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REVIEW

CHROMATOGRAPHY OF URINARY INDOLE DERIVATIVES

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CONTENTS

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LIST OF ABBREVIATIONS

5-S-CD	5-S-Cysteinyl-DOPA
COMT	Catechol-O-methyltransferase
5,6DHI	5,6-Dihydroxyindole
5,6DHI2C	5,6-Dihydroxyindole-2-carboxylic acid
5,6DMI2C	5,6-Dimethoxyindole-2-carboxylic acid
DOPA	3,4-Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
GC	Gas chromatography
5-HIAA	5-Hydroxyindole-3-acetic acid
5H6MI	5-Hydroxy-6-methoxyindole
6H5MI	6-Hydroxy-5-methoxyindole
5H6MI2C	5-Hydroxy-6-methoxyindole-2-carboxylic acid
6H5MI2C	6-Hydroxy-5-methoxyindole-2-carboxylic acid
HVA	Homovanillic acid
HPLC	High-performance liquid chromatography
IAA	Indoleacetic acid
I.S.	Internal standard
MAO	Monoamine oxidase
MS	Mass spectrometry
NAD	Nicotinamide-adenine dinucleotide, oxidized
NADP	Nicotinamide-adenine dinucleotide phosphate, oxidized
TLC	Thin-layer chromatography
VMA	Vanillylmandelic acid

1. INTRODUCTION

1.1. Indoles

Indoles are part of a complex mixture of compounds, present in cells and extracellular fluids of all living organisms, and belong to the group of heterocyclic amine compounds, found in hundreds of natural products ranging from the amino acid tryptophan (2-amino-3-indolylpropionic acid) to the more than 600 indole alkaloids derived from tryptophan. The parent ring system (Fig. 1) consists of a benzene ring fused to a five-membered heterocycle containing nitrogen. The ring systems are assigned rigidly defined numbering schemes, in which nitrogen is given the number 1.

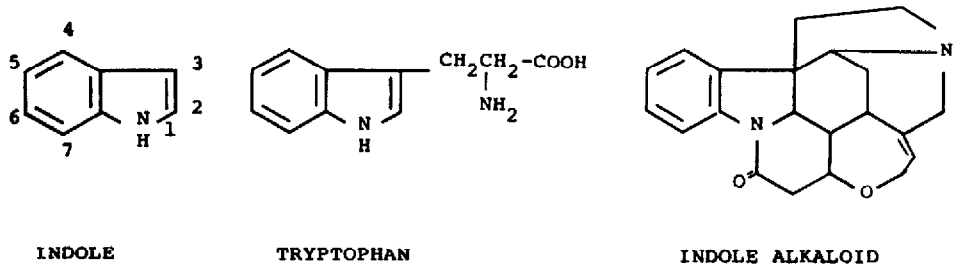

Fig. 1. Chemical structures of indoles.

1.2. Tryptophan-related indole derivatives

Tryptophan metabolism has recently been reviewed by Jepson [1]. A large portion of tryptophan is retained by tissue protein. The major breakdown route is via kynurenine/3-hydroxyanthranilic acid (Fig. 2). This pathway is important not only for degradation, but also for conversion of tryptophan into nicotinic acid, the precursor of the coenzyme NAD and its congeners. Other routes of tryptophan metabolism involve indolic compounds, which are the subject of this review.

The analysis of indole-group-related tryptophan metabolites is of great interest for various clinical and fundamental biochemical reasons, such as their suggested or proven involvement in many diseases and mental abnormalities, including carcinoid tumours, Down's syndrome, malignant melanoma, (familial) tryptophanuria and indoleketonuria, Hartnup disease, depression, schizophrenia, anorexia nervosa, diabetes mellitus, gallstone formation, malignant hyperthermia, dementia, autism and uremia.

From the neurochemical point of view, tryptophan is the precursor of two important biogenic amines, 5-hydroxytryptamine (serotonin) and tryptamine (Fig. 2). The first is an established neurotransmitter and vasoconstrictor and the second a possible neuromodulator of the former. Serotonin has been implicated in a variety of physiological processes, including blood pressure and its regulation (for a review, see ref. 2). The amine itself apparently does not cross the blood–brain barrier, and as a result serotonin concentration in urine does not reflect brain serotonin activity. Its precursors tryptophan and 5-hydroxytryptophan (Fig. 2) may exert physiological and behaviourial effects, which cannot entirely be ascribed to forthcoming serotonin (for a review, see ref. 3). Normally only 1% of tryptophan is converted into serotonin. Serotonin is mainly present in the enterochromaffin cells of the intestine, in serotonergic neurons of the brain and in blood platelets. Diurnal variations of urinary serotonin occur [4], the night level being higher than the day-time level, a pattern of diurnal rhythm the reverse of that of the catecholamines. Differences in peak times may be caused by dissimilar neurophysiological roles of these amines.

From the biochemical point of view, tryptophan is also a precursor of the hormone melatonin and 5-methoxytryptophol (Fig. 3), both derived chiefly from the pineal gland and implicated in the physiology of circadian rhythms, reproductive development and behaviour (for reviews, see refs. 5 and 6). A universal physio-

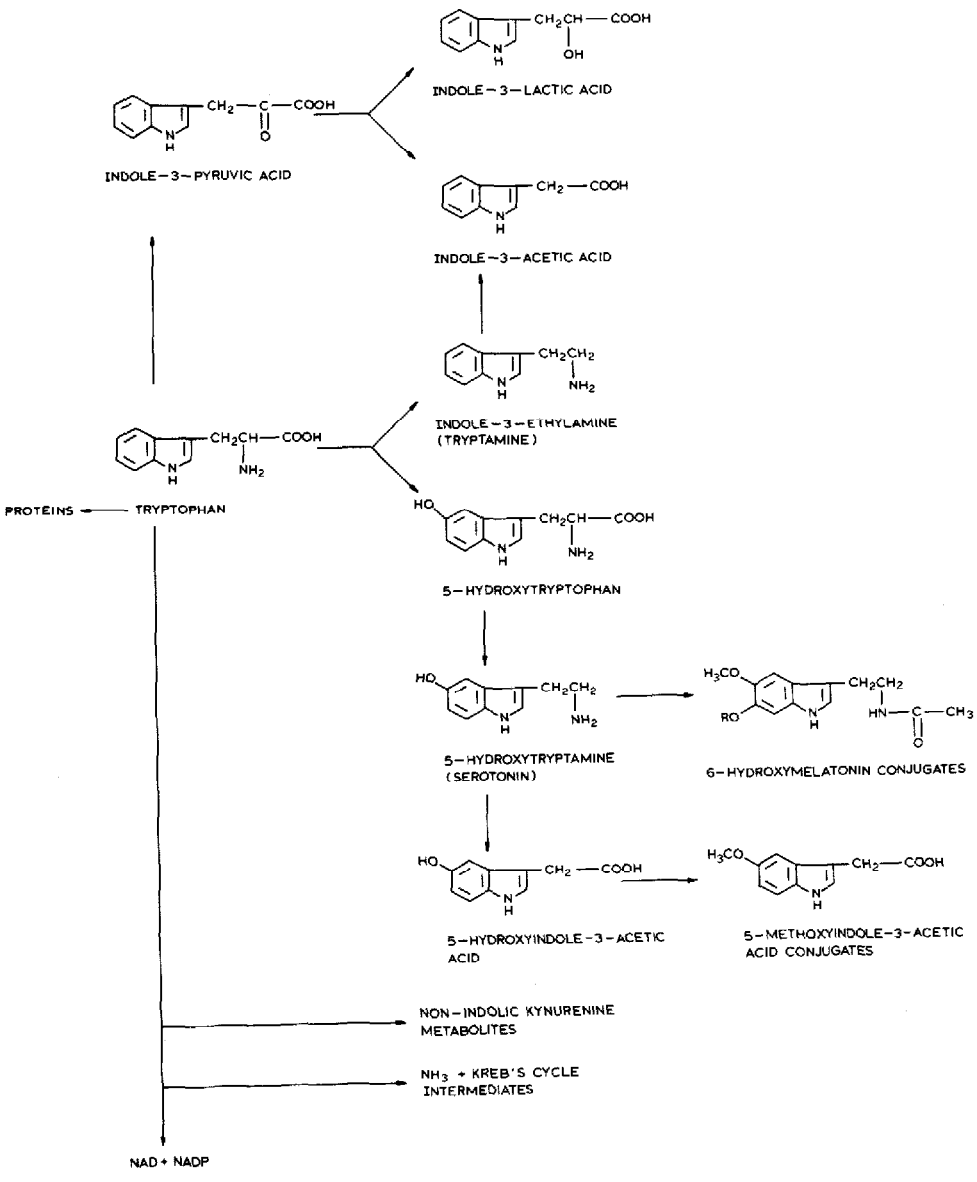

Fig. 2. Metabolic pathways of tryptophan.

logical role of melatonin in mammals has not been established. Diurnal variation in plasma melatonin levels occurs, concomitantly with high urinary excretion levels of 6-hydroxymelatonin sulphates and glucuronidates (Fig. 2) occurring at night, which are the main metabolites [7]. Very small amounts of melatonin and 5-methoxytryptophol are found in urine [8].

1.3. Eumelanin-related indole derivatives

Also of indolic nature are compounds found in the urine of patients suffering from malignant melanoma. On exposure to oxygen they can blacken urine by

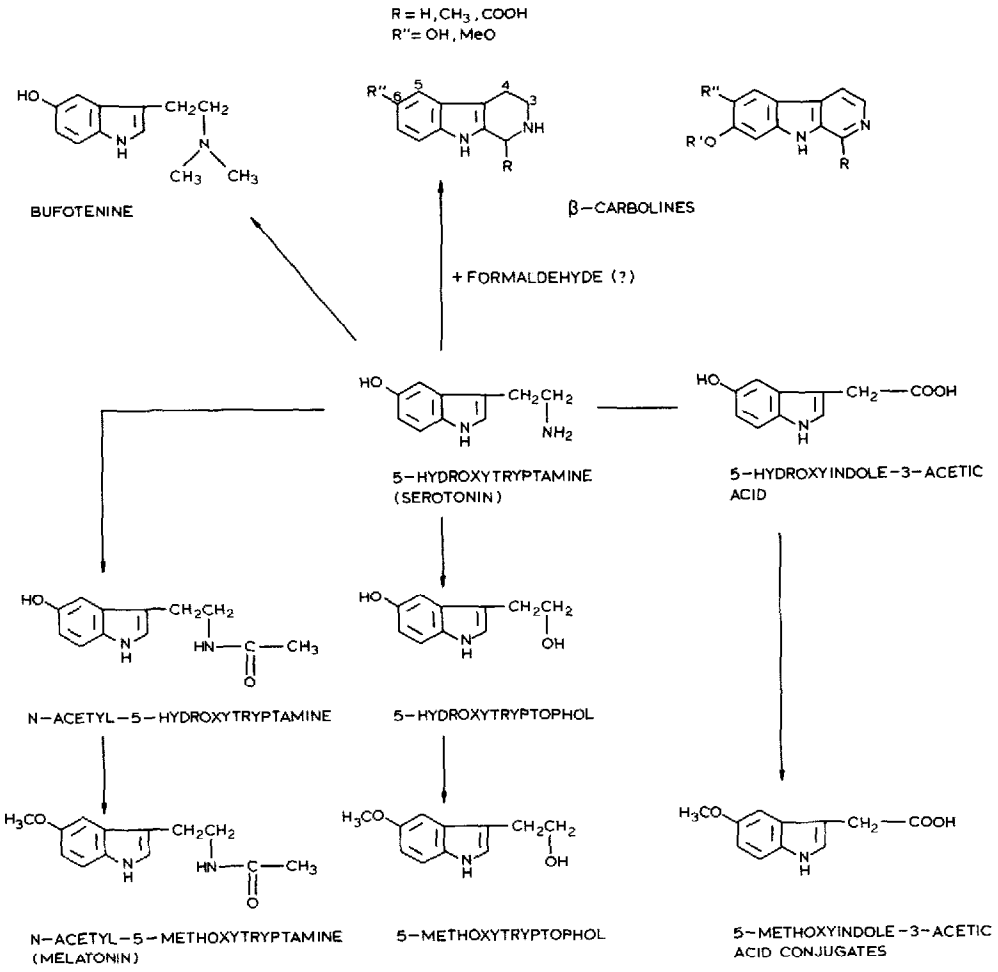

Fig. 3. Biosynthesis and metabolism of melatonin.

formation of urinary melanin. This group of indolic compounds is probably the result of overproduction of precursors of the skin pigment eumelanin, a polymer that shares with catecholamines the common precursor tyrosine and with tryptophan the indolic ring system (Fig. 4). This polymer should not be confused with polymeric products arising from the instability of catecholamines [9]. It is general biochemical knowledge that tyrosine is normally metabolized via *p*-hydroxyphenylpyruvic acid to link up with the intermediary metabolism of the cell. Melanogenesis (melanin synthesis) is a special branch of tyrosine metabolism based on the presence of the enzyme tyrosinase, responsible for catalysing (at least) two steps in melanin formation (Fig. 4). These initial steps in melanin biosynthesis include hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and subsequent conversion into dopaquinone. While the former reaction appears to be common for all the cells of neuroectodermal origin, the latter can be considered specific for melanin-forming cells, the melanocytes. All metab-

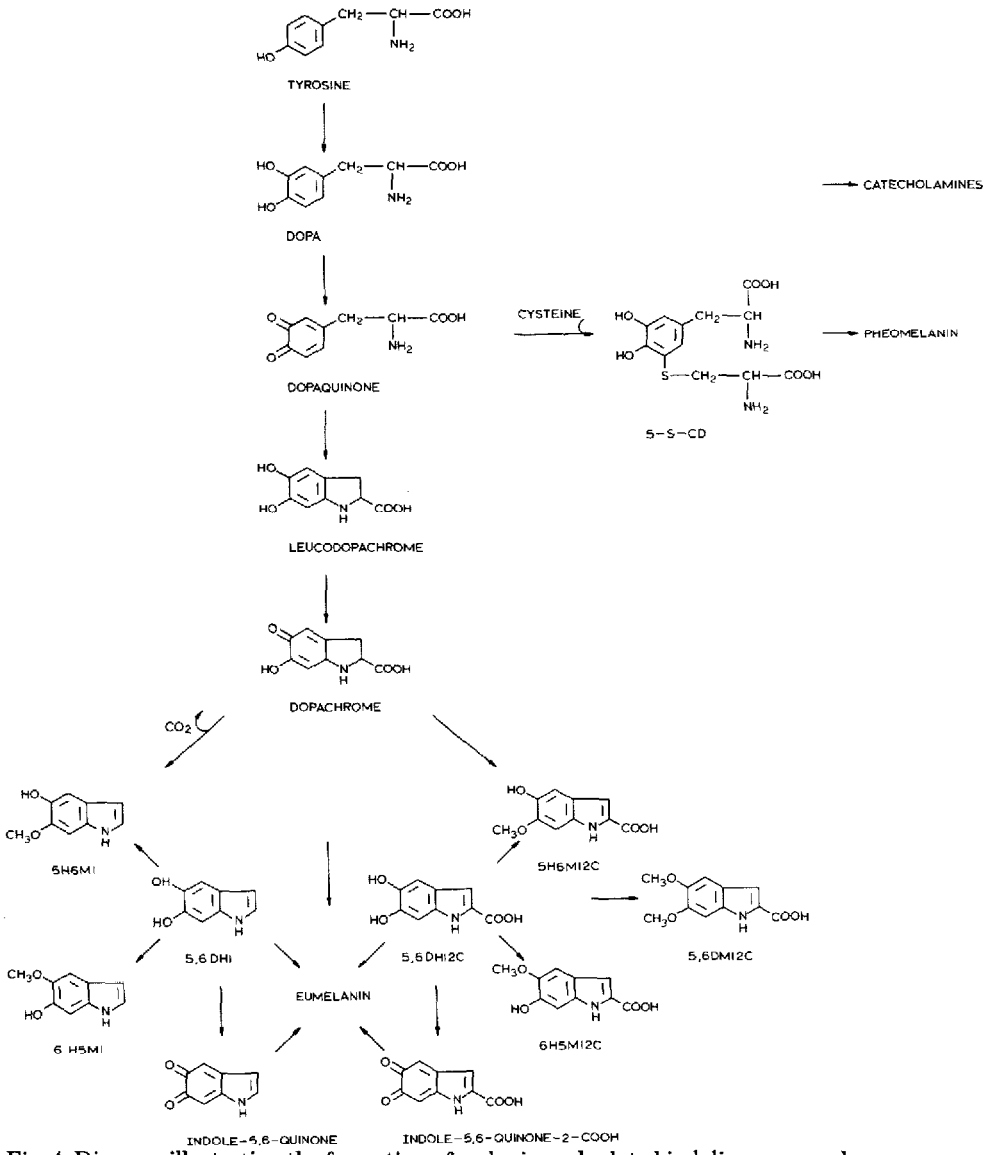

Fig. 4. Diagram illustrating the formation of melanin and related indolic compounds.

olites arising from the metabolic pathway after dopaquinone can therefore be regarded as specific melanin-related compounds. Dopaquinone is also a divergence point for the syntheses of two types of melanin, i.e. eumelanin and pheomelanin.

Like most quinones, dopaquinone is a highly reactive molecule. Its further metabolism, i.e. spontaneous reductive cyclization to cyclo-DOPA (known also as leucodopachrome) or conjugation with cysteine or glutathione, can be seen as detoxification steps. Leucodopachrome (Fig. 4) thus formed, however, has never been isolated. Under appropriate conditions, the spontaneous rearrangement of cyclo-DOPA followed by the loss of the carbon dioxide leads to the formation of

indolic structures of 5,6-dihydroxyindole (5,6DHI) and 5,6-dihydroxyindole-2-carboxylic acid (5,6DHI2C). Both these indolic compounds are believed to be basic monomer units of the polymer eumelanin (for reviews, see refs. 10–12). The application of gas chromatography (GC) in combination with mass spectrometry (MS) has allowed the identification of the entire class of indolic melanin-related substances. This class was shown to consist of seven compounds: 5,6DHI, 5-hydroxy-6-methoxyindole (5H6MI), 6-hydroxy-5-methoxyindole (6H5MI), 5,6DHI2C, 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MI2C), 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2C) and 5,6-dimethoxyindole-2-carboxylic acid (5,6DMI2C). Two of them, namely 5H6MI2C and 6H5MI2C were described in 1967 by Duchon and Matous [13].

The entire class can be divided into two sets: (a) indole-2-carboxylic acids and (b) indoles without a carboxyl group. In accordance with general biochemical rules for conjugation, it was found that indolic substances without the 2-carboxyl group are excreted as sulphate or glucuronic acid conjugates, whereas the more hydrophilic indole-2-carboxylic acids are excreted in unconjugated form [14]. It has recently been proposed that an increased concentration of indolic eumelanin precursors in bile could lead to the formation of a nucleus for future formation of gallstones in humans [15]. Analyses in urine of two compounds, i.e. conjugated 5H6MI2C and 5H6MI, seem to be promising in the follow-up of treatment of malignant melanoma and in screening for pigmentation disorders and gallstone formation.

1.4. Miscellaneous

Reactions between indoleethylamines and aldehydes or α-keto acids may result in the formation of tetrahydro-β-carbolines (Fig. 3). Such reactions may occur under physiological conditions and produce compounds that can function as neurotransmitters or neuromodulators (for a review, see ref. 16). Tetrahydro-β-carbolines and derivatives have been proposed to play an important role in alcoholism and in the pathophysiology of depression. Bufotenine (N,N-dimethylserotonin; Fig. 3) has been described as an endogenous hallucinogenic compound in humans, connected with anxiety states, dysperceptions, periodic destructiveness and compulsive suicide. When it is present, 6% of bufotenine is excreted in urine. Other N-methylated indoles have been suggested to occur in humans with various hallucinogenic effects [17].

In faeces, during bacterial fermentation and putrefaction, tryptophan undergoes a series of reactions resulting in formation of (among others) indole and 3-methylindole (skatole), substances particularly responsible for the odour of the faeces. These compounds are partly absorbed and metabolized, and may be recovered in urine.

1.5. The routine clinical laboratory

The study of structural, metabolic and functional relationships between tryptophan-related indole derivatives and other physiologically important com-

pounds, non-indolic tyrosine derivatives in particular, frequently requires the determination of several individual compounds in a biological sample. Although from a clinical point of view interest was and (sometimes unfortunately) still is focused exclusively on a single compound (e.g. 5-hydroxyindoleacetic acid in urine from patients suffering from carcinoid tumours) there was a need for methods that allow the determination of a particular indolic compound in the presence of many other (related) compounds of comparable or even greater concentration. Of methods of this type, gas–liquid, liquid–liquid and liquid–solid chromatography have been shown to be particularly useful.

The concentration of indoles in human samples is generally low. The reported urinary concentrations of major indole derivatives are given in Table 1. For comparison, concentrations of major (related) tyrosine derivatives are included.

Achieving the quantitative determination of picomole or even femtomole amounts of indoles is one criterion, i.e. sensitivity for the suitability of a method in neurochemistry. However, the application of very advanced high-performance liquid chromatography (HPLC) with electrochemical detection, using arrays of electrodes in series in the assay of indoles in cerebrospinal fluid [18], revealed that actual concentrations were lower by orders of magnitude than originally thought. This indicates that specificity (accuracy) is another, more important

TABLE 1

NORMAL LEVELS OF INDOLE DERIVATIVES IN URINE, FOUND BY DIFFERENT METHODS

Levels of non-conjugated compounds are given, unless stated otherwise. Data on tyrosine derivatives are added for comparison.

Compounds	Concentration (μmol per 24 h)	References
Tyrosine	<150	109
Norepinephrine	<0.47	33,63,76,118
Epinephrine	<0.11	33,63,76,118
Vanillylmandelic acid	<45	33,63
Dopamine	<3	63,76,118
5-Hydroxytryptophan	$\ll 0.7$	103
Normetanephrine	<0.13	76,118
5-Hydroxytryptamine (serotonin)	<1.3	47,56,103
Tryptophan	<200	86
5-Hydroxyindoleacetic acid	$\ll 54$	32,33,42,47,54
Metanephrine	$\ll 0.3$	76,118
Eumelanin-related indole derivatives	$\ll 1$	29
6-Hydroxymelatonin conjugates	<0.13	6
Melatonin	<28 pmol/h	8
Homovanillic acid	<39	63
Bufotenine	<0.01	17
Tryptophol	<28 pmol/h	8
Indoleacetic acid	$\ll 10$	86
Indolepyruvic acid	$\ll 20$	86
Indolelactic acid	$\ll 10$	86

criterion. Thus, although less sensitive methods may be required for monitoring urine samples, the clinical laboratory is more and more confronted with problems of selectivity and specificity. Improvements in specificity are more important than increases in sensitivity. For instance, the study of infections in which abnormalities in tryptophan excretory patterns or metabolism have been noticed, but in which no real insight into the relationship of cause and effect has yet been achieved (e.g. in biological psychiatry; for a review, see ref. 19), requires analytical techniques that are not time-consuming (avoiding sampling errors), but sensitive (avoiding false relationships) and totally specific (avoiding false diagnoses). The application of MS, although confined to larger and/or governmental laboratories, in combination with other separation methods in analytical biochemistry, is aimed at improving specificity and establishing reference methods (for an excellent review, see ref. 20).

The routine clinical laboratory may be merely interested in supporting clinical diagnosis with data derived from few compounds secreted in urine. The laboratory, however, is confronted with the results of studies on the metabolism of the amino acids tryptophan and tyrosine, involving the analytical monitoring of tryptophan, 5-hydroxytryptophan, tryptamine, N,N-dimethyltryptamine, 5-hydroxytryptamine (serotonin), N,N-dimethylserotonin (bufotenine) N-acetyl-5-methoxytryptamine (melatonin), 5-methoxytryptophol, indole-3-acetic acid, 5-hydroxyindoleacetic acid, β-carbolines, eumelanin precursors and derivatives, metabolites of kynurenine (non-indoles) and conjugates (sulphates, acetates and glucuronidates) of metabolites and peptides. In other words, the laboratory has to cope with clinical relevances (i.e. which compounds are markers for suspected pathology), methodological relevances (i.e. which method can accommodate markers for different pathologies) and cost and efficiency aspects (e.g. which system can accommodate various methods and different clinical investigations) when drawing conclusions about what to analyse, how, and for what investment.

1.6. Strategies

Based on metabolic studies, a recent trend in analytical biochemistry concerned the development of chromatographic methods allowing the concurrent determination of functionally related compounds (e.g. tryptophan and tyrosine derivatives) and metabolically related compounds (e.g. indole-group-related tryptophan derivatives) from the same sample.

HPLC seems to be the method of choice for (direct) fingerprinting of samples for the elucidation of rare diseases and/or for quantitative estimation of single or selected classes of compounds, but when the overall profile of metabolites is more informative, GC is used. Consequently, separation and multi-detection strategies for multi-component mixtures are being explored. When screening the literature on HPLC analyses of indole derivatives, it becomes evident that those strategies will result ultimately in the use of a single stationary phase, a limited number of mobile phase solvents and two detection systems.

The scope of this review is to present the latest methods for the separation and quantitation of the most clinically significant indole derivatives in urine.

2. URINE COLLECTION AND PRESERVATION

When determining any compound in urine, accuracy will not be achieved, despite sensitive and specific instrumentation and efficient separation power, if certain basic conditions are neglected, such as urine collection protocol and compound stability.

Excretion data for indole derivatives may be influenced by several factors, e.g. food intake, as reported for 5-hydroxyindoleacetic acid (nuts, bananas [21]), flushing frequency in patients with carcinoid tumours, drugs, cooperation of patients in urine sampling and the difficulty of sampling in patients with diarrhea. Moreover, complete sampling of 24-h urine in new-borns and young children is practically impossible.

Generally, it is recommended that 24-h samples are collected and that excretion data are expressed both as amounts per period and as amounts per mmol of creatinine. However, although circadian rhythm data on individual compounds are still scanty, urine collected after the onset of clinical symptoms can be used for screening purposes, and it is recommended that this is followed by complete 24-h collection, preferably before medication or therapy is started. Indications of objective pathology progression or regression should be based on reference values, established for random portions, early-morning urine or 24-h collected urine samples, obtained from a large number of healthy persons of various ages.

Factors affecting the stability of indole derivatives in solution have been studied. Although data on the stability of various indole derivatives in urine samples or urine extracts are still scanty, the efficiency of preservation and storage techniques have been reported for 5-hydroxyindoleacetic acid [22–25], tryptophan [26], serotonin [22,23], 5-hydroxytryptophan [22], β-carbolines [16], catecholamines, indoles and their derivatives [9,23,24,27], indolecarboxylic acids, melatonin and related compounds [8,28] and eumelanin-related indole derivatives [29].

In summary, the degradation of indole derivatives in any solution can be prevented or delayed by the following measures:

(1) (Sun)light filtering or, even better, exclusion.

(2) Immediate refrigeration of each collected urine specimen, especially when studying melatonin metabolites.

(3) Freezing of samples, without thawing and refreezing.

(4) Air exclusion, especially when studying β-carbolines and eumelanin-related indole derivatives.

(5) Slight acidification (pH > 2, at all times), preferably by acetic acid, sulphosalicylic acid or boric acid, e.g. in order to avoid artifactual formation of β-carbolines.

(6) Addition of an anti-oxidant, preferably sodium metabisulphite or ascorbic acid, the latter also being useful for the acidification of standard solutions and stabilization of compounds during chromatography.

To add a few notes, urine collection in concentrated (hydrochloric) acid solution, as still practised according to randomly referred literature [30–33] should be discontinued because it is detrimental to indole derivatives. For example, Fig.

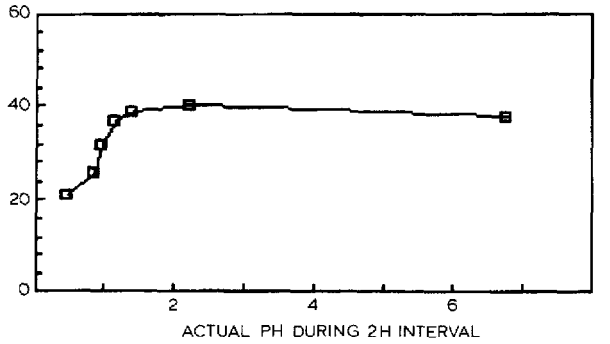

Fig. 5. Stability of 5-hydroxyindoleacetic acid (μmol/l) in urine during collection on strong acid solution. Urinary (pH 6.8) aliquots of 200 ml were added subsequently to 10 ml of 6 mol/l hydrochloric acid, the pH was monitored and samples were adjusted to pH 3 before HPLC analysis according to ref. 42.

5 shows that, 5-hydroxyindoleacetic acid is partly destroyed in urine in the presence of (hydrochloric) acid until pH 2 is reached. It is tempting to assume that other indole derivatives are destroyed or partly deconjugated during collection of urine on (any) acid as long as the pH is below 2.

It has also been experienced that under slightly acidic conditions urinary indole derivatives (and catecholamine metabolites), if protected from light, degrade by less than 10% during storage at 4°C for one week and at −20°C for more than three months, even without preservation [24].

As for standard solutions, indole derivatives seem to be stable in methanol for at least six months if stored at −20°C. Alternatively, aqueous stock solutions of indole derivatives seem to be stable for at least six months when stored at 4°C in 0.1% ascorbic acid [27,28] and other acidic solutions (pH > 2). Combinations of alcohols and acids should be avoided, since esterification of carboxylic acids may occur.

It is recommended that separate aliquots of stock solutions are stored, from which working standards can be prepared freshly and appropriately when needed. If a relatively strong acidic solution (pH < 2) has to be used as a solvent or extraction liquid, it is recommended that antioxidants [22], which can be removed if necessary [34], are added.

3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

3.1. Sample pretreatment

A variety of pretreatment techniques (none truly specific) have been described for the HPLC analysis of indole derivatives, using electrochemical detection (amperometric and coulometric mode), (spectro)fluorometric detection and UV–visible spectrophotometry. An integrated scheme for the consecutive isolation of indole derivatives and related precursors and metabolites from one urine sample has not yet been reported, although methods have been described for tissue anal-

ysis [35], employing four analytical separation systems and one detector. The quantification, after computer-designed and optimized separation of all clinically important indole derivatives, following "direct" injection of one urine sample has not yet been published, although strategies have been designed for clinical samples [36–38] or for general use [39], using one detection system. Multi-detection after direct injection of biological samples has been explored using an array of electrochemical reaction detectors [18], multi-wavelength detection [40] or variable systems in series [28,41].

For rapid analyses of multiple samples it is necessary to combine easy, yet accurate sample pretreatment with problem-free separation and detection techniques. Production expenses and instrumentation investments are factors to be considered by each laboratory but, with a given set of equipment and personnel, reliable reproducibility of quantitative and qualitative profiles of urinary indole derivatives should be aimed at. Accuracy and reproducibility criteria are more stringent for a single component than for classes of compounds. Because the compounds under study function in normal metabolism and are excreted in pathological cases in a dynamic fashion, strategies are aimed at sample pretreatment (including on-line enrichment and derivatization techniques), separation methodology or post-column techniques. In the following paragraphs, major recently reported strategies will be discussed.

3.1.1. Direct analysis

For routine screening of urine samples for elevated excreted amounts of 5-hydroxyindoleacetic acid (tentatively serotonin, tryptophan and 5-hydroxytryptophan), reports indicate that sample pretreatment, apart from pH adjustment, is confined to centrifugation [31,42,43] and/or filtration [30,44] and/or dilution [41,45,47]. No problems were encountered with regard to urinary protein concentrations up to 3 g/l, or density in the range 1.010–1.030 kg/l, when urine was "directly" injected [24,30,42,43]. The same holds true for the "direct" injection of urine samples in the concurrent determination of catecholamine metabolites and 5-hydroxyindoleacetic acid, when spectrofluorometric or serial coulometric detection is used [24,30,43]. However, for the qualitative and/or quantitative profiling of indole derivates, with the exception of 5-hydroxyindoleacetic acid, sample pretreatment is necessary, as can also be seen from the normal ranges found in urine (Table 1). Specific extractions, such as are common for catecholamines, do not apply to indole derivatives.

3.1.2. Liquid–liquid extraction

Diethyl ether is most successfully employed as an extraction solvent for various indole derivatives in urine. Specifically, 5-hydroxyindoleacetic acid is extracted with diethyl ether from urine samples, adjusted to pH 1–2 shortly before. It was shown that analytical recoveries were better than 90% after reconstitution of extracts in eluent or acetic acid solution [32,42,48]. At the low pH value applied no salting out is necessary. Slightly acidified (pH 5–6.5) urine is also satisfactorily extracted with diethyl ether before profiling of eumelanin-related indole derivatives [29].

Ethyl acetate is often used for extracting urine (e.g. at pH 1) for profiling urinary excretion of catecholamine metabolites [31,49] or aromatic acids in general [40]; this solvent is certainly not recommended (even with salting out) for accurate determination of individual indole derivatives, since analytical recoveries are below 90% [49]. Beneficial effects on accuracy claimed for the application of complex extraction solvents are not convincing, with regard to 5-hydroxyindoleacetic acid [50] and eumelanin-related indole derivatives [29].

Melatonin-related indole derivatives have been selectively extracted from urine saturated with boric acid using dichloromethane [8], and from other physiological fluids using chloroform [6]. Washing the extracts with pH 10 buffer may selectively remove indoles containing carboxyl and phenolic hydroxyl groups [6,8]. Analytical recoveries of melatonin, tryptophol and 5-methoxytryptophol were below 80%, but fairly constant. Relatively large amounts of 5-hydroxytryptophan, 5-hydroxytryptamine, tryptophan, 5-methoxytryptophan, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, N-acetylserotonin, tryptamine, 5-methoxytryptamine, indole-3-acetaldehyde, N-acetyltryptophan and N-methyltryptamine can be recovered in the aqueous phase from boric acid-saturated urine samples, extracted with dichloromethane including an alkaline washing step [8].

Serotonin was extracted from samples adjusted to pH 8–11 with heptanol [51], chloroform–pentanol (60:20, v/v) or with ion-pairing di-(2-ethyl)hexyl-phosphoric acid in chloroform–isoamyl alcohol [52,53], although no applications to urine samples have been described. It has been reported [16] that the determination of tetrahydro-β-carbolines (tetrahydro-β-carboline, 1-methyltetrahydro-β-carboline, 6-hydroxytetrahydro-β-carboline, 6-hydroxy-1-methyltetrahydro-β-carboline, tetrahydro-β-carboline-3-carboxylic acid and 1-methyltetrahydro-β-carboline-3-carboxylic acid) in biological samples necessitates careful sample pretreatment in order to avoid artifactual formation of β-carbolines or oxidation of these compounds. (For the structures and atom numbering scheme, see Fig. 3.) Under aldehyde-scavenging conditions, tetrahydro-β-carbolines and their precursors (tryptamine, tryptophan and 5-hydroxytryptamine) are converted into carbamates and carbonates by methylchloroformate under acidic followed by alkaline conditions. The derivatized precursors, tryptamine and 5-hydroxytryptamine, and non-acidic tetrahydro-β-carbolines were extracted from the reaction mixture at pH 9.5 using dichloromethane. The extracts, reconstituted in pH 7.4 buffer, were used for determination of carbamate derivatives of tryptamine, tetrahydro-β-carboline and 1-methyltetrahydro-β-carboline or, after treatment with esterase (to selectively free the phenolic group), for determination of carbamate derivatives of 5-hydroxytryptamine, 6-hydroxytetrahydro-β-carboline and 6-hydroxy-1-methyltetrahydro-β-carboline. The derivatized acidic compounds tryptophan, tetrahydro-β-carboline-3-carboxylic acid and 1-methyltetrahydro-β-carboline-3-carboxylic acid were isolated from the remaining aqueous phase after acidification and extraction with dichloromethane. After reconstitution in eluent, the carbamate derivatives of acidic compounds were analysed. Unfortunately, no application to urine samples was reported. The acetylation of phenolic indoles or phenolic tetrahydro-β-carbolines resulted in complete loss of their native fluorescence [16], a fact that may be used for identification purposes.

3.1.3. Liquid–solid extraction

Serotonin has been satisfactorily "extracted" from urine (pH 0.8) by the application of liquid–solid extraction with Amberlite XAD-2 [47], with adsorbents being recycled up to 50 times. Serotonin was desorbed quantitatively from 200 mg of adsorbent after draining 1 ml of urine, in 3 ml of water–methanol (80:20, v/v). For profiling purposes, the authors [47] suggested that one part of untreated urine be mixed before analysis with 10 parts of XAD-2 eluate containing 5-hydroxytryptamine, using preferably spectrofluorometry.

It has been shown that 5-hydroxyindoleacetic acid can be satisfactorily extracted from urine (pH < 5) using C_{18} cartridges [54], before analysis with electrochemical detection. Desorption was performed using ammonium acetate (pH 5.1)–methanol (1760:325, v/v) and void volume and eluates used for analysis. Various tryptophan-related indoles have been also extracted employing C_{18} cartridges before their thin-layer chromatographic (TLC) determination [55]. By contrast, the liquid–solid extraction of urinary serotonin using cation-exchange material is rather tedious, as employed for physiological fluids and blood platelets by Kwarts et al. [56], who applied fluorometry. The method was later used with minor modifications for urine by Jouve et al. [57], who applied electrochemical detection.

Although they are a challenge, on-line enrichment procedures for determination of serotonin [41,58] seem to be still far from the routine laboratory, coping as it is with budgetary constraints. Moreover, information on long-term reliability of such systems is scarce.

3.2. Separation and detection

Much information in the field of indole derivatives was acquired by planar techniques, such as paper chromatography and TLC. It is our feeling that these techniques are still in use for screening purposes even now. However, reports on the application of these methodologies are scarce after 1982. Most of the planar techniques are lengthy and lack resolving power, which is reflected in distorted chromatograms. When samples are not pretreated (cleaned-up) and even when samples are pretreated using modern clean-up techniques, differences in retention times between reference compounds and analytes still occur, and these reduce the sensitivity and specificity of the methods. Detection limits for clinically important tryptophan-related indole derivatives, stained with van Urk–Salkowski reagent for example, are generally too high to allow for quantitation or even qualitative assay, if occurring at normal concentrations in urine samples. The existence of indole (melanin-related) conjugates in melanotic urine has been proposed in older studies (before 1960) that employed planar methods. These conjugated compounds give a specific colour reaction (Thormählen reaction), after separation using paper chromatography. However, for screening purposes planar separation techniques are still useful if combined with modern clean-up methods.

HPLC offers, in terms of general strategy, great potential in the (routine) clinical laboratory for the assay of urinary indole derivatives. Specificity criteria,

however, call for a combination with MS. Factors that need to be considered are: (1) clinical goals (see Sections 1 and 5); (2) flexibility and speed in terms of meeting various clinical goals and of efficient use of chromatographic systems; (3) specificity, including items such as sample pretreatment, quality assessment (comparison studies) and evaluation of the sensitivity of total assays to changing experimental conditions (e.g. eluent composition); (4) costs of personnel and instrumentation, which should meet the budgetary constraints of the laboratory; (5) sensitivity, related to excretory reference values, linear dynamic ranges and the selectivity of the chromatographic and detection equipment.

Once samples have been treated correctly and stored adequately, it is necessary to decide on sample pretreatment, separation and detection mode(s). Internal standards (e.g. 5-methoxyindole-2-carboxylic acid, 5-hydroxyindole-3-propionic acid, 5-hydroxyindole-2-carboxylic acid, 5-hydroxy-N-methyltryptamine, 5-methoxytryptamine) may be added to meet various quantitative and qualitative requirements. In general, at Delft we prefer the method of standard addition, with or without internal standard addition (for a review on quantitation methods, see ref. 59).

3.2.1. The stationary phase

Octadecylsilane (C_{18}) reversed-phase packing materials are the most satisfactory for separation of urinary indole derivatives as free compounds, or even in derivatized form, as shown for tetrahydro-β-carbolines. The next important step (and this is certainly important if "direct" injection is to be used) is picking the right reversed-phase C_{18} packing (and column design) out of the numerous commercially available substances. It cannot be said [38] and shown [36] too often that chromatographic supports vary in performance for the various compounds excreted in urine, including indole derivatives. Although classification of column packings is underway [60,61], the chromatographer either should test several of the available supports or scan the literature to pick out a suitable material for the separation of amines, acids and other compounds in urine. Even once a support has been chosen, selectivity of separation may be influenced by many factors, such as eluent composition, temperature and column design. Consequently, the laboratory should follow a separation strategy, including factors such as column dimensions, separation mode (eluent composition) and speed (isocratic or gradient). For example, in accordance with a philosophy of modern separation strategy, we manage at Delft, using a ternary type of HPLC pump, a spectrofluorometer and automated sampler, to employ one type of reversed-phase C_{18} packing in steel columns (150–250 mm $\times$ 4.6 mm I.D.) for the analysis of water-soluble vitamins [62], catecholamines [63], catecholamine metabolites [24], 5-hydroxyindoleacetic acid [42], porphyrins [64], amino acids (prolines excluded [65]), pteridines [66], some purine and pyrimidine derivatives [67] and creatinine [66], among others in supporting clinical diagnosis. We have had experience with this packing for more than three years, evaluating five different batches of 100 g each. During continued use of each column, one day a week, the tops of column packings are replaced by hand when chromatographic evidence, e.g. of voids, indicates the need. Organic modifier concentrations are chosen empirically at the start-up

of each laboratory-made column, in order to maintain resolution between diagnostically important and background peaks through at least 800 injections.

3.2.2. The mobile phase

As judged from various reports, it seems to be general strategy to use eluents composed of (ammonium) acetate buffer (pH 4–5.5) and methanol for profiling clinic-related classes of indole derivatives. This eluent combination has been shown (or judged) to be satisfactory when used to separate eumelanin-related indole derivatives [29], melatonin-related indole derivatives [8,28,68,69], tryptophan-related indole derivatives [28,42,44,45,68,69] and β-carbolines [16] or "single" compounds [32,42,45,54,57,70]. Additives vary from EDTA (in electrochemical detection), citrate (silanol masking and ion-pairing), alkylamines (peak symmetry improvement) to ascorbic acid (stabilization of compounds during separation). Catecholamines do not interfere with the most polar indole derivatives, since they are only slightly retained.

So, in terms of strategy, there is no obvious need for an eluent composed of monochloracetic acid [33,46], oxalate or perchlorate in combination with tributylphosphate [47] or even citrate–phosphate in combination with ion-pairing agents [23,27,36–38,71–73], if the laboratory aims mainly for separation of urinary indole derivatives, after class pretreatment. However, if concurrent analyses of some indole derivatives and catecholamine metabolites are needed, dynamic ion-exchange/ion-pairing separation modes should be explored too and results compared with hydrophobic modes (for excellent discussions, see refs. 36,38,58 and 74).

To avoid problems with citric acid (complexing residual silica; corrosion of equipment; high background currents in electrochemical detection), phosphate buffer (insolubility problems mainly), tributylphosphate (loading problems), acetonitrile and monochloroacetic acid (environment), ion-pairing agents (stabilization time, electrode or column poisoning, insolubility problems with gradient and potassium ions), it seems to make good sense to use ammonium acetate as a buffer, EDTA as a metal scavenger and methanol as the organic modifier in the analysis of indole derivatives. In addition, ammonium acetate serves as a masking agent for residual silanol groups, is compatible with electrochemical detection and spectrofluorometry and, being volatile, is compatible with MS and flame-ionization detectors.

Retention of indole derivatives, separated in hydrophobic mode using ammonium acetate–methanol combinations, is simply controlled by pH, buffer concentration ($<0.5\ M$) and methanol addition, and is comparable with the use of plain citrate buffer [58].

The degassing of any eluent should be considered only if it can be maintained continuously, does not alter the eluent composition within a relevant period and/or if it is obligatory in terms of sensitivity.

3.2.3. Detection mode

Selection of detection mode for the analysis of indole derivatives is relatively straightforward. If the whole range of indole derivatives is to be analysed in sep-

arate assays, the spectrofluorometer (excitation wavelength 280–320 nm, emission wavelength 300–370 nm, roughly) seems a good choice [8,28,29,44,75]. For concurrent analyses of indole derivatives and catecholamine metabolites in urine [30,43], or catecholamines and their derivatives [76], these wavelength settings will again be sufficient in terms of sensitivity and selectivity. A spectrofluorometer can be simply checked for general sensitivity by determining the signal-to-noise ratio of the Raman band of water and plotting peak heights and widths-at-half-height for test compounds versus time of use.

Concerning detection, it is heart-warming to read critiques on electrochemical detection, arising from the routine clinical laboratory [77]. In general, electrochemical detection needs longer stabilization times than spectrofluorometric detection. Carbon-paste electrodes last from one week to two months before requiring repacking, depending on the eluent composition [49,71]. Glassy carbon electrodes seem to be more stable but less sensitive [58,71]. At high potentials, necessary for detection of tryptophan and some of its derivatives, electrode life-times may even decrease to days [27,69]. No repacking, but tedious cleaning is necessary using modern coulometric flow-through cells (ESA, Coulochem), whereas these cells remain fairly stable in sensitivity for empirically established periods and are used, in our experience, for one application each [24,78]. Still, hydrodynamic voltammograms for every analyte have to be (re)constructed for each new cell or cleaned cell, and even at any change in eluent composition, as can be deduced from various plots in the literature [27,50,54,71,73,79].

In the analysis of indole derivatives in urine the choice of UV detection remains very unattractive, except when multi-wavelength detection, gradient elution and very sophisticated data handling is feasible [40]. A few authors still try to convince others that UV detection is feasible in determining 5-hydroxyindoleacetic acid [50,70], albeit after extensive sample pretreatment.

Detection limits for electrochemical detection (oxidation potentials above 0.8 V) and spectrofluorometric detection (excitation wavelength range 280–320 nm; emission wavelength range 300–370 nm) are comparable in the analysis of indole derivatives. They are in the range 5–200 pg (on-column; signal-to-noise ratio 2) and vary depending on several factors, including the capacity ratio of each compound [28,47,68,69,75,80–82]. Comparison data for eumelanin-related indole derivatives are lacking, but can be expected to be similar.

However, phenolic indoles are more readily oxidized than non-phenolic compounds making the electrochemical detector somewhat more selective (and sensitive) than the spectrofluorometer in indole class analysis. Detection limits can be strongly influenced when serial electrochemical detection and reaction is applied, in which the first cell on-line with the column pre-oxidizes phenolic compounds and the second cell in series post-oxidizes, as was shown in an excellent recent paper [36]. Dual electrochemical detection therefore may serve both in selectivity and accuray, since it enables the chromatographer to detect (or delete with respect to the following cell) easily oxidized compounds on the first cell and detect high-potential responding compounds and left-overs of the first cell on the second cell. Accuracy may be enhanced by the fact that signal ratios can be calculated for every analyte and compared with data for standard compounds. This

trend, although rather expensive in terms of equipment, is followed in several recent papers [18,24,36,37,41,71,72,83].

Perhaps more relevant, the use of a spectrofluorometer and a variable electrochemical detector in series [28] is comparable with this trend and shows truly that detectors in essence are complementary. Unfortunately, modern coulometric detectors cannot be used downstream of a spectrofluorometer, owing to their intrinsic back-pressure.

4. GAS CHROMATOGRAPHY

4.1. Sample pretreatment

Direct injection methods, such as headspace and gas-phase stripping, do not seem applicable to clinically important indole derivatives in urine. Therefore, sample pretreatment techniques are necessary, and these seem to be confined to solvent extraction techniques, typically the same as those used in HPLC analysis (see Section 3.1).

Like many other compounds of biomedical interest containing polar functional groups, indole derivatives are thermally labile at GC separation temperatures. So derivatization is used to improve their thermal stability and volatility.

For "routine" GC analysis of urinary 5-hydroxyindoleacetic acid, concurrently with catecholamine metabolites, extractions were performed at pH 2–6.5, using ethyl acetate [84,85]. Derivatization (sometimes combined with oximation) is commonly performed using *tert.*-butyldimethylchlorosilane–imidazole [84], N-methyl-N-trimethylsilyl-trifluoroacetamide [86], N,O-bis(trimethylsilyl) trifluoroacetamide [85] or fluoronated agents such as pentafluoropropionic anhydride [87,88], the latter when electron-capture detection is used. Alternative methods, involving tandem MS for the direct analysis of lyophilized urine samples, are being explored [89]. For more detailed information on derivatization, see the excellent compilation made by Poole and Schuette [90].

Melatonin and related compounds have been extracted and derivatized for negative-ion chemical ionization and selected-ion monitoring MS, using pentafluoropropionic anhydride in trifluoroethanol [91] and ethyl acetate [92]. The effect of solvents on derivatization efficiency has been studied [92]. For a comparison of methods, see the review by Namboodiri et al [6]. No (routine) application to urine has been reported.

Eumelanin-related indolic compounds in urine are difficult to study because of their marked tendency to undergo polymerization [93]. Application of GC in the analysis of these compounds requires the preparation of volatile thermostable derivatives, e.g. by using pentafluoropropionic anhydride. During these studies it became clear that, under chromatographic conditions, these derivatives were more stable than the original compounds and so could be more easily analysed.

The analysis of indole derivatives, when incorporated in organic acids profiling, is commonly preceded by extraction with ethyl acetate and/or diethyl ether [86,94] and by derivatization using trimethylsilylating agents.

4.2. Separation and detection

Although packed columns are still widely used, fused-silica capillary columns coated (and chemically bound) with liquid phases such as SE-54, SE-52, CP-SIL 5, SE-30, OV-17 and DB1 are being applied increasingly in the separation of indole derivatives derived from biomedical samples. Routine analysis of 5-hydroxyindoleacetic acid, separated on CP-SIL 5 and detected with flame ionization, has been described [85]. It is tempting to assume that most separations in the field of indole derivatives in urine, including catecholamine metabolites, can be accomplished on WCOT capillary columns coated with relatively non-polar phases (e.g. SE-30 or SE-54, OV-1 or OV-3). Despite the high resolution power of modern GC columns, analysis should be performed on two columns with stationary phases of different polarities (e.g. SE-30 and OV-17) to enhance identification reliability if no mass spectrometer is available.

Flame ionization is most commonly used in the routine analysis of indole derivatives, closely followed by MS (selected-ion monitoring or chemical ionization mode). Since the purpose of this review is mainly to discuss routine portraits of indole analyses, we shall not go into further detail on MS applications because, as stated before, MS does not lend itself to routine serial analysis.

5. SELECTED CLINICAL AND BIOMEDICAL APPLICATIONS

5.1. Carcinoid syndrome

Increased concentrations of indole derivatives, especially 5-hydroxyindoleacetic acid, serotonin and 5-hydroxytryptophan, are found in urine from patients suspected of suffering from carcinoid tumours, which occur most frequently in the small bowel. Primary malignant tumours of the small bowel are relatively rare and show no specific clinical features. Early recognition is desirable, however, owing to the high death rates. In the Western world frequencies are 0.6–1.5 per 100 000 inhabitants [95–97]. These tumours never occur at young age [98], and their frequency is highest at age 50–70 years.

All types of small bowel tumours evoke complaints in the patient such as weight loss, blood loss, intermittent stomach aches, obstructions and general psychic depression, but carcinoid tumours may also provoke cutaneous flushing, diarrhea and cardiac valvular lesions. Only 10% of patients with carcinoid tumours, however, eventually show these symptoms. The name carcinoid has been introduced to differentiate this less aggressive malignant small bowel tumour from the adenocarcinoma, which occurs more frequently. Carcinoids grow submucosally, are less than 20 mm in diameter, and occur in 30% of cases in multiple form. Malignant processes in the small bowel are diagnosed most reliably by enteroclysis.

Carcinoids may occur throughout the gastrointestinal tract, but most frequently in that part originating from the embryonic midgut, between the duodenum and the colon transverse, i.e. jejunum, ileum and cecum. Carcinoids of the appendix are often found during operation by coincidence and are in general non-malignant. Carcinoids of the ileum are usually malignant and metastasize to the

liver. Other carcinoids occur in tissues derived from the embryonic foregut (bronchus, stomach, pancreas) and hindgut (transverse colon, left colon, rectum). Since carcinoids are relatively small tumours, in 70–80% of cases no physical complaints are evoked. If so, complaints may follow recidive obstructions, due to bowel enclosure by events secondary to tumour growth. The symptoms of the carcinoid syndrome may be caused by secretion of several biologically active substances from the tumour, i.e. serotonin, histamine, bradykinin, tachykinins, prostaglandins and dopamine.

Not all malignant carcinoid tumours, however, secrete these substances; for example those arising in the hindgut characteristically do not and some arising in the midgut may first present themselves with massive liver metastases before manifestations become overt. Although most serotonin is converted by the ubiquitous enzyme monoamine oxidase (MAO, EC 1.4.3.4) into 5-hydroxyindoleacetic acid, which is excreted in urine, the pharmacological effects evoked from midgut and foregut carcinoids are caused by (among others) serotonin, circulating outside the blood platelets. However, foregut carcinoids frequently secrete ectopically neuroendocrine polypeptide hormones and 5-hydroxytryptophan, the latter compound because foregut tumours lack the ability to convert it into serotonin. The 5-hydroxytryptophan released is converted in the intestine, the kidney and other tissues partly into serotonin, causing humoral effects; the free compound, serotonin and the main metabolite, 5-hydroxyindoleacetic acid, are excreted in urine.

Midgut carcinoid tumours, by contrast, contain and secrete only serotonin, often after development of extensive liver metastases, which is mainly metabolized to 5-hydroxyindoleacetic acid. These differences between carcinoid tumours have important analytical diagnostic implications: (1) high levels of 5-hydroxytryptophan, next to 5-hydroxyindoleacetic acid in urine provide strong evidence of a carcinoid originating in tissues derived from foregut (bronchus, stomach, pancreas); (2) administration of methyl-DOPA, an inhibitor of non-selective (aromatic) amino acid decarboxylase (EC 4.1.1.28, converting 5-hydroxytryptophan into serotonin), will afford relief of symptoms in patients with 5-hydroxytryptophan-secreting carcinoids, since the mediator of manifestations, serotonin, is diminished in concentration.

By contrast, methyl-DOPA will not inhibit formation of serotonin within the midgut carcinoid tumours, and is not effective in the relief of symptoms there. For detailed information on the studies and reviews of the carcinoid syndrome, see refs. 99–102.

In patients with less pronounced disease, there may be considerable clinical difficulty in differentiating carcinoid syndrome from other disorders. Biochemical techniques are required to characterize the disease process. The assay of 5-hydroxyindoleacetic acid is probably the most frequently used procedure for diagnosis of carcinoid syndrome, but analysis of 5-hydroxytryptophan and serotonin in urine may help to differentiate carcinoids of foregut and midgut origin.

It can be of interest for a clinical chemist that other metabolites have also been identified in urine of patients with carcinoids. These include 5-hydroxyindoleaceturic acid (the glycine conjugate of 5-hydroxyindoleacetic acid), N-acetyl-

serotonin, conjugated with glucuronic acid, and small amounts of oxidation products of indican (3-indoxyl sulphate) nature.

From a biochemical point of view, a carcinoid may be considered to be an abnormality in tryptophan metabolism in which a greater proportion of tryptophan than normal is metabolized by the hydroxyindole pathway. This metabolic diversion markedly reduces the production of nicotinic acid (and so NAD, NADP and their congeners) as well from tryptophan. Consequently, symptoms of pellagra as well as negative nitrogen balance may be observed in patients suffering from carcinoid tumours. The extensive study of Feldman [103] recently showed, however, that it may be sufficient to measure only the serotonin excretion in the urine of patients with carcinoids. Moreover, Feldman and Lee [21] showed that the excretion of serotonin in healthy subjects was not influenced by the ingestion of serotonin-rich foods, of which the serotonin content had been determined. Abnormal liver function should be excluded in the diagnosis.

5.2. Malignant melanoma, pigmentation disorders and gallstone formation

It is well known that some patients in a far advanced state of malignant melanoma excreted compounds that on exposure to air blacken urine by secondary formation of melanin. Attempts to elucidate the structures of these precursors of melanin (urinary melanogens) have been numerous, but not all conclusive. Considerable recent progress has been made, however, with more sophisticated chromatographic techniques. The diagnostic value of profiling melanin-related indolic compounds in (melanotic) urine appears to be limited by the fact that in most cases patients with primary tumours or local metastases excrete normal amounts of these urinary metabolites. It is very probable that at least some of the indolic melanin-related compounds are excreted in bile. This possibility, experimentally confirmed in hamsters [104] led to the formulation of a new hypothesis on possible pathogenesis of some gallstones [15].

Although the group of eumelanin-related indolic substances consists of seven metabolites (see Section 1), not all of them seem to be suitable for routine laboratory determination. Experience has shown that one of these compounds, 5,6DHI2C, is excreted in unprotected, i.e. unconjugated, form, as are other indolecarboxylic acids. Owing to its instability this compound may initiate secondary formation of melanin in melanotic urine during collection and storage. That is why measurements of this substance in urine may lead to unreliable results. However, the same holds true for 5,6DHI, a melanin precursor that is known to be excreted as its sulphate conjugate [105]. Its determination is hampered by several difficulties, such as (1) partial oxidation and polymerization after hydrolysis, (2) instability of natural and deuterium-labelled 5,6DHI during storage and (3) by a small difference in the masses of the dideuterated and natural analogues, which causes a non-linear calibration curve owing to overlap of masses.

Another compound, 5,6DMI2C, seems not to be useful for routine determination either. This recently discovered metabolite [106] was found in normal urine in very low, sometimes undetectable amounts, and the differences in excreted amounts between normal and melanotic samples were always very small [93].

The presence of 5,6DMI2C in melanotic urine is an interesting phenomenon. Analogous to the isomeric compounds 5H6MI2C and 6H5MI2C, one can presume that formation of O-methylated indoles results from a very efficient methylating activity of catechol-O-methyltransferase (COMT, EC 2.1.1.6), which detoxifies reactive *ortho*-dihydroxyindole compounds and protects the cell against self-destruction. Inhibition of O-methylation could therefore lead to destruction of the pigment cell. However, O-methylated indoles are more suitable for routine measurements than their non-methylated precursors. In particular, 5-hydroxy-6-methoxyindole exhibited the largest differences between pre- and post-therapeutic stadia in melanoma patients. This compound was found in melanotic urines in considerably higher amounts than its isomer 6-hydroxy-5-methoxyindole, a fact which, together with similar results from measurements in melanoma cell cultures [107], gave indirect evidence that 5,6DHI is preferentially methylated at the 6-position.

On the other hand, excretions of 5H6MI2C and its isomer 6H5MI2C run parallel. It seems ,therefore, sufficient to measure one of these compounds. In a recent study [108], the urinary excretion of 5H6MI2C, together with 5H6MI, 5-S-cysteinyl-DOPA and DOPA, was determined in people with different capacities to produce melanin, i.e. persons with black skin, white skin, vitiligo and albinism. The results show that the excretion of the two indolic substances corresponds very well with the degree of pigmentation, being particularly true of 5H6MI2C. It was concluded that of all the measured melanin-related compounds, the indolic metabolites appear to be the best markers of skin pigmentation.

To summarize, malignant melanoma, pigmentation disorders and possibly gallstone formation can be most promisingly followed by determination of two indolic melanin-precursors, 5H6MI2C and 5H6MI, the normal urinary excretion values of which still have to be established more accurately. Low melanogenic activity of tumour cells and the existence of alternative modes of excretion (e.g. bile) should be taken into consideration.

5.3. Hartnup disorder, tryptophanuria, intestinal flora overgrowth and tryptophan malabsorption

Congenital defects in tryptophan metabolism are relatively rare. Hartnup disorder is a hereditary transport defect of a group of amino-monocarboxylic acids (not small peptides) in kidney and small bowel. Of paramount diagnostic value is the increased urinary excretion of neutral amino acids, including tryptophan. It has been reported that patients excrete variable increased amounts of intestinally well absorbed compounds (up to 38 different entities), including indoxyl-sulphate (indican), indoxyl-β-glucuronide, indoleacetic acid, indoleacetic-L-glutamine, indoleacetylglucuronide, indolepropionic acid, indoleacryloylglycine, indoleacetamide and indoleacrylic acid. Compounds originate from oxidation and intestinal bacterial metabolism of malabsorbed tryptophan, as deduced from findings after intravenous tryptophan loads and oral antibiotics medication. The intestinal absorption defect of tryptophan can be demonstrated by oral loads of the amino acid and monitoring of plasma tryptophan levels. Affected patients do

not always show the symptoms, consisting of dermatological, neurological and psychological signs. Hartnup disease should be differentiated from true pellagra.

Increased excretion of indoles is sometimes seen in patients with severe malabsorption of tryptophan, unusual flora or the blind loop syndrome, but these patients do not have a renal transport defect as seen in Hartnup disorder. These and other disorders of amino acid metabolism have been extensively described by Bremer et al. [109].

Patients have been described with abnormal tryptophan catabolism, as shown by marked hypertryptophanuria, increased excretion of indoleacetic acid, indolelactic acid, 5-hydroxyindoleacetic acid and indolepyruvic acids, the latter not caused by intestinal bacterial action. These facts point at an enzymatic catabolism defect of tryptophan, possibly in the intestinal conversion of tryptophan into kynurenin. The consequence is a much higher conversion of tryptophan via serotonin and an increase in the amount of 5-hydroxyindoleacetic acid excreted in urine [86].

5.4. Applications of HPLC analysis

Systematic screening of the urinary excretion of 5-hydroxyindoleacetic acid, concomitantly with serotonin and 5-hydroxytryptophan, in supporting the clinical diagnosis of carcinoid tumours of the small bowel, calls for reliable and automated techniques. In 1986, Baars et al. [42] compared the HPLC analysis of 5-hydroxyindoleacetic acid (according to ref. 45) with colorimetric assays. Typical chromatograms and relevant data for the direct injection technique are given in Fig. 6. Baseline separation between 5-hydroxyindoleacetic and other urinary peaks of various concentrations was feasible. Accuracy was checked by comparing data obtained from the direct injection technique with those obtained after injecting reconstituted acidic ether extracts [42]. In accordance with the method of Skrinska and Hahn [45], we employed sodium acetate buffer (pH 4.5–4.7) and methanol in order to quantitate 5-hydroxyindoleacetic acid using a spectrofluorometer (excitation wavelength 295–305 nm; emission wavelength 345–355 nm) at low sensitivity settings. The clinically important compounds 5-hydroxytryptophan and serotonin eluted, at the pH range used, in front of 5-hydroxyindoleacetic acid on two reversed-phase packings, and also, in accordance with data presented in the reference paper [45], on another packing. The method was applied routinely for more than four years without serious problems or interferences. Elrod et al. [46] injected diluted urine samples, using monochloroacetic acid (pH 3) as a buffer and applying electrochemical detection, and also showed that accurate quantitation of 5-hydroxyindoleacetic acid is feasible at this low pH.

However, in view of the proposal that screening for increased excretion of serotonin (and 5-hydroxytryptophan) might be more relevant and less influenced by diet, and also enables differentiation between carcinoid tumours (see Section 5.1 for details), we re-evaluated the method. In the profiling mode, quantitation of 5-hydroxyindoleacetic acid remained unaffected and qualitative information on serotonin and 5-hydroxytryptophan becomes more apparent when the eluent

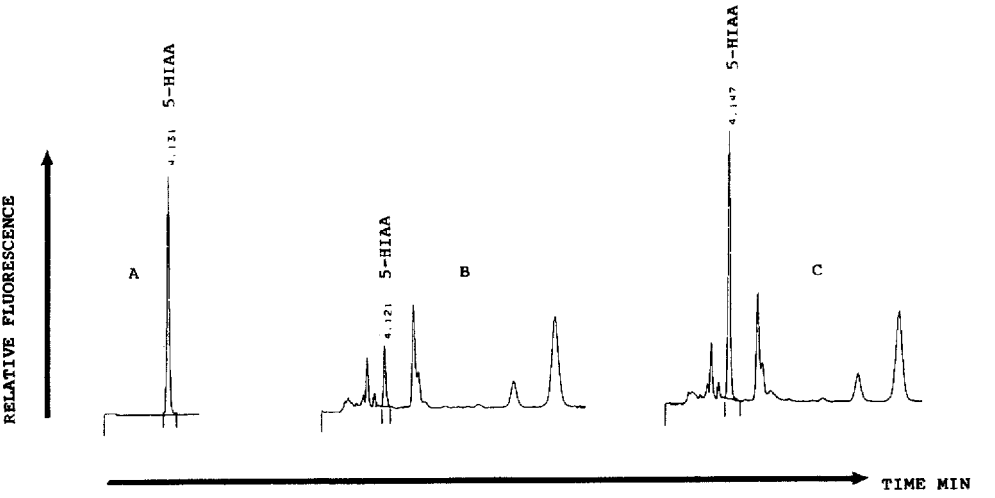

Fig. 6. Chromatograms of a 5-HIAA standard (50 μM) (A), a negative patient urine (B) and the same patient sample spiked with 45 μM 5-HIAA (C). Conditions: column, Hypersil 5C18 (Shandon, Zeist, The Netherlands, 150 mm×4.6 mm I.D.); mobile phase, 0.01 M sodium acetate buffer (pH 4.7)–methanol (90:10, v/v); flow-rate, 2 ml/min; excitation wavelength, 295–305 nm; emission wavelength, 345–355 nm. Aliquots of 20 μl were automatically injected from a 250-μl loop. (Contributed by Van Haard and used in ref. 42.)

is altered to contain ammonium acetate and the pH is changed to 5.5, leaving the methanol concentration in the range 5–10% (v/v). As shown in Fig. 7, serotonin now elutes after 5-hydroxyindoleacetic acid, making it feasible to screen for elevated concentrations of this compound, provided that the detector sensitivity is increased by a factor of 4. Although 5-hydroxytryptophan (see Fig. 7) is not baseline-separated from other peaks, we found that the selectivity was maintained between this compound and near-eluting peaks during routine analysis of patient samples. Another question bothering us was whether it is feasible to frame the whole profile within 10 min without using a gradient. We explored the use of a methanol gradient but were not able to profile the three target compounds within 10 min and also re-equilibrate the system.

A challenging technique was published recently by Kodama et al. [110], who injected highly eluotropic methanol or (better) acetonitrile after the peak(s) of interest in order to clean the column. Since we employ constant-loop (250 μl) automated samplers in our routine reversed-phase systems, but are able to inject various volumes (up to 250 μl) into the column, we combined the technique of Kodama et al. [110] with the updated assay described before. As Fig. 8 shows, non-diagnostic peaks eluting behind serotonin are "blown away" by injecting acetonitrile (run time 5 min) as soon as 5-hydroxyindoleacetic acid (retention time 5.1 min) has been eluted. The autosampler takes another 1 min, roughly, to inject the acetonitrile. The baseline is stable beyond 10 min and remains virtually "clean" for up to 60 min, as tested.

Pretreated urine samples (see Section 3.1) can be incorporated into the assay to investigate questions on recoveries, routine sensitivity and quality control on

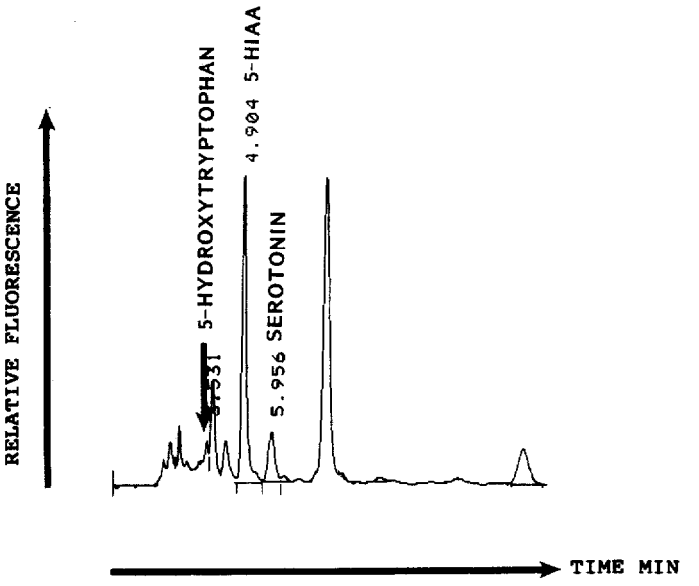

Fig. 7. Chromatogram of a negative patient urine, spiked with 5 μM serotonin. Conditions: column, Hypersil 5C18 (250 mm×4.6 mm I.D.); mobile phase, 0.1 M ammonium acetate buffer (pH 5.5)–methanol (90:10, v/v); flow-rate, 1.5 ml/min. For further details see legend to Fig. 6. (Contributed by Van Haard, unpublished data.)

single compounds. If concurrent screening for tryptophan and related indole derivatives is needed, e.g. in Hartnup disorder, tryptophan malabsorption and other rare diseases (see Section 5.3), the proposed direct injection technique, omitting the "blow", may be applicable too, but since disturbances in amino acid metabolism are more commonly screened first by amino acid analysis (e.g. according to refs. 65 and 111) or GC profiling, we do not make use of it that way.

Screening urine samples in order to differentiate chronic and acute renal failure seems to be feasible too, using ammonium acetate–methanol as the eluent and a spectrofluorometer (emission wavelength 395–415 nm) for detection, as reported for serum [112]. Indican (3-indoxyl sulphate), a tentative marker for chronic renal disease, should be converted into 3-hydroxyindole before analysis, since the latter compound shows enhanced retention compared with indican, making its quantitation more reliable. This application should be further explored, since it enlarges the application field of the simple chromatographic system used for analysis of indole derivatives in urine. Hartnup disease and tryptophan malabsorption [86], however, should be excluded.

The use of gradient elution for rapid selection of isocratic conditions (e.g. according to ref. 113), is recommended for the routine clinical laboratory equipped with a gradient HPLC system. Laboratories employing isocratic systems, unfortunately, must try to obtain optimal isocratic conditions by time-consuming "trial-and-error" procedures. However, isocratic elution is ultimately more reliable in routine laboratory performance.

The profiling of melanin-related indole derivatives in urine with the use of

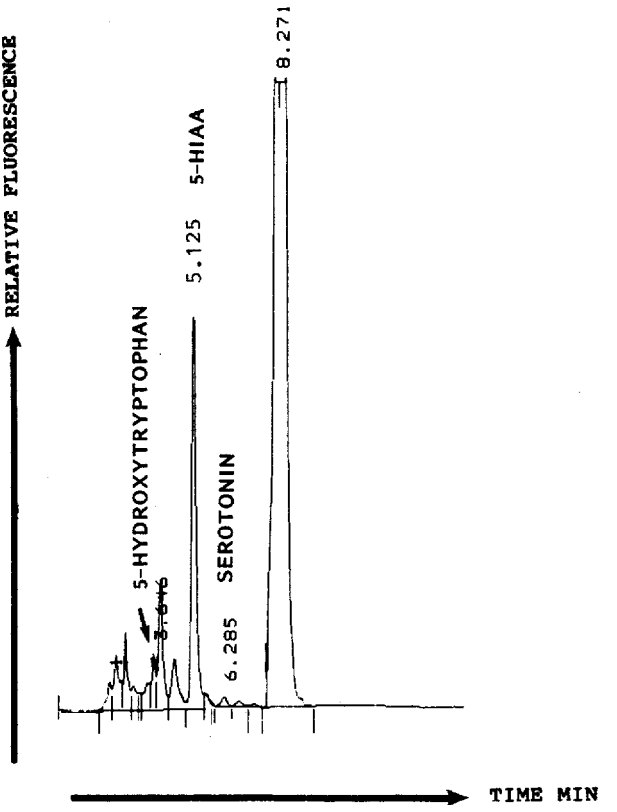

Fig. 8. Chromatogram of negative patient sample, showing combination of isocratic analysis according to the conditions described in Fig. 7 with injection of acetonitrile at 5 min, according to the technique proposed by Kodoma et al. [110]. Aliquots of 200 μl of acetonitrile were injected to elute any retarded components. (Contributed by Van Haard, unpublished data.)

HPLC was described in 1986 [29]. This method employed reversed-phase chromatography with sodium acetate buffer (pH 4.3), methanol gradient elution and spectrofluorometric detection (excitation wavelength 290–320 nm; emission wavelength 345–375 nm). *Helix pomatia* juice was used to deconjugate the indole derivatives. Compounds were extracted (pH > 5) with diethyl ether before reconstitution in starting buffer solution and injection into the column. In normal unhydrolysed urine the isomeric compounds 5H6MI2C and 6H5MI2C are always found (Fig. 9). After enzymatic hydrolysis a few, originally conjugated compounds, e.g. 5H6MI and 6H5MI, appear in the chromatogram. Melanotic urine profiles provide only quantitatively different pictures, as can be seen in Fig. 10. Instabilities of other compounds that are sometimes found make their quantitation unreliable. The figures show that it is feasible to measure 5-hydroxyindoleacetic acid simultaneously in samples not treated with *Helix pomatia* juice, as was confirmed with MS [29]. In fact, the method is a gradient variant of the one designed by Skrinska and Hahn [45], which has been routinely applied by one of us [42]. However, extraction with diethyl ether is a prerequisite to avoid in-

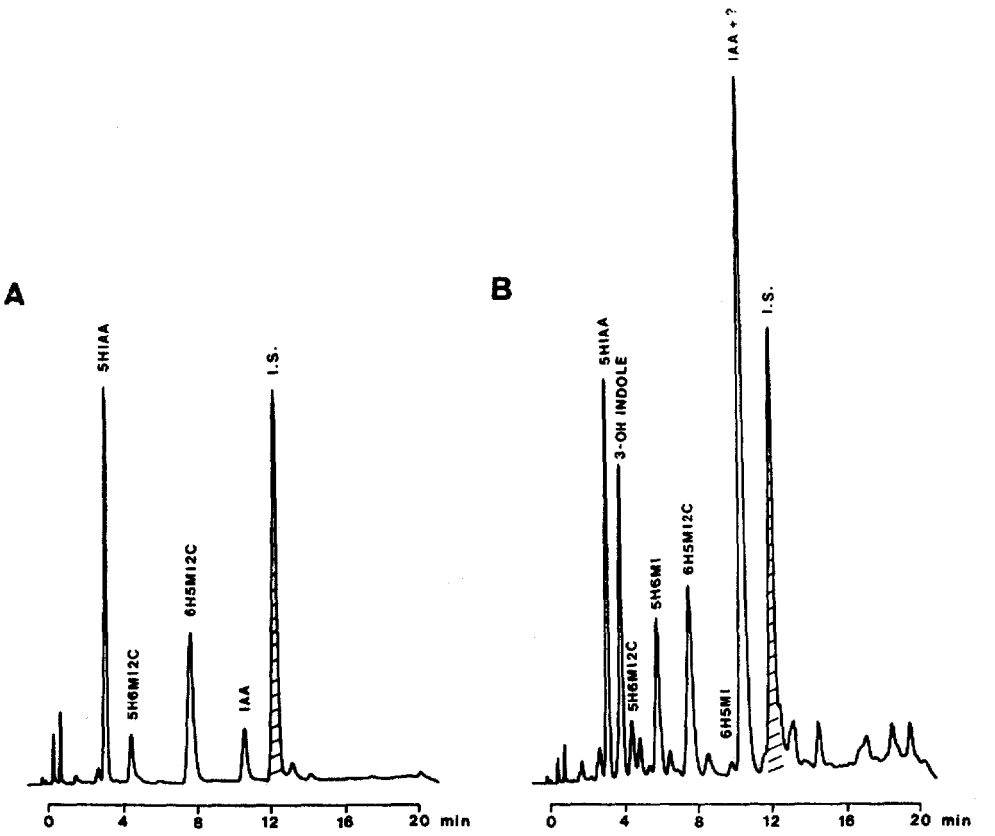

Fig. 9. Typical chromatogrm of a diethyl ether extract of normal urine without (A) and with (B) enzymatic hydrolysis of glucuronic and sulphuric acid conjugates. Conditions: column, Spherisorb 5 ODS (100 mm×4.6 mm I.D.); guard column, Brownlee (30 mm×4.6 mm I.D.); mobile phase, 0.05 *M* sodium acetate buffer (pH 4.3)–methanol (85:15, v/v), containing ascorbic acid and EDTA isocratically for 5 min, followed by linear gradient up to 40% (v/v) methanol for 15 min; flow-rate, 1.5 ml/min; excitation wavelength, 290–320 nm; emission wavelength, 345–375 nm. (Taken from ref. 29, with permission.)

terferences in the estimation of total eumelanin-related indole derivatives. The method [29] makes it possible to separate all eumelanin-related indole derivatives in less than 30 min (Figs. 9 and 10). Since HPLC measures the free indolic compounds, strict precautions have to be taken to prevent losses of compounds during their processing and so to make the method sufficiently reliable. As well as the GC–MS method [93], the HPLC technique may find application in studies concerning melanin pigmentation, malignant melanoma and, to a lesser extent, in screening for gallstone formation.

The increased catecholamine formation in neural crest tumours, such as neuroblastoma, pheochromocytoma and ganglioneuronoma, is accompanied by increased catabolism, in which the end-products homovanillic acid (HVA), vanillylmandelic acid (VMA) and others are excreted in urine. Since clinical symptoms arising from the presence of such tumours partly resemble those oc-

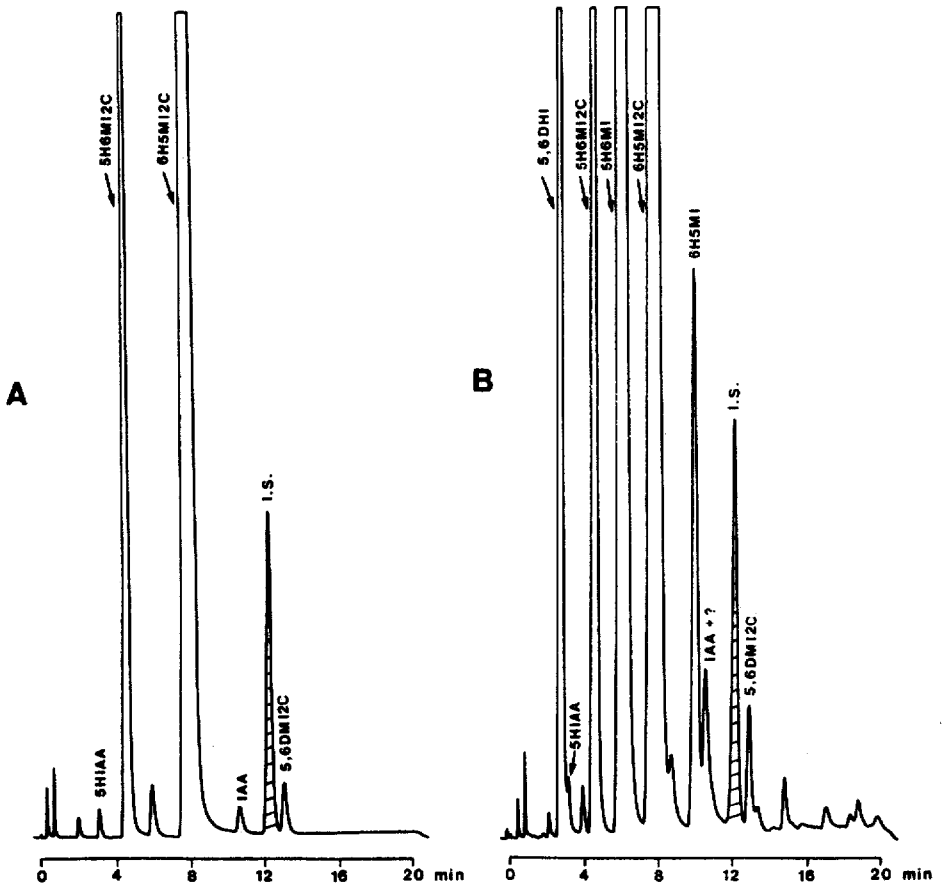

Fig. 10. Chromatogram of a diethyl ether extract of a ten times diluted urine sample of a melanoma patient with liver metastases, without (A) and with (B) hydrolysis of conjugates. For experimental conditions see legend to Fig. 9. (Taken from ref. 29, with permission.)

curring in patients suffering from carcinoid tumours (this review), it is worth simultaneously determining indole derivatives (e.g. 5-hydroxyindoleacetic acid) and the catecholamine metabolites HVA and VMA in urine. The three compounds are, to a major extent, excreted in non-conjugated form. Few methods have been published for the profiling of these clinically important compounds, and recent ones make use of direct injection of urine [30,43]. In a recent study [24], we compared direct injection techniques that involve gradient elution and spectrofluorometry [30,43] with one that requires linear gradient elution and serial coulometric electrochemical detection [24]. The method of Van Haard et al. [24] combines the feasibilities of the method designed by Saller and Salama [71] with those of the method described by Holly and Patel [114]. As shown in Fig. 11, it is possible to quantitate 5-hydroxyindoleacetic acid after direct injection of urine using electrochemical detection at a low potential, concomitantly with VMA and HVA at higher potential (down-stream cell) fully automated within 60 min. The operational system (Fig. 11) incorporates a linear gradient ap-

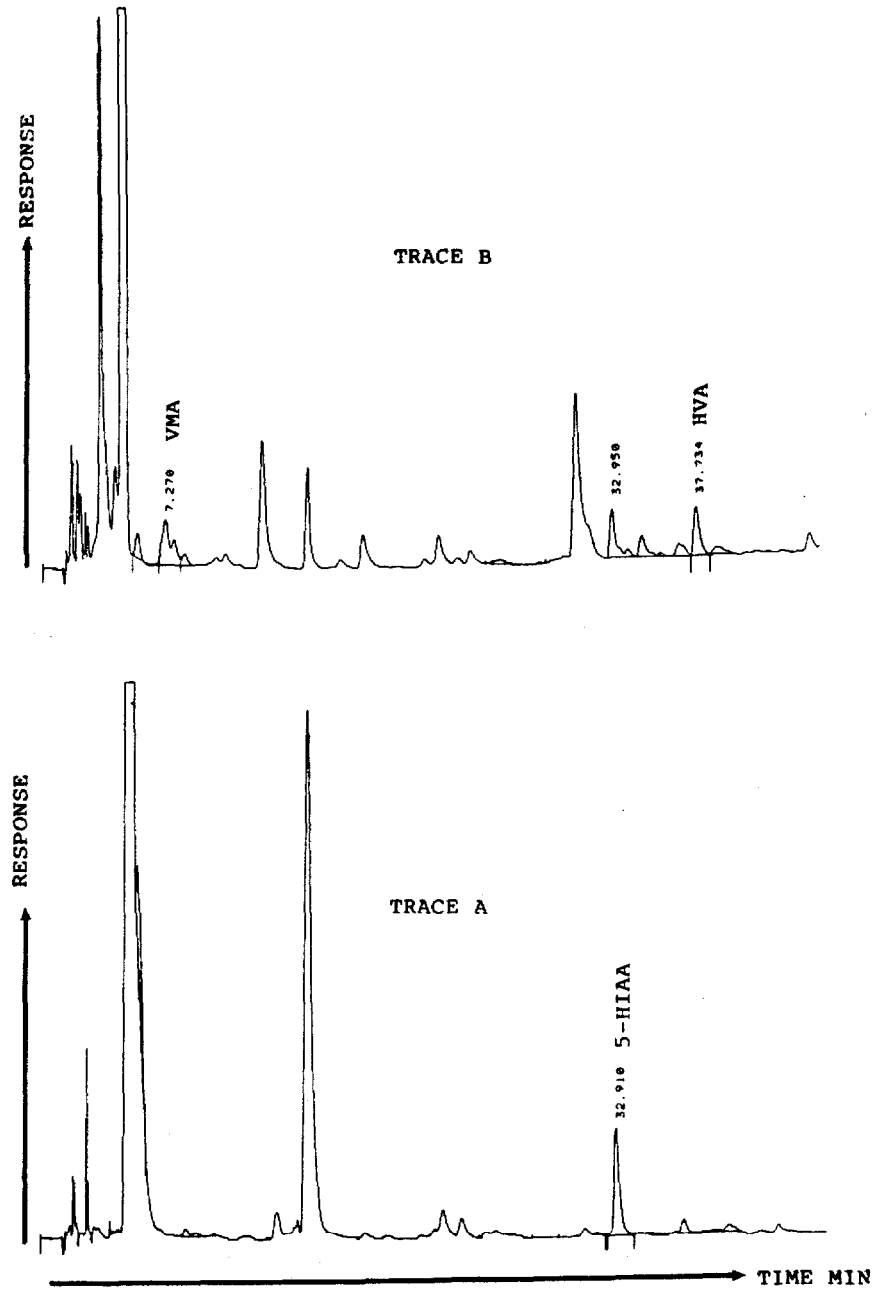

Fig. 11. Chromatogram of a negative patient sample, directly injected on a reversed-phase column (Hypersil 5C18, 250 mm × 4.6 mm I.D.). Detection was performed using a serial coulometric electrochemical detetor (ESA Coulochem, cell type 5010). The first cell on-line with the column was operated at +0.25 V (trace A) and the second cell in series at +0.55 V (trace B). Gradient elution was performed using 0.01 *M* citric acid and 0.05 *M* disodium hydrogenphosphate (pH 2.2) containing 0.005 *M* heptanesulphonate isocratically for 5 min; linear gradient started at 5 min up to 25% methanol (v/v) till 50 min. Next injection feasible after 60 min. Flow-rate, 1.5 ml/min. (Contributed by Van Haard and used in comparison report ref. 24.)

proach, based on the pK_a values of VMA, 5-hydroxyindole-3-acetic acid (5-HIAA) and HVA. As a result VMA is eluted at pH 2.2, whereas 5-HIAA and HVA are eluted near pH 5 in the presence of methanol. The ion-pairing agent added serves as a retarding agent for amines, which would otherwise interfere with fast-eluting VMA (among other effects, empirically established). The use of two electrochemical cells (in coulometric mode) enables the chromatographer to check for peak purities. The method has been in routine use for more than two years now, without any problems as judged from quality assessment programs and drug interference check. Regularly exchanging the graphite filter in front of the cells, and cleaning the cells by eluting acids, organic solvents and bases in order to remove remnants from the cell electrode, enhances routine use up to at least thirty injections, one day per week for at least four months.

A simple, routine-compatible procedure for separation and detection of tetrahydro-β-carbolines and their indolic precursors, based on aqueous derivatization (protection) with methylchloroformate has been described [16], but without application to urine samples. A more extensive study on the separation of indolealkylamines and their analogous tetrahydro-β-carbolines has been reported, involving the use of reversed-phase and strong cation-exchange columns in combination with fluorescence detection [75]. Routine application of these methods awaits further study and clinical relevances [16,75].

Melatonin has been quantitated in urine after sample pretreatment [8], through the application of hydrophobic-interaction chromatography and spectrofluorometry. Findings for related compounds in urine (e.g. 5-methoxytryptophol and tryptophol) are still tentative or not reliable owing to detection limits. Routine application awaits further studies and clinical relevances [6–8,68].

5.5. Applications of GC analysis

There have been more than 500 organic acids (other than amino and fatty acids) detected in urine. Catecholamine and indole derivatives add a relatively small group of compounds to this number of urinary components. Although HPLC and TLC are used to some extent, only high-resolution GC really meets the demands for separation of such a large number of compounds. This technique, combined with MS, remains the accepted standard and most widely used method for quantitation and identification of urinary compounds, including indole derivatives. As such, the application of GC to biomedical problems in the field of indole derivatives in urine is confined to validation of other (chromatographic) methods, and on a routine basis seems to be employed (only) for the analysis of 5-hydroxyindoleacetic acid [84,85]. Specificially, congenital defects of tryptophan metabolism have been studied using GC–MS [86], showing the complexity of such screening programmes. Melatonin and related compounds have not been studied in urine [6,91,92] with GC alone. Direct analysis of lyophilized urine samples by means of tandem MS is being explored [86]. Progress has been made in recent years, in the use of GC (also combined with MS) in the field of eumelanin-related indole derivatives in urine.

The use of GC–MS has led to the identification of the entire class of indolic

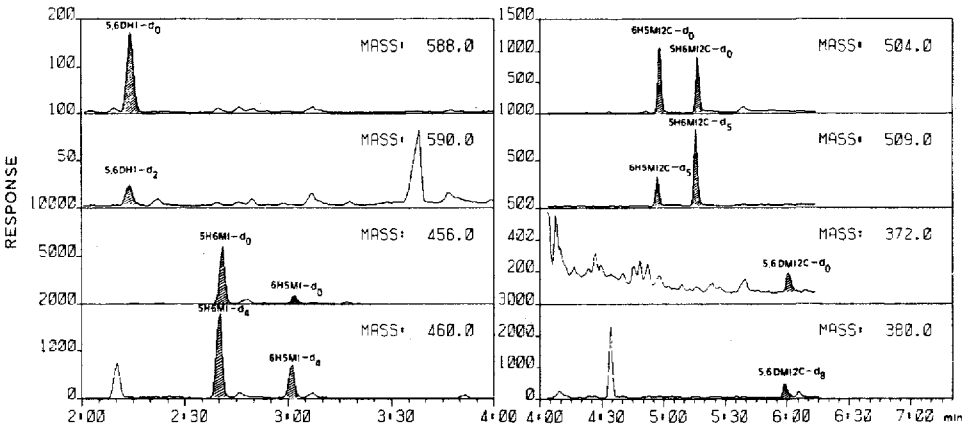

Fig. 12. Mass fragmentograms obtained from a derivatized extract of melanotic urine to which deuterated analogues were added as internal standards. Mass spectrometry was performed in chemical ionization mode. Abbreviations: 5,6DHI-d_0 and -d_2 = natural and dideuterated 5,6-dihydroxyindole; 5H6MI-d_0 and -d_4 = natural and tetradeuterated 5-hydroxy-6-methoxyindole; 6H5MI-d_0 and -d_2 = natural and dideuterated 6-hydroxy-5-methoxyindole; 6H5MI2C-d_0 and -d_5 = natural and pentadeuterated 6-hydroxy-5-methoxyindole-2-carboxylic acid; 5H6MI2C-d_0 and -d_5 = natural and pentadeuterated 5-hydroxy-6-methoxyindole-2-carboxylic acid; 5,6DMI2C-d_0 and -d_8 = natural and octadeuterated 5,6-dimethoxyindole-2-carboxylic acid. (Taken from ref. 93, with permission.)

eumelanin-related substances, which was shown to consist of seven compounds (see Fig. 4). Investigations during the past few years led at length to the successful clarification of the structure, the extent and steric preference of conjugation [105,115,116]. GC has also made possible the measurement of the whole profile of all reported indolic compounds in melanotic urines. It failed, however, to detect the substances in urine samples of healthy persons, owing to the low concentrations and peak interferences with other compounds. GC–MS increased the selectivity of the measurements of the indolic compounds extracted from urine (see Section 3.1), and also made the measurement of normal values possible. A GC–MS method in chemical ionization mode was used for a follow-up of the urinary excretion of eumelanin-related indolic compounds in melanoma patients (see Section 5.2.) before, during and after cytostatic treatment [93]. Deuterium-labelled analogues [117] were used as internal standards (Fig. 12). In several cases, a clear decrease of the excretion of the measured indolic substances was recorded after the treatment. However, the diagnostic value of these determinations appears to be limited by the fact that, in most cases, the patients with a primary tumour or local metastases excreted normal amounts of those urinary metabolites.

6. ADVANTAGES AND LIMITATIONS

Since there is a lack of data on planar techniques, it seems at present that the majority of chromatographic analyses involving indole derivatives in urine are carried out by reversed-phase HPLC and include direct injections or laborious pretreatment techniques (including amino acid analyses, which are not reviewed

here). Direct injections of urine are suitable for screening tests, mostly in combination with spectrofluorometry. The use of filter fluorometers is not recommended for direct analyses. When fine-tuned simple eluent compositions, such as ammonium acetate–methanol, are used for chromatography, direct injection techniques are also suitable for multi-component analyses. However, to shorten analysis times, gradient elution is often needed, even after sample pretreatment.

The best strategy for the determination of indole derivatives in urine would be to shorten the time involved in sample pretreatment and chromatography. Promising in sample pretreatment is the satisfactory and relatively cheap application of C_{18} cartridges, used for the removal of fast- and late-eluting components in the ultimate separation model. Time-consuming, however, remains the concurrent concentrating step, e.g. evaporation of solvent and reconstitution of extracts. The on-line enrichment techniques reported in the literature are challenging, but are not suitable for most of the chromatography-oriented routine clinical laboratories equipped with few HPLC systems. Flexibility is low when complex pre-column techniques have to be switched on and off using a single multi-purpose HPLC system, and when relatively small numbers of samples have to be analysed each week.

Promising also seems to be the injection of highly eluotropic solvents after elution of peaks of interest [110], as reported for the direct analysis of 5-hydroxyindoleacetic acid in urine (tentatively, concurrently with serotonin). Whether automated samplers can be programmed to inject very small volumes of urine samples, followed by (variably) large volumes of highly eluotropic solvents, remains to be seen. Ternary HPLC systems have, in our routine experience, the advantage that, beyond the gradient and mixing facilities, automated column clean-up and flow reduction can be programmed in order to maintain a 24-h routine clinical service in serial analyses.

The spectrofluorometer is best suited for attacking the various clinical questions on indole derivatives (and even catecholamines [63]) in urine, both in view of routine reliability and sensitivity assessment and overall selectivity. The attendant costs are limited to those for new excitation sources. However, electrochemical detection (especially serial coulometry) is, when used and viewed as a reactor array, very promising in profiling urinary compounds that simultaneously relate to different clinical questions and are excreted to different dynamic extents. By fine-tuning the reactor array, it is possible to concurrently screen (or quantitate) various clinical markers with comparable detection limits and linear dynamic ranges. The disadvantage of flow-through solid-state serial coulometric detectors at first sight is the relatively high costs of cell replacement. However, costs should be compared with those involved with the time-consuming frequent replacement and cleaning of amperometric electrodes, and the costs of replacement of modern light sources for spectrophotometers and spectrofluorometers.

More information is needed on the routine analysis of eumelanin-related indoles, β-carbolines and melatonin-related indoles, in order to discuss the advantages and disadvantages of the (now) separate assays.

The significance of high-resolution techniques for the analysis of urinary constituents has been well recognized. GC profiling and concomitant MS analysis

have been particularly useful for the characterization of inborn errors of metabolism. It would also be desirable to obtain further identification of peaks of interest by tandem operation of the mass spectrometer with the liquid chromatograph. Despite limitations, the higher speed of analysis makes HPLC more useful in the clinical laboratory than GC. Criticisms of the routine GC investigation of urinary organic acids have been expressed [94]. To date, much of the work on organic acids, phenolic compounds and indole derivatives has been performed in a few highly specialized centres.

Although it may be desirable to have more locally based services to cope with clinical demands and to reduce the delay in obtaining results, the GC technique of analysing organic acids and indole derivatives in urine remains time-consuming and highly specialized. Therefore, the routine analysis of indole derivatives is at present confined chiefly to 5-hydroxyindoleacetic acid, which is determined concurrently but not optimally with organic acids, including catecholamine metabolites. Moreover, in order to cope with the complex problems it is apparent that high-resolution capillary GC should be used and, even then the analyses should be performed with two stationary phases of different polarity to enhance identification. Interpretation of chromatograms can still be complicated by interferences from drugs and other artefacts, and thus requires much skill and experience. Many would suggest that metabolic profiling of urine using GC should not be done without MS, or at least access to a mass spectrometer.

GC seems to be more suitable for the analyses of organic acids and catecholamine metabolites than for the profiling of indole derivatives in urine and will remain so, since the lengthy pretreatment necessary to make compounds more volatile and specifically derivatized cannot be omitted. On the other hand, GC equipment does not need such a lengthy start-up procedure as HPLC.

GC–MS is clearly the most attractive method for the validation of chromatographic procedures. However, it does not lend itself to the routine analysis of large numbers of samples that relate to major clinical questions on indole derivatives.

Comparing the individual separation procedures involved with urinary indole derivatives, it seems clear that direct injection techniques in HPLC are most advantageous for the routine laboratory, followed by solid-phase extraction-preceded techniques.

7. SUMMARY

Latest strategies are discussed for the routine chromatographic analysis of clinically important indole derivatives in urine. Analysis of 5-hydroxyindoleacetic acid and, perhaps more importantly, serotonin and 5-hydroxytryptophan remains attractive in the screening for carcinoid tumours and their differentiation.

Analyses of two precursors of the skin pigment eumelanin seem to be promising in the monitoring of treatment of malignant melanoma and screening for pigmentation disorders and gallstone formation.

Studies on the clinical relevance of the determination of tetrahydro-β-carbolines and melatonin-related indoles await routine application of chromatographic

methods designed to take into consideration the relative instability of these compounds.

Application of GC–MS, although confined to larger and/or governmental laboratories remains attractive as a way of improving the specificity of analyses and in establishing reference methods. As for HPLC, the recent development of chromatographic and detection methods for the concurrent determination of different clinically important and metabolically related compounds from the same sample, preferably by direct injection techniques, seems to be fruitful and should be continued.

REFERENCES

1 J.B. Jepson, in J.B. Stanbury, J.B. Wijngaarden and D.S. Frederickson (Editors), The Metabolic Basis of Inherited Disease, McGraw Hill, New York, 4th ed., 1978, p. 1567.
2 D.M. Kuhn, W.A. Wolf and W. Lovenberg, Hypertension, 2 (1980) 243.
3 W.F. Byerley, L.L. Judd, F.W. Reimherr and B.I. Grosser, J. Clin. Psychopharmacol., 7 (1987) 127.
4 A. Kojima-Sudo, Industrial Health, 15 (1977) 109.
5 P. Pevet, in J. Axelrod, F. Fraschini and G.P. Velo (Editors), The Pineal Gland and Its Endocrine Role, Plenum, London, 1983, p. 331.
6 M.A.A. Namboodiri, D. Sugden, I.N. Mefford and D.C. Klein, in S. Parvez, T. Nagatsu, I. Nagatsu and H. Parvez (Editors), Melatonin and Related Indole Compounds, Elsevier, Amsterdam, 1983, Ch. 24, p. 549.
7 S.P. Markey, S. Higa, M. Shih, D.N. Danforth and L. Tamarkin, Clin. Chim. Acta, 150 (1985) 221.
8 M.H. Mills, M.G. King, N.G. Keats and R.A. MacDonald, J. Chromatogr., 377 (1986) 350.
9 K. Thoma and M. Struve, Pharm. Acta Helv., 61 (1986) 2.
10 H.S. Mason, J. Biol. Chem., 172 (1948) 83.
11 G.A. Swan, Progr. Chem. Natur. Org. Prod., 31 (1974) 521.
12 H.S. Raper, J. Biol. Chem., 21 (1927) 89.
13 J. Duchon and B. Matous, Clin. Chim. Acta, 16 (1967) 397.
14 S. Pavel, F.A.J. Muskiet, A. Budesinska and J. Duchon, Tumori, 67 (19981) 325.
15 S. Pavel, Med. Hypotheses, 14 (1984) 285.
16 T.R. Bosin and C.A. Jarvis, J. Chromatogr., 341 (1985) 287.
17 W.R. McLeod, Acta Psych. Scand., (1985) 447.
18 W. Matson, P. Langlais, L. Volicer, P.H. Gamache, E. Bird and K.A. Mark, Clin. Chem., 30 (1984) 1477.
19 D.P. van Kammen, Biol. Psychiatry, 22 (1987) 1.
20 B.G. Wolthers, F.A.J. Muskiet, S. Pavel, J.J. Keyzer, B.J. Koopman, G.A. van den Berg, L.D. Dikkeschei, R. Berger, D.J. Reyngoud, T.E. Chapman, H.J. Derks, E.P. Kraan, R.J. Vonk and A.W. Teelken, Tijdschr. NVKC, 12 (1987) 137 (in English).
21 J.M. Feldman and E.M. Lee, Am. J. Clin. Nutr., 42 (1985) 639.
22 N. Verbiese-Genard, M. Hanocq, C. Alvoet and L. Molle, Anal. Biochem., 134 (1983) 170.
23 A. Jacubovic, D. Fu and H.C. Fibiger, J. Pharmacol. Methods, 17 (1987) 1.
24 P.M.M. van Haard, J.P.M. Wielders and J.B.W. Wikkerink, Biomed. Chromatogr., 2 (1987) in press.
25 N. Fornstedt, Anal. Chem., 50 (1978) 1342.
26 E. Grushka, E.J. Kikta, Jr. and E.W. Naylor, J. Chromatogr., 143 (1977) 51.
27 S.M. Lasley, I.A. Michaelson, R.D. Greenland and P.M. McGinnis, J. Chromatogr., 305 (1984) 27.
28 G.M. Anderson, J.G. Young, D.K. Batter, S.N. Young, D.J. Cohen and B.A. Shaywitz, J. Chromatogr., 223 (1981) 315.
29 S. Pavel and W. van der Slik, J. Chromatogr., 375 (1986) 392.

30 J.P.M. Wielders and Chr.J.K. Mink, J. Chromatogr., 310 (1984) 379.
31 K. Fujita, K. Maruta, S. Ito and T. Nagatsu, Clin. Chem., 29 (1983) 876.
32 T.G. Rosano, J.M.Meola and T.A. Swift, Clin. Chem., 28 (1982) 207.
33 N.C. Parker, C.B. Levtzow, P.W. Wright, L.L. Woodard and J.F. Chapman, Clin. Chem., 32 (1986) 1473.
34 L. McKay, C. Bradberry and A. Oke, J. Chromatogr., 311 (1984) 167.
35 K. Oka, K. Kojima, A. Togari, T. Nagatsu and B. Kiss, J. Chromatogr., 308 (1984) 43.
36 P. Wester, J. Gottfries, K. Johansson, F. Klintebäck and B. Winblad, J. Chromatogr., 415 (1987) 261.
37 P. Wester, J. Gottfries and B. Winblad, J. Chromatogr., 415 (1987) 275.
38 P. Kontur, R. Dawson and A. Monjan, J. Neurosci. Methods, 11 (1984) 5.
39 M. De Smet, G. Hoogewijs, M. Puttemans and D.L. Massart, Anal. Chem., 56 (1984) 2662.
40 H. Todoriki, T. Hayashi and H. Naruse, J. Chromatogr., 310 (1984) 273.
41 P.O. Edlund, J. Pharm. Biomed. Anal., 4 (1986) 641.
42 J.D. Baars, P.M.M. van Haard and A.J.P.F. Lombarts, Clin. Chim. Acta, 158 (1986) 173.
43 K. Breebaart and H. Grave, Tijdschr. Ned. Ver. Klin. Chem., 1 (1983) 13 (in Dutch).
44 A. Iwatani and H. Nakamura, J. Chromatogr., 309 (1984) 145.
45 V. Skrinska and S. Hahn, J. Chromatogr., 311 (1984) 380.
46 L. Elrod, Jr., R.E. Shoup and G.S. Mayer, Clin. Chem., 32 (1986) 1590.
47 J. de Jong, U.R. Tjaden, E. Visser and W.H. Meijer, J. Chromatogr., 419 (1987) 85.
48 P. Riederer and G.P. Reynolds, J. Chromatogr., 225 (1981) 179.
49 M.M. Joseph, B.V. Kadam and D. Risby, J. Chromatogr., 226 (1981) 361.
50 Z.K. Shihabi and J. Scaro, Clin. Chem., 26 (1980) 907.
51 G.P. Jackman, V.J. Carson, A. Bobik and H. Skews, J. Chromatogr., 182 (1980) 277.
52 M. Picard, D. Olichon and J. Gombert, J. Chromatogr., 341 (1985) 445.
53 P. Herregodts, Y. Michotte and G. Ebinger, J. Chromatogr., 345 (1985) 33.
54 P.P. Chou and P.K. Jaynes, J. Chromatogr., 341 (1985) 167.
55 D. Tonelli, E. Gattavecchia and M. Gandolfi, J. Chromatogr., 231 (1982) 283.
56 E. Kwarts, J. Kwarts and H. Rutgers, Ann. Clin. Biochem., 21 (1984) 425.
57 J. Jouve, J. Martineau, N. Mariotte, C. Barthelemy, J.P. Muh and G. Lelord, J. Chromatogr., 378 (1986) 437.
58 R.B. Mailman and C.D. Kilts, Clin. Chem., 31 (1985) 1849.
59 S.T. Balke, Quantitative Column Liquid Chromatography: A Survey of Chemometric Methods, J. Chromatogr. Library, Vol. 29, Elsevier, Amsterdam, 1984, p. 157.
60 M.J. Walters, J. Assoc. Anal. Chem., 70 (1987) 465.
61 B. Walczak, M. Lafosse, J.R. Chretien, M. Dreux and L. Morin-Alloy, J. Chromatogr., 369 (1986) 27.
62 E. Vervloet, P.M.M. van Haard, Tijdschr. Ned. Ver. Ziekenh. Apoth., 2 (1986) 1 (in Dutch).
63 P.M.M. van Haard, R. Engel and T. Postma, in preparation.
64 G.J.J. Beukeveld, G.T. Nagel, A.W. de Ruyter-Buitenhuis, E.W. Kwarts and B.G. Wolthers, Tijdschr. Ned. Ver. Klin. Chem., 10 (1985) 223 (in Dutch).
65 D.C. Turnell and J.D.H. Cooper, Clin. Chem., 28 (1982) 527.
66 A. Hausen, D. Fuchs, K. Grunewald, H. Huber, K. König and H. Wachter, Clin. Chim. Acta, 117 (1981) 297.
67 A.H. van Gennip, Screenings for Disorders of Purine and Pyrimidine Metabolism, Thesis, University of Utrecht, Utrecht, 1981, p. 59.
68 G.M. Anderson, J.G. Young, D.J. Cohen and S.N. Young, J. Chromatogr., 228 (1982) 155.
69 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
70 P.S. Draganac, S.J. Steindel and W.G. Trawick, Clin. Chem., 26 (1980) 910.
71 C.F. Saller and A.I. Salama, J. Chromatogr., 309 (1984) 287.
72 C.G. Honegger, R. Burri, H. Langemann and A. Kempf, J. Chromatogr., 309 (1984) 53.
73 C.D. Kilts, G.R. Breese and R.B. Mailman, J. Chromatogr., 225 (1981) 347.
74 W.A. Bartlett, J. Liq. Chromatogr., 8 (1985) 719.
75 B.R. Sitaram, R. Talomsin and G.L. Blackman, J. Chromatogr., 275 (1983) 21.
76 N.G. Abeling, A.H. van Gennip, H. Overmars and P.A. Voute, Clin. Chim. Acta, 137 (1984) 211.

77 A.H. Anton and A.I. Berk, Clin. Chem., 33 (1987) 600.
78 P.M.M. van Haard, R. Engel and A.L.J.M. Pietersma-de Bruyn, Clin. Chim. Acta, 157 (1986) 221.
79 T. Bottiglieri, C.K. Lim and J.J. Peters, J. Chromatogr., 311 (1984) 354.
80 S. Hori, K. Ohtani, S.Ohtani, K. Kayanuma and T. Ito, J. Chromatogr., 231 (1982) 161.
81 D.E. Mais, P.D. Lahr and T.R. Bosin, J. Chromatogr., 225 (1981) 27.
82 W.A. Wolf and D.M. Kuhn, J. Chromatogr., 275 (1983) 1.
83 A. Minegishi and T. Ishizaki, J. Chromatogr., 308 (1984) 55.
84 F.A.J. Muskiet, M.C. Stratingh, G.J. Stob and B.G. Wolthers, Clin. Chem., 27 (1980) 1.
85 E.B.M. de Jong, B.P.M. Horsten and H.M.J. Goldschmidt, J. Chromatogr., 279 (1983) 563.
86 W. Snedden, C.S. Mellor and J.R. Martin, Clin. Chim. Acta, 131 (1983) 247.
87 B.A. Davis, D.A. Durden and A.A. Boulton, J. Chromatogr., 374 (1986) 227.
88 G.B. Baker, V.K. Yeraganil, W.G. Dewhurst, R.T. Coutts, R.N. MacDonald and T.F.J. Wong, Biochem. Arch., 3 (1987) 257.
89 D.F. Hunt, A.B. Giordani, G. Rhodes and D.A. Herold, Clin. Chem., 28 (1982) 2387.
90 C.F. Poole and S.A. Schuette, Contemporary Practice of Chromatography, Elsevier, Amsterdam, 1984, p. 485.
91 O. Beck and P. Pévet, J. Chromatogr., 311 (1984) 1.
92 D.J. Skene, R.M. Leone, I.M. Young and R.E. Silman, Biomed. Mass Spectrom., 10 (1983) 655.
93 S. Pavel, H. Elzinga, F.A.J. Muskiet, J.M. Smit, N.H. Mulder and H. Schraffordt-Koops, J. Clin. Chem. Clin. Biochem., 24 (1986) 167.
94 M.J. Bennett, A. Green, R.J. Pollitt and E. Worthy, Ann. Clin. Biochem., 21 (1984) 45.
95 T.H.C. Barclay and D.V. Schapira, Cancer, 51 (1983) 878.
96 C.M. Swinson, E.C. Coles, G. Slavin and C.C. Booth, Lancet, i (1983) 111.
97 J.D. Godwin, Cancer, 36 (1975) 560.
98 A.N. Campbell, H.S.L. Chan, A. O'Brien, C.R. Smith and L.E. Becker, Arch. Dis. Childh., 62 (1987) 19.
99 S.L. Aleshire, C.A. Bradley and F.F. Parl, in P.J. Howanitz (Editor), Clinics in Laboratory Medicine: The Carcinoid Syndrome, Vol. 4, Saunders, Philadelphia, PA, 1984, p. 803.
100 G. Griffioen and M.E.C. Jeurissen, Ned. Tijdschr. Geneesk., 130 (1986) 528 (in Dutch).
101 S.A. Metz and R.J. Levine, in L. Landsberg (Editor), Clinics in Endocrinology and Metabolism: Catecholamines, Vol. 6, Saunders, London, 1977, Ch. 8, p. 719.
102 A. Lasson and A. Alwmark, Am. Chir. Gynecol., 74 (1985) 219.
103 J.M. Feldman, Clin. Chem., 32 (1986) 840.
104 S. Pavel, unpublished results.
105 S. Pavel, R. Bovenhof and B.G. Wolthers, J. Invest. Dermatol., 82 (1984) 577.
106 S. Pavel, H. Elzinga, F.A.J. Muskiet and B.G. Wolthers, Acta Dermatovenerol. (Stockholm), 63 (1983) 340.
107 S. Pavel, F.A.J. Muskiet, L. de Ley, T.H. The and W. van der Slik, J. Cancer Res. Clin. Oncol., 105 (1983) 275.
108 W. Westerhof, S. Pavel, A. Kammeyer, F.D. Beusenberg and R. Cormane, J. Invest. Dermatol., 84 (1987) 78.
109 H.J. Bremer, M. Duran, J.P. Kamerling, H. Przyrembel and S.K. Wadman, Disturbances of Amino Acid Metabolism: Clinical Chemistry and Diagnosis, Urban and Schwarzenberg, Munich, 1981.
110 K. Kodama, K. Yamanaka, T. Nakata and M. Aoyama, Clin. Chem., 32 (1986) 1944.
111 D.C. Turnell, Clin. Chem., 30 (1984) 588.
112 J.S. Swan, E.Y. Kragten and H. Veening, Clin. Chem., 29 (1983) 1082.
113 P.J. Schoenmakers, H.A.H. Billiet and L. de Galan, J. Chromatogr., 205 (1981) 13.
114 J.M.P. Holly and N. Patel, Ann. Clin. Biochem., 23 (1986) 447.
115 S. Pavel, F.A.J. Muskiet, G.T. Nagel, Z. Schwippelová and J. Duchon, J. Chromatogr., 222 (1981) 329.
116 S. Pavel, R. Bovenhof and W. van der Slik, Arch. Dermatol. Res., 276 (1984) 156.
117 S. Pavel and F.A.J. Muskiet, J. Labelled Compd. Radiopharm., 20 (1983) 101.
118 B.H.C. Westerink and N. ten Kate, J. Clin. Chem. Clin. Biochem., 24 (1986) 513.