Fractional excretion of sodium (FE_{Na}) was calculated as renal clearance of sodium divided by GFR (C_{In}) and expressed as a percentage.

Results are expressed as means ± SD. The variables measured serially on the 4 experimental days were compared by repeated measures ANOVA, with the different treatment regimens and time as the within-subject repeated measures. If there was a significant overall difference between the 4 experimental days, follow-up paired comparison between the treatment regimens were made to determine where the differences lie, with correction of the P value by the Bonferroni method to allow for

multiple comparisons and protect against a type 1 error. Two-way ANOVA was employed to compare the cumulative data on the four study days. When significant differences were found, relevant pairs of data were compared by Student's t-test for paired observations. The P values were adjusted by the Bonferroni method and the associated 99%, rather than 95%, confidence interval of difference (CI_{diff}) between the means quoted where appropriate. A value of zero was assumed when a measured variable was below the limit of detection of the assay technique used to allow statistical comparisons to be made. Differences were considered to be statistically significant when P values were less than 0.05.

6.3. Results

The urinary excretion rates of 5-HTP, 5-HT, 5-HIAA and dopamine for each 30 min period and the 3-6 h cumulative excretion for the three metabolites on the four study days are shown in Table 6-1 and Table 6-2 respectively. The urinary excretion of 5-HT was approximately 0.4 nmol min^{-1} at baseline (period 1.5-2.0 h). This rose to a peak value of 278 ± 44 nmol min^{-1} after glu-5-HTP infusion but did not change significantly after placebo infusion. The cumulative urinary 5-HT excretion over the 3 h period after the start of glu-5-HTP infusion was 430-fold higher than that after placebo infusion (32.4 ± 3.6 μmol *vs* 75.6 ± 12.7 nmol; $P < 0.001$). Similarly, 5-HTP and 5-HIAA which were undetectable in urine during placebo infusion, increased markedly after administration of glu-5-HTP. Carbidopa suppressed urinary 5-HT and dopamine excretion to undetectable levels in all subjects during placebo infusion. It markedly attenuated the increase in 5-HT excretion after glu-5-HTP. Compared with placebo pretreatment, carbidopa reduced the 3-6 h cumulative 5-HT excretion by 99% from 32.4 ± 3.6 μmol to 0.3 ± 0.1 μmol ($P < 0.001$). The amount of infused glu-5-HTP recovered in the urine as 5-HT was 17.4 ± 2.2% and this was reduced to 0.1 ± 0.1% after pretreatment with carbidopa (Table 6-3). Carbidopa increased the 3 h cumulative 5-HTP excretion by 60% from 45.6 ±

10.9 μmol to 72.8 ± 14.8 μmol ($P < 0.001$) and reduced 5-HIAA excretion by 74% from 30.8 ± 6.3 μmol to 7.9 ± 3.2 μmol ($P < 0.001$) (Table 6-2).

There was a steady increase in urinary sodium excretion from 278 ± 77 μmol min^{-1} (1.5-2.0 h period) to 379 ± 144 μmol min^{-1}(5.5-6.0 h period) during the placebo day in response to infusion of saline (Table 6-4; Figure 6-1). Glu-5-HTP produced a significant attenuation of sodium excretion when compared with placebo infusion ($P < 0.01$). The 3-6 h cumulative sodium excretion values after placebo and glu-5-HTP administration were 63.3 ± 19.1 and 45.9 ± 9.2 mmol respectively (99% CI_{diff}: 2.0 to 32.8 mmol; $P < 0.05$). Pretreatment with carbidopa abolished the antinatriuretic effect of glu-5-HTP. The 3-6 h cumulative sodium excretion when the subjects received carbidopa and glu-5-HTP infusion was 63.5 ± 15.6 mmol which is similar to that during placebo infusion only (99% CI_{diff}: -19.8 to 20.2 mmol). Carbidopa had no significant effect on cumulative sodium excretion during placebo infusion (63.3 ± 5.2 mmol with carbidopa pretreatment *vs* 63.3 ± 6.7 mmol without carbidopa; 99% CI_{diff}: -10.6 to 10.6 mmol). The changes in cumulative sodium excretion, relative to the respective pre-infusion (period 2.5-3.0 h) excretion rates, on the 4 experimental days were: +7.6 ± 5.3 mmol after placebo + placebo; –10.3 ± 4.0 mmol after placebo + glu-5-HTP; +10.8 ± 7.3 mmol after carbidopa + placebo; and +13.2 ± 16.7 mmol after carbidopa + glu-5-HTP. Analysis of the cumulative sodium data using these values did not alter the conclusions reached. There were no differences in urinary potassium excretion (Table 6-4; Figure 6-2) and urine output (Table 6-4; Figure 6-3) between the 4 experimental days.

Glu-5-HTP significantly increased plasma aldosterone when compared with placebo infusion ($P < 0.001$) (Figure 6-4). Pretreatment with carbidopa attenuated the increase in plasma aldosterone concentrations produced by glu-5-HTP ($P < 0.005$). Carbidopa had no effect on the steady fall in plasma aldosterone concentration during placebo infusion. PRA declined progressively in response to saline loading during all experimental days (Figure 6-4).

Repeated measures ANOVA revealed no significant differences in GFR values between the 4 experimental days (Figure 6-5) although there appears to be a trend towards a fall in GFR after infusion of glu-5-HTP. Similarly, ERPF (Figure 6-5), blood pressure and pulse rate (Figure 6-6) did not differ between the four study days. No side effects were reported or recorded.

Table 6-1. Mean (SD) urinary excretion rates of 5-HTP, 5-HT, 5-HIAA and dopamine for each 30 min period on the 4 study days (n = 8). Carbidopa or placebo was given at 2 h and time 3-4 h represents the infusion (glu-5-HTP or placebo) period. A value of 0 is entered where a variable was below the limit of detection of the assay used. * = dopamine was not detectable in 7 out of 8 subjects.

	Time (h)								
	1.5-2.0	2.0-2.5	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
5-HTP excretion (nmol min^{-1})									
Placebo + placebo	0	0	0	0	0	0	0	0	0
Placebo + glu-5-HTP	0	0	0	232.6 (69.0)	436.5 (132.3)	368.9 (134.9)	246.9 (55.8)	149.8 (48.0)	84.3 (23.6)
Carbidopa + placebo	0	0	0	0	0	0	0	0	0
Carbidopa + glu-5-HTP	0	0	0	264.6 (71.1)	682.5 (133.5)	593.3 (173.7)	410.1 (79.6)	260.9 (85.4)	209.9 (42.8)
5-HT excretion (nmol min^{-1})									
Placebo + placebo	0.3 (0.2)	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	0.4 (0.0)	0.4 (0.2)	0.5 (0.1)
Placebo + glu-5-HTP	0.5 (0.1)	0.4 (0.1)	0.4 (0.0)	113.1 (42.6)	274.3 (51.5)	278.4 (43.9)	198.8 (41.7)	126.1 (21.9)	88.4 (11.2)
Carbidopa + placebo	0.5 (0.2)	0.4 (0.1)	0.1 (0.1)	0	0	0	0	0	0
Carbidopa + glu-5-HTP	0.4 (0.2)	0.3 (0.2)	0.2 (0.1)	0.6 (0.4)	2.0 (1.1)	2.6 (1.6)	2.2 (1.0)	1.7 (0.7)	1.5 (0.9)
5-HIAA excretion (nmol min^{-1})									
Placebo + placebo	0	0	0	0	0	0	0	0	0
Placebo + glu-5-HTP	0	0	0	73.7 (27.8)	199.2 (100.9)	222.8 (46.7)	213.2 (46.2)	171.4 (32.7)	144.9 (27.2)
Carbidopa + placebo	0	0	0	0	0	0	0	0	0
Carbidopa + glu-5-HTP	0	0	0	32.1 (35.3)	29.1 (15.2)	49.2 (21.1)	60.4 (35.7)	49.4 (21.3)	43.5 (16.9)
Dopamine excretion (nmol min^{-1})									
Placebo + placebo	1.3 (0.2)	1.3 (0.3)	1.3 (0.2)	1.3 (0.2)	1.3 (0.3)	1.4 (0.3)	1.4 (0.3)	1.4 (0.3)	1.4 (0.2)
Placebo + glu-5-HTP	1.4 (0.2)	1.3 (0.3)	1.2 (0.2)	1.3 (0.1)	1.2 (0.2)	1.2 (0.2)	1.2 (0.4)	1.2 (0.1)	1.2 (0.1)
Carbidopa + placebo	1.6 (0.2)	1.6 (0.4)	0.7 (0.6)	0.2 (0.3)	0.1 (0.2)	0	0	0	0
Carbidopa + glu-5-HTP	1.6 (0.5)	1.6 (0.3)	0.9 (0.6)	0.2 (0.5)	0.3 (0.8)	0.3* (0.7)	0.2* (0.6)	0.2* (0.6)	0.2* (0.5)

Table 6-2. The 3-6 h cumulative excretion values (μmol) of 5-HTP, 5-HT and 5-HIAA after administration of placebo + placebo (P + P), placebo + glu-5-HTP (P + G), carbidopa + placebo (C + P), and carbidopa + glu-5-HTP (C + G). Values shown are means ± SD (n = 9).

	Treatment combination				*P + G* vs *C + G*
	P + P	*P + G*	*C + P*	*C + G*	*99% CI_{diff}; P value*
5-HTP	0	45.6 ± 10.9	0	72.8 ± 14.8	16.6 to 37.8; P < 0.001
5-HT	0.08 ± 0.01	32.4 ± 3.6	0	0.3 ± 0.1	22.8 to 36.4; P < 0.001
5-HIAA	0	30.8 ± 6.3	0	7.9 ± 3.2	16.9 to 28.9; P < 0.001

Table 6-3. Percentage urinary recoveries (%) of infused glu-5-HTP as 5-HTP, 5-HT and 5-HIAA after pretreatment with placebo or carbidopa. Values shown are means ± SD (n = 8).

	Placebo + Glu-5-HTP	*Carbidopa + Glu-5-HTP*	*99% CI_{diff}; P value*
5-HTP	24.3 ± 5.2	38.8 ± 6.8	8.8 to 20.2; P < 0.001
5-HT	17.4 ± 2.2	0.1 ± 0.1	14.5 to 19.9; P < 0.001
5-HIAA	16.5 ± 3.2	4.2 ± 1.6	9.0 to 15.6; P < 0.001
Total	58.1 ± 6.5	43.1 ± 7.5	9.1 to 20.9; P < 0.001

Table 6-4. Mean (SD) urinary sodium excretion rates, urinary potassium excretion rates and urine flow rates for each 30 min period on the 4 study days (n = 8). Carbidopa or placebo was given at 2 h and time 3-4 h represents the infusion (glu-5-HTP or placebo) period.

	Time (h)								
	1.5-2.0	2.0-2.5	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
Sodium excretion (nmol min^{-1})									
Placebo +	278.0	292.9	309.7	320.0	339.4	350.7	346.3	363.8	378.9
placebo	(76.9)	(101.2)	(98.7)	(122.0)	(106.8)	(138.0)	(115.0)	(140.8)	(143.8)
Placebo +	280.0	308.7	312.5	276.4	257.8	216.2	235.3	266.0	277.9
glu-5-HTP	(98.9)	(112.1)	(81.1)	(60.0)	(41.7)	(48.4)	(50.2)	(76.6)	(84.0)
Carbidopa +	293.5	304.8	291.9	303.0	324.2	336.2	370.0	382.7	394.5
placebo	(90.7)	(90.9)	(85.8)	(70.2)	(66.5)	(105.5)	(84.9)	(87.1)	(99.8)
Carbidopa +	279.8	298.2	305.9	320.6	327.9	352.7	375.6	369.0	371.5
glu-5-HTP	(117.3)	(104.8)	(91.0)	(72.3)	(71.3)	(93.5)	(96.8)	(111.6)	(102.3)
Potassium excretion (nmol min^{-1})									
Placebo +	87.0	86.4	90.8	96.8	106.0	107.3	102.1	96.7	94.6
placebo	(33.2)	(36.2)	(25.1)	(36.4)	(36.0)	(33.9)	(29.0)	(20.0)	(17.7)
Placebo +	100.9	105.0	108.2	113.5	118.1	114.9	126.4	126.2	120.1
glu-5-HTP	(37.0)	(39.7)	(31.9)	(24.7)	(30.7)	(22.0)	(21.8)	(28.9)	(29.0)
Carbidopa +	104.1	102.8	102.6	107.5	110.6	107.2	109.7	107.1	103.6
placebo	(36.7)	(38.7)	(39.1)	(24.4)	(23.6)	(33.3)	(16.9)	(22.0)	(26.8)
Carbidopa +	119.4	115.3	111.5	115.4	123.9	130.9	131.8	120.8	117.9
glu-5-HTP	(67.4)	(46.8)	(38.9)	(33.3)	(33.9)	(30.3)	(31.6)	(26.7)	(20.5)
Urine flow (ml min^{-1})									
Placebo +	9.9	8.5	8.2	7.0	7.6	7.5	6.1	7.0	6.6
placebo	(1.9)	(3.4)	(2.2)	(2.9)	(1.9)	(2.7)	(1.6)	(2.9)	(2.6)
Placebo +	9.6	10.0	8.3	7.9	7.2	5.1	5.4	6.6	6.3
glu-5-HTP	(2.9)	(2.8)	(1.8)	(1.1)	(1.7)	(1.3)	(0.8)	(1.7)	(1.3)
Carbidopa +	9.3	9.4	6.7	6.4	7.4	7.0	6.0	6.9	7.4
placebo	(2.6)	(2.1)	(2.8)	(1.4)	(1.1)	(2.9)	(1.9)	(2.0)	(1.3)
Carbidopa +	9.7	10.3	8.6	7.8	7.0	6.7	7.4	7.5	7.8
glu-5-HTP	(2.8)	(2.8)	(4.0)	(1.8)	(2.1)	(2.4)	(2.1)	(2.3)	(1.1)

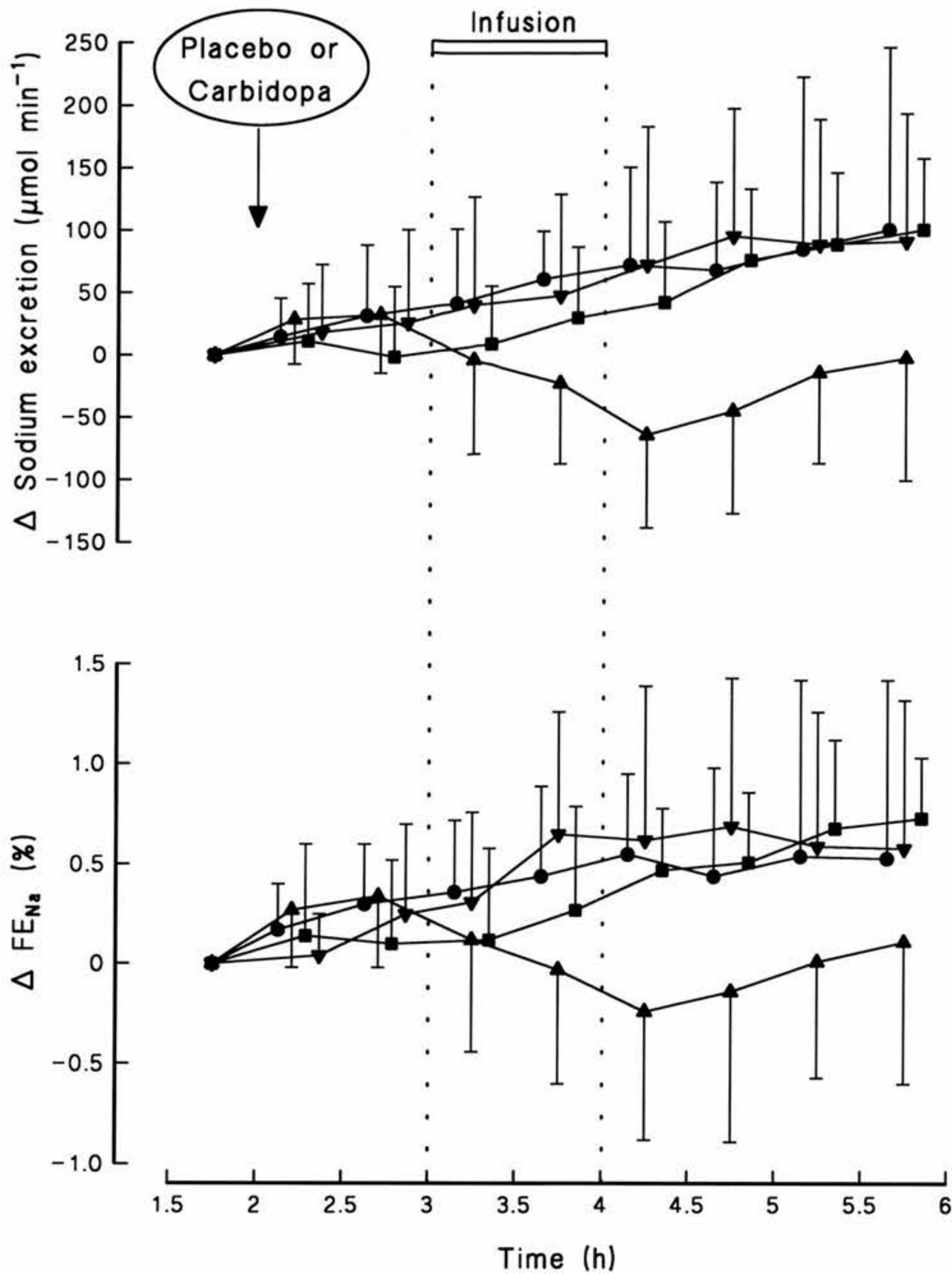


Figure 6-1. Changes (Δ) in urinary excretion rates of sodium and FE_{Na} from the 1.5-2.0 h period on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

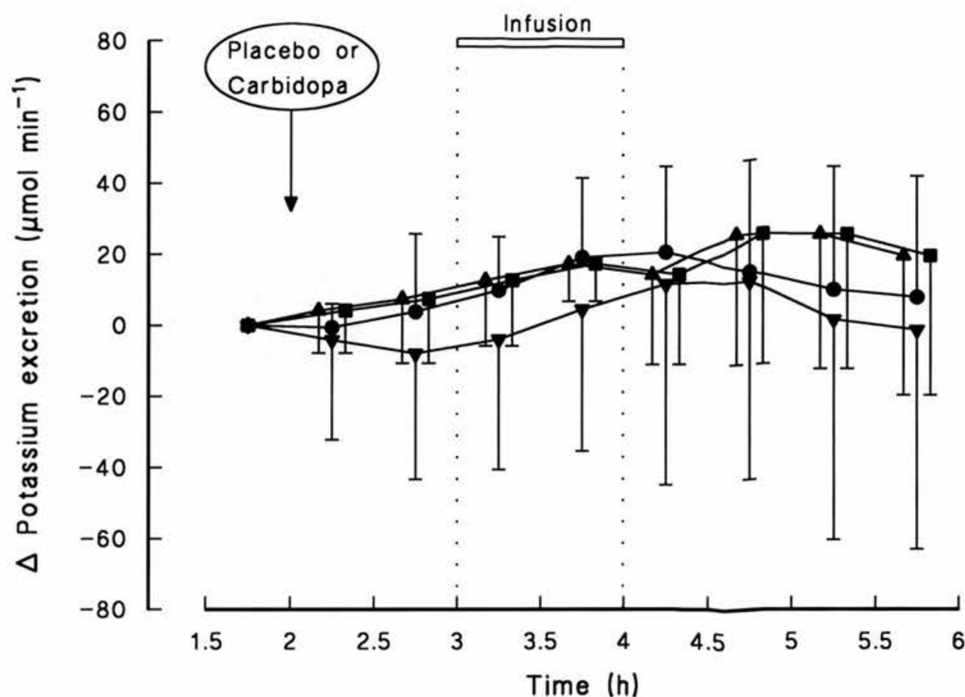


Figure 6-2. Changes (Δ) in urinary excretion rates of potassium from the 1.5-2.0 h period on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

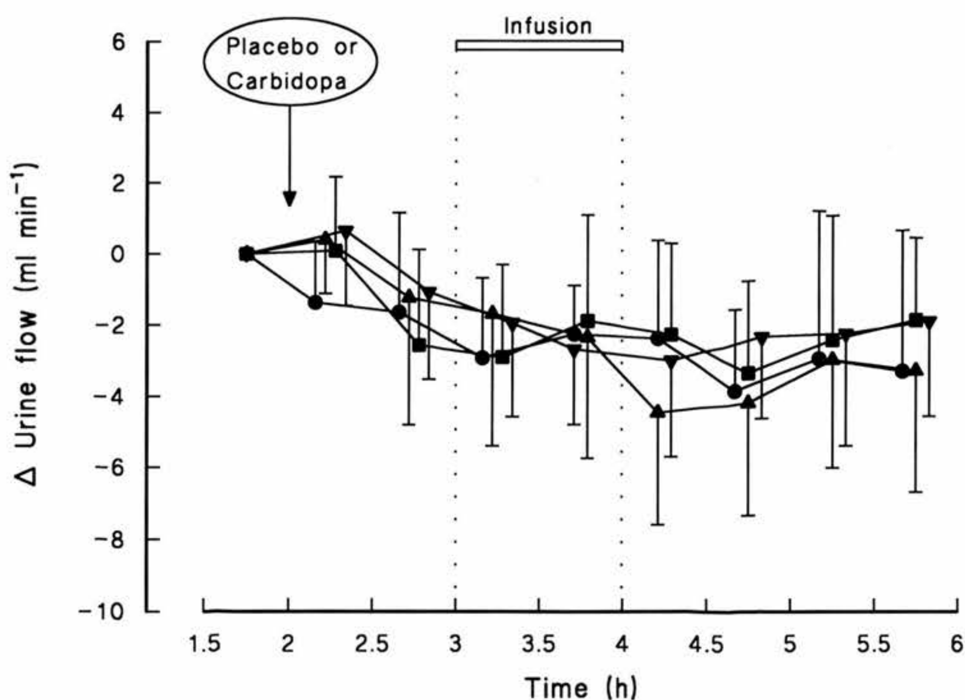


Figure 6-3. Changes (Δ) in urine output from the 1.5-2.0 h period on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

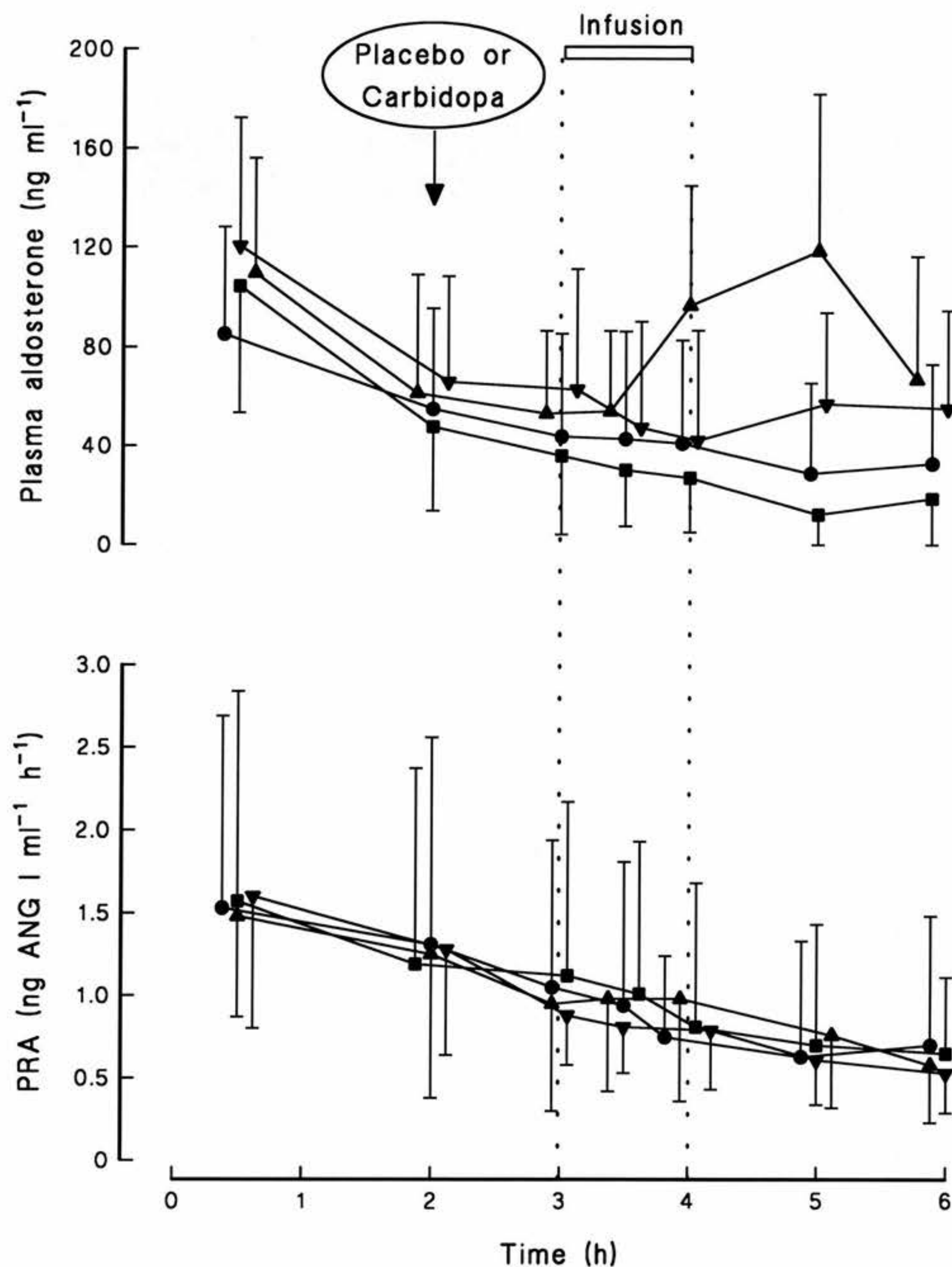


Figure 6-4. Plasma aldosterone and PRA on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

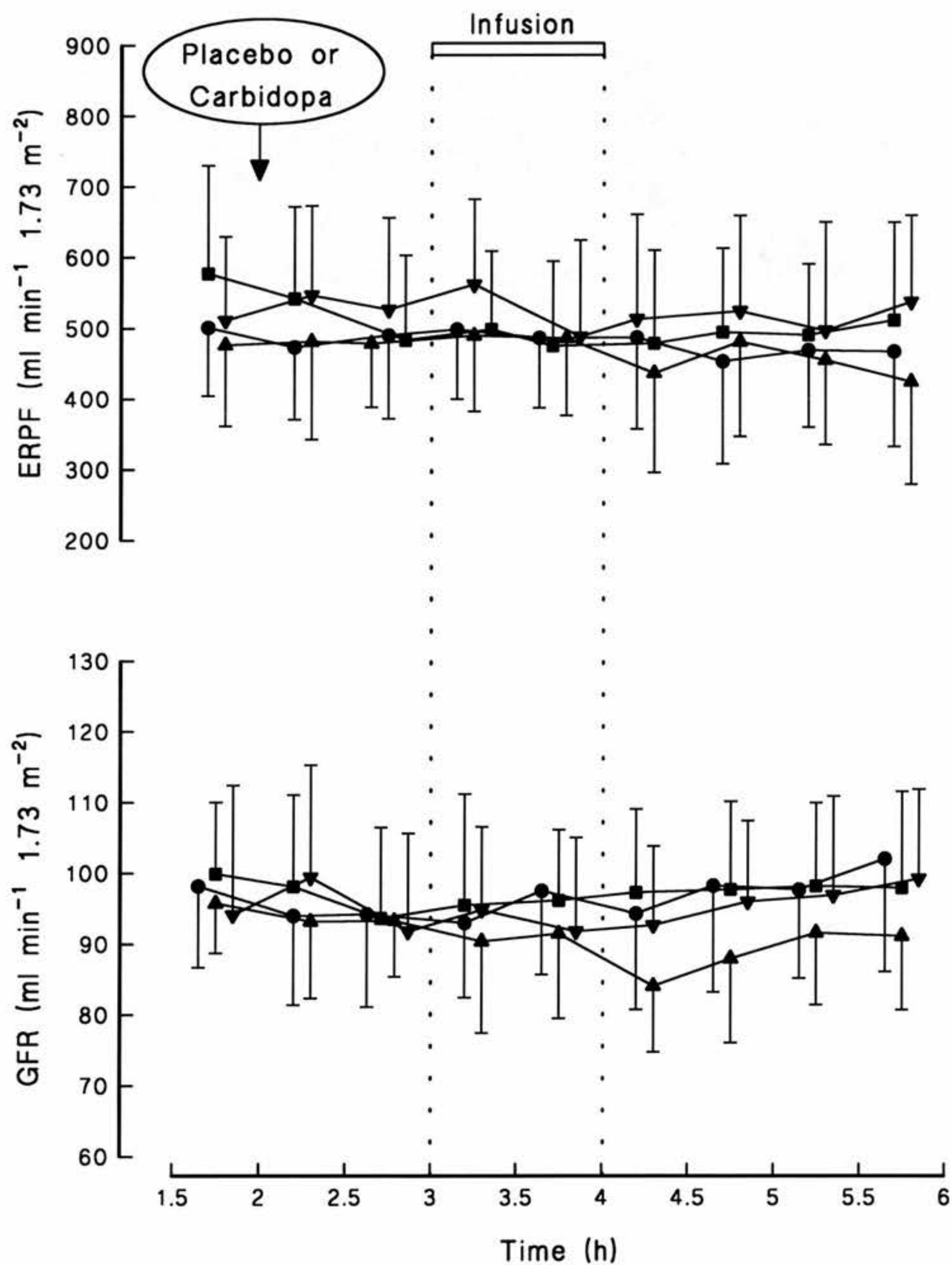


Figure 6-5. ERPF and GFR values on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

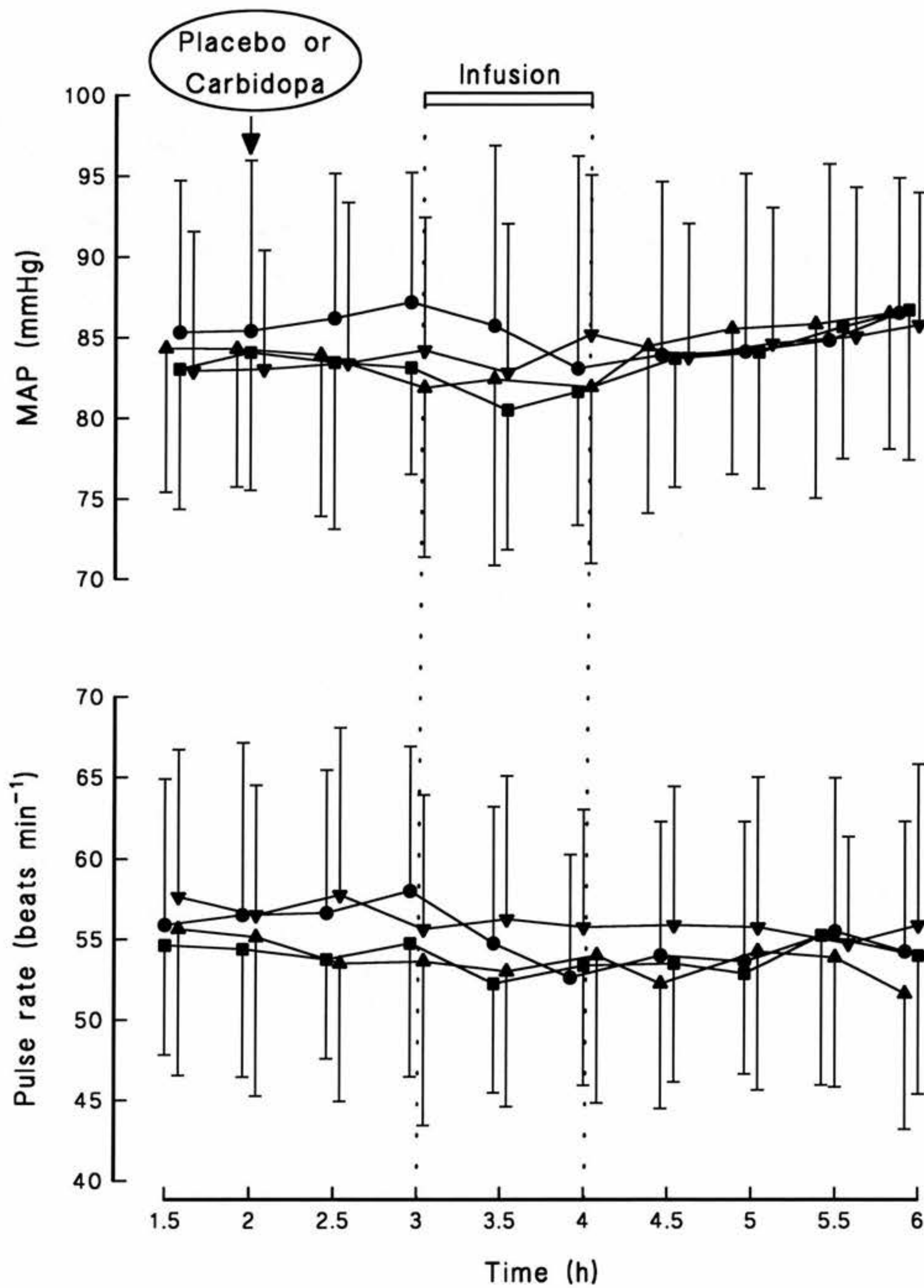


Figure 6-6. Mean arterial blood pressure (MAP) and pulse rate on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

6.4 Discussion

This study confirms our previous observations that glu-5-HTP markedly increases urinary 5-HT excretion and causes retention of sodium without significant alterations in renal haemodynamics in normal man (*see* Chapters 3-5). The prodrug also increases plasma aldosterone levels but the time course of the changes in sodium excretion and the lack of effect on urinary potassium excretion suggest that the antinatriuresis occurs independently of the known actions of aldosterone. These findings have already been discussed in the earlier chapters.

Urinary dopamine results mainly from dopamine synthesis in proximal tubular cells by the renal decarboxylation of L-dopa present in circulating blood [431-436]. Similarly, it has been suggested that urinary 5-HT reflects intrarenal synthesis of 5-HT [35]. In the present study, a single 100 mg dose of carbidopa, an extracerebral LAAD inhibitor, suppressed dopamine and 5-HT excretion to below the levels of detection indicating a very effective inhibition of renal LAAD. We did not observe a significant effect of carbidopa on urinary sodium excretion during saline infusion in agreement with most previous studies [437-440]. A reduction in urinary sodium excretion by carbidopa has, however, also been reported by other investigators and this has been taken as evidence supporting a role for dopamine in regulating renal sodium output [441,442]. The differing results reported may depend on the salt status of the individual subjects. Carbidopa substantially reduced the increment in urinary 5-HT excretion that followed administration of glu-5-HTP. There was an increase in urinary 5-HTP excretion and a reduction in 5-HIAA excretion. These results are consistent with significant inhibition of renal LAAD although we cannot establish (or exclude) a possible role for inhibition of other peripheral decarboxylase enzymes. The lesser reduction in 5-HIAA than 5-HT excretion suggests that there are body compartments where LAAD may still remain active and be capable of 5-HT synthesis, despite carbidopa administration. The decarboxylation of 5-HTP in the brain would be unaffected by the extracerebral

inhibitor, carbidopa, resulting in uninterrupted formation of 5-HT and its metabolism to 5-HIAA, producing a greater relative urinary excretion of 5-HIAA than 5-HT [443]. Transamination of amino acids may also be increased as an alternate pathway of metabolism following inhibition of decarboxylation, and this would allow the continued formation and excretion of 5-HIAA [444-449].

Administration of glu-5-HTP produced a significant reduction in sodium excretion when compared with placebo infusion, in agreement with our previous studies (*see* Chapters 3 and 4). In the present investigation, carbidopa abolished the antinatriuresis induced by glu-5-HTP coincident with the near suppression of 5-HT synthesis. These observations are in keeping with our hypothesis that the sodium retention following administration of glu-5-HTP results from the intrarenal generation of 5-HT and is not due to an effect of the intact dipeptide or of the intermediate metabolite L-5-HTP.

Glu-5-HTP increased plasma aldosterone levels, without a parallel increase in PRA, suggesting that the release of aldosterone does not depend on the activation of the renin-angiotensin system in man (*see* Chapters 3 and 4). It is possible that the adrenal gland may possess γGT [382] and LAAD [31,39] activities. 5-HT could then be produced locally and stimulate aldosterone release since 5-HT has been shown to release aldosterone from the adrenal gland [100-104]. Alternatively, L-5-HTP and 5-HT formed from glu-5-HTP in the kidney may recirculate and act on the adrenal gland. L-5-HTP, unlike 5-HT, can cross the blood-brain barrier and the release of aldosterone could, therefore, also be mediated by central 5-HT pathways [96,97]. Carbidopa does not penetrate the central nervous system to any appreciable extent and would not be expected to inhibit conversion of L-5-HTP to 5-HT in the brain [38,422-424]. It has been reported to increase plasma 5-HTP following administration of L-5-HTP and to increase the stimulatory effect of L-5-HTP on aldosterone suggesting that central 5-HT pathways are involved in the stimulation of aldosterone induced by administration of 5-HTP [96,182]. In the

present study, however, carbidopa attenuated the increase in aldosterone secretion following glu-5-HTP. This finding suggests that release of aldosterone by glu-5-HTP is predominantly a peripheral effect. A possible central effect of 5-HT may, however, partly contribute to the failure of carbidopa to completely suppress the aldosterone secretion induced by glu-5-HTP. In addition, carbidopa had minimal inhibitory effect on the activity of LAAD in the adrenal gland of the rat and guinea pig [429,450,451], and the presence of a blood-adrenal barrier, analogous to the blood-brain barrier, has been suggested [451]. L-5-HTP formed from glu-5-HTP which has penetrated the adrenal gland may, therefore, be less susceptible to the effect of carbidopa and its continued intra-adrenal conversion to 5-HT may thus stimulate aldosterone release. Carbidopa had no effect on the progressive reduction in plasma aldosterone concentrations produced by infusion of saline. This finding contradicts the observation made in the dog by McClanahan *et al.* [452] that carbidopa prevented the suppression of plasma aldosterone levels induced by saline infusion.

We conclude that the results of this work support the proposition that the effect of glu-5-HTP on urinary excretion of sodium is caused by a direct tubular action of 5-HT formed within renal tubular cells by LAAD and is independent of any effect on renal haemodynamics.

CHAPTER SEVEN

COMPARISON OF THE EFFECTS OF γ-L-GLUTAMYL-L-TRYPTOPHAN AND γ-L-GLUTAMYL-5-HYDROXY-L-TRYPTOPHAN ON URINARY EXCRETION OF 5-HYDROXYTRYPTAMINE AND SODIUM

7.1 Introduction

The biosynthesis of 5-HT is a two-step process. L-tryptophan is first hydroxylated by tryptophan 5-hydroxylase to L-5-HTP which is then decarboxylated by LAAD to produce 5-HT. LAAD is present in high concentrations in the renal proximal tubular cells [31,35,141-143]. Renal formation of 5-HT from its immediate amino acid precursor L-5-HTP has been demonstrated in renal tissue homogenates [16,39,42,140], in isolated proximal convoluted tubules [179,180] and in isolated perfused rat kidneys [153,181,453]. Administration of L-5-HTP *in vivo* in the rat [153,166,268,449] and man [162,177,178; Chapters 3-5] produces a marked increase in the urinary excretion of 5-HT, consistent with intrarenal synthesis of 5-HT, and this is accompanied by a reduction in the urinary excretion of sodium (*see* Chapters 3 and 4). Tryptophan 5-hydroxylase activity has also been identified in the rat and human kidney [35,137,138], particularly in the proximal tubules of the cortex [35], suggesting that the kidney might have the capacity to synthesise 5-HT from its first precursor L-tryptophan. This enzyme is not fully saturated by its substrate under normal conditions [31,454,455], and the administration of large doses of L-tryptophan has been employed to investigate the synthesis, and functional significance, of 5-HT in the kidney [35,121]. Tryptophan 5-hydroxylase is, however, present in many other tissues [137,138] and systemic administration of L-tryptophan may, therefore, not be a particularly effective way to enhance the intrarenal formation of 5-HT selectively. In addition, the renal actions of 5-HT produced within the kidney may be modified by confounding extrarenal effects consequent upon increased 5-HT synthesis in other organs (or tissues).

The kidney has a considerable capacity to accumulate, and metabolise, γ-glutamyl derivatives of amino acids and peptides [324,325,329] as a consequence of the high activity of γGT present in the brush border of the renal proximal tubular cells [308-316]. Administration of γ-glutamyl derivatives of drugs offers the possibility of delivering drugs selectively to the kidney. We have previously

employed this approach and administered glu-5-HTP, the glutamyl derivative of L-5-HTP, to increase the delivery of L-5-HTP to the kidney where it can be decarboxylated to 5-HT (*see* Chapters 3-6). Glu-5-HTP produced a large increase in urinary excretion of 5-HT, without a concomitant rise in 5-HT levels measured in platelet-rich plasma, together with a reduction in excretion of sodium. These observations are consistent with intrarenal synthesis of 5-HT following the conversion of glu-5-HTP to L-5-HTP by γGT and the decarboxylation of L-5-HTP by renal LAAD to 5-HT. The glutamyl derivative of L-tryptophan (γ-L-glutamyl-L-tryptophan; glu-TRP) may likewise selectively enhance the delivery of L-tryptophan to the kidney where it can be hydroxylated by renal tryptophan 5-hydroxylase to L-5-HTP, followed by decarboxylation of L-5-HTP by renal LAAD to 5-HT. This approach could provide an alternative strategy to administration of glu-5-HTP to augmenting 5-HT synthesis in the kidney.

The study described in this chapter compared the relative effectiveness of equimolar amounts of glu-TRP and glu-5-HTP in terms of their ability to act as substrates for 5-HT synthesis and also in their actions on urinary sodium excretion.

7.2. Methods

Nine healthy male subjects, aged 19-38 years (mean 30.7 years) and weighing 53.2-82.4 kg (mean 71.6 kg), were recruited to this randomised, single-blind, placebo-controlled, cross-over study. Each subject attended on 3 separate days, at least 1 week apart. They refrained from alcohol for 24 h, abstained from xanthine-containing drinks from 18.00 h, and fasted from 22.00 h the evening before each study day. Salt intake was not strictly controlled but dietary advice was given to avoid excess salt over the 36 h prior to each study day.

The subjects arrived at the clinical investigation unit at about 08.00 h having drunk 500 ml of tap water one hour previously. They received intravenous 0.9% NaCl at a constant rate of 5 ml min^{-1} for the next 7 h. After a 3 h run-in period

(time = 3 h), an equimolar amount (45 nmol kg^{-1} min^{-1}) of glu-5-HTP (16.6 μg kg^{-1} min^{-1}) or glu-5-TRP (15.5 μg kg^{-1} min^{-1}) in 0.9% saline, made up to a total volume of 30 ml, was infused intravenously at 0.5 ml min^{-1} for 1 h. Placebo was 30 ml of saline alone. Accurately timed consecutive urine collections of about 30 min duration were started half an hour before and continued until 3 h after the completion of the infusion of the test compounds. In addition to the saline administered intravenously, the subjects drank 200 ml of water half-hourly. These measures were used to promote an adequate natriuresis and diuresis. The subjects remained supine or semi-recumbent throughout the experiment except when standing up to micturate.

The volume of each urine collection was recorded and aliquots stored at -40° C for analysis of sodium, glu-TRP, tryptophan, 5-HTP, 5-HT and 5-HIAA. Venous blood samples were collected at 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7 h for measurement of plasma glu-TRP, L-tryptophan and aldosterone. Plasma and urinary glu-TRP and L-tryptophan; plasma aldosterone; and urinary 5-HTP, 5-HT and 5-HIAA were analysed as described in section 2.5.

All results are expressed as means ± SD. Variables measured serially on the three experimental days were analysed by three-way repeated measures ANOVA giving the effect of subjects, treatment (type of infusion), time and the interaction of treatment and time. The differences from baseline (preinfusion) values were used in the analyses to allow for variable baseline values. The 3-7 h cumulative urinary metabolite and sodium data following infusion of glu-5-HTP and glu-TRP were compared with those following placebo by Student's t-test for paired observations and the 95% CI_{diff} of the means presented where appropriate. Effects were considered to be statistically significant when the P values were less than 0.05.

7.3 Results

Table 7-1 shows the urinary excretion rates of tryptophan, 5-HTP, 5-HT, 5-HIAA and sodium for each 30 min period before, during and after infusion of placebo, glu-5-HTP and glu-TRP. Urinary excretion of 5-HTP, 5-HT and 5-HIAA increased markedly after administration of glu-5-HTP. The 3-7 h cumulative 5-HT excretion after infusion of glu-5-HTP was 34.8 ± 8.0 μmol compared to 0.10 ± 0.02 μmol after placebo infusion ($P < 0.001$) and this increase in urinary 5-HT excretion represented 18 ± 3% of the infused dose of glu-5-HTP (on a molar basis). At the end of the study period, 64 ± 6% of infused glu-5-HTP was recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA.

In contrast, after infusion of glu-TRP, there were no increases in urinary excretion of 5-HTP, 5-HT and 5-HIAA (Table 7-1). The urinary tryptophan excretion data are presented (and analysed) as changes from baseline (2.5-3.0 h period) (Figure 7-1) to allow for the different baseline excretion rates before infusion of placebo and glu-TRP (Table 7-1). Mean urinary tryptophan excretion values were slightly greater after infusion of glu-TRP than after placebo and there was a significant treatment x time interaction ($P < 0.05$) in the urinary tryptophan excretion rates after administration of glu-TRP when compared with placebo infusion. Cumulative tryptophan excretion over the 4 h period was 17.7 ± 7.7 μmol after infusion of glu-TRP and 13.4 ± 7.0 μmol after placebo (95% CI_{diff}: 0.8 to 7.8 μmol; $P < 0.05$). The increase in tryptophan excretion represented less than 3% of the infused dose of glu-TRP. If allowance is made for the different urinary tryptophan excretion rates just before infusion, the mean cumulative urinary tryptophan excretion after glu-TRP was only 1.8 μmol (95% CI_{diff}: -3.2 to 6.8 μmol; $P = 0.4$) greater than after placebo infusion. There were no significant increases in plasma tryptophan concentrations after glu-TRP compared with placebo. Plasma glu-TRP reached a peak level at the end of glu-TRP infusion and declined with time (Figure 7-1). Glu-TRP was undetectable in urine.

The urinary excretion of sodium increased progressively on the placebo day in response to infusion of saline (Table 7-1; Figure 7-2) as has been seen in other studies (*see* Chapters 3-6). Glu-5-HTP, but not glu-TRP, significantly attenuated the increase in sodium excretion when compared with placebo ($P < 0.001$). Cumulative urinary sodium excretion was reduced by 16.2 ± 7.9 mmol following glu-5-HTP compared with placebo infusion (95% CI_{diff}: 10.1 to 22.3 mmol; $P < 0.001$). The cumulative sodium excretion after glu-TRP was not different from that after placebo (95% CI_{diff}: -2.4 to 12.2 mmol; $P = 0.2$).

Glu-5-HTP produced a significant increase in plasma aldosterone concentration when compared with placebo infusion ($P < 0.01$), whereas glu-TRP was without effect (Figure 7-3). There were no significant changes in blood pressure or pulse rate between the 3 study days (Figure 7-4), and there were no reported or observed side effects from either dipeptide.

Table 7-1. Mean (SD) urinary excretion rates of tryptophan, 5-HTP, 5-HT, 5-HIAA and sodium before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4 h) and after (period 4.0-4.5 h to period 6.5-7.0 h) infusion of placebo, glu-5-HTP and glu-TRP (n = 9). A value of 0 is entered where a variable was below the limit of detection of the assay used.

	Time (h)								
	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0	6.0-6.5	6.5-7.0
Tryptophan excretion (nmol min^{-1})									
Placebo	58	53	56	55	56	53	56	59	59
	(29)	(30)	(33)	(32)	(30)	(26)	(31)	(27)	(30)
Glu-TRP	68	69	82	89	83	61	71	70	63
	(40)	(41)	(51)	(39)	(36)	(23)	(35)	(29)	(26)
5-HTP excretion (nmol min^{-1})									
Placebo	0	0	0	0	0	0	0	0	0
Glu-5-HTP	0	204	473	416	235	146	85	64	49
		(35)	(56)	(42)	(74)	(42)	(16)	(20)	(13)
Glu-TRP	0	0	0	0	0	0	0	0	0
5-HT excretion (nmol min^{-1})									
Placebo	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)
Glu-5-HTP	0.4	100	240	279	193	137	90	67	60
	(0.1)	(31)	(50)	(59)	(43)	(43)	(29)	(23)	(23)
Glu-TRP	0.4	0.4	0.4	0.5	0.5	0.4	0.4	0.5	0.6
	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.3)	(0.2)
5-HIAA excretion (nmol min^{-1})									
Placebo	0	0	0	0	0	0	0	0	0
Glu-5-HTP	0	81	191	251	244	209	155	120	103
		(83)	(91)	(57)	(51)	(66)	(49)	(38)	(51)
Glu-TRP	0	0	0	0	0	0	0	0	0
Sodium excretion (μmol min^{-1})									
Placebo	261	279	290	296	295	315	340	346	343
	(122)	(138)	(144)	(162)	(99)	(147)	(184)	(164)	(134)
Glu-5-HTP	277	272	230	216	241	270	268	282	308
	(75)	(105)	(75)	(61)	(54)	(88)	(83)	(83)	(82)
Glu-TRP	270	297	310	353	362	331	338	370	377
	(107)	(122)	(164)	(158)	(169)	(130)	(139)	(121)	(143)

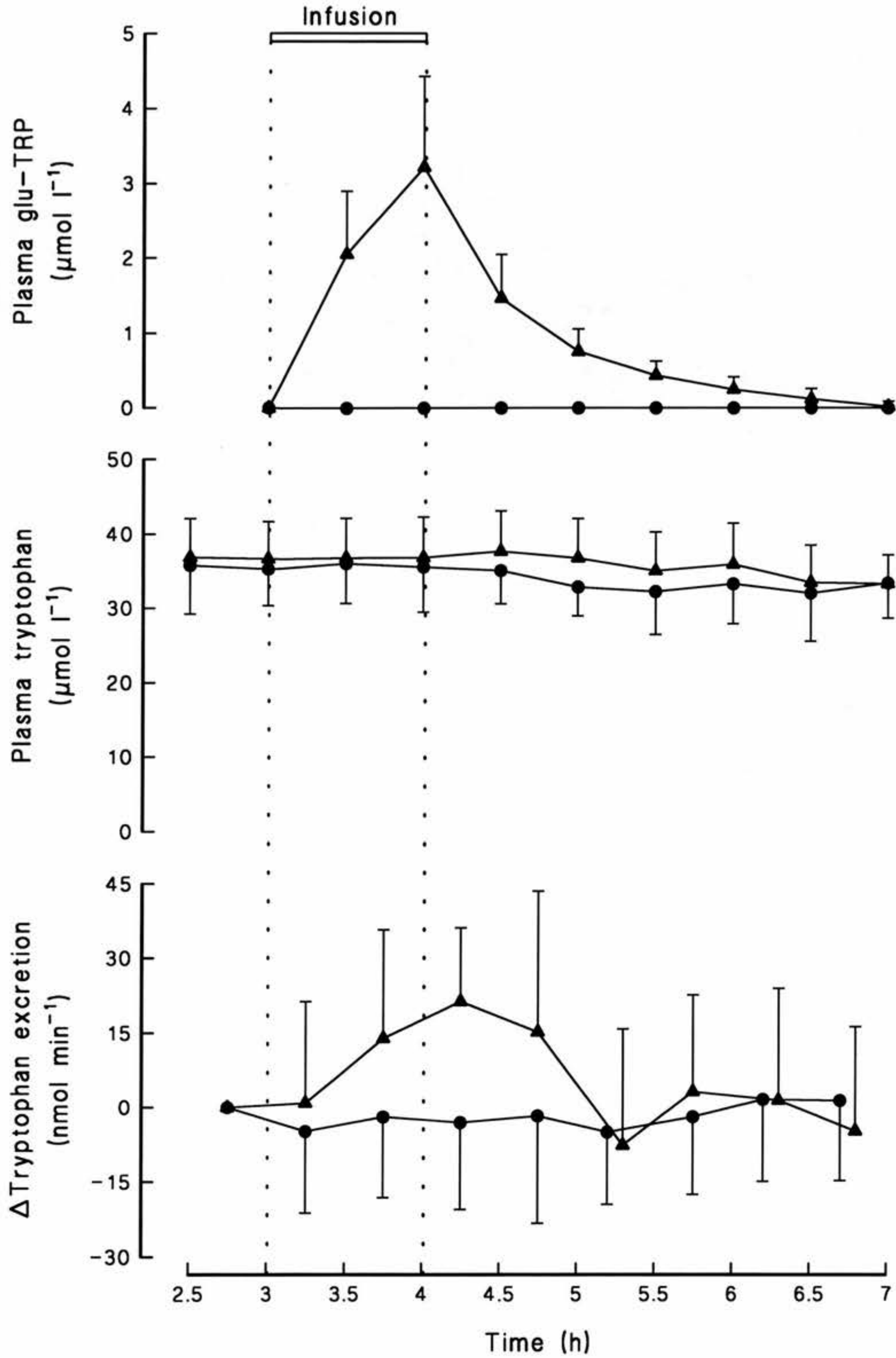


Figure 7-1. Plasma glu-TRP concentrations, plasma tryptophan concentrations, and changes (Δ) in urinary excretion rates of tryptophan from baseline (period 2.5-3.0 h) after infusion of placebo (●) and glu-TRP (▲). Values shown are means ± SD (n = 9).

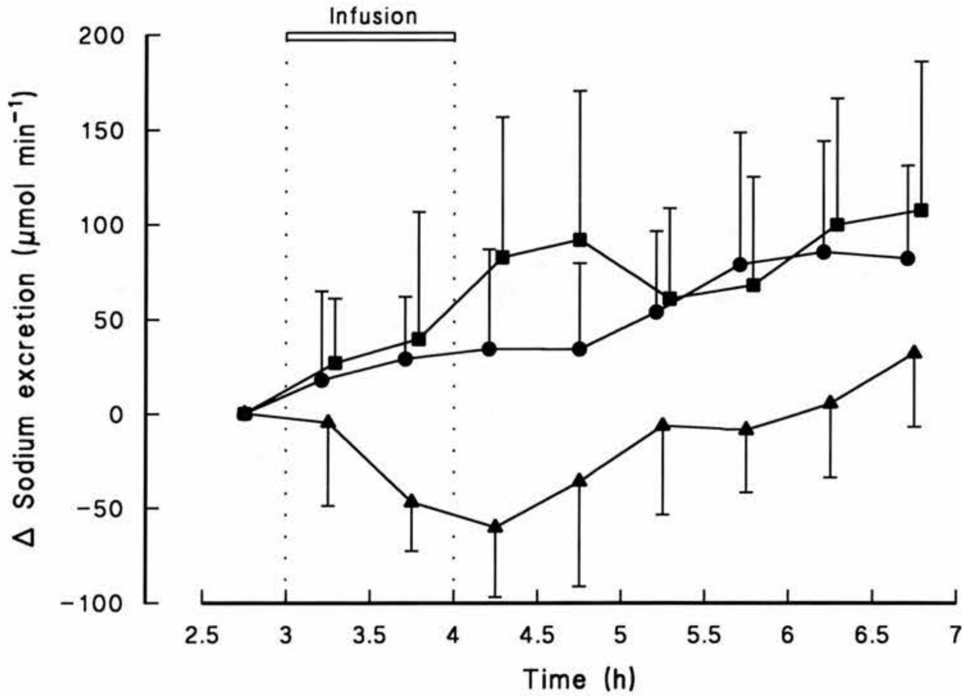


Figure 7-2. Changes (Δ) in urinary excretion rates of sodium from baseline (period 2.5-3.0 h) following infusion of placebo (●), glu-5-HTP (▲) and glu-TRP (■). Values shown are means ± SD (n = 9).

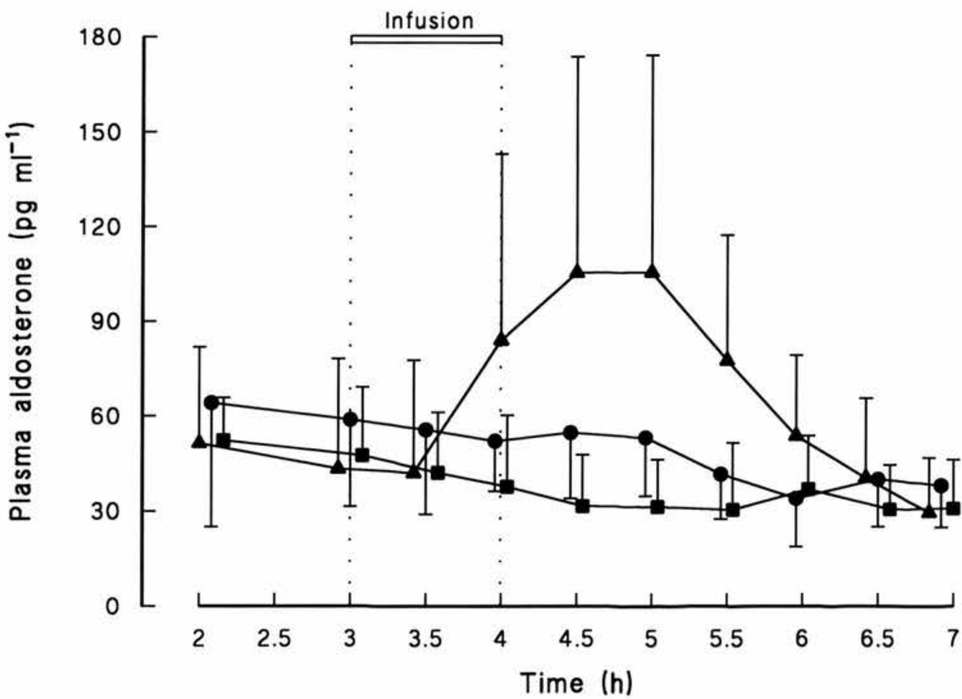


Figure 7-3. Plasma aldosterone concentration before, during and after infusion of placebo (●), glu-5-HTP (▲) and glu-TRP (■). Values shown are means ± SD (n = 9).

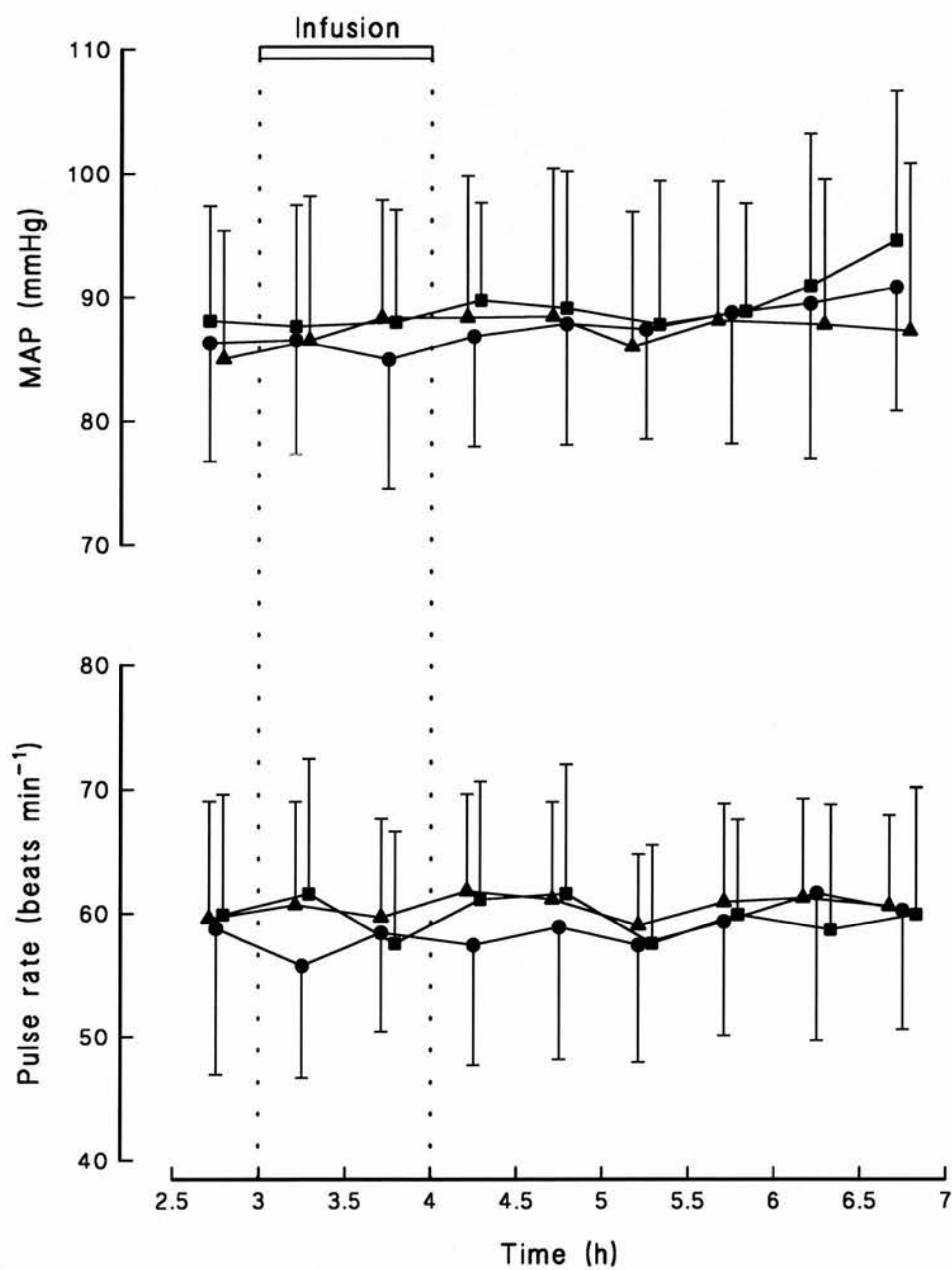


Figure 7-4. Mean arterial blood pressure (MAP) and pulse rate before, during and after infusion of placebo (●), glu-5-HTP (▲) and glu-TRP (■). Values shown are means ± SD (n = 9).

7.4 Discussion

Administration of glu-5-HTP was associated with increases in the urinary excretion of 5-HTP, 5-HT and 5-HIAA together with a reduction in excretion of sodium in agreement with our earlier studies (*see* Chapters 4-6). This sodium retaining effect was previously shown to be dependent on the conversion of glu-5-HTP to 5-HT (*see* Chapter 6). In contrast, infusion of an equimolar dose of glu-TRP did not increase urinary excretion of 5-HTP, 5-HT or 5-HIAA, and it had no effect on urinary sodium excretion. As the kidney is highly active in the uptake and metabolism of γ-glutamyl derivatives of amino acids and peptides [324,325,329], it would have been expected that glu-TRP would be taken up actively by the proximal tubules in an analogous manner to glu-5-HTP or gludopa [329-331,333,334,341]. It should be readily converted by γGT to L-tryptophan thus releasing large amounts of the free amino acid at the proximal tubules. L-tryptophan is known to be actively and efficiently reabsorbed in the proximal tubules [185,186], and it should therefore be available for the onward synthesis of 5-HT by the sequential actions of tryptophan 5-hydroxylase and LAAD present in the tubular cells. The absence of a rise in urinary 5-HTP or 5-HT and of an effect on sodium excretion after glu-TRP infusion in the present study suggests that there was no effective renal conversion of glu-TRP to L-5-HTP and 5-HT.

We subsequently estimated plasma and urinary levels of glu-TRP and L-tryptophan in view of the above findings to exclude the possibility that glu-TRP may not have been metabolised and was excreted in an intact form. Plasma glu-TRP reached a peak level at the end of glu-TRP infusion and then declined with time but we were unable to detect glu-TRP in urine. There was a small increase in urinary tryptophan excretion after administration of glu-TRP without concomitant changes in plasma tryptophan concentrations. These additional observations indicate that the glutamyl linkage of glu-TRP was cleaved by renal γGT but the kidney was unable to utilise the L-tryptophan released from glu-TRP as a substrate

for the synthesis of 5-HT. It appears that for some reason, the enzyme tryptophan 5-hydroxylase, which has been shown to be present in the human cadaveric kidney [35], was not available to the L-tryptophan formed intrarenally from glu-TRP under the conditions of the study. The increment in urinary L-tryptophan excretion accounted for only 3%, at most, of the infused dose of glu-TRP. L-tryptophan, like other essential amino acids, is rapidly and almost totally reabsorbed by the renal tubules [185,186,456,457] and this may explain partly our failure to detect a more substantial increase of tryptophan excretion in urine. The usual clearance of L-tryptophan in the dog, for instance, was about 0.2 ml min^{-1} and the maximal clearance attained was 0.47 ml min^{-1} when blood level increased 12-fold [456]. In man, the renal clearance of L-tryptophan after its oral administration (100 mg kg^{-1}) was reported to be 0.4-0.8% of the creatinine clearance value in one study [174]. The failure of plasma tryptophan concentrations to rise and the fact that there were no significant increases in the urine of the metabolites of 5-HT suggests that much of L-tryptophan released from glu-TRP was converted to products which were not measured in this study. This would not be totally unexpected since L-tryptophan is metabolised along a number of alternative metabolic pathways. Previous studies have suggested that only approximately 1% of dietary tryptophan is normally metabolised via the 5-HT pathway [33,458-460].

Tryptophan 5-hydroxylase activity was identified in the frog [36], but not in the rat [35], adrenal gland, and the enzyme LAAD is known to be present in this organ [31,36,39]. An increase in plasma aldosterone concentration has been demonstrated in man following administration of the 5-HT precursors, L-tryptophan [95] and L-5-HTP [96,97,105; *see* Chapters 3 and 4]. The infusion of glu-5-HTP increased plasma aldosterone levels in keeping with observations from our previous studies (see Chapters 3, 4 and 6). This may be due to recirculation of L-5-HTP or 5-HT produced within the kidney, acting on the adrenal gland, or to the conversion of glu-5-HTP to 5-HT within this organ. Glu-TRP, unlike glu-5-HTP, exerted no effect on

aldosterone release in the present study. This finding should be interpreted cautiously but could indicate that there was no appreciable increase in 5-HT concentration within the adrenal gland after the administration of this dipeptide.

The results of the present investigation are reminiscent of the study by Jeffrey *et al.* [338] which showed that γ-L-glutamyl-L-dopa (gludopa), but not γ-L-glutamyl-L-tyrosine, increased the urinary excretion of dopamine and sodium. The kidney was unable to use L-tyrosine, released from its glutamyl precursor, for the synthesis of dopamine and the study supported the contention that circulating L-dopa, rather than L-tyrosine, is the main physiological source for the generation of renal and, in turn, urinary dopamine. Similarly, it is conceivable that L-5-HTP, rather than L-tryptophan, is the principal precursor of 5-HT formed locally in the kidney.

In conclusion, glu-TRP, unlike glu-5-HTP, was not converted to 5-HT intrarenally and probably, as a result, had no effect on the urinary excretion of sodium. Glu-5-HTP differs from glu-TRP only in the presence of an additional hydroxyl group at the C5 position of the indole ring. This group would appear to be important in the active uptake of the amine precursor by the renal tubules and its subsequent conversion to 5-HT.

CHAPTER EIGHT

THE RENAL METABOLISM AND EFFECTS OF SEPARATE AND SIMULTANEOUS INFUSIONS OF THE GLUTAMYL DERIVATIVES OF L-DOPA AND 5-HYDROXY-L-TRYPTOPHAN IN SALT-REPLETE HEALTHY MAN

8.1 Introduction

The same enzyme, aromatic LAAD, catalyses the decarboxylation of L-dopa and L-5-HTP to produce dopamine and 5-HT respectively [31]. These biogenic amines are both primarily metabolised by MAO. 5-HT is converted by MAO-A to 5-hydroxyindoleacetaldehyde which is promptly degraded to produce 5-HIAA. Dopamine, on the other hand, is readily metabolised by both MAO-A and MAO-B to form DOPAC. Both LAAD and MAO are present in high concentrations in the mammalian kidney and there is evidence that dopamine [432-436] and 5-HT (*see* section 1.2.3) can be synthesised intrarenally from their immediate precursors. Increased renal dopamine production is associated with a natriuresis whereas enhanced 5-HT synthesis is accompanied by a reduction in urinary sodium output. Dopamine and 5-HT therefore share common synthetic and metabolic pathways but have opposite effects on sodium excretion. It is possible that these amines are generated within the kidney as reciprocal regulatory paracrine substances for the local control of sodium excretion, and more, or less, dopamine and 5-HT are synthesised depending on the need to excrete or conserve sodium [268,436]. This proposition is supported by the observations that a reduction in 5-HT excretion [461] and an increase in dopamine excretion [462-466] occur with salt-loading, whereas a low sodium diet results in decreased urinary excretion of dopamine [467] and an increased excretion of 5-HT [461] in man. An increased urinary excretion of 5-HT was, however, observed in rats which were fed on a high salt diet [469].

The kidney is highly active in the uptake and metabolism of γ-glutamyl derivatives of amino acids and peptides [324,329] as a result of the high activity of γGT in the renal tissues, particularly in the proximal tubules [308-316]. These observations suggest that the γ-glutamyl group may provide a convenient carrier for directing compounds containing an amino group into kidney metabolism resulting in relative renal selectivity and absence of systemic effects. Administration of gludopa, the glutamyl derivative of L-dopa, has been employed to increase the

delivery of L-dopa to the kidney for decarboxylation to dopamine [329,334-342]. We have applied a similar approach and administered glu-5-HTP, the glutamyl derivative of L-5-HTP, to enhance the renal delivery of L-5-HTP and demonstrated a marked increase in the renal synthesis of 5-HT (*see* Chapters 3-7). Renal γGT catalyses the cleavage of the γ-glutamyl linkage of these glutamyl compounds. The resulting L-dopa and L-5-HTP are then decarboxylated to dopamine and 5-HT respectively by LAAD which is also present in high concentrations in the proximal tubules of the kidney [31,35,139,141-143]. Administration of gludopa and glu-5-HTP has the advantage that L-dopa and L-5-HTP are largely released within the kidney thus allowing the renal effects of locally formed dopamine and 5-HT to be studied with a minimum of confounding systemic effects whereas L-dopa and L-5-HTP, infused intravenously, may exert systemic and confounding effects.

In the present study, we have infused equimolar doses of gludopa and glu-5-HTP separately, and together, in salt-replete healthy male subjects to investigate whether the administration of one amine precursor affects the metabolism of the other given that similar enzymes are involved in the synthesis and degradation of dopamine and 5-HT. We were also interested as to whether dopamine or 5-HT is generated preferentially and the overall effect on sodium excretion.

8.2 Methods

Eight male subjects, aged 19-37 years (mean 27.9 years) and weighing 54.4-86.2 kg (mean 70.3 kg), took part in this randomised, single-blind, placebo-controlled, within-subject cross-over study. The subjects were each studied on 4 occasions, separated by at least 10 days. They had been given dietary advice to avoid salty and highly processed food and to avoid adding salt to their food over the 3 days before each study day. During that period, they took 5 tablets of Slow Sodium 4 times daily at 08.00 h, 12.00 h, 16.00 h and 20.00 h, giving a total of 200 mmol additional sodium intake per day. These measures were taken in an attempt to ensure that the subjects were salt-replete (or salt-loaded) and to reduce the variability in dietary sodium intake between the study days. The subjects refrained from alcohol for 24 h, abstained from xanthine-containing drinks from 18.00 h, and fasted from 22.00 h the evening before each study day.

They arrived at the clinical investigation unit at about 08.00 h having drunk 500 ml of tap water 1 h previously. They received loading doses of 0.5 g of PAH and 3.5 g of polyfructosan (Inutest) intravenously at the start of the study (time = 0 h) followed by a maintenance infusion of PAH (3.75 g l^{-1}) and polyfructosan (4.5 g l^{-1}) in 0.9% NaCl at a constant rate of 5 ml min^{-1} for the next 6 h to allow estimation of ERPF and GFR. After a 3 h run-in period (time = 3 h), they received placebo or gludopa from one infusion pump and placebo or glu-5-HTP from another pump intravenously into the same arm. Equimolar amounts (54.4 nmol kg^{-1} min^{-1}) of gludopa (18.7 μg kg^{-1} min^{-1}) and glu-5-HTP (20 μg kg^{-1} min^{-1}) were given over 1 h. Each solution was made up to a total volume of 30 ml with saline and infused at 0.5 ml min^{-1}. Placebo was 30 ml of saline alone. Each subject therefore received the following four combinations in a randomised sequence: placebo + placebo; gludopa + placebo; placebo + glu-5-HTP; and gludopa + glu-5-HTP.

Accurately timed consecutive urine collections of about 30 min duration were started half an hour before and continued for 3 h after the start of the infusion of the

test compounds. In addition to the saline administered intravenously, the subjects drank 200 ml of water half-hourly to promote an adequate urine flow. They remained semi-recumbent or supine throughout the experiment except when standing to micturate. The volume of each urine collection was recorded and aliquots removed and stored at -40° C for analysis of sodium, potassium, PAH, polyfructosan, dopa, dopamine, DOPAC, 5-HTP, 5-HT, and 5-HIAA. Urine samples for dopamine, DOPAC, 5-HT and 5-HIAA were acidified (pH < 3.0) with 5 M HCl acid to prevent their oxidation. Venous blood samples were collected at 0, 2.5, 3, 3.5, 4, 4.5, 5 and 6 h for measurement of plasma sodium, PAH, and polyfructosan, and at 2, 3, 3.5, 4, 4.5, 5, 5.5 and 6 h for determination of PRA and plasma aldosterone. Plasma was separated after centrifugation at 4° C and stored at -40° C until analysis. Plasma aldosterone and PRA; plasma and urinary electrolytes, PAH and polyfructosan; and urinary dopa, dopamine, DOPAC, 5-HTP, 5-HT and 5-HIAA were measured as described previously in section 2.5.

Renal clearance was calculated using the standard formula UV/P, where U is the urine concentration, V is the urine flow rate and P is the mean of the plasma levels at the beginning and end of each clearance period (*see* section 2.6). ERPF and GFR were estimated by calculating the renal clearances of PAH (C_{PAH}) and polyfructosan (C_{In}) respectively and the values were corrected to a body surface area of 1.73 m^2. The filtration fraction (FF) was calculated as (C_{In}/C_{PAH}) x 100%. Fractional excretion of sodium (FE_{Na}) was calculated as renal clearance of sodium divided by GFR (C_{In}) and expressed as a percentage.

All results are expressed as means ± SD. Variables measured serially on the 4 experimental days were compared by repeated measures ANOVA, with the different treatment (infusion regimens) and time being the within-subject repeated measures. If there was a significant overall difference between the four experimental days, follow-up paired comparison between the infusion regimens were made to determine where the differences lie, with correction of the P value by the Bonferroni

method to allow for multiple comparisons and protect against a type 1 error. The differences from baseline (preinfusion) values were used in the analyses to allow for variable baseline values. Similarly, the changes in 3-6 h cumulative sodium, potassium and urine output, relative to baseline excretion rates, were calculated and used in the analysis rather than the absolute 3-6 h cumulative values to take into account the differences in baseline excretion values between study days. The cumulative data between the different treatment regimens were compared using two-way ANOVA, followed by Student's t test for paired observations if ANOVA revealed a significant overall difference. The P values were adjusted by the Bonferroni method, and the associated 99%, rather than 95%, confidence interval of the difference between the means (CI_{diff}) quoted where appropriate. Effects were considered to be statistically significant when the P values were less than 0.05.

8.3 Results

Urinary excretion of dopa, dopamine and DOPAC

Table 8-1 shows the urinary excretion rates of dopa, dopamine and DOPAC for each 30 min period before, during and after administration of the four infusion regimens. Urinary dopamine excretion remained relatively constant during infusion of saline on the placebo day. The administration of gludopa produced a marked increase in the rate of dopamine excretion from a baseline value of 1.6 nmol min^{-1} during the preinfusion clearance period (2.5-3.0 h) to a peak value of 622 nmol min^{-1} during the collection period (3.5-4.0 h) at the end of the infusion. The 3-6 h cumulative dopamine excretion after gludopa was 215-fold higher than that following placebo infusion only (Table 8-2). This increase in urinary dopamine excretion represented 27 ± 7% of the infused dose of gludopa (on a molar basis) (Table 8-3). Administration of gludopa also produced marked increases in the urinary excretion rates of dopa and DOPAC. At the end of the study period, 54 ± 11% of infused gludopa was recovered in the urine as the sum of dopa, dopamine and DOPAC. The urinary excretion values of these 3 metabolites following the simultaneous infusion of gludopa and glu-5-HTP were not significantly different from those observed after the infusion of gludopa alone (Tables 8-1 to 8-3). Thus, the 3-6 h cumulative excretion of dopamine was 60.4 ± 11.3 μmol after infusion of gludopa and 61.8 ± 11.4 μmol after the combined infusion of the two dipeptides (mean difference: 1.4; 99% CI_{diff}: −5.1 to 7.8 μmol). The administration of glu-5-HTP alone was without effect on the urinary excretion of dopa, dopamine and DOPAC when compared with infusion of placebo.

Urinary excretion of 5-HTP, 5-HT and 5-HIAA

Table 8-4 shows the urinary excretion rates of 5-HTP, 5-HT and 5-HIAA on the 4 experimental days. Urinary 5-HT excretion rate increased markedly from 0.4 nmol min^{-1} during the run-in period to a peak value of 291 nmol min^{-1} at the end of glu-5-HTP infusion whereas it remained fairly constant on the placebo day. The 3-6 h

cumulative 5-HT excretion after glu-5-HTP was about 430-fold higher than that following placebo infusion (Table 8-2) and this increment in urinary 5-HT excretion was equivalent to 15 ± 2% of the infused dose of glu-5-HTP (on a molar basis) (Table 8-5). There were also marked increases in the urinary excretion rates of 5-HTP and 5-HIAA after the administration of glu-5-HTP. During the 3-6 h period, 56 ± 5% of the dose of glu-5-HTP infused was recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA. The urinary excretion of these 3 metabolites following the simultaneous infusion of gludopa and glu-5-HTP were not significantly different from those measured after infusion of glu-5-HTP alone (Tables 8-2, 8-4, 8-5). The 3-6 h cumulative excretion of 5-HT, for instance, was 34.7 ± 9.0 μmol after glu-5-HTP and 34.8 ± 7.4 μmol after the combined infusion (mean difference: 0.2; 99% CI_{diff}: −7.3 to 7.6 μmol). The infusion of gludopa alone produced an increase in the urinary excretion rate of 5-HT from an average baseline value of 0.5 nmol min^{-1} to a mean peak value of 4.3 nmol min^{-1}. The 3-6 h cumulative 5-HT excretion was 481 ± 125 nmol after gludopa infusion and 81 ± 23 nmol after placebo (mean difference: 400; 99% CI_{diff}: 242 to 558 nmol; $P < 0.001$). The increase in 5-HT excretion induced by gludopa occurred in every subject.

Urinary excretion of sodium, potassium and water

Gludopa significantly increased urinary sodium excretion ($P < 0.05$) and FE_{Na} ($P < 0.02$) when compared with infusion of placebo only (Table 8-6; Figure 8-1). The changes in the 3-6 h cumulative sodium excretion values, relative to the baseline excretion rates (Table 8-7), after infusion of placebo and gludopa were +2.3 ± 15.6 and +36.1 ± 20.6 mmol respectively (mean difference: 33.8; 99% CI_{diff}: 1.4 to 66.1 mmol; $P < 0.05$). In contrast, the mean urinary excretory rates of sodium and FE_{Na} values after glu-5-HTP infusion were lower than those observed on the placebo day but the differences were not statistically significant (mean difference in cumulative sodium excretion: −8.7; 99% CI_{diff}: -33.1 to 15.6 mmol). The simultaneous infusion of gludopa and glu-5-HTP produced an increase in the urinary excretion of sodium

and FE_{Na} when compared with administration of glu-5-HTP only ($P < 0.05$). The changes in the 3-6 h cumulative sodium excretion relative to baseline values after the combined infusion (gludopa + glu-5-HTP) and infusion of glu-5-HTP only were +26.3 ± 22.4 mmol and −6.5 ± 16.5 mmol respectively (mean difference: 32.8; 99% CI_{diff}: 0.3 to 65.2 mmol; $P < 0.05$). The mean urinary sodium excretion rates after the combined infusion of gludopa and glu-5-HTP were greater than those measured on placebo day but the differences were not statistically significant (mean difference in cumulative sodium excretion: +24.0; 99% CI_{diff}: −4.6 to 52.6 mmol). The changes in FE_{Na} values after the combined infusion of gludopa and glu-5-HTP, however, nearly reached statistical significance ($P = 0.06$) when compared with placebo infusion. The urinary sodium excretion rate and FE_{Na} values after the concomitant infusion of gludopa and glu-5-HTP were not significantly different from those observed on gludopa infusion day (mean difference in the cumulative sodium excretion: −9.8; 99% CI_{diff}: −39.9 to 20.4 mmol).

The urinary excretion rate values of potassium were not significantly different between the 4 experimental days (Table 8-6; Figure 8-2). Similarly, there were no significant differences in the changes in cumulative potassium excretion between the 4 infusion regimens (Table 8-7; $F(3,21) = 0.27$, $P = 0.9$). There was a trend towards an increase in urine flow rate after infusion of gludopa and a reduction in urine output after glu-5-HTP but these changes were not statistically significant when compared with those observed after infusion of placebo (Table 8-6; Figure 8-3). There was, however, a significant treatment x time interaction ($P < 0.02$) when the urine flow rate values after gludopa infusion were compared with those after glu-5-HTP infusion. There were no significant differences in the changes in cumulative urine output between the 4 study days (Table 8-7; $F(3,21) = 0.97$, $P = 0.4$). The variances of the changes in urine output on the 4 days were, however, wide. The mean difference in cumulative urine output between gludopa infusion and placebo infusion, for instance, was 180 ml (95% CI_{diff}: −330 to 690 ml).

ERPF, GFR and FF

Glu-5-HTP produced a significant reduction in ERPF when compared with placebo infusion ($P < 0.02$; Table 8-8; Figure 8-4). The changes in ERPF after infusion of gludopa alone or after the combined infusion of gludopa and glu-5-HTP were not significantly different from those measured on placebo day. There were no significant differences in GFR or FF values between the 4 experimental days. There was a trend towards a decrease in GFR after infusion of glu-5-HTP but this reduction just failed to reach statistical significance when compared with placebo ($P = 0.08$). As a result, the FF values after infusion of glu-5-HTP, in contrast to the ERPF values, were not significantly different from those observed after infusion of placebo only.

Plasma aldosterone and PRA

Compared with infusion of placebo only, glu-5-HTP ($P < 0.05$) and the combined infusion of gludopa and glu-5-HTP ($P < 0.05$) produced significant increases in plasma aldosterone levels whereas infusion of gludopa alone was without effect (Figure 8-5). The mean plasma aldosterone concentrations during the simultaneous infusion of gludopa and glu-5-HTP appeared lower than those after infusion of glu-5-HTP alone but the differences were not statistically significant. PRA was suppressed to below the limit of detection of the assay (< 0.35 ng ANG I ml^{-1} h^{-1}) on all 4 study days in 5 subjects and on 3 of the study days in another subject. Statistical analysis was deemed inappropriate since only two subjects had detectable PRA values on all 4 experimental days. The mean PRA values in these two subjects are shown in Figure 8-6.

There were no differences in pulse rate and blood pressure between the 4 study days (Figure 8-7). No adverse effects were reported during the study.

Table 8-1. Mean (SD) urinary excretion rates of dopa, dopamine and DOPAC for each 30 min period before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) administration of the 4 infusion regimens (n = 8). A value of 0 is entered when the measured variable was below the limit of detection of the assay employed.

	Time (h)						
	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
Dopa excretion (nmol min^{-1})							
Placebo +	0	0	0	0	0	0	0
placebo							
Gludopa +	0	160	436	314	163	92	53
placebo		(43)	(74)	(64)	(34)	(37)	(13)
Placebo +	0	0	0	0	0	0	0
glu-5-HTP							
Gludopa +	0	143	343	309	159	86	49
glu-5-HTP		(31)	(72)	(117)	(86)	(39)	(21)
Dopamine excretion (nmol min^{-1})							
Placebo +	1.6	1.7	1.7	1.5	1.5	1.6	1.5
placebo	(0.5)	(0.4)	(0.4)	(0.2)	(0.3)	(0.3)	(0.2)
Gludopa +	1.6	272	622	533	274	179	120
placebo	(0.4)	(92)	(129)	(119)	(48)	(53)	(13)
Placebo +	1.6	1.9	1.7	1.8	1.8	1.9	1.8
glu-5-HTP	(0.3)	(0.2)	(0.3)	(0.3)	(0.3)	(0.3)	(0.3)
Gludopa +	1.6	262	597	566	312	194	124
glu-5-HTP	(0.3)	(67)	(140)	(109)	(92)	(51)	(20)
DOPAC excretion (nmol min^{-1})							
Placebo +	3.7	3.6	3.9	3.6	3.5	3.5	3.2
placebo	(2.5)	(2.0)	(2.2)	(1.4)	(1.3)	(1.0)	(1.0)
Gludopa +	3.4	89	248	239	131	88	57
placebo	(1.1)	(20)	(50)	(47)	(22)	(31)	(9)
Placebo +	2.9	2.9	3.1	3.3	3.3	3.0	2.8
glu-5-HTP	(0.8)	(1.0)	(1.8)	(1.4)	(1.2)	(0.7)	(0.7)
Gludopa +	3.6	113	260	264	156	98	61
glu-5-HTP	(1.7)	(83)	(60)	(43)	(40)	(26)	(14)

Table 8-2. The 3-6 h cumulative excretion values (μmol) of dopa, dopamine, DOPAC, 5-HTP, 5-HT and 5-HIAA on the 4 experimental days. A value of 0 is entered when the measured variable was below the limit of detection of the assay employed. Values shown are means ± SD (n = 8).

	Infusion			
	Placebo+Placebo	*Gludopa+Placebo*	*Placebo+Glu-5-HTP*	*Gludopa+Glu-5-HTP*
Dopa	0	36.89 ± 3.76	0	32.76 ± 9.32
Dopamine	0.28 ± 0.04	60.39 ± 11.30	0.32 ± 0.04	61.75 ± 11.36
DOPAC	0.64 ± 0.26	25.69 ± 4.42	0.55 ± 0.20	28.61 ± 6.01
5-HTP	0	0	58.62 ± 11.95	62.64 ± 6.65
5-HT	0.08 ± 0.02	0.48 ± 0.13	34.67 ± 9.02	34.83 ± 7.38
5-HIAA	0	0	34.83 ± 5.33	35.16 ± 4.64

Table 8-3. Percentage urinary recoveries (%) of infused gludopa as dopa, dopamine and DOPAC after infusion of gludopa alone and after the simultaneous infusion of gludopa and glu-5-HTP. Values shown are means ± SD (n = 8). The 99% CI values of the differences between the means of the 2 regimens are quoted.

	Infusion		
	Gludopa	*Gludopa + Glu-5-HTP*	*95% CI of difference*
Dopa	16.2 ± 1.9	14.3 ±3.6	-1.0 to 4.7
Dopamine	26.7 ± 7.1	27.2 ± 6.5	-2.3 to 1.4
DOPAC	11.1 ± 2.4	12.2 ± 1.7	-2.5 to 0.2
Total	54.0 ± 10.7	53.6 ± 8.8	-4.4 to 5.0

Table 8-4. Mean (SD) urinary excretion rates of 5-HTP, 5-HT and 5-HIAA for each 30 min period before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) administration of the 4 infusion regimens (n = 8). A value of 0 is entered when the measured variable was below the limit of detection of the assay employed.

	Time (h)						
	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
5-HTP excretion (nmol min^{-1})							
Placebo + placebo	0	0	0	0	0	0	0
Gludopa + placebo	0	0	0	0	0	0	0
Placebo + glu-5-HTP	0	241 (54)	580 (96)	481 (70)	321 (154)	193 (96)	124 (42)
Gludopa + glu-5-HTP	0	261 (73)	605 (174)	547 (57)	325 (76)	211 (70)	138 (53)
5-HT excretion (nmol min^{-1})							
Placebo + placebo	0.4 (0.1)	0.5 (0.2)	0.5 (0.1)	0.4 (0.1)	0.4 (0.1)	0.4 (0.2)	0.5 (0.1)
Gludopa + placebo	`0.5 (0.1)	2.0 (0.7)	3.8 (1.4)	4.3 (1.3)	2.4 (0.6)	2.0 (0.5)	1.4 (0.5)
Placebo + glu-5-HTP	0.4 (0.1)	98 (28)	291 (77)	291 (75)	221 (69)	152 (64)	101 (33)
Gludopa + glu-5-HTP	0.5 (0.1)	92 (37)	303 (64)	333 (84)	209 (67)	134 (57)	88 (37)
5-HIAA excretion (nmol min^{-1})							
Placebo + placebo	0	0	0	0	0	0	0
Gludopa + placebo	0	0	0	0	0	0	0
Placebo + glu-5-HTP	0	75 (18)	210 (65)	242 (65)	253 (50)	201 (47)	180 (37)
Gludopa + glu-5-HTP	0	92 (24)	208 (64)	255 (37)	248 (37)	204 (44)	169 (47)

Table 8-5. Percentage urinary recoveries (%) of infused glu-5-HTP as 5-HTP, 5-HT and 5-HIAA after infusion of glu-5-HTP alone and after the simultaneous infusion of gludopa and glu-5-HTP. Values shown are means ± SD (n = 8). The 99% CI values of the differences between the 2 regimens are quoted.

		Infusion	
	Glu-5-HTP	*Gludopa + Glu-5-HTP*	*95% CI of difference*
5-HTP	25.6 ± 3.9	27.5 ±2.7	-4.8 to 1.0
5-HT	15.0 ± 2.3	15.2 ± 2.4	-2.3 to 1.9
5-HIAA	15.3 ± 2.6	15.5 ± 2.5	-2.1 to 1.8
Total	55.9 ± 4.9	58.2 ± 5.4	-5.7 to 1.1

Table 8-6. Mean (SD) sodium excretion rates, FE_{Na}, potassium excretion rates and urine flow rates for each 30 min period before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) administration of the 4 infusion regimens (n = 8).

				Time (h)			
	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
Sodium excretion (μmol min^{-1})							
Placebo +	389.2	373.9	392.9	425.1	418.2	409.8	390.8
placebo	(132.0)	(113.3)	(122.5)	(155.0)	(109.7)	(94.4)	(87.0)
Gludopa +	397.2	475.0	663.3	725.3	593.9	577.8	547.1
placebo	(153.6)	(127.9)	(194.7)	(154.5)	(125.5)	(136.7)	(116.7)
Placebo +	368.5	343.0	311.2	295.5	312.1	350.7	382.3
glu-5-HTP	(95.5)	(89.3)	(67.1)	(48.5)	(69.5)	(73.5)	(89.4)
Gludopa +	405.2	478.9	557.7	650.3	588.3	558.7	480.1
glu-5-HTP	(100.5)	(90.6)	(129.0)	(219.5)	(138.0)	(138.1)	(114.8)
FE_{Na} (%)							
Placebo +	2.8	2.7	3.0	3.0	3.0	2.9	2.8
placebo	(0.8)	(0.8)	(0.9)	(0.7)	(0.7)	(0.6)	(0.7)
Gludopa +	2.8	3.4	4.8	5.0	4.3	4.1	3.8
placebo	(0.8)	(1.0)	(1.5)	(1.2)	(1.1)	(1.0)	(0.9)
Placebo +	2.6	2.4	2.2	2.4	2.4	2.6	2.8
glu-5-HTP	(0.7)	(0.5)	(0.5)	(0.5)	(0.6)	(0.7)	(0.7)
Gludopa +	2.8	3.3	4.1	4.6	4.4	4.0	3.6
glu-5-HTP	(0.7)	(0.9)	(1.3)	(1.8)	(1.5)	(1.2)	(1.0)
Potassium excretion (μmol min^{-1})							
Placebo +	87.7	90.2	95.0	97.5	97.0	91.7	90.3
placebo	(45.3)	(33.5)	(36.7)	(42.3)	(34.5)	(25.8)	(27.7)
Gludopa +	74.3	75.0	81.3	92.0	91.3	91.8	89.5
placebo	(28.1)	(25.3)	(32.3)	(30.8)	(31.2)	(27.5)	(27.5)
Placebo +	76.4	83.5	90.3	82.7	87.0	88.6	82.8
glu-5-HTP	(44.5)	(37.7)	(34.5)	(34.5)	(20.8)	(16.1)	(18.8)
Gludopa +	87.8	90.1	86.6	98.3	97.3	100.2	90.5
glu-5-HTP	(31.5)	(29.2)	(26.7)	(30.3)	(18.0)	(19.4)	(24.1)
Urine output (ml min^{-1})							
Placebo +	10.2	8.8	8.9	9.2	8.3	8.5	7.3
placebo	(2.3)	(2.0)	(2.8)	(3.1)	(1.4)	(2.0)	(1.2)
Gludopa +	10.3	10.4	11.7	10.2	8.2	8.5	8.7
placebo	(2.6)	(2.4)	(3.5)	(1.3)	(1.5)	(2.7)	(1.9)
Placebo +	9.4	8.2	8.3	5.5	5.5	8.0	8.6
glu-5-HTP	(2.6)	(2.6)	(2.0)	(2.2)	(1.6)	(2.3)	(2.2)
Gludopa +	9.8	12.2	10.9	8.0	7.4	9.7	8.6
glu-5-HTP	(2.1)	(1.5)	(3.5)	(2.4)	(2.6)	(2.9)	(1.9)

Table 8-7. The 3-6 h cumulative sodium excretion (mmol), potassium excretion (mmol) and urine output (ml) on the 4 experimental days, and the changes in these values relative to the baseline excretion rates. Values shown are means ± SD (n = 8).

	Infusion			
	Placebo+Placebo	*Gludopa+Placebo*	*Placebo+Glu-5-HTP*	*Gludopa+Glu-5-HTP*
Absolute cumulative excretion				
Sodium	72.4 ± 18.0	107.6 ± 23.8	59.9 ± 11.1	99.2 ± 22.1
Potassium	16.9 ± 5.6	15.6 ± 4.6	15.4 ± 4.5	16.9 ± 3.7
Urine	1527 ± 240	1737 ± 257	1333 ± 158	1706 ± 226
Change in cumulative excretion from baseline				
Sodium	+2.30 ± 15.6	+36.1 ± 20.6	-6.5 ± 16.5	+26.3 ± 22.4
Potassium	+1.1 ± 5.2	+2.2 ± 5.5	+1.7 ± 3.9	+1.1 ± 3.6
Urine	-301 ± 435	-121 ± 443	-354 ± 421	-60 ± 404

Table 8-8. Mean (SD) ERPF, GFR and FF values for each 30 min period before (period 2.5-3.0 h), during (period 3.0-3.5 h and period 3.5-4.0 h) and after (period 4.0-4.5 h to period 5.5-6.0 h) administration of the 4 infusion regimens (n = 8).

	Time (h)						
	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.0-5.5	5.5-6.0
ERPF (ml min^{-1} $1.73m^{-2}$)							
Placebo +	459.9	488.7	464.0	479.2	472.2	472.5	422.1
placebo	(87.7)	(129.8)	(137.2)	(97.4)	(112.9)	(127.7)	(68.2)
Gludopa +	546.6	532.2	565.5	595.4	551.9	556.2	527.4
placebo	(161.2)	(91.3)	(127.3)	(132.2)	(122.6)	(138.6)	(78.7)
Placebo +	537.0	534.4	516.0	456.3	500.6	488.8	471.6
glu-5-HTP	(121.8)	(134.7)	(120.3)	(66.1)	(146.8)	(102.2)	(47.4)
Gludopa +	512.7	526.6	480.8	517.5	510.5	496.9	503.4
glu-5-HTP	(99.6)	(109.1)	(90.8)	(96.8)	(106.9)	(94.6)	(96.2)
GFR (ml min^{-1} $1.73m^{-2}$)							
Placebo +	95.1	96.0	91.2	94.6	96.5	98.3	94.4
placebo	(9.0)	(18.0)	(20.8)	(14.8)	(17.2)	(13.9)	(5.3)
Gludopa +	96.6	95.0	94.5	99.1	95.1	96.2	100.5
placebo	(16.8)	(8.3)	(7.0)	(6.7)	(6.3)	(10.0)	(9.3)
Placebo +	96.9	97.8	96.5	85.9	88.7	93.1	93.6
glu-5-HTP	(7.4)	(16.5)	(11.8)	(5.0)	(11.6)	(15.6)	(8.2)
Gludopa +	98.1	100.4	93.9	98.2	94.6	97.3	94.0
glu-5-HTP	(8.9)	(9.5)	(5.7)	(11.1)	(11.0)	(10.9)	(6.1)
FF (%)							
Placebo +	21.3	20.3	20.5	20.2	20.9	21.7	22.9
placebo	(3.9)	(4.1)	(5.0)	(4.1)	(3.2)	(4.4)	(3.7)
Gludopa +	18.5	18.2	17.3	17.3	17.8	18.0	19.4
placebo	(3.8)	(2.2)	(3.3)	(3.4)	(3.0)	(3.5)	(2.7)
Placebo +	18.7	18.8	19.3	19.2	18.6	19.3	20.1
glu-5-HTP	(3.6)	(3.3)	(3.5)	(2.8)	(3.9)	(2.7)	(3.1)
Gludopa +	19.6	19.5	20.2	19.3	19.0	19.9	19.1
glu-5-HTP	(3.1)	(2.9)	(4.4)	(2.4)	(2.8)	(2.3)	(2.6)

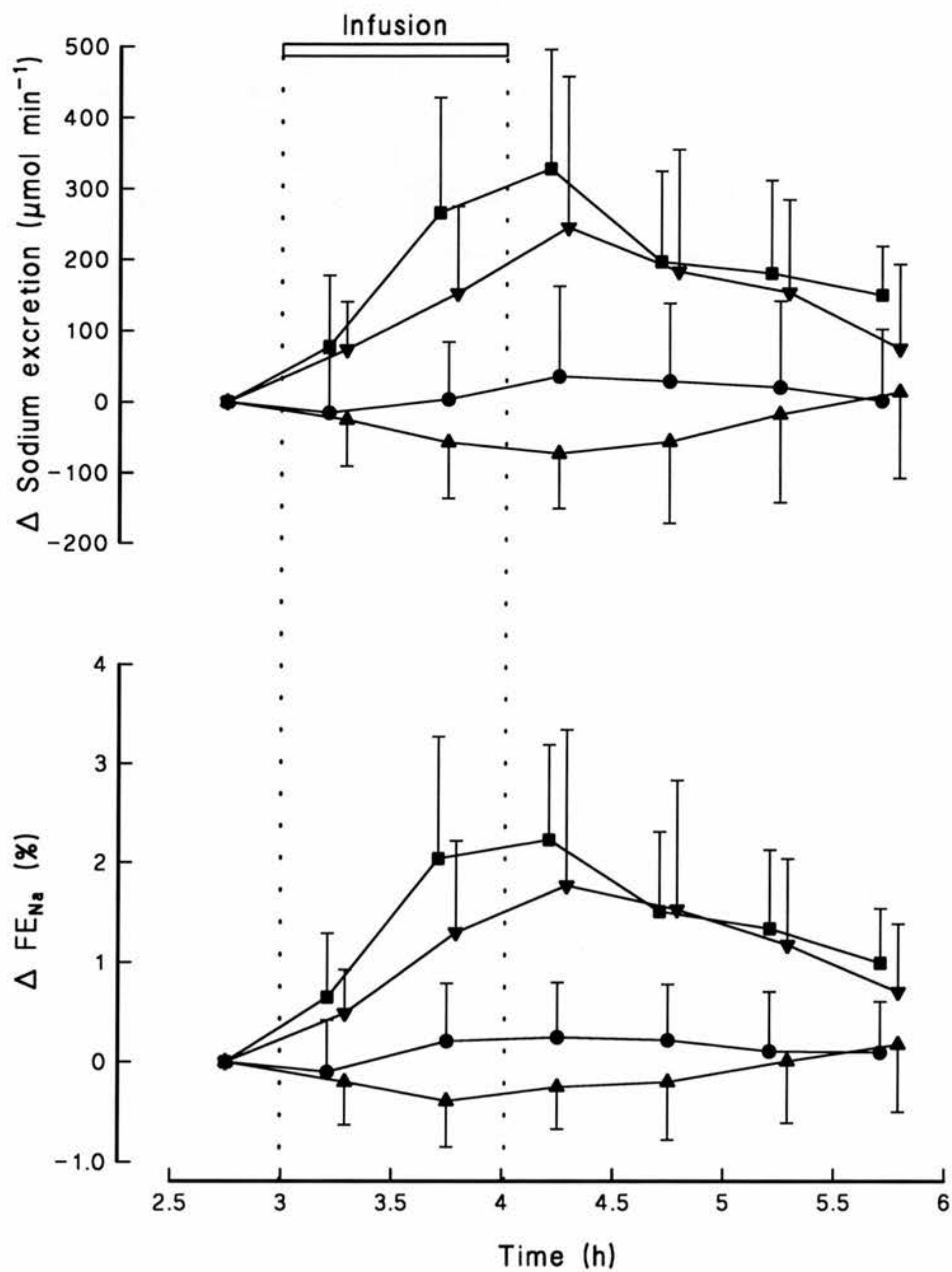


Figure 8-1. Changes (Δ) in urinary excretion rates of sodium and FE_{Na} from the baseline period (2.5-3.0 h) following infusion of placebo + placebo (●); gludopa + placebo (■); placebo + glu-5-HTP (▲); and gludopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

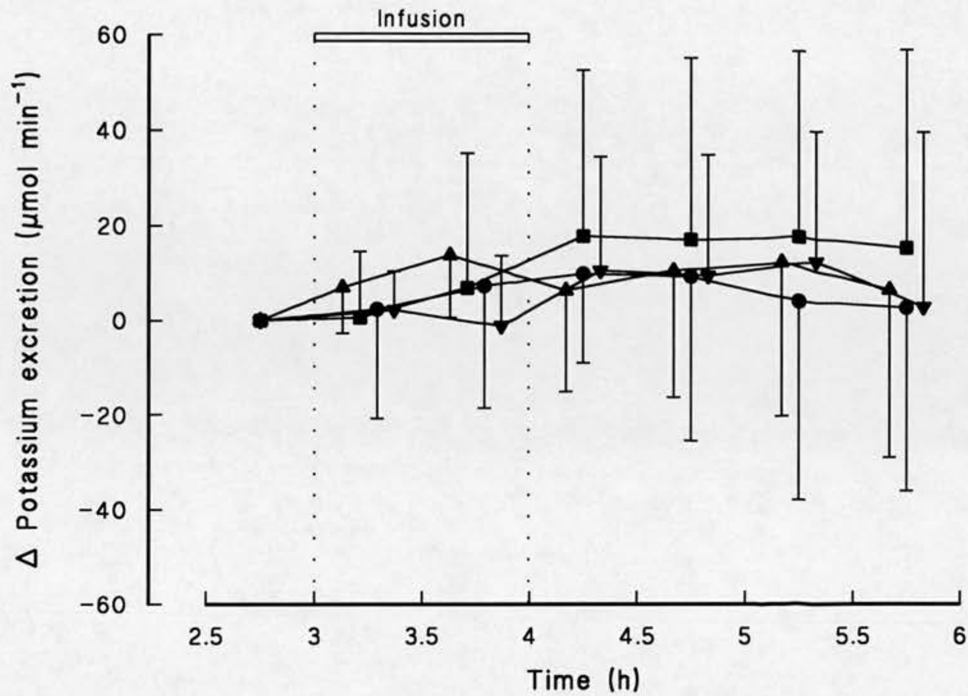


Figure 8-2. Changes (Δ) in urinary excretion rates of potassium from the baseline period (2.5-3.0 h) following infusion of placebo + placebo (●); gludopa + placebo (■); placebo + glu-5-HTP (▲); and gludopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

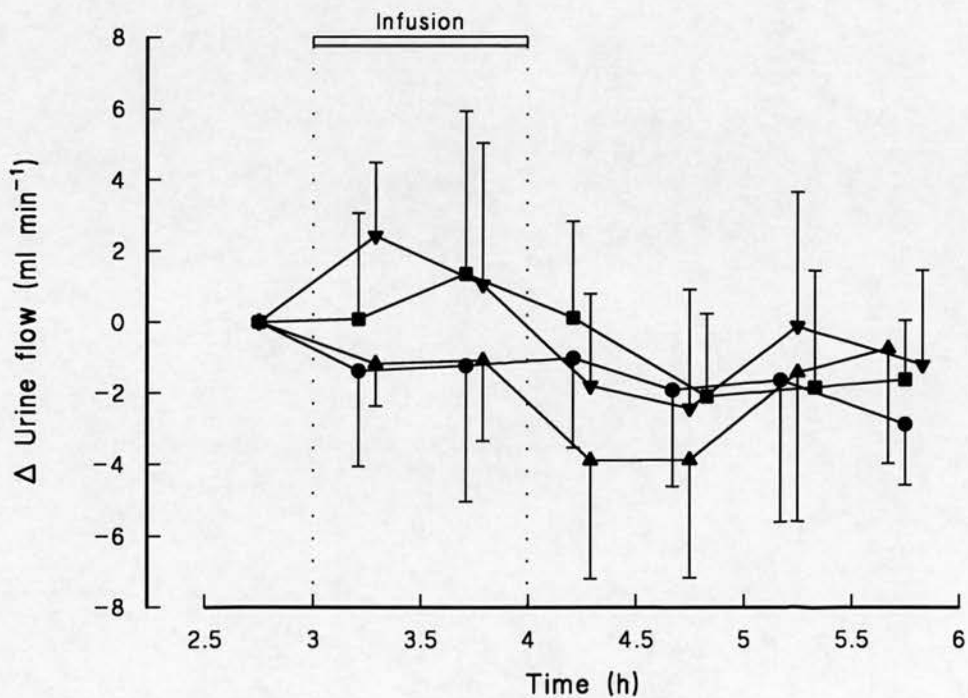


Figure 8-3. Changes (Δ) in urine output from the baseline period (2.5-3.0 h) following infusion of infusion of placebo + placebo (●); gludopa + placebo (■); placebo + glu-5-HTP (▲); and gludopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

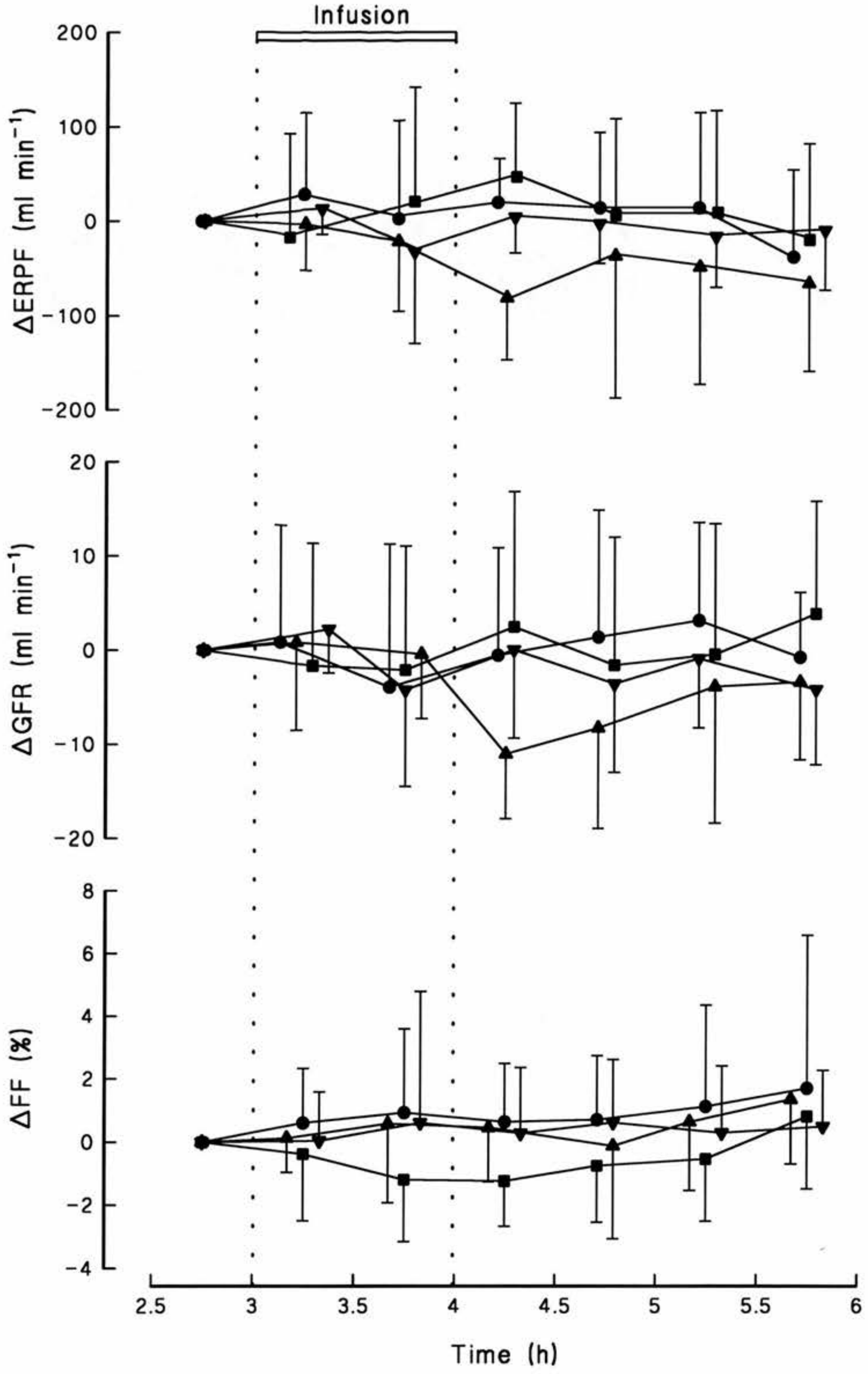


Figure 8-4. Changes (Δ) in ERPF, GFR and FF from the baseline period (2.5-3.0 h) following infusion of placebo + placebo (●); gludopa + placebo (■); placebo + glu-5-HTP (▲); and gludopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

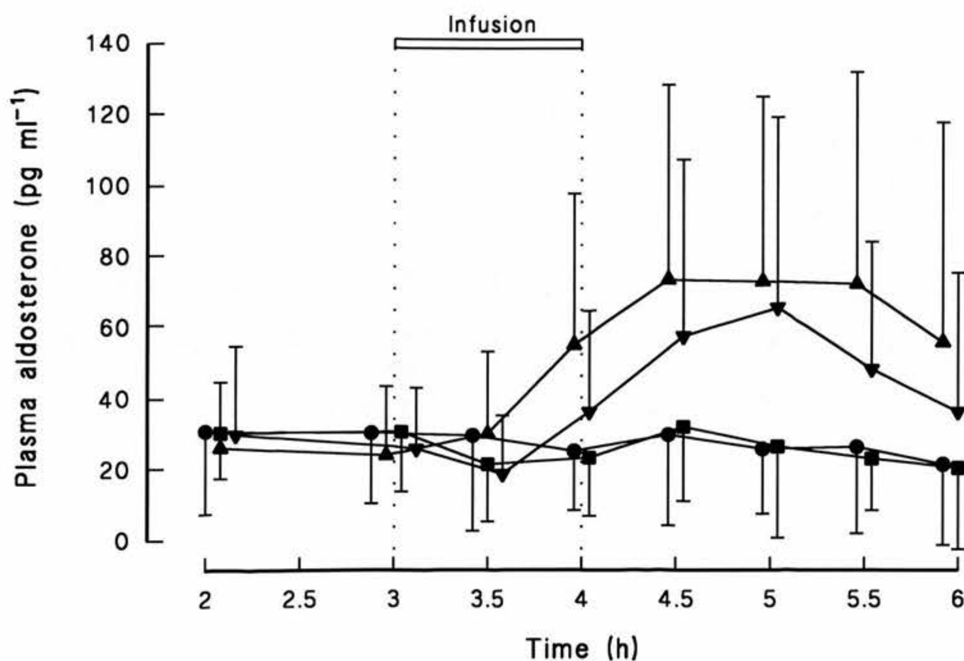


Figure 8-5. Plasma aldosterone [illegible] before, during and after infusion of placebo + placebo (●); gludopa + placebo (■); placebo + glu-5-HTP (▲); and gludopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

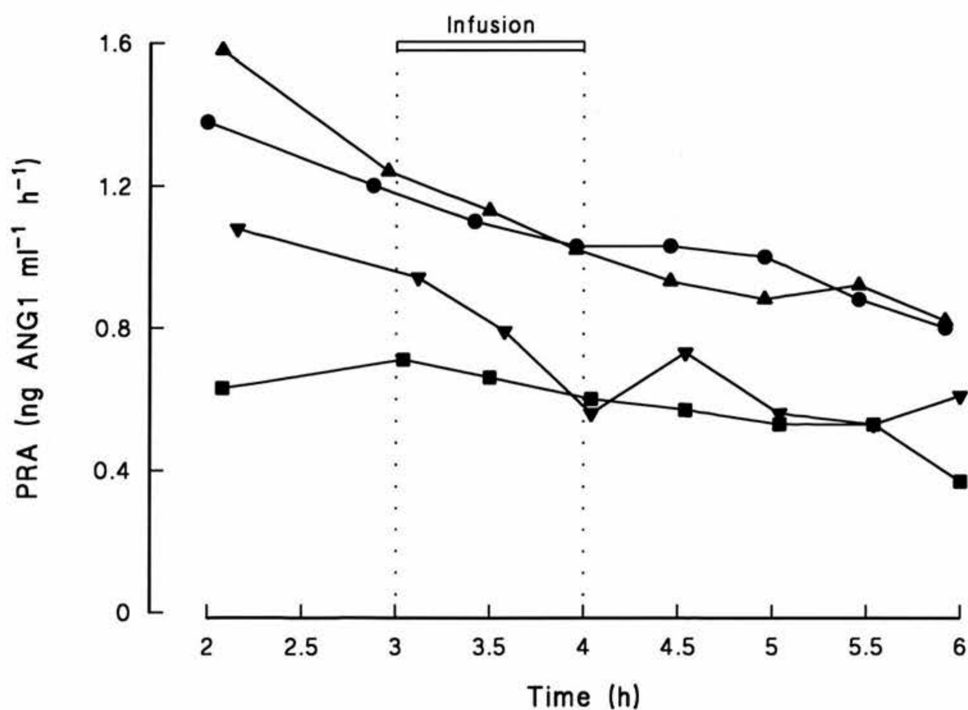


Figure 8-6. Mean PRA after infusion of placebo + placebo (●); gludopa + placebo (■); placebo + glu-5-HTP (▲); and gludopa + glu-5-HTP (▼) in 2 subjects. No SD values are shown because PRA was only detected in 2 subjects on all 4 days (*see* text).

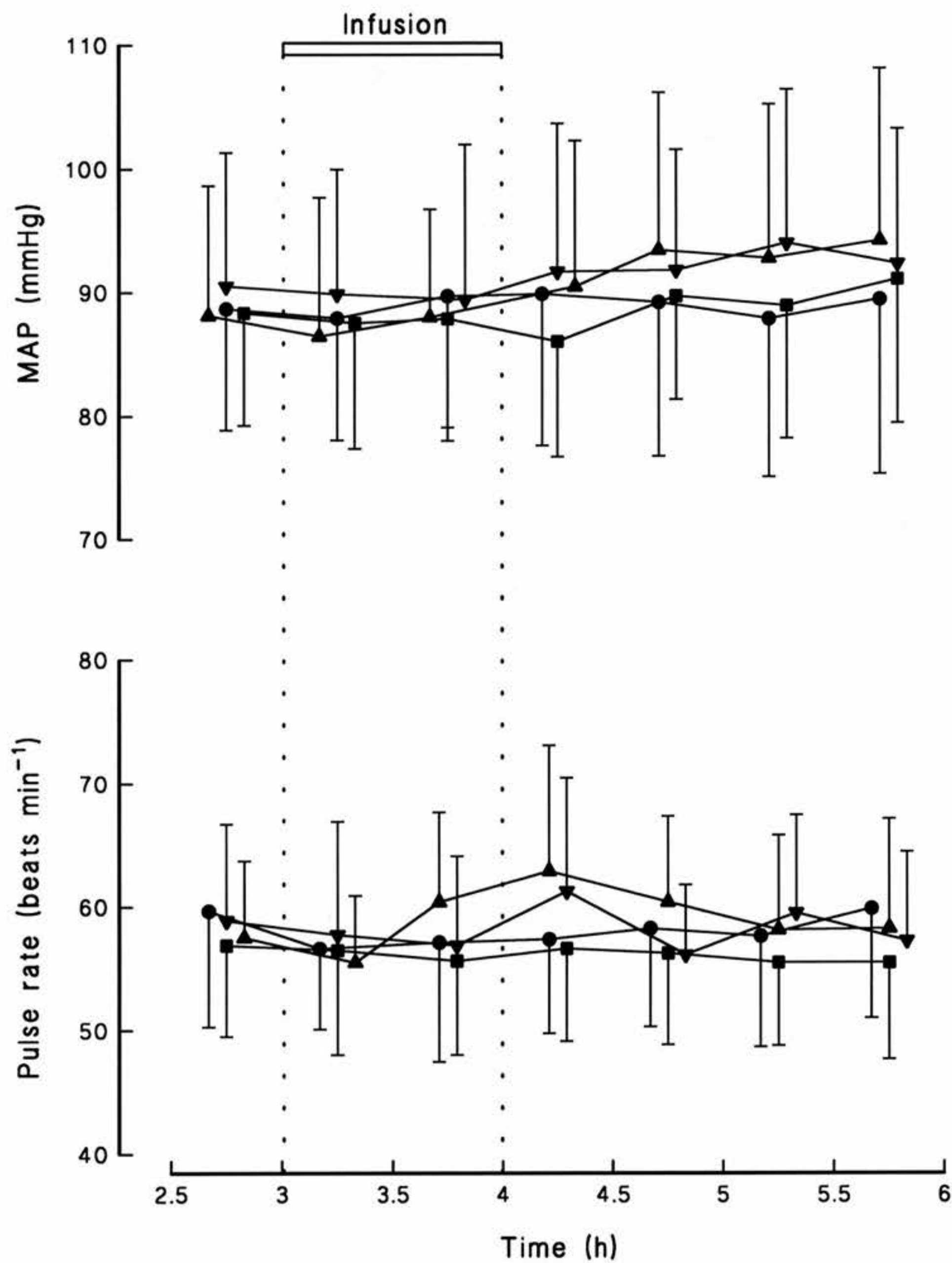


Figure 8-7. Mean arterial blood pressure (MAP) and pulse rate before, during and after infusion of placebo + placebo (●); gludopa + placebo (■); placebo + glu-5-HTP (▲); and gludopa + glu-5-HTP (▼). Values shown are means ± SD (n = 8).

8.4 Discussion

Administration of gludopa was associated with marked increases in the urinary excretion of dopa, dopamine and DOPAC together with a rise in the urinary excretion of sodium in agreement with published studies [334-341,468]. A diuresis as well as a small increase in both ERPF and GFR have also been reported [334,335,340]. In the present study, we were unable to demonstrate significant differences in these variables between gludopa and placebo infusion days but the highest mean urinary flow rates were observed on the day the subjects received gludopa. The absence of an effect on renal haemodynamics may be partly due to the shorter duration of gludopa infusion employed, and hence the total dose of gludopa administered. In addition, our subjects were volume expanded and salt-replete, and the renal vasculature may exhibit background vasodilatation in such conditions making it more difficult to demonstrate an additional vasodilator effect of dopamine.

The renal vasodilatation, natriuresis and diuresis produced by gludopa appears to be mediated by locally generated dopamine activating dopamine-1 (DA_1) receptors [331,333,340,470]. These receptors have been identified in several areas of the kidney including arterioles, proximal convoluted tubules and cortical collecting ducts [470-474]. The evidence for the presence of DA_1 receptors in the glomeruli has been controversial [470]. The increased dopamine excretion after gludopa can be observed in the isolated perfused rat kidney [333] and occurs with minimal increases in circulating plasma dopamine levels when given in low doses in the rat [331] and in man [334,475] indicating direct intrarenal synthesis of dopamine and its subsequent excretion. The enhanced sodium excretion induced by gludopa is mainly a result of the inhibitory effect of dopamine (probably formed within the cells of the proximal tubules) upon the tubular reabsorption of this cation, since it can occur in man without detectable changes in renal haemodynamics [336,341]. This is supported by observations in the isolated perfused rat kidney that dopamine-

induced natriuresis and diuresis could persist even when renal blood flow, GFR and perfusion pressure were kept constant [476]. In the present study, the increases in FE_{Na} in the absence of significant changes in renal haemodynamics after infusion of gludopa would also be in agreement with a tubular site (or sites) for the action of dopamine. The latter may act by inhibiting Na^{+}-K^{+}-ATPase at the basolateral membrane [477,478] as well as Na^{+}-H^{+} antiport activity at the brush border membrane in the proximal tubular segments [470,474,479]. Dopamine may also decrease sodium transport at more distal segments of the nephron [480] and it has also been shown to antagonise both the effects of mineralocorticoids and the hydrosmotic effect of vasopressin on the rabbit cortical collecting duct [481].

Glu-5-HTP produced marked increases in the urinary excretion of 5-HTP, 5-HT and 5-HIAA. We have demonstrated these changes in our previous studies and have shown that the marked increase in urine 5-HT excretion occurred without concomitant increases in circulating 5-HT levels measured in platelet-rich plasma, consistent with the proposition that it was primarily due to the intrarenal synthesis of 5-HT (*see* Chapters 3-7). Glu-5-HTP was also shown to reduce urinary excretion of sodium and FE_{Na} without affecting ERPF or GFR in the previous studies and this sodium retaining effect was dependent on the conversion of glu-5-HTP to 5-HT. In the present study, glu-5-HTP produced a significant reduction in ERPF and a trend towards a fall in GFR. These effects may be due to the slightly higher dose of glu-5-HTP employed in this study (20 *vs* 16.6 $\mu g\ kg^{-1}\ min^{-1}$). A trend towards a reduction in GFR was also observed in an earlier study (see Chapter 6) and the failure to demonstrate a significant effect on renal haemodynamics may be due to a type 2 error.

The absence of a significant antinatriuretic effect following glu-5-HTP in the present investigation is at variance with our previous results (*see* Chapters 3 to 7). The mean urinary sodium excretion rates after glu-5-HTP were, however, lower than those observed on the placebo day and a type 2 error may account for the

failure to observe a significant effect. In addition, the sodium excretion values during the baseline periods were much higher than in previous studies, and the antinatriuretic action of 5-HT may be less easily demonstrable in this state. It is also possible that increased salt intake may have led to a down-regulation of the tubular 5-HT receptors in an attempt to facilitate renal sodium excretion. The changes in urinary excretion of potassium and urine output after infusion of glu-5-HTP were not significantly different from those observed after infusion of placebo alone, in agreement with observations from our previous studies (*see* Chapters 4 and 6).

The release of dopamine or 5-HT from the glutamyl compounds results from the sequential enzymatic actions of γGT and LAAD. The activity of γGT in mammalian tissues is at its highest in the kidney [308,309]. This membrane-bound glycoprotein is heavily concentrated on the brush border of the proximal tubular cells [309-316] but it is also found at the antiluminal border of these tubules in lesser concentration [312,316,318-320]. At these sites, the enzyme is orientated in the membranes so as to react with substrates present in the extracellular milieu [317]. γGT will cleave the γ-glutamyl moieties of gludopa and glu-5-HTP releasing the free amino acid precursors, L-dopa and 5-HTP respectively, in relatively high quantities on both sides of the proximal tubular cells for subsequent decarboxylation. The enzyme has a tremendous capacity to metabolise γ-glutamyl compounds as has been shown by the very low recovery of unmetabolised gludopa in the urine (less than 1%) following its intravenous infusion in man [468,475]. Administration of the two glutamyl compounds therefore offers the possibility of investigating whether there is competition between L-dopa and 5-HTP for renal LAAD *in vivo* in man. This cytosolic enzyme is present in high concentrations within the cells of the proximal tubules [31,35,141-143]. The locally released L-dopa and L-5-HTP can enter these cells from the basolateral and luminal sides to be decarboxylated by LAAD to produce dopamine and 5-HT respectively

[142,179,180,192,432,482]. These two amino acids could compete with each other for the enzyme and there is evidence that 5-HTP inhibits the decarboxylation of L-dopa [37,483]. In addition, experiments performed in isolated intact proximal convoluted tubules of the rat suggest that the precursors share the same transporter for entry into the renal tubular cells and L-dopa was shown to exert inhibition of a competitive type on the tubular uptake of L-5-HTP [180]. The formed amines may then compete for deamination by MAO-A as well as for secretion or transport out of the cells.

In the present study, the urinary excretion values of dopa, dopamine, and DOPAC following the simultaneous infusion of equimolar amounts of gludopa and glu-5-HTP were not significantly different from those observed after the infusion of gludopa only. Similarly, the urinary levels of 5-HTP, 5-HT and 5-HIAA following the coinfusion were similar to those measured after the administration of glu-5-HTP alone. These observations would suggest that, under the conditions of our study, there was no competition between the two compounds for the renal enzymes or transporters. These systems may, however, not be readily susceptible to saturation at the substrate load that we supplied. This may explain the failure to demonstrate an inhibition of synthesis of either amine. That enzyme saturation may not occur at the doses employed in this study is supported by a previous report which demonstrated that the mean percentage conversion of administered gludopa to urine free dopamine did not fall when the dose of gludopa was increased from 12.5 to 100 $\mu g\ kg^{-1}\ min^{-1}$ [334]. An alternative explanation is that the prodrugs are metabolised in different compartments within the renal tubular cells and this will need to be investigated by experiments in the animal. The amount of dopamine produced, as measured in the urine, was approximately 75% greater than the quantity of urinary 5-HT generated following infusion of equimolar amounts of the two glutamyl compounds. This suggests that LAAD in the kidney preferentially decarboxylates L-dopa and is consistent with previous *in vivo* and *in vitro* studies in the rat

demonstrating that L-dopa is the preferred substrate for renal LAAD [46,453]. Itskovitz *et al.* [268] also reported that comparatively more dopamine than 5-HT was excreted when L-5-HTP and L-dopa were infused together into the renal artery of the rat. In addition, there was no evidence from the study by Itskovitz *et al.* that L-dopa acted as a competitive inhibitor and diminished the renal synthesis of 5-HT during the combined infusion of L-5-HTP and L-dopa.

There was an unexpected rise in urinary 5-HT excretion after infusion of gludopa. We did not however observe an effect on 5-HIAA excretion but this may be due to the failure of our spectrophotometric assay to detect low concentrations of 5-HIAA. In contrast, glu-5-HTP infusion had no effect on dopamine excretion consistent with our previous finding (*see* Chapter 4). The reason for the increased 5-HT excretion is unclear. It may result from displacement of endogenous renal 5-HT by dopamine derived from gludopa analogous to the situation in the brain of the rat where administration of L-dopa leads to cerebral accumulation of dopamine, together with a reduction of cerebral 5-HT, probably caused by a release or displacement (or both) of endogenous 5-HT from its storage sites by the large excess of dopamine [484-488]. Similarly, *in vitro* studies using the rat brain slices suggested that dopamine may be released from vesicle storage sites by 5-HT derived from decarboxylation of 5-HTP in catecholaminergic neurones [489,490]. We did not, however, observe an effect of glu-5-HTP on urinary dopamine excretion in the present study. The increased 5-HT excretion after gludopa may also be related to the natriuresis induced by gludopa, perhaps as a renal response to the need to conserve sodium. It is also possible that it is merely a flow-dependent phenomenon and represents a wash-out of pre-formed 5-HT from the tubular lumen related to the increased urinary flow rate induced by gludopa. Interestingly, an increase in 5-HT excretion has been reported during frusemide-induced diuresis in rats and this was associated with a reduction in renal 5-HT content [295]. However, although the urinary flow rate values tended to be higher on gludopa infusion day,

they were not significantly different from those measured following infusion of placebo only in the present study.

Renal dopamine is believed to be formed in the proximal tubular cells by the action of LAAD on L-dopa derived from the circulation [432,433,435,436,466]. It has been suggested that locally produced dopamine may act as an endogenous natriuretic factor in the kidney and that it may have a physiological role in the regulation of sodium excretion [434-436,441,491,492]. This is supported by the close correlation between the concentration of sodium and that of dopamine in urine [493]; the increased urinary excretion of dopa and dopamine with salt loading in laboratory animals [143,494-496] and humans [462-467]; the attenuation by carbidopa [452,497] and by dopamine antagonists [498,499] of the natriuresis produced by saline infusion; and the observation that carbidopa inhibited renal dopamine production and reduced sodium excretion in man [441,500]. Dopamine may act either as a paracrine or an autocrine substance since it is synthesised within (or in close proximity to) cells which are endowed with dopamine receptors [436,470,491,492,501]. Renal 5-HT may play an antagonistic role to the natriuretic dopamine by reducing urinary sodium excretion [268].

Gludopa has been shown to increase urinary sodium excretion [334-342] whereas glu-5-HTP has the opposite effect (*see* Chapters 3-7). In the present study, the overall pharmacological effect of infusing gludopa and glu-5-HTP simultaneously was towards increased sodium excretion. This suggests that, under the conditions of the study, the natriuretic effect of intrarenally generated dopamine was more potent than the sodium retaining action of 5-HT. This may be due to the greater amount of dopamine than 5-HT synthesised after infusion of equimolar amounts of the two glutamyl derivatives. It is of interest in this respect that studies have shown that the rate of uptake of L-dopa and formation of dopamine by renal cortical slices varied with the concentration of sodium in the incubation medium [502-504]. In addition, the natriuretic effect of dopamine has been shown to be more evident with

salt loading or extracellular fluid volume expansion [492,505]. Whether this is as a result of a volume-induced up-regulation of DA_1 receptors or an increased efficiency of coupling to signal transduction is unclear. There may also be down-regulation of the 5-HT receptors, as we suggested earlier, since we were unable to demonstrate the sodium retaining effect of glu-5-HTP, when infused alone, in contrast to our previous studies (*see* Chapters 3 to 7). This may explain why there was no significant blunting of the natriuretic effect of gludopa by the concomitant administration of glu-5-HTP. It would be of great interest to repeat this study in salt-depleted individuals to see if the sodium intake did have an effect on the sensitivity to the antinatriuretic action of 5-HT and whether there would be any alteration in the relative amounts of dopamine and 5-HT excreted. It is of interest to note that urinary excretion rates of dopa and dopamine after infusion of L-dopa was reported to be higher following dietary salt loading in man [466].

Glu-5-HTP increased plasma aldosterone concentrations without affecting PRA consistent with our previous observations (*see* Chapters 4, 6 and 7). The aldosterone release may be due to recirculation of intrarenally produced L-5-HTP or 5-HT acting on the adrenal gland, formation of 5-HT from glu-5-HTP within the adrenal gland, or a central effect of glu-5-HTP. Dopamine, in contrast to 5-HT, has been shown to exert a tonic inhibitory influence on aldosterone secretion [506-510], an effect which is mediated via adrenal DA_2 dopamine receptors [511,512]. A study using isolated bovine adrenal cells suggested a direct inhibitory role of dopamine on the late phase of aldosterone synthesis [513]. We investigated the effect of gludopa on aldosterone release as it is possible that L-dopa or dopamine, formed intrarenally from gludopa, may recirculate to act on the adrenal gland or that dopamine may be produced within the adrenal gland from its glutamyl prodrug. Gludopa had no effect on plasma aldosterone levels nor did it significantly alter the release of aldosterone induced by glu-5-HTP although there was a tendency for the plasma aldosterone concentrations to be lower (Figure 8-5).

There is evidence from *in vitro* and *in vivo* studies in animals that exogenous dopamine stimulates renin secretion or release by acting on DA_1 receptors [470,514-520] but a lack of effect of the amine has also been reported [476]. Similarly, studies in man demonstrated that an intravenous infusion of dopamine increased plasma renin activity [521,522] but this effect was not observed by other investigators [523]. In contrast, dopamine synthesised intrarenally following administration of L-dopa [524] or gludopa [334-340] was previously found to lower plasma renin activity in our laboratory. The exact mechanism underlying gludopa-induced renin suppression is unclear but it has been suggested that it may reflect either a response to the increased tubular sodium load at the macula densa or a direct inhibitory effect of dopamine at the juxtaglomerular cells. In the present study, PRA was suppressed throughout the experiment in the majority of subjects probably as a result of the salt loading protocol employed [364,383-390] and we were unable to demonstrate any additional effect of gludopa on PRA.

In conclusion, the present study in salt-replete (or -loaded) subjects has demonstrated that dopamine was the preferred amine to be synthesised. The overall effect on sodium excretion was to produce a natriuresis with these equimolar pharmacological doses of gludopa and glu-5-HTP when they were administered simultaneously. It is not clear whether similar effects would be observed under physiological conditions. It is of interest, however, to note that reciprocal changes in urinary excretion of endogenous dopamine and 5-HT have been shown to occur in man when salt intake is altered [461-467]. The present experiment should be repeated in man under conditions of sodium deprivation to ascertain whether opposite results would be obtained. It would also be valuable to repeat both sets of experiments under metabolic balance conditions where compliance with dietary sodium (and potassium) intakes could be assured and isocaloric conditions maintained, since mineral and calorie status could well affect the results of sensitive investigations such as these.

CHAPTER NINE

EFFECTS OF FRUSEMIDE ON THE URINARY EXCRETION OF DOPAMINE AND 5-HYDROXYTRYPTAMINE

9.1 Introduction

Frusemide (4-chloro-*N*-furfuryl-5-sulphamoyl-anthranilic acid), a potent loop diuretic [525], has been reported to increase the urinary excretion of dopamine in man [526-530] and in the rat [531-533]. The renal responses following frusemide of natriuresis, kaliuresis and diuresis in the anaesthetised rat were reduced by the non-selective dopamine antagonist haloperidol and by the DA_1 receptor antagonist SCH 23390, but not by ±sulpiride, a preferential DA_2 receptor antagonist, indicating that endogenous dopamine may contribute to the sodium, potassium and water excretion produced by the loop diuretic, possibly through an effect on tubular DA_1 dopaminergic receptors [532]. Pretreatment with intravenous benserazide (25 mg kg^{-1}), an LAAD inhibitor, resulted in a marked inhibition of the rise in urine dopamine after frusemide and this was associated with at least 60% reduction in frusemide-induced diuresis, natriuresis and kaliuresis [533]. The increase of dopamine in urine following frusemide appeared, therefore, to be an effect on recent synthesis, rather than being a release from renal stores. These findings suggest that the effects of frusemide described above may be partially dependent on endogenous dopamine. Benserazide reduced frusemide excretion in urine during the period of maximal diuretic response by 60%, whereas infusion of L-dopa, the immediate precursor of dopamine, increased its excretion by 62%, and it is possible that endogenous dopamine may also facilitate the transport of frusemide to its luminal site of action [525,533]. In contradistinction to these observations in the rat, Jeffrey *et al.* [529] had demonstrated in healthy human subjects that a single oral 100 mg dose of carbidopa, another LAAD inhibitor, lowered urinary dopamine to undetectable levels but had no effect on the natriuretic response to frusemide. These studies, however, did not consider the possible effect of frusemide or LAAD inhibition on urinary 5-HT production which may influence sodium excretion.

There is evidence that 5-HT, like dopamine, is synthesised within the kidney and previous studies described in this thesis (*see* Chapters 3,4,6,7,8) together with other

published work in the rat [166,268,] suggest that intrarenally generated 5-HT has an opposite effect to dopamine on urinary sodium excretion. In man, a single oral 100 mg dose of carbidopa reduced not only the urinary excretion of dopamine [529], but also that of 5-HT, to undetectable levels (*see* Chapter 6). We also demonstrated an increase in urinary 5-HT excretion when the kidney was primed to increase dopamine synthesis by administration of gludopa, a renal dopamine prodrug (*see* Chapter 8). In addition, the diuretics, frusemide, chlorothiazide and mersalyl, have been shown to increase the urinary excretion of 5-HT and to simultaneously decrease the renal content of 5-HT in the rat [295,534]. In rats treated with 1.32 mg kg^{-1} of frusemide intramuscularly, the concentration of 5-HT in the urine at the time of maximal diuresis was 576 times the value in untreated animals. The elevation in urine 5-HT occurred not only during the period of maximum diuresis but continued for a longer time. The cumulative 5-HT excretion over an 8 h time interval was 1000 times that in control rats. There have been no reports on the effect of frusemide on urinary 5-HT excretion in man.

The present study examined the effects of acute intravenous administration of frusemide on the urinary excretion of both dopamine and 5-HT in man.

9.2 Methods

Eight healthy male subjects, aged 20-41 years (mean 31.6 years), attended on 2 separate occasions, at least 1 week apart, in this randomised, open, placebo-controlled, cross-over study. They were given general dietary advice to avoid excessive intake of dietary salt over the 36 h prior to each study day. They refrained from alcohol for 24 h, abstained from caffeine-containing beverages from 18.00 h, and fasted from 22.00 h the evening before each of the study days. They reported to the clinical investigation unit at about 08.00 h, having drunk 200 ml of tap water one hour previously. They emptied their bladder on arrival and drank a further 200 ml of water. This was followed by 200 ml of water every half hour for the next 7 h.

They emptied their bladder after an hour and the urine collected during this period was taken as representing the baseline urine collection. The subjects then received either 40 mg of frusemide (Lasix®; 10 mg ml^{-1}), made up to a total volume of 30 ml with 0.9% NaCl, or placebo (30 ml of saline only) (time = 0 h). These solutions were infused intravenously over 10 min using a Braun Perfusor VI infusion pump. The subjects emptied their bladder at hourly intervals over the next 6 h. Any urine passed before the end of an hour period was summated. In four subjects, accurately timed urine collections were made at 20 min intervals during the first hour after frusemide administration. The subjects remained semi-recumbent or supine throughout the experiment except for rising to pass urine. The volume of each urine collection was measured and aliquots stored at -40°C for analysis of sodium, potassium, dopamine and 5-HT. Urine samples for dopamine and 5-HT were acidified ($pH < 3.0$) with 5 M HCl to prevent their oxidation. The concentrations of sodium, potassium, dopamine and 5-HT in urine were determined as described in section 2.5.

All results are expressed as mean ± SD. The urinary dopamine and 5-HT data on the two experimental days were compared by repeated measures ANOVA for overall statistical significance. The urinary excretion rates during the first hour on the two study days were compared by Student's t test for paired observations. Differences were considered to be statistically significant when the P value was less than 0.05.

9.3 Results

Frusemide produced the expected natriuresis, kaliuresis and diuresis (Figure 9-1). The sodium excretion during the first hour after frusemide administration was 13 times that after placebo infusion. The potassium excretion during the first hour was 2.7 times and the urine output 8.4 times the corresponding values after placebo infusion. The urinary excretion of sodium, potassium, and water remained elevated during the second hour but tended to be lower afterwards.

Compared with placebo, frusemide produced a significant increase in the urinary excretion of dopamine ($P < 0.001$). The baseline dopamine excretion on the placebo day (1.34 ± 0.30 nmol min^{-1}) was not significantly different from that on the frusemide day (1.39 ± 0.34 nmol min^{-1}). The urinary dopamine excretion during the first hour after frusemide administration (1.81 ± 0.25 nmol min^{-1}) was, however, significantly higher than that observed after placebo (1.10 ± 0.37 nmol min^{-1}; 95% CI_{diff}: 0.51 to 0.91 nmol min^{-1}; $P < 0.01$). The increased dopamine excretion and the natriuretic, kaliuretic and diuretic responses evoked by frusemide were maximal during the 20-40 min period in the 4 subjects in whom urine collections were made at 20 min intervals during the first hour (Figure 9-3).

The 5-HT excretion values before infusion of placebo and frusemide were not significantly different at 0.42 ± 0.14 nmol min^{-1} and 0.45 ± 0.13 nmol min^{-1}. The urinary 5-HT excretion rate decreased during the first hour after placebo and then remained relatively constant. Similarly, there was an initial drop in 5-HT excretion rate after frusemide. There were no significant differences between the changes of 5-HT excretion from baseline on the two study days at corresponding time periods. The 6 h cumulative 5-HT excretion after frusemide was 112.5 ± 12.8 nmol and 126.2 ± 13.0 nmol after placebo (95% CI_{diff}: -1.9 to 29.2 nmol).

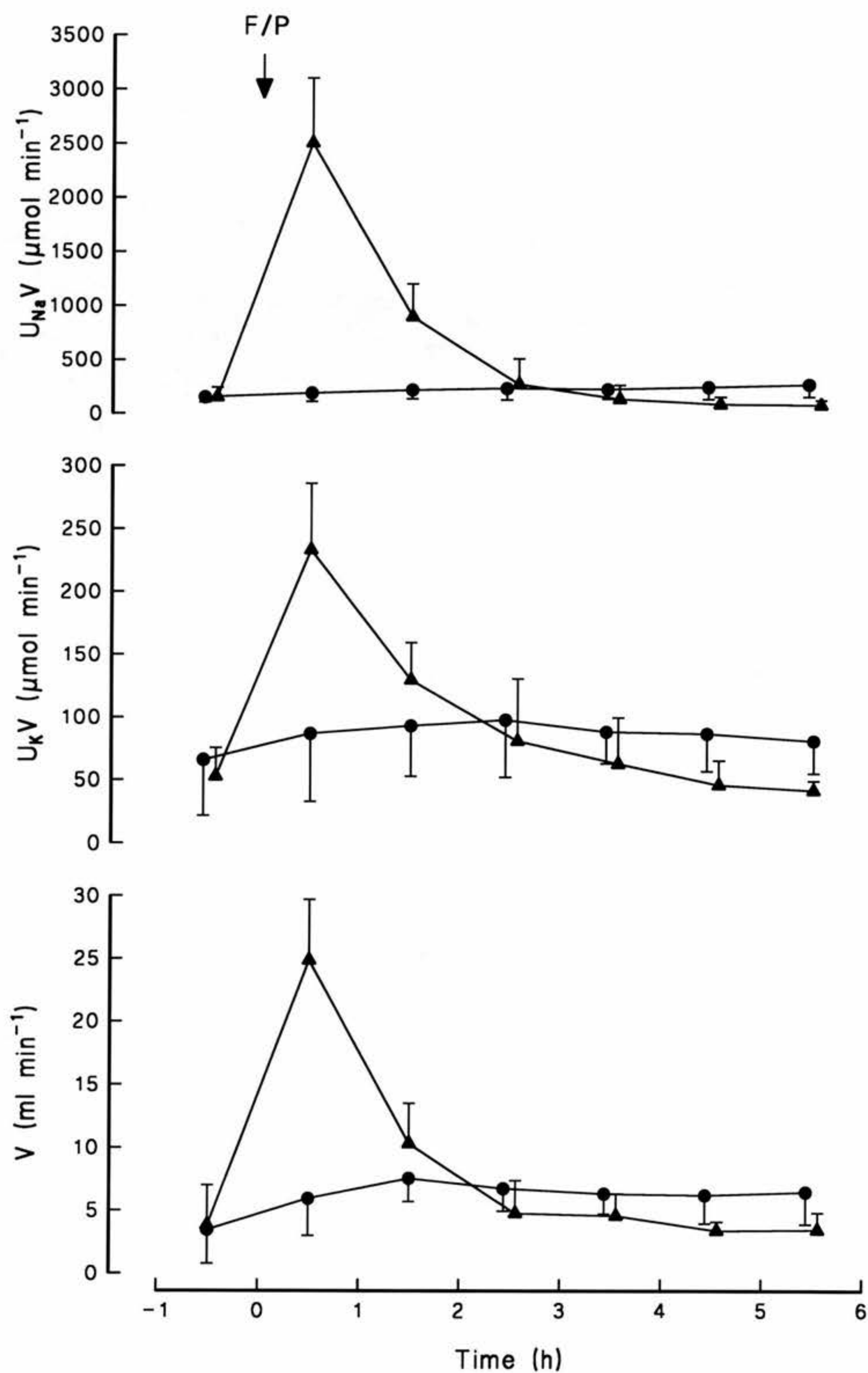


Figure 9-1. Urinary excretion rates of sodium ($U_{Na}V$), urinary excretion rates of potassium (U_kV) and urine flow rates (V) before and after administration of placebo (●) and frusemide (▲). Placebo (P) or frusemide (F) was administered at time 0 h. Values shown are means ± SD (n = 8).

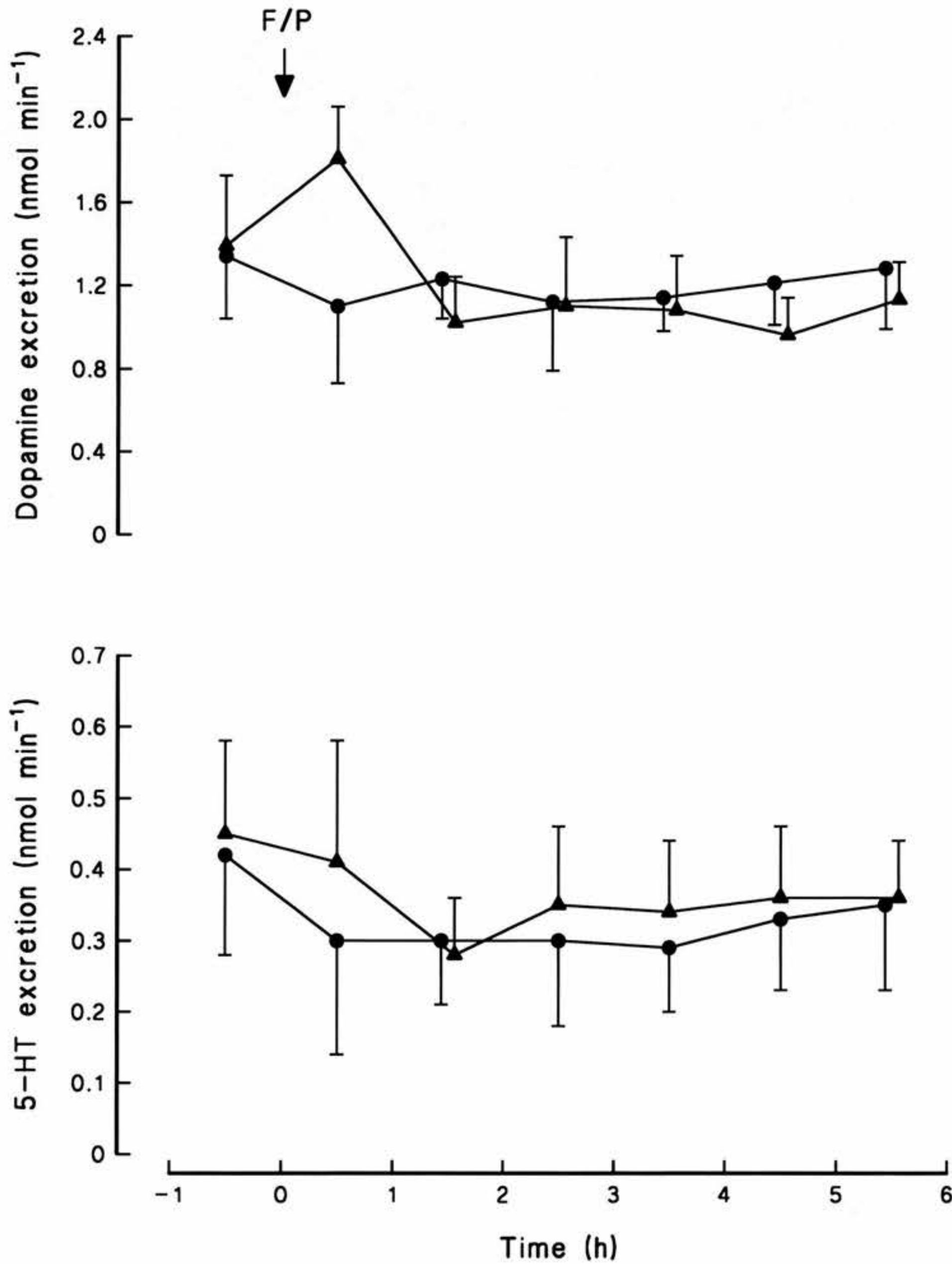


Figure 9-2. Urinary excretion rates of dopamine and 5-HT before and after administration of placebo (●) and frusemide (▲). Placebo (P) or frusemide (F) was administered at time 0 h. Values shown are means ± SD (n = 8).

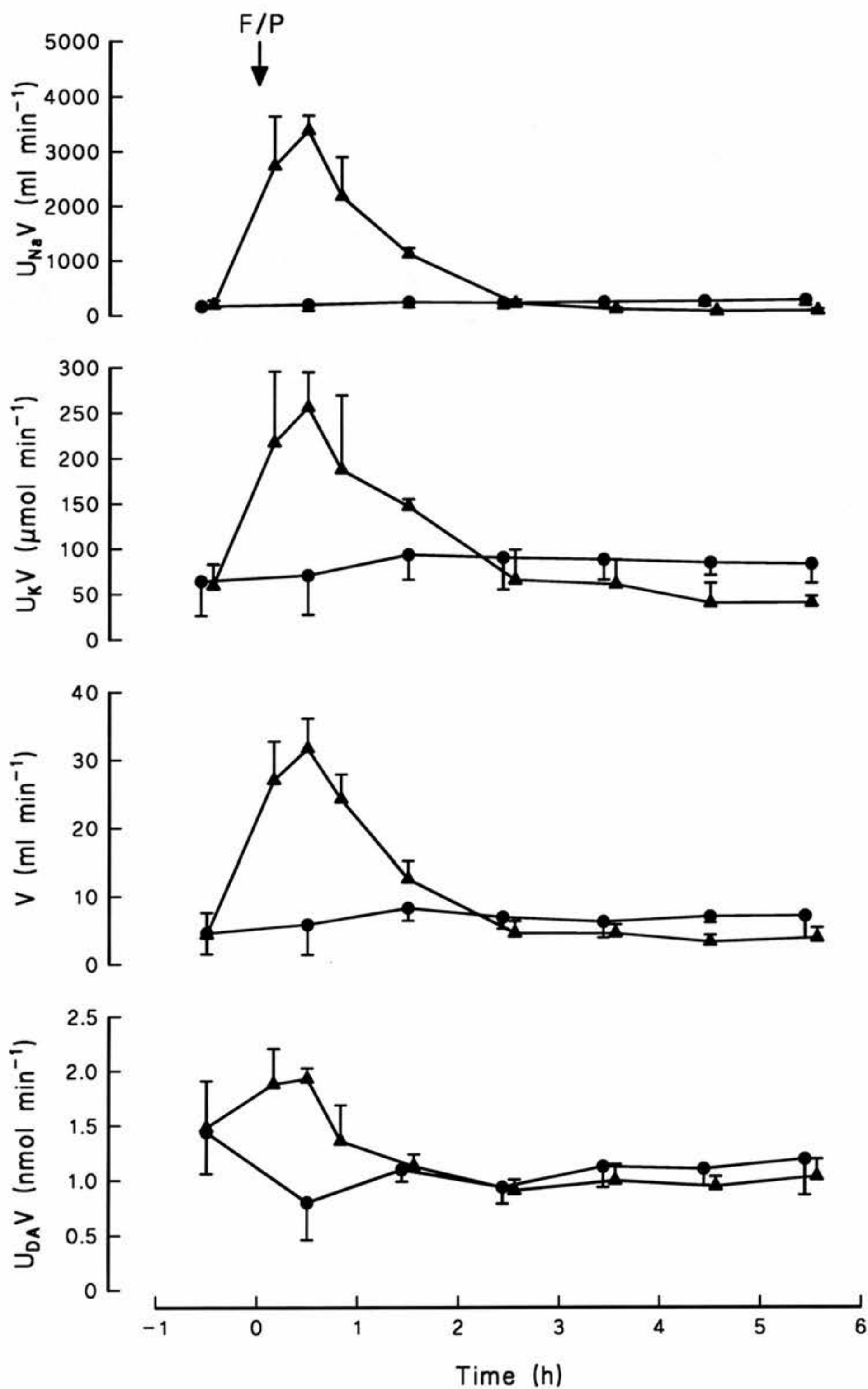


Figure 9-3. Urinary excretion rates of sodium ($U_{Na}V$), urinary excretion rates of potassium (U_kV), urine flow rates (V) and urinary excretion rates of dopamine ($U_{DA}V$) before and after administration of placebo (●) and frusemide (▲) in 4 subjects. Placebo (P) or frusemide (F) was administered at time 0 h. Values shown are means ± SD.

9.4 Discussion

In the present study, frusemide produced the expected diuresis, natriuresis and kaliuresis [525]. It increased urinary dopamine excretion as reported by other investigators in previous studies [526-530]. At variance with these results is the study of Okaniwa *et al.* [535] which reported that frusemide reduced dopamine excretion in healthy female human subjects. Increased urinary excretion of dopamine has also been observed in man after administration of torasemide, a similar loop diuretic [528], and also after hydrochlorothiazide and triamterene in the conscious rat [531]. In contrast, spironolactone had no effect on dopamine excretion in man [527]. Studies with the LAAD inhibitor carbidopa showed that release of renal dopamine does not contribute to the natriuretic effect of frusemide in man, and Jeffrey *et al.* [529] concluded that the enhanced excretion must be regarded as an epiphenomenon. Different findings were, however, observed in the rat pretreated with benserazide [533]. The reason for the discrepancy between rat and man is unexplained.

There was an initial lowering of urinary 5-HT excretion after both frusemide and placebo in the present study. The reason for this is unclear but it may be that the subjects had not yet achieved a steady state, and a longer run-in phase would have been more appropriate. Nevertheless, there was no significant difference in the urinary 5-HT excretion on the two experimental days and there was certainly no suggestion of a rise after frusemide. The absence of an effect of frusemide on urinary 5-HT excretion in man in the present study contradicts studies in rats which demonstrated marked increases in the urinary excretion of 5-HT after the administration of frusemide, hydrochlorothiazide and mersalyl [295,534].

In the previous study (*see* Chapter 8), an increase in urinary 5-HT excretion was observed after infusion of gludopa. The reason for this was not clear. We mentioned that one possible explanation was that it could be a flow-dependent 'wash-out' phenomenon of preformed 5-HT from the tubular lumen resulting from

the diuresis caused by gludopa. The absence of a rise in urinary 5-HT excretion during the marked diuresis induced by frusemide could be cautiously taken as providing evidence that this was unlikely to be the case.

In conclusion, the present study demonstrated that frusemide increased urinary dopamine excretion, but for only a short period, and had no effect on 5-HT excretion in man. It is most unlikely, therefore, in man that either amine contributes materially to an increase in sodium output, or alternatively acts to limit the natriuresis produced by frusemide.

CHAPTER TEN

FINAL DISCUSSION AND CONCLUSIONS

The concept of drug targeting to the kidney via γ-glutamyl prodrugs is based on the work of Orlowski and Wilk [324]. They demonstrated that the kidney is highly active in the uptake and metabolism of γ-glutamyl derivatives of amino acids and peptides. This property is probably related to the high activity of γGT in the renal proximal tubular cells [308-316]. Their observations prompted them to examine the possibility of using γ-glutamyl derivatives of pharmacologically active agents as kidney specific prodrugs [329]. Their work and that of others demonstrated that gludopa, the glutamyl derivative of L-dopa, produces a relatively selective generation of dopamine in the kidney via the sequential actions of renal γGT and LAAD [329-331,333,334; *see* section 1.3]. It was suggested that the γ-glutamyl group can provide a convenient carrier for directing compounds containing an amino group into kidney metabolism and that the γ-glutamyl derivatives of certain drugs may be useful as kidney specific prodrugs [329].

In the work described in this thesis, I have employed the γ-glutamyl prodrug approach to targeting drugs to the kidney in an attempt to explore the renal formation and actions of 5-HT in man. There is little information in this area in man, and species differences in the pattern of distribution, metabolism and actions of 5-HT mean that one has to be cautious when applying the findings obtained in one animal species to another and to man (*see* section 1.2). Previous human studies on the renal effects of 5-HT focused largely on the pharmacological reactions to intravenous administration of the amine and these produced variable results (*see* sections 1.2.6 and 1.2.7). Most studies suggested that 5-HT reduced both sodium and water excretion but these responses were not consistently related to changes in the renal circulation. It was unclear, therefore, whether the observed changes were related to alterations in renal haemodynamics, redistribution of intrarenal blood flow or an action on tubular transport. 5-HT also has a wide spectrum of actions in the body and its extrarenal effects may secondarily alter renal function. Furthermore, there is evidence in the rat that the kidney, in addition to being a target

influenced by 5-HT, is itself capable of locally producing and degrading 5-HT [35,153,166,181,268; *see* section 1.2.3]. Indeed, 5-HT access to its target receptors may be more dependent upon its local metabolism (synthesis and catabolism), and its delivery through the circulation may play only a minor role in its effects on target tissues in the kidney. It can be argued that the systemic administration of 5-HT does not reproduce the normal pattern of 5-HT synthesis in the kidney and its effects may differ from those following endogenous intrarenal 5-HT generation. Systemic infusion studies to a large extent represent the effect of 5-HT on the renal vasculature since exogenous 5-HT reaches the renal arterioles before the tubular structures and its effects on the vasculature may obscure any effects on specific segments of the nephron. The proximal tubule appears to be the main site for 5-HT production in the kidney [35,142,179,180,192] and it is conceivable that it may produce local concentrations of the amine sufficiently high to directly alter tubular reabsorption of sodium or water without affecting the vascular system or 'spilling over' into the systemic circulation. The actions of endogenous renal 5-HT may, therefore, be better investigated using strategies which stimulate renal 5-HT synthesis since this may be more relevant to the physiological situation *in vivo*.

The γ-glutamyl prodrug approach to targeting the kidney offers the possibility of achieving this and thereby controlling the extraneous influences that may complicate the interpretation of the local renal actions of 5-HT. We postulated that glu-5-HTP, the glutamyl derivative of L-5-HTP, could be a relatively selective 5-HT prodrug for the kidney in the same way that gludopa is a renally selective dopamine prodrug [329-331,334,341,343], and that it would be converted to 5-HT via the sequential actions of the same enzymes involved in the formation of dopamine from gludopa, i.e. γGT and LAAD. We compared glu-5-HTP with L-5-HTP, the immediate precursor of 5-HT, which has been shown to increase intrarenal formation of 5-HT in the rat (*see* Chapters 3 and 4). We demonstrated marked increases in the urinary excretion of 5-HT after both compounds and we argued that

these largely resulted from intrarenal generation of 5-HT. There was a reduction in urinary excretion of sodium after both compounds and a reduction in urine output after L-5-HTP. These changes occurred without significant alterations in ERPF or GFR suggesting a predominantly tubular effect of intrarenally generated 5-HT on sodium reabsorption although we could not exclude (or establish) the possibility of an intrarenal redistribution in blood flow partially accounting for the result. The absence of an effect on ERPF and GFR may not be surprising given the likely tubular site of production of 5-HT. There was, however, a trend towards a reduction in GFR in some studies (*see* Chapters 6 and 8) and it is possible that a significant effect would be observed with a higher dose (or longer infusion) of glu-5-HTP or with studying a larger number of subjects. A significant reduction of ERPF was observed in the study described in Chapter 8 when a slightly higher dose (20 *vs* 16.6 $\mu g\ kg^{-1}\ min^{-1}$ for 1 h) of glu-5-HT was infused in salt-replete individuals. Increases in plasma aldosterone concentrations, without changes in PRA, occurred after infusion of both glu-5-HTP and L-5-HTP but the rise in aldosterone levels was smaller after glu-5-HTP (*see* Chapters 3 and 4). The time course of the changes in plasma aldosterone and lack of effect on potassium excretion argued against the antinatriuresis being due primarily to an effect of aldosterone. The aldosterone response may be mediated by an effect within the central nervous system or by a peripheral effect of 5-HT directly on the adrenal gland. The urinary metabolite data and reduced extrarenal effects, as evidenced by changes in growth hormone and aldosterone release, blood pressure and incidence of adverse effects, supported the hypothesis that glu-5-HTP is relatively more selective for the kidney than L-5-HTP.

We then measured the circulating levels of 5-HT and 5-HTP in platelet-rich plasma (PRP) after infusion of L-5-HTP and glu-5-HTP (*see* Chapter 5). We showed that the marked increases in urinary 5-HT after both compounds occurred without significant increases in the concentrations of 5-HT in PRP providing supporting evidence that 5-HT was produced intrarenally. There was the expected

increase in 5-HTP in PRP after L-5-HTP but an increase was also observed after glu-5-HTP. This suggests that glu-5-HTP metabolism was not completely confined to the kidney and that the delivery of 5-HTP via its glutamyl derivative was not entirely selective for the kidney. This finding is not unexpected since γGT is present at extrarenal sites. Its activity is, however, highest in the renal tissue [308,309] and it can be assumed that 5-HTP will be predominantly released in the kidney. This is supported by the observations that the peak level of 5-HTP and the AUC of its concentration-time curve following glu-5-HTP infusion were lower than those after administration of L-5-HTP. Gastrointestinal side effects after L-5-HTP appear to be related to plasma 5-HTP concentrations [360,421] and the absence of such adverse effects after infusion glu-5-HTP (except in one subject) may be related to the lower circulating levels of 5-HTP after administration of this dipeptide. The amount of 5-HTP in urine after glu-5-HTP was, however, higher than that after L-5-HTP infusion. It was greater than can be accounted for by the total clearance of 5-HTP from the plasma perfusing the kidney providing strong support for the intrarenal synthesis of L-5-HTP from glu-5-HTP. L-5-HTP produced intrarenally may be excreted into urine, converted to 5-HT or 5-HIAA, or reabsorbed into the recirculation. Recirculation of renally produced L-5-HTP, in addition to L-5-HTP formed outside the kidney, may be responsible for the rise in circulating 5-HTP after administration of glu-5-HTP. Exogenous L-5-HTP, or L-5-HTP formed from glu-5-HTP, may be converted to 5-HT extrarenally. The absence of a rise in the concentration of 5-HT in PRP suggests that the assay used was unable to detect this increase in 5-HT or that any 5-HT produced was rapidly metabolised.

The sodium retention following the infusion of glu-5-HTP was most likely due to an effect of 5-HT on the renal tubules. We could not, however, exclude from the earlier studies (*see* Chapters 3 and 4) the possibility of the antinatriuresis being due to an effect of the intact amide or of the intermediate metabolite L-5-HTP. We therefore studied the effect of the peripheral LAAD inhibitor carbidopa on the renal

metabolism and actions of glu-5-HTP (*see* Chapter 6). Pretreatment with carbidopa produced a 99% reduction in urinary 5-HT after glu-5-HTP and an abolition of its antinatriuretic effect. This indicates that the 5-HT prodrug (or L-5-HTP) has no effect *per se* and requires conversion to 5-HT to exert its antinatriuretic action. The reduction in glu-5-HTP-induced aldosterone release by carbidopa would also suggest that the aldosterone response is predominantly mediated by a peripheral effect of 5-HT on the adrenal gland.

We attempted to apply the glutamyl prodrug approach to increase the delivery of L-tryptophan to the kidney by administration of glu-TRP, the glutamyl derivative of L-tryptophan (*see* Chapter 7). This compound is structurally very similar to glu-5-HTP and differs from it in the presence of one hydroxyl group in the C5 position of the indole ring. Tryptophan 5-hydroxylase has been localised in the proximal tubules suggesting that the kidney has the capacity to synthesise 5-HT from its first precursor L-tryptophan [35]. Given that the kidney is highly active in the uptake and metabolism of γ-glutamyl derivatives of amino acids or peptides [324,329], it would have been expected that glu-TRP would be taken up by these tubules in a way similar to glu-5-HTP or gludopa [329-331,333,334,341]. It should be readily converted by γGT to L-tryptophan thus increasing delivery of L-tryptophan to the proximal tubular cells and be available for hydroxylation by tryptophan 5-hydroxylase to L-5-HTP, followed by decarboxylation by LAAD to produce 5-HT. Glu-TRP, in contrast to glu-5-HTP, failed to increase urinary 5-HTP or 5-HT and to reduce sodium excretion. The small rise in urinary tryptophan and the undetectable levels of glu-TRP in urine, in contrast to measurable levels of glu-TRP in plasma which declined with time, suggests that glu-TRP was cleaved by γGT to L-tryptophan. L-tryptophan has been shown to be reabsorbed efficiently by the proximal tubules [185,186,456,457] and would, therefore, be available in the cells for the onward synthesis of 5-HT. The absence of a rise in urinary 5-HTP or 5-HT suggests an absence of significant tryptophan 5-hydroxylase activity or that the

L-tryptophan formed from glu-TRP cannot gain access to the enzyme compartment. It is also possible that L-tryptophan was metabolised via other metabolic pathways [33,458-460]. L-tryptophan, like other essential amino acids, has a low renal clearance value, being efficiently reabsorbed in the renal tubules, and this may explain the low levels of urinary tryptophan [174,456,457]. The results of this study are reminiscent of a previous study in our department which showed that the glutamyl derivative of L-tyrosine, γ-L-glutamyl-L-tyrosine, unlike gludopa, failed to increase urinary dopamine, again demonstrating the importance of the additional hydroxyl group on the amine precursor [338]. That study suggested that the kidney is unable to utilise L-tyrosine as a source for urine dopamine and provided evidence that circulating L-dopa is the main physiological source for generation of urine dopamine. A parallel may be drawn between our study and that of Jeffrey and colleagues [338]. It may be inferred cautiously perhaps that L-5-HTP, and not L-tryptophan, is the predominant source of urinary 5-HT in the same way that circulating L-dopa rather than L-tyrosine is the source of renal dopamine. The absence of an increase in renal 5-HT production after infusion of glu-TRP suggests that this glutamyl compound, unlike glu-5-HTP, is unsuitable for the study of renal actions and formation of 5-HT.

The enzyme LAAD catalyses the decarboxylation of L-dopa and L-5-HTP to dopamine and 5-HT respectively [31,37,41-47]. These biogenic amines are both metabolised by MAO [31,48-50]. Increased renal dopamine synthesis causes sodium excretion whereas 5-HT synthesis results in the opposite effect, and it has been suggested that dopamine and 5-HT may be generated within the kidney as reciprocal regulatory substances in the local control of sodium excretion [268]. There is also evidence that L-dopa and L-5-HTP share the same transporter for entry into the renal tubular cells [180]. We have administered equimolar amounts of gludopa and glu-5-HTP to increase the delivery of L-dopa and L-5-HTP respectively to the kidney in salt-replete individuals to investigate whether the

administration of one amine precursor affect the other amine synthesis and whether dopamine or 5-HT is preferentially generated (*see* Chapter 8). We demonstrated that relatively more dopamine than 5-HT was synthesised and that the natriuretic effect of dopamine was more potent than the sodium retaining action of 5-HT *under the conditions of this study*. Comparison of the urinary metabolite data after the separate and concomitant infusion of the two compounds provided no evidence of competitive inhibition of synthesis of either amine. Glu-5-HTP failed to reduce sodium excretion in this study at variance with all the previous studies. A type 2 error may account for this unusual result. However, the study was conducted in salt-replete individuals. It is possible that the antinatriuretic action of 5-HT is less easily demonstrable in this state or that the increased salt intake may have led to a down-regulation of the tubular 5-HT receptors in order to facilitate sodium excretion. The study will need to be repeated in salt-restricted or -depleted subjects to establish whether the sodium intake did indeed influence the antinatriuretic action of 5-HT and the relative amounts of dopamine and 5-HT produced. We detected an unexpected increase in urinary 5-HT excretion after infusion of gludopa, but dopamine excretion was not affected by administration of glu-5-HTP. The reason for the finding following gludopa infusion was unclear. Possible explanations include a displacement of endogenous renal 5-HT by dopamine; a compensatory response to the induced natriuresis; or a flow-dependent 'washout' of preformed 5-HT from the tubular lumen resulting from gludopa-induced diuresis. However, gludopa failed to produce a significant increase in urine output when compared with placebo infusion in this particular study, in contrast to the increased diuresis reported in a previous investigation [334]. This observation and the absence of an increase in urinary 5-HT excretion during the marked diuresis induced by frusemide (*see* Chapter 9) could be cautiously taken as evidence that 'washout' is unlikely to be the explanation for the increased 5-HT excretion that occurred after administration of gludopa.

Frusemide increases the urinary excretion of dopamine in man [526-530] but its effect on 5-HT excretion has not been studied. In the rat, frusemide has been reported to increase urinary 5-HT excretion and, at the same time, to decrease the renal content of 5-HT [295,534]. We were able to confirm the effect of frusemide on dopamine excretion but were unable to demonstrate an effect of the loop diuretic on 5-HT excretion in man (*see* Chapter 9), thus contradicting the reported findings in the rat. The disparity in results may reflect species differences in 5-HT response to diuretics and we could study a higher dose of frusemide. Both studies which reported an effect of diuretics on 5-HT excretion in the rat came from the same laboratory [295,534], and it would also be of interest to see if similar results can be produced by other investigators.

Our series of studies suggest that glu-5-HTP could be a valuable experimental tool for manipulating renal 5-HT production and exploring the renal formation and effects of 5-HT in man in the same way that gludopa was useful for investigating the role of renal dopamine [334-342]. Much can be studied in this field using this prodrug. When 5-HT is generated, it acts on the renal tubules to reduce sodium excretion. The tubular site involved is, however, unknown and it will be of interest to explore this. Lithium clearance technique, for instance, can be used as a marker of proximal tubular function [536]. Various 5-HT receptors have been identified in the mammalian kidney [257,258] and in renal cell lines [260-265]. There is little information on the type of 5-HT receptors mediating the antinatriuretic action of 5-HT and this could be investigated in man by studying the effects of selective 5-HT receptor antagonists on the antinatriuretic action of glu-5-HTP. Although the antinatriuretic effect on 5-HT may be due to a direct effect of 5-HT on the renal tubules, our studies do not rule out the participation of other modulators of renal function secondary to increased 5-HT production. The interactions of 5-HT with other systems, such as the renin-angiotensin, kinin-kallikrein, atrial natriuretic peptide or prostaglandin, would be worthy of pursuit. Altered activity of these

systems may modulate the actions of 5-HT, and simultaneous inhibition of their production may, for example, help to unmask any other effect of the amine. There is evidence suggesting that 5-HT is not a specific substrate for MAO-A but is deaminated by both forms of MAO in the pig and rat liver [537-539] and in the rat brain [540]. Whether this is the case in the human kidney can be investigated with relative safety using the new reversible MAO-A and MAO-B inhibitors and studying their effects on the catabolism of 5-HT produced intrarenally from glu-5-HTP. These questions can be answered to a large extent from *in vivo* studies in man using glu-5-HTP. Animal and *in vitro* studies will be required to localise the sites of 5-HT production and destruction in the kidney together with the cell types bearing the 5-HT receptors involved in the antinatriuretic action of 5-HT. It will also be of interest to investigate the production of 5-HT in pathological states affecting the kidney since there is some evidence that 5-HT may play a pathophysiological role in conditions such as hypertension, glomerulonephritis or renal failure (*see* sections 1.1.7 and 1.2.10).

There is little doubt from our work that 5-HT produced intrarenally is capable of reducing sodium excretion in man. Whether renal biosynthesis of 5-HT constitutes an intrarenal control mechanism of sodium excretion under physiological circumstances, however, remains to be established. The possibility is raised that endogenous renal 5-HT may act as a paracrine or autocrine substance in the control of sodium excretion in a way analogous to that demonstrated for renal dopamine. The observation that 5-HT excretion may vary with salt intake supports a role for 5-HT as physiological regulator of sodium excretion [461].

In conclusion, the glutamyl prodrug approach to renal drug targeting has been applied to 5-HT with positive results in man. Our work suggests that glu-5-HTP will prove a useful pharmacological experimental tool for the study of the formation and function of 5-HT in the human kidney.

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APPENDIX I

LIST OF ABBREVIATIONS

5-CT	5-Carboxamidotryptamine
5-CT	5-Carboxamidotryptamine
5-HIAA	5-Hydroxy-3-indoleacetic acid
5-HT	5-Hydroxytryptamine (serotonin)
5-HTOL	5-Hydroxytryptophol
5-HTP	5-Hydroxytryptophan
5-MeOT	5-Methoxytryptamine
8-OH-DPAT	8-Hydroxy-2-(di-*n*-propylamino)tetralin
CI_{diff}	Confidence interval of the difference between the means
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
ANG I	Angiotensin I
ANG II	Angiotensin II
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BMY 7378	8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]-decane-7,9-dione
BSA	Bovine serum albumin
cAMP	Adenosine 3′:5′-cyclic phosphate
cDNA	Complementary DNA (deoxyribonucleic acid)
CGS 12066	7-Trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo [1,2-*a*]quinoxaline
C_{In}	Renal clearance of polyfructosan (Inutest)
CNS	Central nervous system
CP 93,129	3-(1,2,5,6-Tetrahydropyrid-4-yl)pyrrolo[3,2-*b*]pyrid-5-one
C_{PAH}	Renal clearance of PAH
DAG	Diacylglycerol
DAU 6285	Endo-6-methoxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxylate
DBP	Diastolic blood pressure
DHBA	3,4-Dihydroxybenzylamine
DOI	1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane
DOPAC	3,4-Dihydroxyphenylacetic acid (dopacetic acid)
Dopamine	3,4-Dihydroxyphenylethylamine
DP-5-CT	Dipropyl-5-carboxamidotryptamine
EDTA	Ethylenediaminetetra-acetic acid

ERPF	Effective renal plasma flow
FE_{Na}	Fractional excretion of sodium
FF	Filtration fraction
FW	Formula weight
GFR	Glomerular filtration rate
Glu-5-HTP	γ-L-Glutamyl-5-hydroxy-L-tryptophan
Glu-TRP	γ-L-Glutamyl-L-tryptophan
Gludopa	γ-L-Glutamyl-L-3,4-dihydroxyphenylalanine
GR 113808	[1-[2-(Methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate
GR 127935	N-[4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide
HCl	Hydrochloric acid
hGH	Human growth hormone
HPLC	High performance liquid chromatography
ICI 169369	2-(2-Dimethylaminoethylthio)-3-phenylquinoline
i.m.	Intramuscular
i.p.	Intraperitoneal
IP_3	Inositol (1,4,5)-triphosphate
IRMA	Immunoradiometric assay
i.v.	Intravenous
L 694247	2-[5-[3-(4-Methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine
L-Dopa	L-3,4-Dihydroxyphenylalanine
LAAD	L-Amino acid decarboxylase
LSD	(+)-Lysergic acid diethylamide
LY 53857	4-Isopropyl-7-methyl-9-(2-hydroxy-1-methylpropoxycarbonyl)-4,6,6A,7,8,9,10,10A-octahydroindolo[4,3-FG]quinolone
MAO	Monoamine oxidase
MAP	Mean arterial blood pressure
mCPP	1-(3-Chlorophenyl)piperazine
MDL 72222	1αH,3α,5αH-Tropan-3-yl-3,5-dichlorobenzoate
MDL 72832	8-(4-[1,4-Benzodioxan-2-ylmethylamino]butyl)-8-azaspiro[4,5]decane-7,9-dione
MDMA	Methylenedioxymethamphetamine ('ecstasy')
MK 212	6-Chloro-2-(1-piperazinyl)pyrazine
mRNA	Messenger RNA (ribonucleic acid)
MW	Molecular weight

n	Number of subjects
NaCl	Sodium chloride (saline)
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NAN 190	1-(2-Methoxyphenyl)-4-[4-(2-phthalimmido)butyl]piperazine
NaOH	Sodium hydroxide
PAH	*Para*-aminohippurate
PRA	Plasma renin activity
PRP	Platelet-rich plasma
RIA	Radioimmunoassay
RS 23597-190	3-(Piperidin-1-yl)propyl 2-methoxy-4-amino-5-chlorobenzoate
RU 24969	5-Methoxy-3(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole
SB 200646	N-(1-Mehtyl-5-indolyl)-N-(3-pyridyl)urea
SB 204070	(1-Butyl-4-piperidinylmethyl)-8-amino-7-chloro-1,4-benzodioxan-5-carboxylate
SBP	Systolic blood pressure
SD	Standard deviation
SDZ 205557	2-Methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino)ethyl ester
SDZ 21009	4(3-Terbutylamino-2-hydroxypropoxy)indol-2-carbonic acid-isopropylester
SDZ 216525	Methyl-4(4-[4-(1,1,3-trioxo-2H-1,2-benziosothiazol-2-yl)butyl]-1-piperazinyl)1H-indole-2-carboxylate
SSRI	Selective serotonin reuptake inhibitors
TFMPP	N-(3-Trifluoromethyl-phenyl)piperazine
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
TSH	Thyroid stimulating hormone
v/v	Volume (of solute) per volume (of solvent)
w/v	Weight (of solute) per volume (of solvent)
WAY 100135	N-tert-butyl-3-(4-[2-methoxyphenyl]piperazin-1-yl)-2-phenylpropanamide

APPENDIX II

PUBLICATIONS RELATED TO THIS THESIS

1. Li Kam Wa TC, Freestone S, Samson RR, Johnston NR, Lee MR. A comparison of the effects of two putative 5-hydroxytryptamine renal prodrugs in normal man. *British Journal of Clinical Pharmacology* 1993; **36**: 19-23.

2 Li Kam Wa TC, Freestone S, Samson RR, Johnson NR, Lee MR. A comparison of the renal and neuroendocrine effects of two 5-hydroxytryptamine renal prodrugs in normal man. *Clinical Science* 1993; **85**: 607-14.

3. Li Kam Wa TC, Freestone S, Samson RR, Johnston NR, Lee MR. The antinatriuretic action of γ-L-glutamyl-5-hydroxy-L-tryptophan is dependent on its decarboxylation to 5-hydroxytryptamine in normal man. *British Journal of Clinical Pharmacology* 1994; **38**: 265-9.

4. Li Kam Wa TC, Burns NJT, Williams BC, Freestone S, Lee MR. Blood and urine 5-hydroxytryptophan and 5-hydroxytryptamine levels after administration of two 5-hydroxytryptamine precursors in normal man. *British Journal of Clinical Pharmacology* 1995; **39**: 327-9.

Br J clin Pharmac 1993; **36**: 19–23

A comparison of the effects of two putative 5-hydroxytryptamine renal prodrugs in normal man

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1 The effects of 1 h intravenous infusions of equimolar amounts of two putative 5-hydroxytryptamine (5-HT) renal prodrugs, 5-hydroxy-L-tryptophan (5-HTP, 10 μg kg^{-1} min^{-1}) and γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP, 16.6 μg kg^{-1} min^{-1}) were examined in five healthy male volunteers in a randomised, placebo-controlled, cross-over study.

2 Both compounds increased urinary excretion of 5-HT and there was greater extra-renal formation of 5-HT following 5-HTP administration than after glu-5-HTP.

3 Glu-5-HTP was significantly antinatriuretic. 5-HTP reduced mean urinary sodium excretion but this effect was not statistically significant.

4 5-HTP, but not glu-5-HTP, significantly increased plasma aldosterone. There was no increase in plasma renin activity with either compound.

5 There were no significant changes in pulse rate or blood pressure. Two subjects complained of nausea at the end of 5-HTP infusion but none had any adverse reactions with glu-5-HTP.

6 The results of this study suggest that both prodrugs generate 5-HT in man and that glu-5-HTP is antinatriuretic. The glutamyl derivative may have greater renal specificity than 5-HTP and, as a result, causes less systemic side effects.

Keywords 5-hydroxytryptamine 5-hydroxy-L-tryptophan γ-L-glutamyl-5-hydroxy-L-tryptophan kidney sodium excretion renin aldosterone

Introduction

The mammalian kidney is rich in each of the enzymes required for the synthesis of 5-hydroxytryptamine (5-HT; serotonin) from its essential amino-acid precursor L-tryptophan as well as those necessary for its degradation [1]. It has been demonstrated in rats that 5-HT is formed from its immediate precursor, 5-hydroxy-L-tryptophan (5-HTP), under the action of renal aromatic L-amino acid decarboxylase (LAAD) and the amine reduces renal blood flow, urine output and sodium excretion [2–4]. γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP) may be another putative 5-HT prodrug, sequentially converted by γ-glutamyl transferase (γGT) to 5-HTP and then decarboxylated by LAAD to 5-HT. Given the high activities of both γGT and LAAD in the renal tissues [1,5], the conversion of glu-5-HTP to 5-HT should be relatively specific for the kidney, more so than with 5-HTP, and increase local production of 5-HT in the kidney. This is analogous to the conversion of γ-L-glutamyl-L-dopa to L-dopa by γGT and then to dopamine by LAAD thus increasing endogenous renal production of dopamine [6,7]. Administration of a relatively renally selective 5-HT prodrug may enable the renal effects of 5-HT to be assessed with less interference from the effects on other systems that could be produced by an increase in circulating 5-HT.

The present study compared the effects of intravenous infusions of equimolar amounts of glu-5-HTP and 5-HTP with infusion of saline (placebo) in normal man. We were interested in observing changes in urinary excretion of sodium, 5-HTP, 5-HT and 5-hydroxyindole acetic acid (5-HIAA), plasma renin activity (PRA) and plasma aldosterone.

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Methods

Five healthy male volunteers, aged 22 to 32 years (mean 28 years) and weighing 61.5 to 99.5 kg (mean 71.3 kg), took part in this randomised, placebo-controlled, crossover study. They were normal on clinical examination and none had laboratory evidence of hepatic, renal or haematological abnormality. They were on no medication for at least 2 weeks before the start of the study and no drugs apart from the study medications were allowed during the experimental period. They refrained from alcohol for 24 h before each study day. The study was approved by the Medical and Clinical Oncology Ethics of Medical Research sub-committee, Lothian Health Board, and written informed consent was obtained from all participants.

Each subject attended on 3 separate days, at least 1 week apart, following an overnight fast. They reported at about 08.15 h having drunk 250 ml of tap water 1 h previously. They received intravenous 0.9% (w/v) sodium chloride (150 mmol l^{-1}; saline) at a rate of 10 ml kg^{-1} h^{-1} for 6 h to increase urine sodium excretion. After a 3 h run-in period, 5-HTP (10 $\mu g\ kg^{-1}\ min^{-1}$) or glu-5-HTP (16.6 $\mu g\ kg^{-1}\ min^{-1}$) in 0.9% saline or placebo (saline alone at 0.5 ml min^{-1}) was administered intravenously for 1 h. The subjects remained semi-recumbent throughout the study except when standing up to pass urine. Accurately timed consecutive urine collections of about 30 min duration were made during the last half-hour of the run-in phase (period 0), during the administration of the test medications (periods 1 and 2) and for 2 h after the completion of the infusion (periods 3 to 6). Urine volumes were recorded and aliquots removed and stored at −40° C for analysis of sodium, 5-HTP, 5-HT and 5-HIAA. Venous blood samples for estimation of PRA and aldosterone were taken just before and at 0.5, 1, 2 and 3 h after the start of the infusion of the test medications. The samples were collected into pre-cooled tubes containing sodium ethylenediamine tetra-acetate and kept on ice. Plasma was separated after centrifugation at 4° C and stored at −40° C until analysis. Pulse rate and blood pressure were measured at regular intervals using a Dinamap semi-automated recorder (Critikon, Inc., Tampa, Florida, USA) and the average values for each 30 min period calculated.

Glu-5-HTP was supplied by Aalto Bio Reagents Ltd, Dublin, Eire, and the preparation contained 95% pure peptide. 5-HTP was obtained from Sigma Chemical Co. Ltd, Poole, UK. Both were prepared for use in 0.9% saline with 0.5% human albumin as a carrier.

Analysis of blood and urine samples

PRA was measured by radioimmunoassay of angiotensin I (AI) generated under standard conditions [8]. The intra- and inter-assay coefficients of variation were 4% and 6% respectively. Plasma aldosterone was measured using radioimmunoassay kits ('Coat-a-count', Diagnostic Products Corporation, Abingdon, UK). Intra- and inter-assay coefficients of variation were 5% and 7% respectively. Urine sodium concentrations were measured by ion-selective electrode (Radiometer KNA1 Analyser). Urine 5-HT was measured using a modification of the method of Jouve *et al.* [9]. Following extraction from urine onto Amberlite CG-50 (BDH), the 5-HT was eluted into 3M ammonium acetate, pH 5.0. *N*-omega-methyl-5-hydroxytryptamine oxalate was used as an internal standard. The intra- and inter-assay coefficients of variation were 5.6% and 11.6% respectively. Measurement was by h.p.l.c. and spectrofluorimetric detection. Urinary 5-HTP was measured by h.p.l.c. and spectrofluorimetric analysis following dilution of the sample with deionised water. Intra- and inter-assay coefficients of variation were 5.5% and 6.5% respectively. Urinary 5-HIAA was measured using the method of Goldenberg [10]. The final extract was measured by spectrophotometry at 590 nM. Intra- and inter-assay coefficients of variation were 4.5% and 7.3% respectively.

Statistical analysis

Results are given as means ± s.d. unless otherwise stated. Differences in the cumulative urinary metabolite data following 5-HTP and glu-5-HTP were analysed by Student's *t*-test for paired observations. The urinary sodium, pulse rate, blood pressure and aldosterone data between the 3 treatment days were compared by repeated measures analysis of variance for overall statistical significance and two-way analysis of variance was used to compare the plasma aldosterone values at each time point. Values of $P < 0.05$ were considered statistically significant.

Results

Urinary excretion rates of 5-HTP, 5-HT and 5-HIAA for each 30 min period are shown in Figure 1. 5-HTP was not detectable in the urine during infusion with saline alone. Marked increases in urine 5-HTP occurred after infusion of either 5-HTP or glu-5-HTP. The 5-HTP excretion was higher for all collection periods after infusion of glu-5-HTP when compared with 5-HTP and the cumulative excretion values over the 3 h following the start of the infusion were 17.1 ± 5.5 μmol after 5-HTP and 50.0 ± 4.4 μmol after glu-5-HTP ($P < 0.001$). The urinary excretion rate of 5-HT was < 0.4 nmol min^{-1} during infusion of placebo. This increased to a peak value of 293 ± 32 nmol min^{-1} after administration of 5-HTP and 215 ± 17 nmol min^{-1} after glu-5-HTP. Cumulative urinary 5-HT excretion values were 0.06 ± 0.01, 26.9 ± 6.2 and 24.2 ± 3.1 μmol for the 3 h after the start of infusion of placebo, 5-HTP and glu-5-HTP respectively; the difference between 5-HTP and glu-5-HTP was not significant. 5-HIAA excretion increased after both compounds and the increment was 43% greater following 5-HTP than after glu-5-HTP ($P < 0.05$). The cumulative 5-HIAA excretion values were 3.1 ± 0.8, 38.1 ± 8.8 and 27.6 ± 3.2 μmol for placebo, 5-HTP and glu-5-HTP respectively. At the end of the 3 h study period, 41 ± 2% (on a molar basis) of admini-

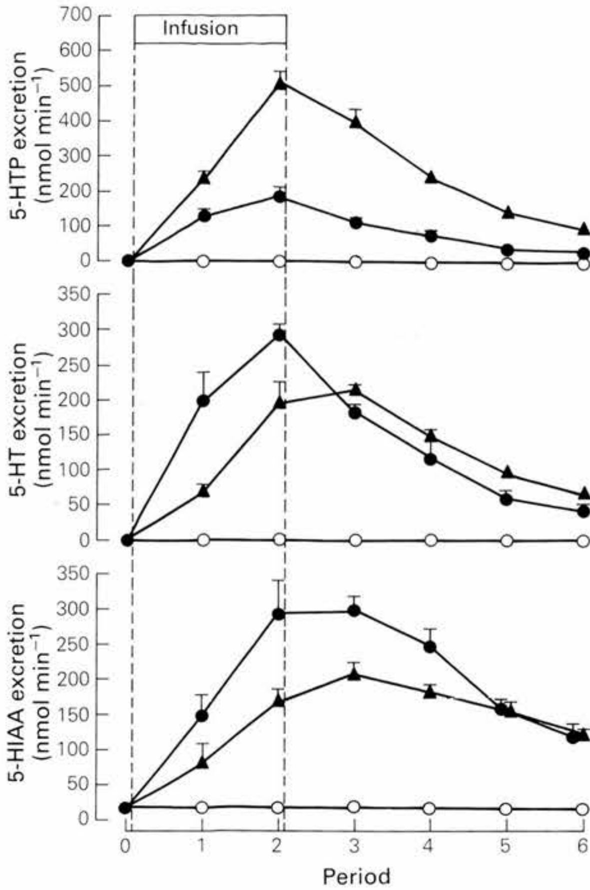

Figure 1 Urine 5-HTP, 5-HT and 5-HIAA excretion rates before (period 0), during (periods 1 and 2) and after (periods 3 to 6) infusion of placebo (saline) (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± s.e. mean (n = 5).

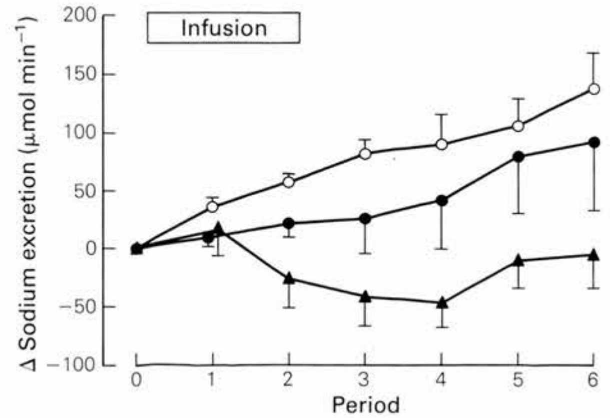

Figure 2 Changes in urinary sodium excretion from baseline (period 0) during (periods 1 and 2) and after (periods 3 to 6) infusion of placebo (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± s.e. mean (n = 5).

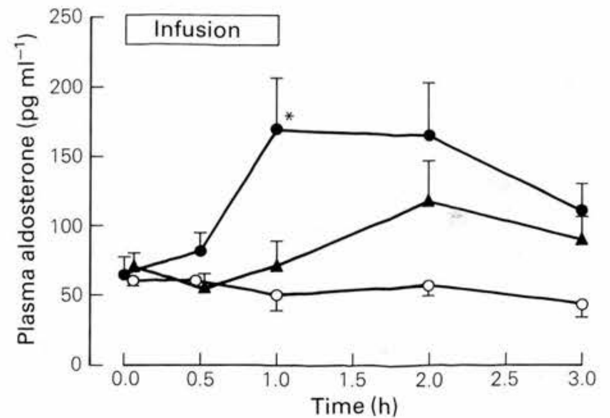

Figure 3 Plasma aldosterone concentration following infusion of placebo (saline) (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± s.e. mean (n = 5). * = $P < 0.05$ *vs* placebo and glu-5-HTP.

stered 5-HTP and 52 ± 8% of glu-5-HTP were recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA. The percentage amounts of infused dose excreted as 5-HTP, 5-HT and 5-HIAA were 9 ± 2%, 14 ± 1% and 18 ± 3% respectively after 5-HTP administration, and 26 ± 5%, 13 ± 1% and 13 ± 2% respectively after glu-5-HTP.

The urinary sodium excretion rates prior to administration of placebo, 5-HTP and glu-5-HTP were 275 ± 115, 210 ± 68 and 280 ± 105 μmol min^{-1} respectively. The sodium excretion values are presented as changes from baseline (period 0) to allow for the lower baseline values prior to infusion of 5-HTP (Figure 2). Urinary sodium excretion increased progressively throughout all clearance periods in response to infusion of saline on the placebo day and the cumulative natriuresis during the 3 h after the start of the placebo infusion was 65.0 ± 19.1 mmol. Glu-5-HTP significantly attenuated the increase in sodium excretion when compared with placebo ($P <$ 0.001) whereas 5-HTP caused a reduction in mean urinary sodium output which was not statistically significant. Cumulative natriuresis was reduced by 19.3 ± 6.2 mmol after glu-5-HTP and by 5.7 ± 11.9 mmol after 5-HTP when compared to placebo infusion.

Plasma aldosterone was significantly increased by 5-HTP infusion ($P < 0.05$) but not after glu-5-HTP (Figure 3). PRA was undetectable (< 0.35 ng AI ml^{-1} h^{-1}) in two subjects on all 3 study days. In the other three subjects, PRA declined from 1.00 ± 0.49 ng AI ml^{-1} h^{-1} at baseline to 0.68 ± 0.28 ng AI ml^{-1} h^{-1} at the end of placebo infusion and 0.61 ± 0.22 ng AI ml^{-1} h^{-1} 1 h later. Equivalent values were 1.21 ± 0.34, 0.91 ± 0.34 and 0.70 ± 0.34 ng AI ml^{-1} h^{-1} on the 5-HTP day, and 1.16 ± 0.20, 0.66 ± 0.22 and 0.48 ± 0.19 ng AI ml^{-1} h^{-1} on the glu-5-HTP day. Statistical analysis was deemed inappropriate due to the small number of subjects with detectable PRA values.

Two subjects complained of nausea at the end of 5-HTP infusion but none had any adverse reactions with glu-5-HTP. There were no significant changes in pulse rate or blood pressure throughout the studies (Table 1).

Discussion

There is evidence from studies in rats that 5-HT can be formed within the kidney and that urinary excretion of 5-HT reflects renal production of 5-HT [2,3,11,12]. The results of the present study are consistent with the hypothesis that 5-HT can be synthesised in the human

Table 1 Pulse rate and blood pressure values before (period 0), during (periods 1 and 2) and after (periods 3 to 6) infusion of placebo, 5-HTP and glu-5-HTP. Values shown are means ± s.d. (n = 5)

	Period 0	1	2	3	4	5	6
Placebo infusion							
Pulse rate (beats min^{-1})	55.2 ±9.1	54.5 ±8.4	54.8 ±8.0	53.5 ±8.9	55.1 ±9.2	54.4 ±9.6	55.0 ±9.9
Systolic blood pressure (mm Hg)	117.8 ±14.7	116.4 ±11.6	117.1 ±12.6	116.1 ±11.4	115.1 ±10.9	120.8 ±13.1	122.4 ±16.5
Diastolic blood pressure (mm Hg)	71.0 ±12.0	68.9 ±9.9	69.2 ±11.6	70.0 ±11.1	70.9 ±11.9	71.2 ±11.1	73.4 ±13.4
5-HTP infusion							
Pulse rate (beats min^{-1})	53.4 ±6.7	53.1 ±6.5	54.4 ±9.0	53.4 ±7.5	52.0 ±6.4	52.6 ±7.2	53.2 ±8.7
Systolic blood pressure (mm Hg)	114.2 ±5.8	110.7 ±7.3	115.8 ±8.4	116.3 ±10.5	116.2 ±8.4	116.4 ±6.4	116.2 ±6.4
Diastolic blood pressure (mm Hg)	67.8 ±8.6	68.6 ±7.8	69.4 ±7.8	70.0 ±8.2	69.0 ±7.3	68.4 ±6.5	72.4 ±7.7
Glu-5-HTP infusion							
Pulse rate (beats min^{-1})	53.0 ±10.0	52.3 ±7.7	52.6 ±7.3	53.5 ±9.8	52.2 ±7.8	52.4 ±7.0	51.6 ±7.1
Systolic blood pressure (mm Hg)	116.2 ±12.1	114.1 ±11.9	113.9 ±10.6	112.9 ±8.7	114.3 ±9.4	116.8 ±10.6	119.0 ±14.3
Diastolic blood pressure (mm Hg)	69.4 ±12.2	66.4 ±6.5	70.2 ±7.2	65.3 ±8.0	70.6 ±9.4	70.0 ±9.3	71.2 ±8.8

kidney. Both putative 5-HT prodrugs produced a marked increase in urine 5-HT excretion. Such levels can only be achieved by generation of 5-HT within the kidney where it would be less accessible to the effects of systemic catabolising enzymes. Any 5-HT produced extrarenally and presented to the blood stream, would be deaminated efficiently by monoamine oxidase (MAO) in the hepatic and pulmonary circulations, or rapidly sequestered into platelets leaving little available for filtration at the renal glomerulus. That circulating 5-HT would be rapidly destroyed is supported by observations that intravenous administration of 5-HT produced only a small increase in blood 5-HT concentration, approximately 2% of the infused amount [13]. Parenteral 5-HT had virtually no effects on the levels of free 5-HT in urine but markedly increased the excretion of 5-HIAA and 5-HT conjugates [13,14]. Furthermore, in the carcinoid syndrome, urine 5-HT excretion is usually less than 1% of 5-HIAA [15].

Urine 5-HTP levels were higher after the administration of glu-5-HTP than with 5-HTP, in our study, suggesting that the amount of 5-HTP presented to the kidney for direct renal decarboxylation is greater following the infusion of glu-5-HTP. Cumulative urinary 5-HT excretion increased by an average of 450-fold with 5-HTP and 400-fold with the glu-5-HTP. If more 5-HT is excreted by the kidney, one would expect that less is available for conversion to 5-HIAA. The urinary 5-HIAA excretion was however higher after 5-HTP than with glu-5-HTP. These observations suggest greater extrarenal formation and metabolism of 5-HT after 5-HTP administration than after glu-5-HTP. About 41% of the infused 5-HTP was recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA during the 3 h observation period in this study whereas the recovery of the three measured metabolites averaged 52% after glu-5-HTP. The quantitative difference indicates that the two compounds are handled differently in the body and is consistent with greater delivery of the glutamyl compound to the kidney. The lower recovery value with 5-HTP may be due to uptake of 5-HTP or its metabolites into other body compartments such as the brain or circulating platelets. It is also possible that more of the 5-HTP than glu-5-HTP was converted to products which were not measured in this study (e.g. sulphate and glucuronide conjugates).

Glu-5-HTP significantly attenuated urine excretion of sodium. Mean urine excretion of sodium was lower after 5-HTP compared with placebo but the difference was not statistically significant (Figure 2). The failure to demonstrate a significant antinatriuresis with 5-HTP in this study may however be due to a type 2 error because of the small number of subjects investigated. The antinatriuresis is presumably mediated by locally synthesized 5-HT, and the greater antinatriuretic effect produced by the glutamyl derivative suggests that 5-HT generated intrarenally, following administration of the dipeptide, is more effective despite the greater increase in mean cumulative 5-HT excretion produced by 5-HTP administration. The antinatriuresis may be a consequence of a reduction in renal blood flow, a fall in glomerular filtration rate or an increased tubular reabsorption of sodium. These questions will be addressed in a future study.

5-HTP caused a significant and prompt increase in aldosterone levels without any increase in PRA in keeping with previous studies in man that the release of aldosterone by 5-HTP is independent of activation of

the renin angiotensin system [16,17]. This effect on aldosterone may be due to direct stimulation of the adrenal cortex [18] or may be mediated by central 5-HT pathways as 5-HTP can cross the blood brain barrier [16]. 5-HTP also stimulates the release of other hormones such as prolactin, ACTH, growth hormone and cortisol, providing further evidence that it can act centrally [19,20]. In contrast, glu-5-HTP caused a small, non-significant delayed rise in aldosterone level in keeping with its effects being largely restricted to the kidney. The delayed rise in aldosterone concentration following glu-5-HTP may be due to conversion in the kidney and recirculation of 5-HTP. Release of aldosterone is unlikely to be responsible for the observed antinatriuresis after glu-5-HTP as the rise in aldosterone occurred later. Suppression of PRA, often to undetectable levels, in the present study probably resulted from the sodium chloride infusion employed in this protocol.

Two out of the five subjects complained of nausea at the end of 5-HTP infusion. This and other gastrointestinal symptoms such as abdominal pain, vomiting and diarrhoea which have been reported with 5-HTP administration are probably mediated by the enteric or central 5-HT receptors [20–22]. The dose of 5-HTP used in our study was chosen in an attempt to minimise adverse gastrointestinal and cardiovascular effects. Glu-5-HTP, in equimolar amount, caused no adverse effects, providing further evidence that it is more renally specific and has less systemic toxicity. There were no significant changes in pulse rate or blood pressure with either compound.

In conclusion, the results of this study indicate that 5-HT can be generated intrarenally in man and that it is antinatriuretic. Glu-5-HTP appears to be more specific for the kidney than 5-HTP. This compound may allow the effects of increased renal synthesis of 5-HT to be evaluated without confounding extrarenal effects. It could provide a valuable pharmacological research tool for the investigation of the renal formation and effects of 5-HT in man.

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A comparison of the renal and neuroendocrine effects of two 5-hydroxytryptamine renal prodrugs in normal man

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1. The effects of 1 h intravenous infusions of equimolar amounts (45 nmol min^{-1} kg^{-1}) of two putative 5-hydroxytryptamine renal prodrugs, 5-hydroxy-L-tryptophan and γ-L-glutamyl-5-hydroxy-L-tryptophan, were investigated in a randomized, placebo-controlled, cross-over study in nine healthy male subjects.

2. Cumulative urinary 5-hydroxytryptamine excretion over the 3 h observation period rose by about 370-fold after 5-hydroxy-L-tryptophan and 390-fold after γ-L-glutamyl-5-hydroxy-L-tryptophan when compared with placebo infusion. Urinary 5-hydroxy-L-tryptophan excretion was three times higher after administration of γ-L-glutamyl-5-hydroxy-L-tryptophan than after 5-hydroxy-L-tryptophan infusion. Urinary 5-hydroxyindole-3-acetic acid excretion after 5-hydroxy-L-tryptophan infusion was significantly greater than that after γ-L-glutamyl-5-hydroxy-L-tryptophan administration. Urinary dopamine excretion was not affected by either compound when compared with placebo.

3. 5-Hydroxy-L-tryptophan significantly reduced urine flow rate and urinary sodium excretion. γ-L-Glutamyl-5-hydroxy-L-tryptophan was antinatriuretic but did not affect urine output. These changes occurred without significant alterations in effective renal plasma flow and glomerular filtration rate.

4. Both 5-hydroxy-L-tryptophan and γ-L-glutamyl-5-hydroxy-L-tryptophan significantly increased plasma aldosterone concentration without a concomitant rise in plasma renin activity. The increase after γ-L-glutamyl-5-hydroxy-L-tryptophan was smaller and delayed. 5-Hydroxy-L-tryptophan, but not γ-glutamyl-5-hydroxy-L-tryptophan, increased serum growth hormone concentration.

5. There was a significant increase in diastolic blood pressure after 5-hydroxy-L-tryptophan administration, but not after γ-L-glutamyl-5-hydroxy-L-tryptophan.

6. These results show that both prodrugs generate 5-hydroxytryptamine. The antinatriuresis after both compounds is presumably mediated by intrarenally generated 5-hydroxytryptamine and this appears to be predominantly a tubular effect. The urinary metabolite data and greater extrarenal effects produced by 5-hydroxy-L-tryptophan indicate that the glutamyl derivative is relatively more selective for the kidney than 5-hydroxy-L-tryptophan.

INTRODUCTION

Parenteral administration in man of 5-hydroxytryptamine (5-HT; serotonin) has been reported in most studies to cause reductions in renal blood flow, glomerular filtration rate (GFR), urine output and sodium excretion [1, 2]. 5-HT, however, has a wide spectrum of actions in the body and the renal responses to infusion of pharmacological doses of 5-HT may be largely modified by and/or possibly be due to the confounding influences produced by effects in other tissues arising from an increase in the circulating amine. In addition, peripherally administered 5-HT may be taken up avidly into platelets and/or efficiently metabolized in the pulmonary and hepatic circulations [3, 4]. It may, therefore, not reach the renal tissues in an adequate concentration to produce effects. Administration of 5-HT precursors which are selectively converted to 5-HT within the kidney may allow an assessment of the renal effects to be assessed separately from any systemic effects caused by the increase in circulating amine that follows the infusion of 5-HT itself. Aromatic L-amino acid decarboxylase (LAAD; EC 4.1.1.28), the enzyme responsible for conversion of 5-hydroxy-L-tryptophan (5-HTP) to 5-HT, is present in large amounts in renal proximal tubular cells [5]. Intrarenal synthesis of 5-HT has been demonstrated in rats given 5-HTP, and this was accompanied by a reduction in renal blood flow, antidiuresis and antinatriuresis [6–9]. Administration of 5-HTP is, however, associated with adverse gastrointestinal effects in man and this may limit the dose of 5-HTP that can be given [10, 11]. Furthermore, unlike 5-HT, it crosses the blood–brain barrier enhancing synthesis of 5-HT in the brain [12–14], and may therefore exert central effects.

Key words: aldosterone, growth hormone, γ-L-glutamyl-5-hydroxy-L-tryptophan, 5-hydroxytryptamine, 5-hydroxy-L-tryptophan, kidney, renal sodium excretion, renin.

Abbreviations: ANG I, angiotensin I; C_{In}, clearance of polyfructosan; C_{PAH}, clearance of *p*-aminohippurate; L-dopa, L-3,4-dihydroxyphenylalanine; DOPAC, 3:4-dihydroxyphenylacetic acid; ERPF, effective renal plasma flow; FE_{Na}, fractional excretion of sodium; FF, filtration fraction; GFR, glomerular filtration rate; glu-dopa, γ-L-glutamyl-3,4-dihydroxyphenyl alanine; Glu-5-HTP, γ-L-glutamyl-5-hydroxy-L-tryptophan; γ-GT, γ-glutamyltransferase; 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxy-L-tryptophan; LAAD, L-amino acid decarboxylase; PAH, *p*-aminohippurate; PRA, plasma renin activity.

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The kidney is highly active in the uptake and metabolism of γ-glutamyl derivatives of amino acids due to the high relative activity of γ-glutamyltransferase (γ-GT; EC 2.3.2.2) in the renal tissues [15, 16]. Extensive previous work in our department has shown that gludopa (γ-L-glutamyl-L-3,4-dihydroxyphenylalanine) is a renal dopamine prodrug which is sequentially converted in the kidney by γ-GT to L-dopa (L-3,4-dihydroxyphenylalanine) and decarboxylated by LAAD to dopamine [17, 18]. γ-L-glutamyl-5-hydroxyl-L-tryptophan (glu-5-HTP) is a glutamyl derivative of 5-HTP, which, in an analogous way, is converted intrarenally to 5-HTP and then decarboxylated to 5-HT [19]. Our hypothesis was that glu-5-HTP may be a relatively more renally selective 5-HT prodrug than 5-HTP, and this may enable the renal actions of 5-HT to be studied with less confounding extrarenal effects. We previously demonstrated in a small study that both 5-HTP and glu-5-HTP produce marked increases in urinary excretion of 5-HT and that glu-5-HTP is significantly antinatriuretic [19]. Our data also suggested that the glutamyl compound has greater renal selectivity than 5-HTP.

The present, more extensive, study, using a modified protocol, was designed to investigate further the effects of intravenous infusions of equimolar amounts of glu-5-HTP and 5-HTP in normal man. In this study, we measured several additional parameters. Effective renal plasma flow (ERPF), GFR and fractional excretion of sodium (FE_{Na}) were estimated in order to elucidate the antinatriuretic actions of these compounds. Urinary dopamine excretion was assessed, since LAAD, the enzyme converting 5-HTP to 5-HT, is also involved in the synthesis of dopamine from L-dopa [20]. In addition, we measured serum growth hormone concentration as a possible index of the central effect of these two compounds, since previous studies demonstrated that 5-HTP causes release of growth hormone [21–23].

METHODS

Subjects

Nine healthy male subjects, aged 21–30 years (mean 25.7 years) and weighing 63.4–96.9 kg (mean 71.2 kg), were studied. All had normal medical examination, ECG, complete blood count, serum urea, creatinine, electrolyte concentrations and liver function tests. They were on no medication for at least 2 weeks before the start of the study. They refrained from alcohol for 24 h, abstained from xanthine-containing drinks from 18.00 hours, and fasted from 22.00 hours the evening before each study day. Smoking was prohibited on the day of the study. Subjects were maintained on their normal diet, but were advised to consume the same diet in the 24 h before each study day. Written informed consent was obtained from all participants, and the study protocol was approved by the Medical and Clinical Oncology Ethics of Medical Research Subcommittee of Lothian Health Board.

Study protocol

Each subject was studied on three separate occasions (5–14 days between study days) and received infusions of placebo, 5-HTP and glu-5-HTP in a single-blind, randomized order. They arrived at the clinical investigation unit at about 08.00 hours on each study day, having drunk 500 ml of tap water 1 h previously. An indwelling intravenous plastic cannula was inserted in each antecubital fossa for blood sampling and administration of infusions. Priming doses of *p*-aminohippurate-sodium (PAH; 0.5 g) and polyfructosan (Inutest; 3.5 g) added to saline (150 mmol/l NaCl) to make up a total volume of 40 ml were infused over 8 min using a Perfusor VI infusion pump (B. Braun, Melsungen AG, Germany). This was followed by a maintenance infusion of PAH (3.75 g/l) and polyfructosan (4.5 g/l) in saline at a constant rate of 5 ml/min using a volumetric infusion pump (IMED 960; IMED Ltd, Abingdon, Oxon, U.K.) throughout the experiment to allow estimation of ERPF and GFR. After a 3 h run-in period, an intravenous infusion of an equimolar amount (45 nmol min^{-1} kg^{-1}) of 5-HTP (10 μg min^{-1} kg^{-1}) or glu-5-HTP (16.6 μg min^{-1} kg^{-1}) in saline made up to a total volume of 30 ml was administered at 0.5 ml/min for 1 h. Placebo was 30 ml of saline alone. In addition to the saline administered intravenously, the subjects drank 150 ml of water half-hourly. These measures were used to promote an adequate natriuresis and diuresis. The subjects remained semi-recumbent throughout the experiment except when standing up to micturate.

Accurately timed consecutive urine collections of about 30 min duration were made during the last hour of the run-in phase (periods 1 and 2), during the administration of the test medications (periods 3 and 4) and for 2 h after the end of their infusions (periods 5–8). Urine volumes were recorded and aliquots were removed and stored at −40°C for determination of sodium, potassium, PAH, polyfructosan, 5-HTP, 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC). Urine samples for measurement of 5-HT, 5-HIAA, dopamine and DOPAC were acidified (5 mol/l HCl) to prevent their oxidation. Venous blood samples were collected into lithium heparin tubes for measurement of plasma electrolytes, PAH and polyfructosan, and into plain tubes for serum growth hormone. Blood for determination of plasma renin activity (PRA) and aldosterone was immediately transferred into chilled glass tubes containing EDTA (sodium salt) and kept on ice. Plasma and serum were separated

after centrifugation at 4°C and were stored at −40°C until analysis.

Objective recordings of blood pressure and pulse rate were made in duplicate using a Dinamap semi-automated recorder (Critikon, Inc., Tampa, FL, U.S.A.) every half hour during the experiment except during and for 1 h after the infusion of the test medications, when they were measured every 15 min. The mean values for each half-hour period were calculated and used in subsequent statistical analysis.

Glu-5-HTP was supplied by Aalto Bio Reagents Ltd, Dublin, Republic of Ireland, and the preparation contained 95% pure peptide. 5-HTP was obtained from Sigma Chemical Co. Ltd, Poole, Dorset, U.K. Both were prepared for use in 0.9% NaCl by the Edinburgh Royal Infirmary Pharmacy and were stored in vials at −40°C.

Analytical methods

Plasma aldosterone concentration was measured by radioimmunoassay with a commercially available kit ('Coat-a-count', Diagnostic Products Ltd, Caernarfon, Gwynedd, U.K.). Intra- and inter-assay coefficients of variation were 5.0% and 7.0%, repectively. PRA was measured by radioimmunoassay of angiotensin I (ANG I) generated under standard conditions [24]. The intra- and inter-assay coefficients of variation were 5.2% and 8.6%, respectively. Growth hormone was measured using an immunoradiometric kit (ELSA-HGH; CIS UK Ltd, High Wycombe, Bucks, U.K.) with intra- and inter-assay coefficients of variation of 2.8% and 4.0%, respectively. Sodium and potassium were measured by an ion-selective electrode analyser (Radiometer KNA1). PAH was measured in protein-free supernate, after precipitation with perchloric acid, by h.p.l.c. and spectrofluorimetric detection. Intra- and inter-assay coefficients of variation were 2.8% and 4.0%, respectively, for plasma, and 3.8% and 7.7%, respectively, for urinary PAH. Deproteinized plasma and urine polyfructosan were measured by spectrophotometry at 480 nm after generation of chromogen by resorcinol in acid. Intra- and inter-assay coefficients of variation were 3.4% and 7.2%, respectively, for plasma polyfructosan, and 3.8% and 7.7%, respectively, for urinary polyfructosan.

Urinary 5-HT was measured using a modification of the method of Jouve et al. [25], 5-HIAA by the method of Goldenberg [26] and 5-HTP as described previously [19]. Dopamine and DOPAC were extracted from urine at pH 8.5 on to alumina previously washed with EDTA [27] and eluted into 0.2 mol/l perchloric acid. They were measured using h.p.l.c. and electrochemical detection using *N*-methyldopamine as internal standard. Intra- and inter-assay coefficients of variation for dopamine were 1.0% and 2.2%, respectively, and those for DOPAC were 2.5% and 2.5%, respectively.

Calculations and statistical evaluation

Renal clearance was calculated using the standard formula UV/P, where U is the urinary concentration, V is the urine flow rate and P is the mean of the plasma levels at the beginning and end of each clearance period. ERPF and GFR were estimated by measuring the renal clearances of PAH (C_{PAH}) and polyfructosan (C_{In}), respectively. All values for ERPF and GFR were corrected to a body surface area of 1.73 m^2. The filtration fraction (FF) was calculated as $(C_{In}/C_{PAH})\times 100\%$. FE_{Na} was calculated as renal clearance of sodium divided by GFR (C_{In}) and expressed as a percentage.

Results are given as means ± SD, except for the Figures where means ± SEM are shown. The data between the three experimental days were analysed by repeated measures analysis of variance for overall statistical significance. When inequality of variances between data sets was detected, the *F*-ratio was corrected using the Greenhouse–Geiser epsilon to give a more conservative significance level. Two-way analysis of variance was employed to identify any differences between treatments at an individual time point. Differences in the cumulative urinary metabolite data after 5-HTP and glu-5-HTP were analysed by Student's *t*-test for paired observations. The growth hormone values were log-transformed before analysis because of the marked skewing of the data. Values of $P<0.05$ were considered statistically significant. SPSS/PC+4.0 statistical software package (SPSS Inc, Chicago, IL, U.S.A.) was employed for all statistical analyses.

RESULTS

Fig. 1 shows the urinary excretion rates of 5-HTP, 5-HT and 5-HIAA for each 30 min period. 5-HTP, which was undetectable in the urine during infusion of saline alone, increased markedly after administration of both 5-HTP and glu-HTP. Excretion of 5-HTP after infusion of glu-5-HTP was higher than that after 5-HTP for all collection periods. Cumulative 5-HTP excretion values over the 3 h after the start of the infusion were 21.0 ± 4.8 μmol after 5-HTP and 66.9 ± 14.1 μmol after glu-5-HTP ($P<0.001$). The mean urinary excretion rates of 5-HT before infusion of placebo, 5-HTP and glu-5-HTP were similar at 0.5 nmol/min. 5-HT excretion rose to a peak value of 349 ± 117 nmol/min during administration of 5-HTP and 301 ± 44 nmol/min after glu-5-HTP. There was no significant change in 5-HT excretion after the infusion of placebo. The 3 h cumulative urinary 5-HT excretion rose 367-fold after 5-HTP (31.4 ± 8.7 μmol) and 390-fold after glu-5-HTP (33.4 ± 9.4 μmol) when compared with placebo infusion (85.6 ± 26.2 nmol). There was no significant difference between the cumulative 5-HT excretion after 5-HTP and glu-5-HTP. 5-HIAA was not detectable in the urine before infusion of the test medica-

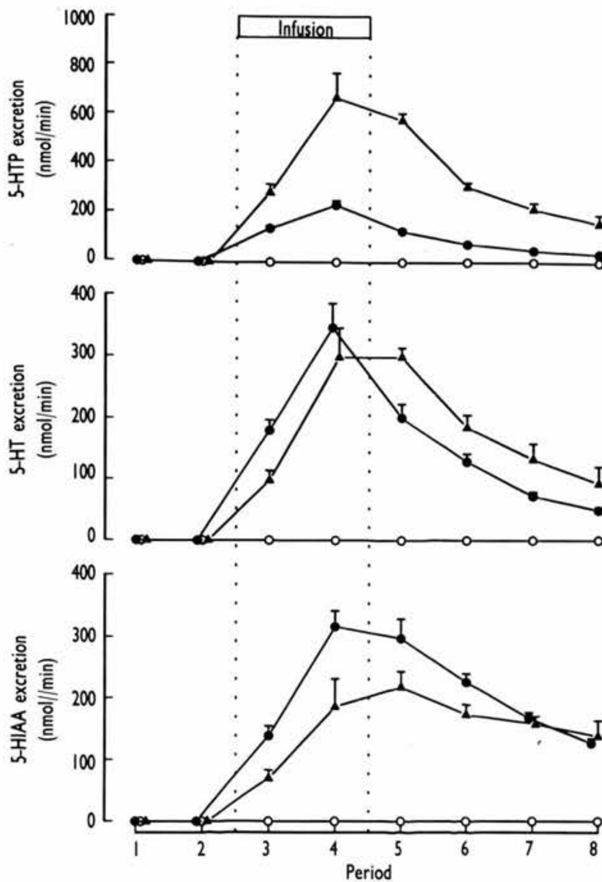

Fig. 1. Urinary 5-HTP, 5-HT and 5-HIAA excretion rates before (periods 1 and 2), during (periods 3 and 4) and after (periods 5–8) infusion of placebo (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SEM ($n=9$). Urinary 5-HTP was below the detection limit of the assay on the placebo day and before infusion of 5-HTP and glu-5-HTP.

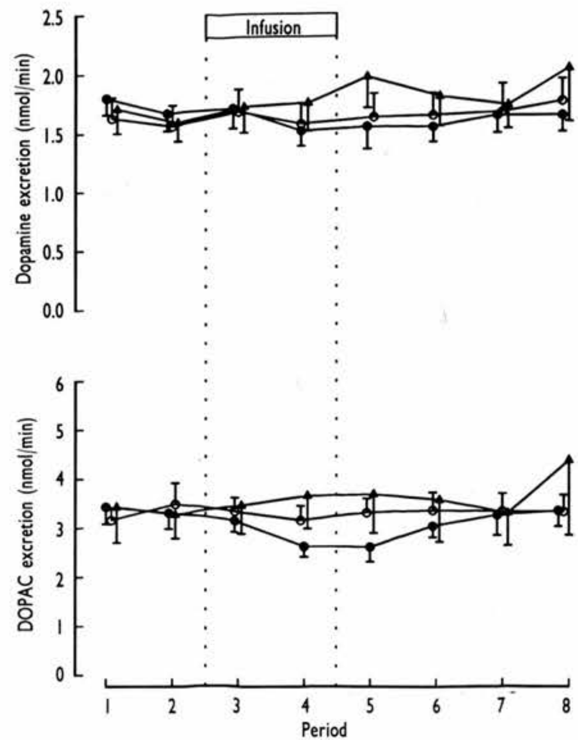

Fig. 2. Urinary dopamine and DOPAC excretion rates before (periods 1 and 2), during (periods 3 and 4) and after (periods 5–8) infusion of placebo (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SEM ($n=9$).

tions. Marked increases in urinary 5-HIAA occurred after administration of 5-HTP and glu-5-HTP. The cumulative 5-HIAA excretion values were higher after administration of 5-HTP (38.8 ± 6.6 μmol) than after glu-5-HTP (28.0 ± 5.8 μmol; $P<0.01$). At the end of the 3 h study period, 47 ± 5% of infused 5-HTP and 66 ± 7% of glu-5-HTP were recovered in the urine as the sum of 5-HTP, 5-HT and 5-HIAA. The urinary excretion of 5-HTP, 5-HT and 5-HIAA was 11 ± 1%, 16 ± 3% and 20 ± 5%, respectively, of the infused dose of 5-HTP. The corresponding values were 35 ± 5%, 17 ± 3% and 15 ± 3% after glu-5-HTP administration. Urinary dopamine and DOPAC excretion rates were unaffected by 5-HTP or glu-5-HTP when compared with placebo (Fig. 2).

Mean urinary sodium excretion rates during the baseline period just before placebo, 5-HTP or glu-5-HTP infusion were not significantly different at 263 ± 130, 323 ± 100 and 272 ± 134 μmol/min, respectively (Fig. 3). The absolute excretion of sodium and FE_{Na} increased progressively on the placebo day throughout all clearance periods in response to infusion of saline. Both 5-HTP ($P<0.01$) and glu-5-HTP ($P<0.005$) significantly attenuated the increase in sodium excretion when compared with placebo. FE_{Na} was likewise reduced by 5-HTP ($P<0.025$) and glu-5-HTP ($P<0.005$) (Table 1). The sodium excretion after 5-HTP infusion was not significantly different from that after glu-5-HTP. The 3 h cumulative sodium excretion values after placebo, 5-HTP and glu-5-HTP administration were 65.3 ± 16.5, 51.1 ± 12.5 and 47.0 ± 13.3 mmol, respectively. There was a significant reduction in urine output after 5-HTP administration ($P<0.01$) but not after glu-5-HTP. There were no significant changes in potassium excretion after 5-HTP or glu-5-HTP when compared with placebo (Fig. 3). Both compounds had no significant effects on ERPF, GFR or FF (Fig. 4), although the mean GFR was lower during and immediately after 5-HTP infusion.

Serial PRA and plasma aldosterone concentration are shown in Fig. 5. Both 5-HTP ($P<0.001$) and glu-5-HTP ($P<0.05$) significantly increased plasma aldosterone concentration when compared with placebo. The increase after glu-5-HTP was, however, smaller ($P<0.005$) and delayed when compared with 5-HTP. PRA was low at baseline and did not increase during infusion of either compound. Compared with placebo, there was a significant increase in serum growth hormone concentrations half an hour after the end of 5-HTP infusion ($P<0.05$) (Fig. 5). There was a large inter-subject variability in the

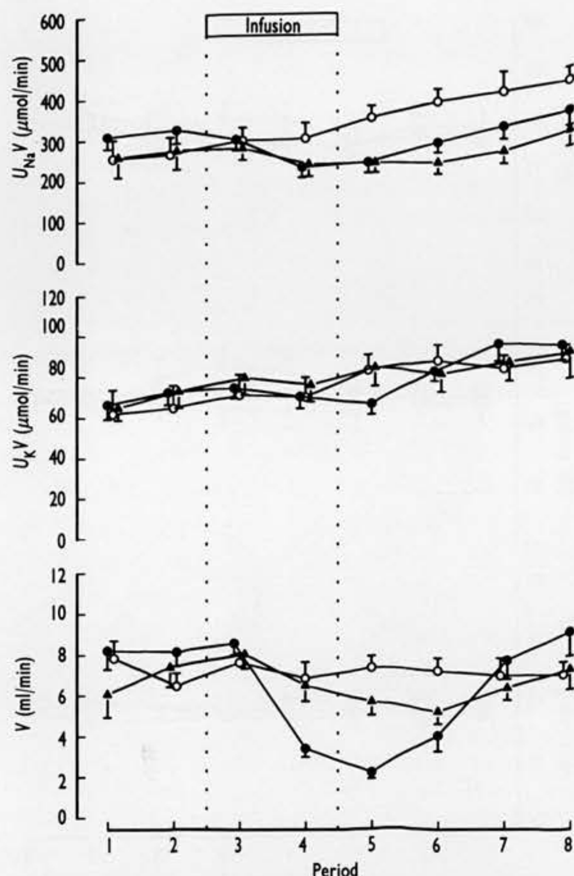

Fig. 3. Urine flow (V), sodium output ($U_{Na}V$) and potassium output (U_KV) before (periods 1 and 2), during (periods 3 and 4) and after (periods 5–8) infusion of placebo (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SEM ($n = 9$).

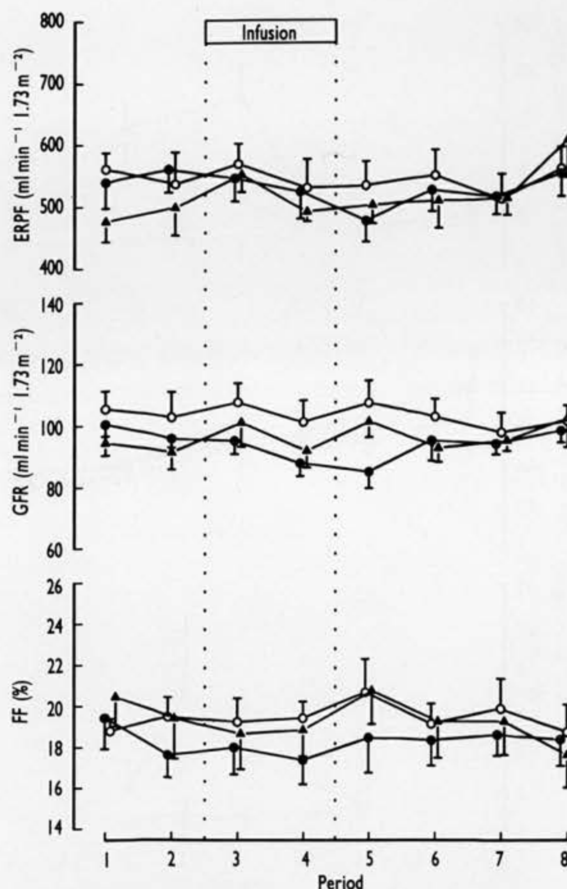

Fig. 4. ERPF (C_{PAH}), GFR (C_{In}) and FF (C_{In}/C_{PAH}) before (periods 1 and 2), during (periods 3) and 4) and after (periods 5–8) infusion of placebo (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SEM ($n = 9$).

Table 1. FE_{Na} before (periods 1 and 2), during (periods 3 and 4) and after (periods 5–8) infusion of placebo, 5-HTP and glu-5-HTP. Values shown are means ± SD ($n = 9$).

	FE_{Na} (%)		
Period	Placebo	5-HTP	Glu-5-HTP
1	1.6 ± 0.7	2.1 ± 0.6	1.9 ± 1.2
2	1.8 ± 0.7	2.3 ± 0.7	2.0 ± 0.7
3	1.9 ± 0.5	2.2 ± 0.6	1.9 ± 0.5
4	2.0 ± 0.6	1.8 ± 0.6	1.7 ± 0.4
5	2.3 ± 0.8	2.0 ± 0.6	1.6 ± 0.4
6	2.6 ± 0.6	2.1 ± 0.6	1.8 ± 0.5
7	2.9 ± 0.8	2.5 ± 1.0	1.9 ± 0.6
8	3.0 ± 0.6	2.6 ± 1.0	2.1 ± 0.6

growth hormone response to 5-HTP. Although there was a significant response for the group *in toto*, a rise in serum growth hormone concentration of >5 ng/ml was present in only four subjects, all of whom experienced nausea. Infusion of glu-5-HTP caused no significant changes in serum growth hormone levels.

5-HTP, but not glu-5-HTP, significantly increased diastolic blood pressure when compared with placebo ($P < 0.05$) (Fig. 6). There were no significant differences between treatments in systolic blood pressure ($0.05 < P < 0.1$) or pulse rate. Six out of the nine subjects complained of nausea at the end of 5-HTP infusion, but only one experienced this effect with glu-5-HTP. No other adverse effect was noted.

DISCUSSION

Urinary excretion of 5-HT increased markedly during and after infusion of its precursors 5-HTP and glu-5-HTP. This suggests that biosynthesis of 5-HT can occur in the human kidney since extrarenal production of 5-HT cannot account completely for the high urinary levels of 5-HT observed in this study. 5-HT produced extrarenally and presented to the blood stream would be rapidly deaminated by monoamine oxidase in the pulmonary and hepatic circulations or sequestered into platelets, leaving little available for filtration at the renal glomerulus [3, 4, 28]. The theory that circulating 5-HT would be rapidly destroyed is supported by previous observations that intravenous administration of 5-HT resulted in only a small increase in blood 5-HT concentration and had virtually no effect on the level of free 5-HT in urine [10, 29]. Moreover, in the carcinoid syndrome, where there is an increase

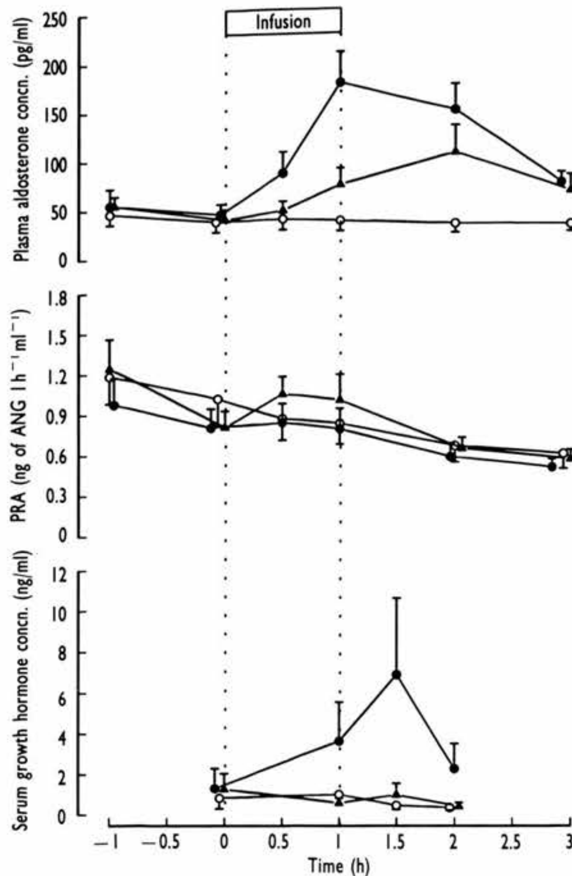

Fig. 5. Plasma aldosterone concentration, PRA and serum growth hormone concentration before, during and after infusion of placebo (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SEM ($n = 9$).

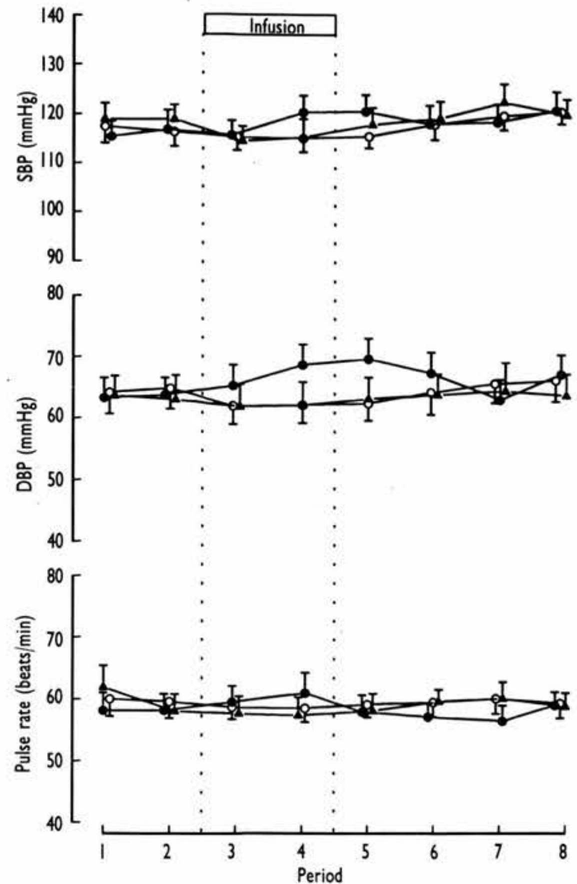

Fig. 6. Systolic (SBP) and diastolic (DBP) blood pressure and pulse rate in nine healthy subjects receiving infusions of placebo (○), 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± SEM.

in circulating 5-HT, urinary 5-HT excretion is usually less than 1% of 5-HIAA excretion [30]. Measurements of 5-HTP and 5-HT in the renal artery and vein, in addition to urinary measurements, will be required to further support the conclusion that 5-HT is generated intrarenally from its precursors. It is difficult to perform these investigations in healthy subjects because of ethical considerations. However, such evidence supporting intrarenal formation of 5-HT from 5-HTP is available from experiments with isolated perfused rat kidneys [6]. Since γ-GT and LAAD are concentrated mainly in the renal tubules [5, 28, 31], our results would seem to indicate renal tubular formation of 5-HT with subsequent urinary excretion. LAAD also converts L-dopa to dopamine [20]. In addition, both 5-HT and dopamine are metabolized by monoamine oxidase to 5-HIAA and DOPAC, respectively [2, 32]. It is therefore possible that administration of 5-HTP may alter the renal formation and/or excretion of dopamine or DOPAC. Indeed, because 5-HT and dopamine could have opposite effects on renal haemodynamics and function, it has been postulated that these biogenic amines may be generated within the kidney under physiological conditions as reciprocal regulators of renal haemodynamics and of salt and water excretion [8, 28]. We did not observe any effect of 5-HTP or glu-5-HTP on urinary excretion of dopamine or its major metabolite DOPAC in our study. The reason for this is unknown, but it would be of interest to infuse equimolar amounts of gludopa and glu-5-HTP to investigate which prodrug is the preferred substrate for the renal enzymes and what is the overall pharmacological effect.

Cumulative urinary excretion of 5-HTP was three times higher after administration of glu-5-HTP than after 5-HTP. This indicates that the amount of 5-HTP delivered to the kidney was greater after infusion of glu-5-HTP and is in keeping with it being relatively more selective for the kidney than 5-HTP. Cumulative urinary 5-HT excretion was not significantly different between 5-HTP and glu-5-HTP infusions. On a molar basis, 16% of the administered dose of 5-HTP and 17% of glu-5-HTP were recovered in the urine as 5-HT. The urinary recoveries, measured as the sum of 5-HTP, 5-HT and 5-HIAA excreted during the 3 h observation

period, were 47% and 66% of the infused dose after 5-HTP and glu-5-HTP, respectively. The higher recovery with glu-5-HTP again suggested greater delivery of the glutamyl compound to the kidney.

Both 5-HTP and glu-5-HTP significantly reduced urinary sodium excretion in the present study. In our previous study, a statistically significant antinatriuretic effect was observed after administration of glu-5-HTP, but not after 5-HTP [19]. The mean urinary excretion of sodium after 5-HTP was, however, lower than that after placebo. A Type 2 error probably explains the failure to demonstrate a significant antinatriuresis after 5-HTP because only five subjects were studied. The reduction in urinary excretion of sodium in the present study occurred without significant alteration in ERPF, GFR or FF, suggesting that the antinatriuresis is mediated by a direct tubular action of 5-HT formed within renal tubular cells by LAAD. The proposition that the retention of sodium results from generation of 5-HT is supported by our observation that carbidopa, a peripheral LAAD inhibitor, markedly reduces the excretion of 5-HT in urine and abolishes the antinatriuretic effect of glu-5-HTP (T. C. Li Kam Wa et al. unpublished work). There was a suggestion of a slight reduction in GFR after 5-HTP in our study, but this effect was inconsistent and not statistically significant. It is possible that an infusion of 5-HTP longer than 1 h could produce a significant effect on GFR. However, the absence of an effect on ERPF and GFR may not be surprising given the tubular site of production of 5-HT. The lack of effect by 5-HTP on renal haemodynamics in this study is in contrast with studies which demonstrated decreases in ERPF and GFR in rats treated with 5-HTP [7–9]. It was suggested that glomerular LAAD activity, although of small degree, formed 5-HT within the glomerulus or that an intrarenal transport system of 5-HT from a tubular site of formation to the blood vessels or glomerulus could explain the observed haemodynamic effects [8].

A significant reduction in urine flow rate was observed after 5-HTP but not after glu-5-HTP. This may be due to release of vasopressin. There is evidence from animal studies that 5-HT acts within the central nervous system to increase plasma vasopressin concentration [33–35]. The antidiuresis induced by 5-HTP may therefore be due to release of vasopressin subsequent to an increase in brain 5-HT, whereas glu-5-HTP did not produce this effect because its conversion to 5-HT occurs mainly in the kidney. An alternative explanation is that the vasopressin release is due to the nausea produced by 5-HTP, since nausea is a potent stimulus to secretion of vasopressin in man [36–38].

There was an early and significant increase in plasma aldosterone concentrations without a concomitant increase in PRA after administration of 5-HTP. This is in agreement with observations by other investigators that the release of aldosterone by 5-HTP does not depend on the activation of the renin–angiotensin system in man [39, 40]. The release of aldosterone may be due to a direct effect on the adrenal cortex [41, 42] and/or be mediated by central 5-HT pathways [39, 40]. Glu-5-HTP caused a smaller and delayed rise in plasma aldosterone level, consistent with its effects being largely confined to the kidney. The delayed rise in plasma aldosterone concentration may be due to the renal formation of 5-HTP from glu-5-HTP and its subsequent recirculation. It is also possible that there may be some γ-GT and LAAD activity in the adrenal gland [43] and 5-HT produced locally could stimulate aldosterone release. The time course of the changes in sodium excretion [44] and the lack of effect on potassium excretion suggest that the antinatriuretic response to 5-HTP or glu-5-HTP infusion did not depend on the known action of aldosterone on the distal convoluted tubule of the kidney.

Serum growth hormone concentrations were significantly increased after infusion of 5-HTP. This is presumably mediated by the conversion of 5-HTP to 5-HT in the central nervous system and is in agreement with studies by other workers which demonstrated a rise in growth hormone after administration of 5-HTP [21–23, 45, 46] or L-tryptophan, the initial precursor of 5-HT [47, 48]. However, variable results were obtained by other investigators [47, 49, 50] and the role of 5-HT in the regulation of growth hormone release remains unclear. It is possible that nausea may be a factor in the release of growth hormone secretion in our study, since all four subjects who showed a rise of more than 5 ng/ml experienced this side effect. Emesis may increase plasma concentrations of growth hormone [36]. On the other hand, an increase in growth hormone has been reported in studies where only a minority of the subjects felt nauseated [21–23, 45–48]. Glu-5-HTP had no effect on growth hormone release in keeping with its conversion to 5-HT being largely limited to the kidney.

Six of the nine subjects experienced nausea at the end of 5-HTP infusion. This is a well-recognized adverse effect associated with 5-HTP administration [10, 11]. Glu-5-HTP, in equimolar amount, caused this reaction in only one subject. In addition, glu-5-HTP did not affect blood pressure, whereas 5-HTP caused a significant increase in diastolic blood pressure after 5-HTP. The low incidence of adverse effects and the lack of effect on blood pressure provide further evidence that glu-5-HTP is more renally selective and has less systemic toxicity.

In conclusion, the results of this study indicate that 5-HT can be generated intrarenally from both putative 5-HT prodrugs in man. When 5-HT is generated, it produces an effect on tubular sodium reabsorption, independent of changes in ERPF or GFR, resulting in sodium retention. The urinary metabolite data and reduced extrarenal effects, as evidenced by changes in growth hormone and aldosterone release, blood pressure and adverse

effects, supports the hypothesis that glu-5-HTP is relatively more selective for the kidney than 5-HTP. This relative selectivity is probably due to the avid uptake and metabolism of glutamyl compounds by the kidney [15, 16], but further work will be required to elucidate the mechanism. Whatever the mechanism involved, the use of glu-5-HTP should allow the effects of increased renal synthesis of 5-HT to be studied with less confounding extrarenal effects.

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The antinatriuretic action of γ-L-glutamyl-5-hydroxy-L-tryptophan is dependent on its decarboxylation to 5-hydroxytryptamine in normal man

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1 The effects of inhibition of peripheral aromatic L-amino acid decarboxylase during infusion of the relatively renally selective 5-hydroxytryptamine (5-HT) prodrug, γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP), were examined in eight healthy male subjects in a randomised, placebo-controlled, cross-over study.

2 Each subject received oral carbidopa (100 mg) or placebo followed, 1 h later, by a 60 min intravenous infusion of glu-5-HTP (16.6 $\mu g\ kg^{-1}\ min^{-1}$) or placebo.

3 After administration of glu-5-HTP, cumulative urinary excretion of 5-HT was 430-fold greater than that after placebo, and was associated with a period of sodium retention.

4 Pretreatment with carbidopa substantially attenuated the increase in 5-HT excretion after glu-5-HTP and abolished its antinatriuretic effect.

5 These results are in keeping with the proposition that the antinatriuretic action of glu-5-HTP is dependent on its decarboxylation to 5-HT.

Keywords 5-hydroxytryptamine γ-L-glutamyl-5-hydroxy-L-tryptophan carbidopa kidney sodium excretion aldosterone

Introduction

The first step in the biosynthesis of 5-hydroxytryptamine (5-HT; serotonin) involves the hydroxylation of the essential amino acid L-tryptophan to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan-5-hydroxylase. 5-HTP is decarboxylated by aromatic L-amino acid decarboxylase (LAAD) to 5-HT. The latter is degraded primarily by monoamine oxidase to produce 5-hydroxyindoleacetic acid (5-HIAA) which is the major catabolic and excretory product of 5-HT metabolism. All these enzymes are present in renal tissue, suggesting that the kidney might have the capacity to synthesise and degrade 5-HT locally [1, 2]. The enzyme γ-glutamyl transferase (γGT) is also present in high concentrations and the kidney is highly active in the uptake and metabolism of γ-glutamyl derivatives of amino acids [3, 4]. We previously demonstrated marked increases in urinary 5-HT excretion after infusion of the 5-HT prodrug, γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP), in keeping with intrarenal synthesis of 5-HT following the conversion of glu-5-HTP to 5-HTP by γGT and its subsequent decarboxylation by renal LAAD to 5-HT [5, 6]. Glu-5-HTP was relatively more selective for the kidney than 5-HTP. It reduced urinary sodium excretion without significant alterations in renal haemodynamics and this was due, presumably, to intrarenally generated 5-HT.

The present study was designed to investigate whether carbidopa, a peripheral inhibitor of LAAD [7], blocks the formation of 5-HT from glu-5-HTP and interferes with the actions of glu-5-HTP in normal volunteers.

Methods

Eight male volunteers, age range 18–39 years (mean 28 years) and weighing 59.9–80.9 kg (mean 69.0 kg),

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gave informed written consent to take part in this randomised, single-blind, placebo-controlled, within-subject cross-over study which was approved by the Healthy Volunteer Studies Ethics of Medical Research Sub-Committee, Lothian Health Board. They were all healthy as judged by medical history and physical examination, ECG, urinalysis, full blood count and biochemical blood analyses. They abstained from medications from at least 2 weeks before the start of the study and until its completion.

Each subject attended on four separate occasions, at least 1 week apart. They refrained from alcohol for 24 h, abstained from caffeine-containing beverages from 18.00 h, and fasted from 22.00 h the evening before each of the study days. They arrived at the clinical investigation unit at about 08.00 h on each study day, having drunk 500 ml tap water 1 h previously. A cannula was inserted into a vein in each antecubital fossa for blood sampling and administration of infusions. The subjects received an intravenous loading dose of 0.5 g *p*-aminohippurate sodium (PAH; Merck Sharp & Dohme, PA, USA) and 3.5 g polyfructosan (Inutest; Laevosan-Gesellschaft, Linz, Austria) at the start of the study (time 0 h) followed by a maintenance infusion of PAH (3.75 g l^{-1}) and polyfructosan (4.5 g l^{-1}) in 0.9% sodium chloride (saline; 150 mmol l^{-1}) at a rate of 5 ml min^{-1} for the next 6 h. They emptied their bladders at 1.5 h and accurately timed consecutive urine collections of about 30 min duration were made thereafter until the end of the study. Two hours after the start of the study, the subjects took either 100 mg carbidopa (Merck Sharp & Dohme Ltd, Hoddesdon, UK) or placebo orally. This was followed 1 h later by a 60 min infusion of glu-5-HTP (Aalto Bio Reagents Ltd, Dublin, Eire), made up to 30 ml with 0.9% saline, at a rate of 16.6 μg kg^{-1} min^{-1} or placebo (saline alone at 0.5 ml min^{-1}). Each subject therefore received the following four combinations in a randomised sequence: placebo + placebo; placebo + glu-5-HTP; carbidopa + placebo; and carbidopa + glu-5-HTP. The subjects remained supine throughout the experiment except when standing to pass urine and drank 150 ml water every 30 min to promote an adequate diuresis. Blood pressure and pulse rate were measured in duplicate by a semi-automated recorder (Dinamap; Critikon Inc., Tampa, FL, USA) every 0.5 h during the study.

Venous blood samples were collected into lithium heparin tubes at 30 min intervals for measurement of plasma PAH and polyfructosan. Blood for determination of plasma renin activity (PRA) and aldosterone were collected at 0.5, 2, 3, 3.5, 4, 5 and 6 h into precooled glass tubes containing sodium ethylenediamine tetra-acetate and kept on ice. Plasma was separated after centrifugation at 4° C and stored at −40° C until analysis. The volume of each urine collection was measured and aliquots removed and stored at −40° C for analysis of sodium, PAH, polyfructosan, 5-HTP, 5-HT, 5-HIAA and dopamine. Urine samples for 5-HT, 5-HIAA and dopamine were acidified (pH < 3.0) with 5M hydrochloric acid to prevent their oxidation.

Blood and urine analyses

Sodium was measured by an ion-selective electrode analyser (Radiometer KNA1). Plasma aldosterone concentrations were measured by radioimmunoassay with a commercially available kit ('Coat-a-count', Diagnostic Products Ltd, Caernarfon, Gwynedd, UK) with intra- and inter-assay coefficients of variation of 5% and 7% respectively. PRA was measured by radioimmunoassay of angiotensin I generated under standard conditions [8]. The intra- and inter-assay coefficients of variation were 5% and 9% respectively. PAH, 5-HTP, 5-HT and dopamine were measured by h.p.l.c. and polyfructosan and 5-HIAA by spectrophotometry as described previously [5, 6]. The values of the lower limit of detection of the assays for 5-HTP, 5-HT, 5-HIAA and dopamine were 45 nmol l^{-1}, 45 nmol l^{-1}, 5.2 μmol l^{-1}, and 26 nmol l^{-1} respectively.

Data analysis

Effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were estimated from the renal clearances of PAH (C_{PAH}) and polyfructosan (C_{In}) respectively using the standard formula UV/P, where U is the urine concentration, V is the urine flow rate and P is the mean of the plasma concentrations at the beginning and end of each clearance period.

Results are expressed as means ± s.d. except for the figures where the mean ± s.e. mean values are shown. Statistical comparisons of the parameters measured serially on the four experimental days were made by repeated measures analysis of variance for overall statistical significance with the pretreatment values included as covariates. Two-way ANOVA was employed to identify any differences between the 3–6 h cumulative data on the 4 days. When significant differences were found, relevent pairs of data were compared by Student's *t*-test for paired observations with Bonferroni correction for multiple comparisons. A value of zero was assumed when the measured variable was below the limit of detection of the assay technique used to allow statistical comparisons to be made. Differences were considered to be statistically significant when the P value was less than 0.05. SPSS/PC + 4.0 statistical software package (SPSS Inc, Chicago, Illinois, USA) was employed for all statistical analyses.

Results

The urinary excretion rates of 5-HTP, 5-HT, 5-HIAA and dopamine for each 30 min period on the 4 study days are shown in Table 1. The urinary excretion of 5-HT was about 0.4 nmol min^{-1} at baseline. This rose to a peak value of 278 ± 44 nmol min^{-1} after glu-5-HTP infusion but did not change significantly after placebo infusion. The cumulative urinary 5-HT excretion over the 3 h period after the start of glu-5-HTP infusion was 430-fold higher than that after placebo

Table 1 Mean (s.d.) urinary 5-HTP, 5-HT, 5-HIAA and dopamine excretion rates for each 0.5 h period on the 4 study days (n = 8). Carbidopa or placebo was given at 2 h and time 3–4 h represents the infusion (glu-5-HTP or placebo) period. * = dopamine was not detectable in seven subjects

	Time (h)								
	1.5–2.0	*2.0–2.5*	*2.5–3.0*	*3.0–3.5*	*3.5–4.0*	*4.0–4.5*	*4.5–5.0*	*5.0–5.5*	*5.5–6.0*
5-HTP excretion (nmol min^{-1})									
Placebo +	0	0	0	0	0	0	0	0	0
placebo									
Placebo +	0	0	0	232.6	436.5	368.9	246.9	149.8	84.3
glu-5-HTP				(69.0)	(132.3)	(134.9)	(55.8)	(48.0)	(23.6)
Carbidopa +	0	0	0	0	0	0	0	0	0
placebo									
Carbidopa +	0	0	0	264.6	682.5	593.3	410.1	260.9	209.9
glu-5-HTP				(71.1)	(133.5)	(173.7)	(79.6)	(85.4)	(42.8)
5-HT excretion (nmol min^{-1})									
Placebo +	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.5
placebo	(0.2)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.0)	(0.2)	(0.1)
Placebo +	0.5	0.4	0.4	113.1	274.3	278.4	198.8	126.1	88.4
glu-5-HTP	(0.1)	(0.1)	(0.0)	(42.6)	(51.5)	(43.9)	(41.7)	(21.9)	(11.2)
Carbidopa +	0.5	0.4	0.1	0	0	0	0	0	0
placebo	(0.2)	(0.1)	(0.1)						
Carbidopa +	0.4	0.3	0.2	0.6	2.0	2.6	2.2	1.7	1.5
glu-5-HTP	(0.2)	(0.2)	(0.1)	(0.4)	(1.1)	(1.6)	(1.0)	(0.7)	(0.9)
5-HIAA excretion (nmol min^{-1})									
Placebo +	0	0	0	0	0	0	0	0	0
placebo									
Placebo +	0	0	0	73.7	199.2	222.8	213.2	171.4	144.9
glu-5-HTP				(27.8)	(100.9)	(46.7)	(46.2)	(32.7)	(27.2)
Carbidopa +	0	0	0	0	0	0	0	0	0
placebo									
Carbidopa +	0	0	0	32.1	29.1	49.2	60.4	49.4	43.5
glu-5-HTP				(35.3)	(15.2)	(21.1)	(35.7)	(21.3)	(16.9)
Dopamine excretion (nmol min^{-1})									
Placebo +	1.3	1.3	1.3	1.3	1.3	1.4	1.4	1.4	1.4
placebo	(0.2)	(0.3)	(0.2)	(0.2)	(0.3)	(0.3)	(0.3)	(0.3)	(0.2)
Placebo +	1.4	1.3	1.2	1.3	1.2	1.2	1.2	1.2	1.2
glu-5-HTP	(0.2)	(0.3)	(0.2)	(0.1)	(0.2)	(0.2)	(0.4)	(0.1)	(0.1)
Carbidopa +	1.6	1.6	0.7	0.2	0.1	0	0	0	0
placebo	(0.2)	(0.4)	(0.6)	(0.3)	(0.2)				
Carbidopa +	1.6	1.6	0.9	0.2	0.3	0.3*	0.2*	0.2*	0.2*
glu-5-HTP	(0.5)	(0.3)	(0.6)	(0.5)	(0.8)	(0.7)	(0.6)	(0.6)	(0.5)

infusion (32.4 ± 3.6 μmol *vs* 75.6 ± 12.7 nmol; P < 0.001). Similarly, 5-HTP and 5-HIAA which were undetectable in urine during placebo infusion, increased markedly after administration of glu-5-HTP. Carbidopa suppressed urinary 5-HT and dopamine excretion to undetectable levels in all subjects during placebo infusion. It markedly attenuated the increase in 5-HT excretion after glu-5-HTP. Compared with placebo pretreatment, carbidopa reduced the 3 h cumulative 5-HT excretion by 99% from 32.4 ± 3.6 μmol to 0.3 ± 0.1 μmol (P < 0.001). It increased the 3 h cumulative 5-HTP excretion by 60% from 45.6 ± 10.9 μmol to 72.8 ± 14.8 μmol (P < 0.001) and reduced 5-HIAA excretion by 74% from 30.8 ± 6.3 μmol to 7.9 ± 3.2 μmol (P < 0.001).

There was a steady increase in urinary sodium excretion from 278 ± 77 μmol min^{-1} (1.5–2.0 h period) to 379 ± 144 μmol min^{-1} (5.5–6.0 h period) during the placebo day in response to infusion of saline (Figure 1). Glu-5-HTP produced a significant attenuation of sodium excretion when compared with placebo infusion (P < 0.01). The 3 h cumulative sodium excretion values after placebo and glu-5-HTP administration were 63.3 ± 19.1 and 45.9 ± 9.2 mmol respectively (P < 0.05). Pretreatment with carbidopa abolished the antinatriuretic effect of glu-5-HTP. The 3 h cumulative sodium excretion when the subjects received carbidopa and glu-5-HTP infusion was 63.5 ± 15.6 mmol which is similar to that during placebo infusion only. Carbidopa had no significant effect on cumulative sodium excretion during placebo infusion (63.3 ± 5.2 mmol).

Glu-5-HTP significantly increased plasma aldosterone when compared with placebo infusion (P < 0.001) (Figure 2). Pretreatment with carbidopa attenuated the increase in plasma aldosterone concentrations produced by glu-5-HTP (P < 0.005). Carbidopa had no effect on the steady fall in plasma aldosterone concentration during placebo infusion. PRA declined progressively in response to saline loading during all experimental days (Figure 2).

GFR, ERPF, blood pressure and pulse rate were not

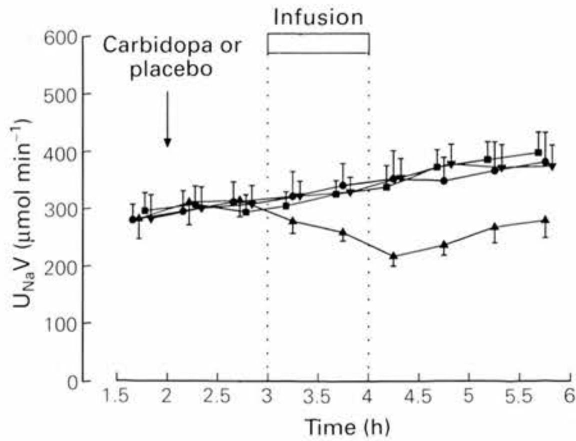

Figure 1 Sodium output ($U_{Na}V$) on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± s.e. mean (n = 8).

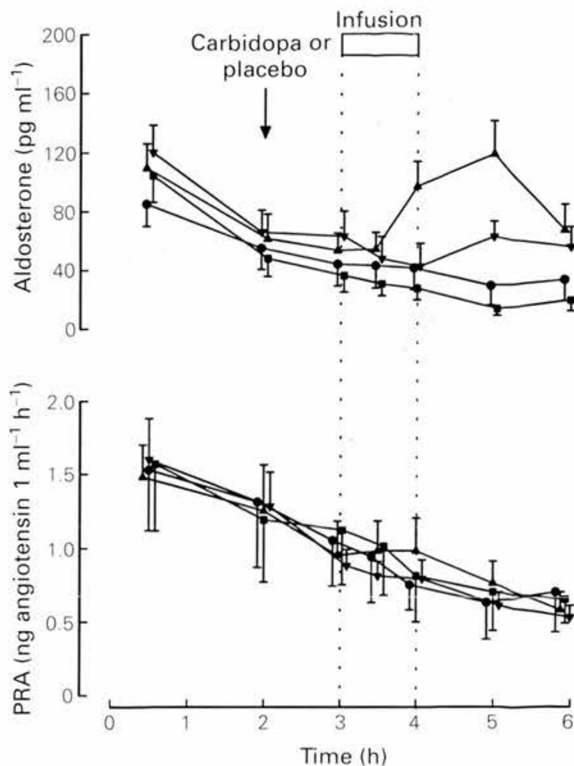

Figure 2 Plasma aldosterone concentrations and PRA on the 4 study days: placebo + placebo (●); placebo + glu-5-HTP (▲); carbidopa + placebo (■); and carbidopa + glu-5-HTP (▼). Values shown are means ± s.e. mean (n = 8).

significantly different between the 4 study days (data available from authors on request). No side effects were reported or recorded.

Discussion

This study confirms our previous observations that glu-5-HTP markedly increases urinary 5-HT excretion and causes retention of sodium without significant alterations in renal haemodynamics in normal man [5, 6]. The prodrug also increases aldosterone production as previously reported but the time course of the changes in sodium excretion and the lack of effect on urinary potassium excretion suggest that the antinatriuresis occurs independently of the known actions of aldosterone [6, 9]. Both the enzymes required for 5-HT synthesis from glu-5-HTP, γGT and LAAD, are highly concentrated in the proximal tubular cells of the kidney [3, 10] and the high urinary levels of 5-HT would indicate that 5-HT is probably formed in the renal tubules and then excreted. That 5-HT is produced intrarenally is further supported by our recently reported findings that the marked increases in urinary excretion of 5-HT occur without significant changes in circulating 5-HT [11].

Urinary dopamine results mainly from dopamine synthesis in proximal tubular cells by the renal decarboxylation of L-dopa [12]. Similarly, it has been suggested that urinary 5-HT reflects intrarenal synthesis of 5-HT [2]. In the present study, a single 100 mg dose of carbidopa, an extracerebral LAAD inhibitor, suppressed dopamine and 5-HT excretion to below the levels of detection of the assays indicating effective inhibition of renal LAAD. We did not observe a significant effect of carbidopa on urinary sodium excretion during saline infusion in agreement with most previous studies [13–15]. Carbidopa substantially reduced the increment in urinary 5-HT excretion that followed administration of glu-5-HTP. There was an increase in urinary 5-HTP excretion and a reduction in 5-HIAA excretion. These results are consistent with significant inhibition of renal LAAD. The lesser reduction in 5-HIAA excretion suggests that there are body compartments, for example the brain, where LAAD may still remain active and be capable of 5-HT synthesis, despite carbidopa administration. Carbidopa abolishes the antinatriuresis induced by glu-5-HTP coincident with the near suppression of 5-HT synthesis, in keeping with the hypothesis that the sodium retention results from intrarenal generation of 5-HT.

Glu-5-HTP increased aldosterone production, without a concomitant increase in PRA, indicating that the release of aldosterone does not depend on the activation of the renin angiotensin system in man [5, 6]. Similar observations have been reported after 5-HTP administration although the rise in aldosterone occurs earlier and is of a greater magnitude [5, 6, 16, 17]. It is possible that the adrenal gland may possess γGT and LAAD activity [18]. 5-HT could then be produced locally and stimulate aldosterone release since 5-HT has been shown to release aldosterone from the adrenal gland [19, 20]. Alternatively, 5-HTP and 5-HT formed from glu-5-HTP in the kidney may recirculate and act on the adrenal gland. 5-HTP, unlike 5-HT, can cross the blood-brain barrier and the release of aldosterone could, therefore, also be mediated by central 5-HT pathways [16, 17]. Carbidopa does not penetrate the central nervous system to any appreciable extent and would not be expected to inhibit conversion of 5-HTP to 5-HT in the brain. It has been reported to increase plasma 5-HTP follow-

ing administration of 5-HTP and to increase the stimulatory effect of 5-HTP on aldosterone suggesting that central 5-HT pathways are involved in the stimulation of aldosterone induced by administration of 5-HTP [17, 21]. In the present study, however, carbidopa attenuated the increase in aldosterone secretion following glu-5-HTP. This finding suggests that release of aldosterone by glu-5-HTP is predominantly a peripheral effect.

We conclude that the results of this work support the proposition that the effect of glu-5-HTP on urinary excretion of sodium is caused by a direct tubular action of 5-HT formed within renal tubular cells by LAAD and is independent of any effect on renal haemodynamics. 5-HT may act as a counter-regulatory paracrine substance to dopamine in the local control of sodium handling in the kidney. It remains to be determined how the balance between 5-HT and dopamine is regulated [22] and whether this is important in the pathogenesis of conditions in which alteration in renal sodium handling may be important such as essential hypertension.

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Blood and urine 5-hydroxytryptophan and 5-hydroxytryptamine levels after administration of two 5-hydroxytryptamine precursors in normal man

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Six healthy male subjects received equimolar amounts of two 5-hydroxytryptamine (5-HT) precursors, 5-hydroxy-L-tryptophan (5-HTP) and γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP), on two occasions in a randomised cross-over study. There were marked increases in urinary 5-HTP and 5-HT excretion after infusion of both compounds. Mean urinary excretion rate of 5-HT, which was < 0.7 nmol min^{-1} before dosing, rose to a peak value of 412 ± 92 nmol min^{-1} at the end of 5-HTP infusion and 303 ± 29 nmol min^{-1} after administration of glu-5-HTP. This occurred without significant changes in blood 5-HT levels measured in platelet-rich plasma. These findings provide further evidence that the increase in urine 5-HT after administration of both 5-HT precursors is largely due to 5-HT synthesised within the kidney.

Keywords 5-hydroxytryptamine 5-hydroxytryptophan γ-L-glutamyl-5-hydroxy-L-tryptophan kidney

Introduction

The mammalian kidney contains all the major enzymes required for the synthesis and degradation of 5-hydroxytryptamine (5-HT) [1, 2]. Intrarenal synthesis of 5-HT occurs in rats given its immediate precursor, 5-hydroxytryptophan (5-HTP), and it has been suggested that 5-HT may act as a counterregulatory paracrine substance to dopamine in the local regulation of sodium excretion [2–4]. We previously administered 5-HTP and its glutamyl derivative, γ-L-glutamyl-5-hydroxy-L-tryptophan (glu-5-HTP), in healthy men and demonstrated a marked increase in urinary 5-HT excretion after both compounds [5, 6]. We argued that this large increment in 5-HT excretion cannot be explained by extrarenal production of 5-HT and that it is principally due to intrarenally generated 5-HT. In the present study, we have estimated 5-HTP and 5-HT concentrations in platelet-rich plasma (PRP), in addition to urinary 5-HTP and 5-HT excretion, after infusion of equimolar amounts of both 5-HT precursors to investigate our hypothesis further. We chose to measure 5-HT in PRP rather than whole blood since processing of whole blood for 5-HT assay inevitably leads to disruption of red blood cells with release of oxyhaemoglobin resulting in oxidation of 5-HT [7].

Methods

Six healthy male volunteers (age range 22–35 years) gave informed written consent to be studied on two separate days, at least 1 week apart, in this randomised cross-over study which was approved by the Lothian Ethics of Medical Research Committee. They abstained from alcohol for 24 h and fasted from 22.00 h the evening before each study day. They attended the clinical investigation unit at 08.00 h after drinking 500 ml of water 1 h previously. The subjects received intravenous 0.9% saline at 5 ml min^{-1} and drank 150 ml of water half-hourly over the next 6 h. They emptied their bladders at 2 h and serial urine collections of 30 min duration were made thereafter. One hour later, an equimolar dose of 5-HTP (10 µg kg^{-1} min^{-1}) or glu-5-HTP (16.6 µg kg^{-1} min^{-1}) was infused intravenously for 60 min. Venous blood samples were collected via a 16 G cannula before and every 30 min for 3 h after the start of the infusion. The blood sample (9 ml) was dispensed into an acid-citrate-dextrose anticoagulant (1 ml) consisting of citric acid (8 g l^{-1}), trisodium citrate (22 g l^{-1}) and glucose (20 g l^{-1}) [8]. The citrated whole blood was centrifuged at 120 *g* for 20 min at room temperature and the upper two-thirds of the supernatant (PRP) were harvested and stored at −40° C in a

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sealed polystyrene tube until analysis. The volume of each urine collection was measured and aliquots stored at −40° C for analysis of 5-HTP and 5-HT. The urine samples were acidified with 5 M hydrochloric acid to prevent their oxidation. PRP 5-HTP and 5-HT were assayed by h.p.l.c. (Waters Associates, Millford, UK) after deproteinisation with perchloric acid (15%) containing cysteine (2 mM) using *N*-methylserotonin as the internal standard [8]. The electrochemical detector operated at a potential of 0.6 V and a sensitivity of 10 nA. The mobile phase (flow rate 2 ml min^{-1}) consisted of phosphate buffer (0.1 M) containing EDTA (1 M), octane sulphonic acid (25 mg l^{-1}) and methanol (5%). Urine 5-HTP and 5-HT were measured by h.p.l.c. as described previously [5]. Glu-5-HTP was supplied by Aalto Bio Reagents Ltd, Dublin, Eire, and 5-HTP was obtained from Sigma Chemical Co. Ltd, Poole, UK.

Results are expressed as means ± s.d. The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule. The apparent renal clearance of 5-HTP was estimated by dividing the amount of 5-HTP excreted in urine by the corresponding area under the concentration-time curve. The data on the 2 experimental days were compared by Student's paired *t*-test and 95% confidence intervals (CI) of the differences between means quoted where appropriate. Differences were considered statistically significant when the P value was less than 0.05.

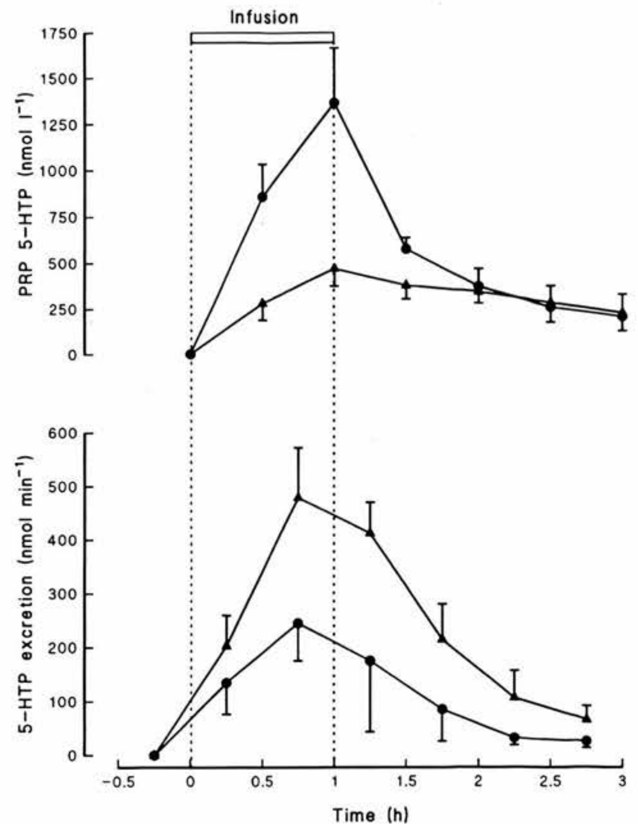

Figure 1 5-HTP concentrations in PRP and urinary 5-HTP excretion rates before, during and after infusion of 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± s.d. (n = 6).

Results

The 5-HTP concentrations in PRP and urinary excretion rates of 5-HTP on the two study days are shown in Figure 1. 5-HTP was undetectable in baseline PRP and urine samples. C_{max} for 5-HTP in PRP was 1365 ± 302 nmol l^{-1} and AUC(0–3 h) was 1763 ± 250 nmol l^{-1} h after administration of 5-HTP. The corresponding values after glu-5-HTP infusion were lower at 471 ± 95 nmol l^{-1} (95% CI of the difference: 539 to 1249, $P < 0.005$) and 934 ± 185 nmol l^{-1} h (95% CI of the difference: 464 to 1193, $P < 0.005$). The 3 h cumulative 5-HTP excretion was 2.5 times greater after glu-5-HTP (44.0 ± 8.6 µmol) than after 5-HTP infusion (17.6 ± 2.1 µmol; 95% CI of the difference: 18.5 to 34.4, $P < 0.001$). The apparent renal clearance of 5-HTP over the first hour was higher after glu-5-HTP (1357 ± 348 ml min^{-1}) than after 5-HTP (246 ± 56 ml min^{-1}; 95% CI of the difference: 734 to 1487, $P < 0.001$).

PRP 5-HT concentration was 812 ± 218 nmol l^{-1} before administration of 5-HTP and 769 ± 140 nmol l^{-1} before glu-5-HTP and did not change significantly after administration of either compound (Figure 2). There were, however, huge increases in urinary 5-HT excretion. Mean urinary excretion rate of 5-HT, which was < 0.7 nmol min^{-1} before dosing, rose to a peak value of 412 ± 92 nmol min^{-1} at the end of 5-HTP infusion and 303 ± 29 nmol min^{-1} after administration of glu-5-HTP. The 3 h cumulative 5-HT excretion values after 5-HTP and glu-5-HTP

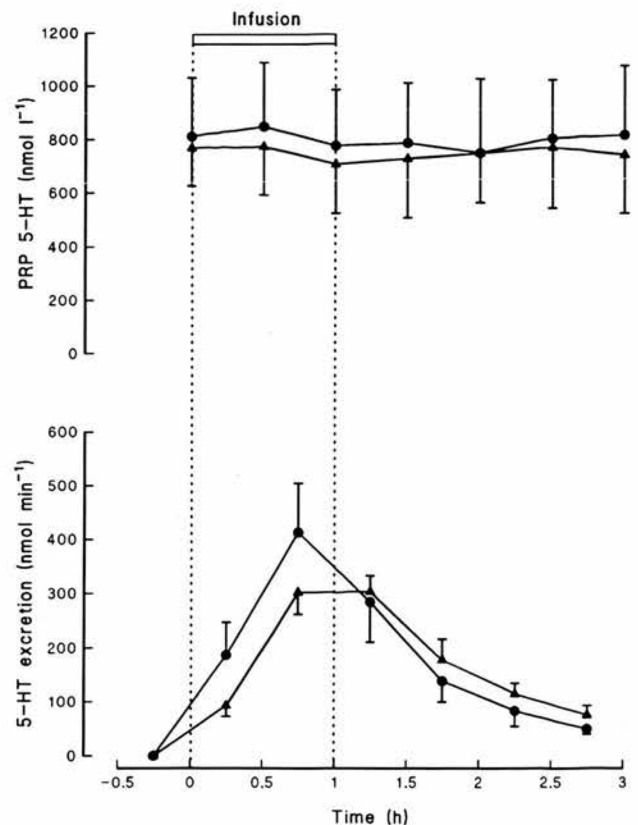

Figure 2 5-HT concentrations in PRP and urinary 5-HT excretion rates before, during and after infusion of 5-HTP (●) and glu-5-HTP (▲). Values shown are means ± s.d. (n = 6).

were not significantly different at 37.4 ± 4.6 µmol and 32.0 ± 4.5 µmol respectively (95% CI of the difference: −0.6 to 11.5).

Two subjects complained of nausea, and one of these two vomited, at the end of 5-HTP infusion. There were no ill-effects following glu-5-HTP infusion.

Discussion

The present study confirms our previous observations that both 5-HTP and glu-5-HTP markedly increase urinary 5-HT excretion [5, 6]. In addition, we have now shown that this occurs without concomitant changes in circulating 5-HT levels. These findings support our hypothesis that urine 5-HT, after infusion of both 5-HT precursors, is largely derived from intrarenal synthesis of 5-HT. Although a placebo day was not included in this study, we previously showed that the saline infusion and water loading employed in the protocol do not affect urinary 5-HTP or 5-HT excretion [5, 6, 9].

The high renal clearance value of 5-HTP observed after administration of glu-5-HTP suggests that urine 5-HTP after glu-5-HTP is also predominantly produced intrarenally. The rise in circulating 5-HTP is probably caused both by its formation within the kidney (followed by recirculation) and extrarenal transformation of glu-5-HTP to 5-HTP since the enzyme γ-glutamyl transferase required for the conversion of glu-5-HTP to 5-HTP is widely distributed in the body although its concentration in renal tissue is considerably greater than elsewhere [10]. Gastrointestinal side effects after administration of 5-HTP appear to be related to the plasma 5-HTP concentrations [11, 12]. In the present study, two subjects developed nausea at the end of 5-HTP infusion at the time when peak circulating levels occurred. In contrast, peak circulating 5-HTP levels were much lower after infusion of the glutamyl compound and no adverse effects were observed. 5-HTP is decarboxylated to 5-HT by aromatic L-amino acid decarboxylase. This enzyme has a ubiquitous distribution with high activity in the kidney and liver [13]. The absence of an increase in circulating 5-HT, particularly after administration of 5-HTP, suggests that 5-HT, if produced extrarenally, is rapidly metabolised and cleared from the circulation.

This study therefore provides further evidence that 5-HT is synthesised intrarenally after administration of both 5-HTP and glu-5-HTP in man. Definitive proof of this will require estimations of 5-HTP and 5-HT in the renal artery and vein, in addition to urinary measurements. The glutamyl compound exhibits greater renal selectivity and is better tolerated [6]. It could be used as a valuable pharmacological research compound for the investigation of the renal formation and effects of 5-HT in man.

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