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Review

Clinical chemistry of serotonin and metabolites

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Abstract

Analyses of serotonin and other 5-hydroxyindoles, such as its precursor 5-hydroxytryptophan and major metabolite 5-hydroxyindoleacetic acid (5-HIAA), are indispensable for the elucidation of their (patho)physiological roles. In clinical chemistry attention is mainly focused on the diagnosis and follow-up of carcinoid tumours. For this most laboratories routinely measure urinary 5-HIAA. More recently, measurements of serotonin in platelets and urine have been advocated. Platelet serotonin may be the most sensitive indole marker for the detection of carcinoid tumours that secrete only small amounts of serotonin and/or its precursor 5-hydroxytryptophan. Although several chromatographic techniques have emerged for the analysis of tryptophan-related indoles, HPLC with either electrochemical or fluorometric detection have become the methods of choice for their quantification. HPLC-based methods combine selectivity, sensitivity and high precision, and enable the simultaneous investigation of several metabolically related indoles. This review aims to place the analysis of indoles in biological matrices in a biochemical, physiological and clinical perspective and highlights several important steps in their chromatographic analysis and quantification. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Serotonin; 5-Hydroxytryptophan; 5-Hydroxyindoleacetic acid

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1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a heterocyclic amine that was first isolated by Ersparmer and Vialli in 1937. The source was enterochromaffin cells originating from the gastric and intestinal mucosa. It was characterised by its ability to cause smooth muscle contraction and named enteramine [1]. In the same period Rapport et al., in search of a humoral agent responsible for hypertension, isolated and characterised a substance from serum that caused vasoconstriction and named it serotonin [2]. After purification, structure elucidation and chemical synthesis enteramine was found to be identical to serotonin [3].

Serotonin proved ubiquitously distributed in nature. In plants it was found in edible fruits, vegetables and nuts, whereas in the animal kingdom it was demonstrated in both invertebrates and vertebrates. Important depots in mammals were enterochromaffin cells in the gastrointestinal mucosa, serotonergic neurones of the brain, pineal gland and platelets [4,5]. Compared with other biogenic amines, e.g., catecholamines and histamine, serotonin has only recently been discovered. Although studies on its physiological role elucidated some of its functions, the significance of its presence in most tissues is still the subject of intensive investigation. Such investigations would not have been possible without the development of accurate, precise and sensitive methods for the analyses of serotonin and its metabolites in biological matrices. This review gives a brief oversight of the present knowledge on the biochemistry, physiology, pathophysiology and current analytical methods of serotonin and its metabolites from the perspective of clinical chemistry.

2. Biochemistry

2.1. Biosynthesis

Serotonin is an indolic compound that is synthesised from the essential amino acid tryptophan (Fig. 1). Biosynthesis of serotonin in the human body represents only a minor metabolic route for tryptophan. Under normal conditions it accounts for not more than 2% of ingested tryptophan, leading to a daily production of about 10 mg serotonin. The major part of tryptophan is utilised for protein synthesis, whereas its major catabolic route is via kynurenine and 3-hydroxyanthranilic acid [6,7].

Hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (EC 1.14.16.4) is the first and rate limiting step in serotonin synthesis. Antibodies against tryptophan hydroxylase demonstrated its presence in several tissues, including enterochromaffin cells of the gastrointestinal tract, serotonergic neurones of the brain, and pineal gland [7]. Although platelets have been reported to contain low tryptophan hydroxylase activity their high serotonin contents originates from enterochromaffin cells of the gastrointestinal tract [8].

Formation of serotonin occurs by decarboxylation of 5-HTP. The reaction is catalyzed by aromatic-L-amino acid decarboxylase (AADC; EC 4.1.1.28) and uses pyridoxal-5-phosphate (the active form of vitamin B_6) as coenzyme. The enzyme has been detected in neoplastic tissues such as carcinoid tumours and phaeochromocytoma [9,10]. Specific types of carcinoid neoplasms may occasionally lack AADC, which gives rise to increased circulating and urinary levels of tumour-derived 5-HTP, instead of serotonin [11,12].

2.2. Regulation of synthesis

Serotonin synthesis rate is obviously dependent on tryptophan hydroxylase and AADC activities, and tryptophan availability. Since AADC activity is about 75-times higher than that of tryptophan hydroxylase, 5-HTP formation is considered to be the rate limiting step [13]. Availability of tryptophan also influences serotonin synthesis rate. High dietary

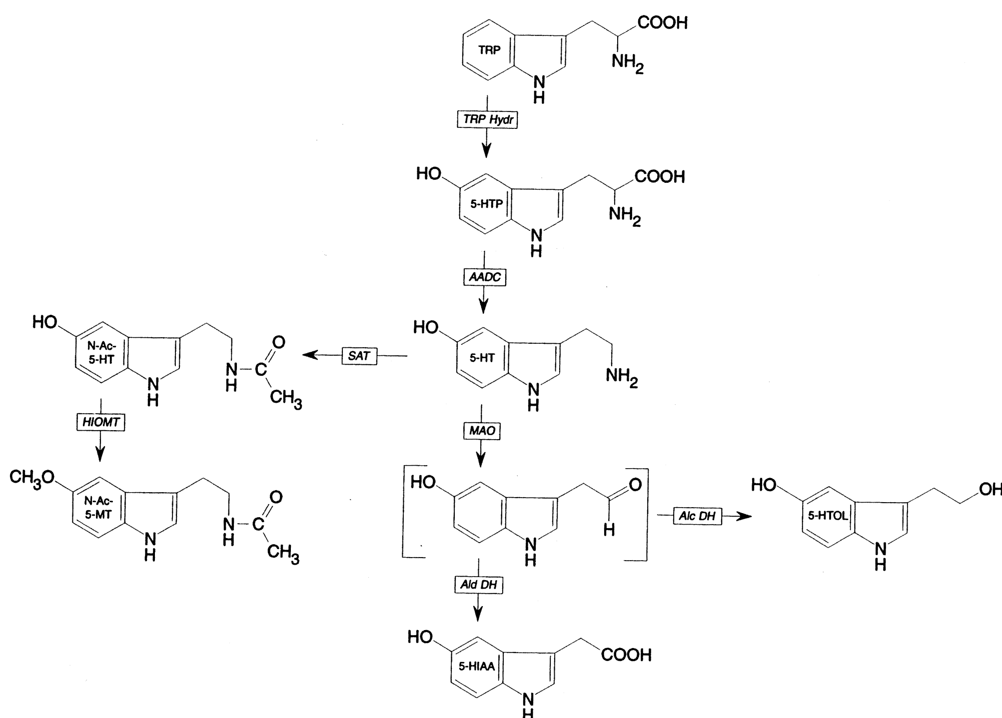


Fig. 1. Biosynthetic and degradative routes in the metabolism of serotonin. Abbreviations: AADC, aromatic-L-amino acid decarboxylase; Alc DH, alcoholdehydrogenase; Ald DH, aldehydedehydrogenase; HIOMT, hydroxyindol-*O*-methyl transferase; MAO, monoamine oxidase; *N*-Ac-5-HT, *N*-acetyl-5-hydroxytryptamine; *N*-AC-5-MT, *N*-acetyl-5-methoxytryptamine, melatonin; TRP, tryptophan; TRP Hydr, tryptophan hydroxylase; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin, 5-hydroxytryptamine; 5-HT, 5-HTOL, 5-hydroxytryptophol; 5-HTP, 5-hydroxytryptophan.

intakes increase tissue serotonin contents, whereas tryptophan deficient diets cause reduced levels [14,15].

Processes that control plasma tryptophan uptake by peripheral cells have not fully been clarified as yet. Because of its amphipathic nature circulating tryptophan is partially bound to plasma albumin. Its uptake in brain is known to be dependent on the plasma free tryptophan concentration [16]. Consequently, processes that influence the equilibrium between free and protein-bound forms of plasma tryptophan (e.g., free fatty acids, certain drugs) modify its availability for uptake in brain and thereby affect serotonin synthesis rate [7,17]. Tryptophan uptake in brain cells is regulated by two saturable systems with high- and low-tryptophan affinities, respectively [18].

Serotonin synthesis in brain is also regulated by mechanisms that activate or inhibit tryptophan hydroxylase. Calcium-induced phosphorylation renders the enzyme active, whereas an intra-neuronal serotonin pool inhibits it via negative feedback. Fluctuations in tetrahydrobiopterin concentrations may also be involved in the control of tryptophan hydroxylation activity [13].

2.3. Metabolism

Serotonin is metabolized via oxidative deamination (monoamine oxidase; MAO), conjugation with sulfuric and glucuronic acids, *N*-acetylation, 5-*O*-methylation and their combinations (Fig. 1). The enzymes are differently distributed among the various organs.

The majority of serotonin is metabolized by the flavoprotein MAO (EC 1.4.3.4). This enzyme is mainly located in mitochondria and catalyses oxidative deamination of several monoamines to their

corresponding aldehydes, ammonia and hydrogen peroxide. At least two forms of MAO, differing in substrate affinities and inhibitors, are known. MAO-A has highest affinity for serotonin and norepinephrine, whereas MAO-B is more active in deaminating benzylamine and phenylethylamine [19].

5-Hydroxyindoleacetaldehyde, the product of oxidative deamination of serotonin, can either be oxidised to 5-hydroxyindoleacetic acid (5-HIAA) or reduced to 5-hydroxytryptophol. The former reaction is quantitative most important and is catalyzed by aldehyde dehydrogenase (EC 1.2.1.3) with NAD as coenzyme. Aldehyde dehydrogenase has been detected in many tissues, including brain and liver [20,19]. The enzyme also catalyses oxidation of catecholamine aldehyde intermediates to their corresponding acidic metabolites. Reduction of 5-hydroxyindoleacetaldehyde to 5-hydroxytryptophol is catalyzed by aldehyde reductase (EC 1.1.1.1). This enzyme uses NADH as coenzyme. Conversion of an intermediate aldehyde to the corresponding alcohol also takes place in catecholamine metabolism [19]. Formation of 5-hydroxytryptophol represents a minor route of serotonin metabolism. Under normal conditions urinary 5-hydroxytryptophol accounts for about 1% of serotonin turnover. In alcoholism and (other) liver pathology the proportion of 5-hydroxytryptophol may considerably increase by a metabolic shift from 5-HIAA to 5-hydroxytryptophol [21,22].

Conjugation of serotonin with sulfuric acid (formation of serotonin-*O*-sulfate) or glucuronic acid (serotonin-*O*-glucuronide) represents a minor metabolic pathway [7]. Both types of conjugates have been detected in urine of healthy subjects and patients with carcinoid tumours [23–26]. Serotonin and 5-HIAA are predominantly excreted in the free form, whereas 5-hydroxytryptophol is mainly excreted as conjugate [7,22]. Serotonin-containing foods can substantially increase urinary excretion of serotonin conjugates [27].

Two highly specific enzymes that subsequently catalyse serotonin *N*-acetylation and 5-*O*-methylation are involved in the synthesis of melatonin (5-*O*-methyl-*N*-acetylserotonin) in the pineal gland. Melatonin is a hormone implicated in the physiology of circadian rhythm, reproductive development and behaviour [28,29]. Serotonin-*N*-acetyltransferase has been found in both brain and liver. In the pineal gland it shows typical circadian rhythmicity that causes considerably higher plasma melatonin levels at night [30]. Hydroxyindole-*O*-methyltransferase (EC 2.1.1.4) catalyses the second step in melatonin formation. This enzyme gives rise to 5-*O*-methylation of *N*-acetylserotonin with *S*-adenosylmethionine as methyl donor [31].

2.4. Peripheral serotonin

The majority of serotonin is found in enterochromaffin cells of the gastrointestinal tract. In these structures it accounts for approximately 80% of total body serotonin content [32,33]. Serotonin can be released from these cells by stimulation with acetylcholine, noradrenergic nerve stimulation, increased intra-luminal pressure and a decline of intestinal pH. Released serotonin can be detected in the intestinal lumen, portal blood and systemic circulation [33]. Serotonin is a potent vasoactive amine. In the circulation it is almost entirely confined to platelets and thereby rendered functionally inactive. Since elevated plasma serotonin concentrations may be hazardous, several rapid clearance mechanisms have evolved. Platelets possess an active serotonin uptake system, the liver catabolises serotonin, pulmonary endothelial cells take up serotonin and specific macromolecules bind free serotonin.

2.5. Platelet uptake and storage

Circulating plasma serotonin is taken up by platelets mainly by an active transport mechanism [8,34]. The majority of platelet serotonin originates from enterochromaffin cells, and is accumulated during the platelet's life cycle [35]. Platelet serotonin content is elevated in patients with serotonin-secreting carcinoid tumours [12,36] and during long-term serotonin ingestion [37]. It is unaffected by short-term consumption of serotonin-rich foods [27]. Platelet serotonin half life is about 4.2 days, which approximately equals that of platelets [37,38]. In platelets, serotonin is stored in dense granules. The platelet membrane contains two types of serotonin binding sites. One site mediates uptake, whereas the other causes platelet aggregation [39]. Apart from active uptake a passive uptake process has been noted. Passive uptake occurs at high extracellular serotonin concentrations and is proportional to

serotonin levels. It may play a role in patients with serotonin-secreting carcinoid tumours [40,36].

Compared with inactivation in liver and lung, platelet serotonin uptake is a relatively slow process. Animal experiments showed that after 60–120 s approximately 50% of non-protein bound plasma serotonin is taken up by platelets [41]. Despite the slowness of the uptake process, long-term uptake by platelets contributes significantly to overall clearance of circulating non-protein bound plasma serotonin.

2.6. Liver and lung

The liver is capable of removing substantial amounts of plasma serotonin, with subsequent formation of 5-HIAA. From animal studies it is estimated that serotonin removal between portal and hepatic veins amounts to about 30%. Following continuous serotonin infusion, its metabolic clearance by the liver can increase to up to 80% [42,41]. Such studies mimic the catabolic adaptation that occurs during increased intestinal serotonin release, such as in patients with ileum carcinoid tumours. In patients with serotonin-releasing metastases in the liver, hepatic serotonin inactivation does not necessarily compensate for the released amounts, since most of it is directly secreted in the effluent vascular system. Consequently, these patient may have high plasma serotonin concentrations in hepatic veins [43]. Long-term exposure of the heart may lead to endocardial fibrosis, primarily of the right side [44].

Pulmonar removal of circulating plasma serotonin is a very rapid and efficient process. Over 90% of intravenously injected or endogenously released serotonin is removed within a single transpulmonar passage. The primary metabolite of serotonin captured in endothelial cells of the lungs is 5-HIAA. MAO-A inhibitors prevent oxidative deamination of serotonin in the lungs, but do not affect its pulmonary removal rate. Thus, uptake of serotonin in endothelial cells, rather than intra-endothelial catabolism, is the rate limiting step in its overall pulmonar removal [45].

3. Physiology

Serotonin is involved in a variety of physiological processes, including smooth muscle contraction, blood pressure regulation and both peripheral and central nervous system neurotransmission. Its exact role in most of these processes is still to be elucidated [4,5]. Synthesis occurs in brain, spinal cord, enterochromaffin cells of the gastrointestinal tract, bronchi, thyroid, pancreas and thymus. Circulating serotonin does not enter the brain by crossing the blood–brain barrier. In the central nervous system it acts as a neurotransmitter–neuromodulator that is implicated in sleep pattern regulation, appetite control, sexual activity, aggression and drive (a driven tendency or instinct, especially of sexual or aggressive origin) [46]. Central nervous system serotonin exerts its actions in concert with other neurotransmitters [5,47]. In the periphery serotonin acts as a vasoconstrictor and proaggregator when released from aggregating platelets, as a neurotransmitter in the enteric plexuses of the gut and as an autocrine hormone when released from enterochromaffin cells from the gut, pancreas and elsewhere [5,33]. Its precise function in the gastrointestinal tract is not fully understood [48].

Serotonergic receptors are found throughout the body, and are composed of at least three distinct types of molecular structures: (1) guanine nucleotide-binding protein-coupled receptors that are subdivided into the 5-HT_{1-} (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}) and 5-HT_{2-} (5-HT_{2}, 5-HT_{1C}) families, (2) ligand-gated ion channels (5-HT_{3}) and (3) transporters (5-HT uptake site) [49]. Receptors of the 5-HT_{1} family are found in several areas of the brain and blood vessels, those of the 5-HT_{2} family in platelets, brain and vascular smooth muscle, and those of the 5-HT_{3} family in peripheral efferent and afferent autonomic neurones [5].

4. Pathophysiology

Abnormalities of serotonin-related processes give rise to various pathological conditions. Aberrations in its central nervous system function are thought to be involved in anorexia, anxiety, depression and schizophrenia, whereas degeneration of serotonergic neurones have been noted in Alzheimer's and Parkinson's diseases [5,17,47,50]. Peripheral aberrations in serotonin-related processes have been implicated in drug-induced emesis, hypertension, migraine, genesis of cardiac arrhythmias, Raynaud's disease,

fibrotic syndromes and some symptoms of the carcinoid syndrome [5,44,51,52]. The quantitatively most pronounced aberration in serotonin production and metabolism is encountered in patients with carcinoid tumours.

4.1. Carcinoid tumours

Carcinoid tumours are APUD-omas (characterised by amine precursor uptake and decarboxylation) that arise from enterochromaffin cells. Embryologically, these cells are derived from the primitive neuronal ectoderm [53–55]. Foregut carcinoids arise from the respiratory tract, pancreas, stomach and duodenum; midgut carcinoids from ileum and appendix; and hindgut carcinoids from left colon and rectum [56]. The tumours appear most frequently in midgut (about 70% of cases) and to a lesser extent in fore- and hindguts (13% and 17%, respectively). Depending on origin and size of the primary tumour, distant metastases (usually to liver) can occur. The predilection to deliver metastases amounts to 14–50, 2–60 and 3% for fore-, mid- and hindgut carcinoids, respectively [57]. Carcinoid tumours have been reported to occur in all age groups, but appear most frequently in adults aged 60–70 years [58].

The endocrine manifestation of enterochromaffin cell neoplasm is the carcinoid syndrome. Its occurrence and severity is directly related to tumour bulk in an area that drains into the systemic circulation [55]. Carcinoid syndrome is characterised by cutaneous flushing, diarrhoea, valvular lesions of the right side of the heart, and bronchoconstriction [59,60,44]. Notably midgut carcinoids with distant metastases and bronchus carcinoids are known to cause the syndrome. Depending on tissue origin and presence of metastases, these tumours can cause excessive synthesis, storage and release of both peptides and biogenic amines. Serotonin is the most prominent biogenic amine. Production of catecholamines (notably dopamine) and histamine [61] has also been reported [62–64]. Characteristically, midgut carcinoid tumours readily produce and secrete serotonin [43,65]. Some foregut carcinoids are AAD-C-deficient and may therefore secrete 5-HTP and to a lesser extent serotonin [11,12,66]. Carcinoid tumours from hindgut usually do not secrete substantial amounts of serotonin [57,67,36]. Serotonin plays an important role in the aetiology of some symptoms of the carcinoid syndrome, although other mediators are also implicated [5]. Carcinoid patients may convert as much as 60% of dietary tryptophan to serotonin. Long-term augmentation of the serotonin biosynthetic pathway may result in serious reduction of the free-tryptophan body pool, causing niacin deficiency and subsequent development of pellagra-like symptoms [67].

5. Analysis

A wide variety of analytical methods has been used for the qualitative and quantitative determination of serotonin in blood. Early methods made use of bioassays by taking advantage of its smooth muscle contractile properties [7].

All 5-hydroxyindoles fluoresce at neutral pH at an absorbance wavelength of 300 nm with an emission wavelength of 340 nm, which enables quantitation by fluorescence detection [68,69]. Fluorometric methods for the determination of serotonin in biological matrices are, however, rather aspecific and have to be preceded by its separation from interfering compounds. For this solvent extraction, ion-exchange chromatography and gel filtration have been used [7]. Detection limits of approximately 1 nmol can be reached and lower limits (50 pmol) are feasible using *o*-phtaldialdehyde or ninhydrin [70]. Rapid developments in analytical techniques founded the introduction of more specific and precise methods. Employing thin-layer chromatography [71], radio immunoassay [72,73], enzyme immunoassay [74], gas chromatography [75], gas chromatography–mass spectrometry [76–79], and high-performance liquid chromatography (HPLC) with ultraviolet [80], fluorometric [68,69,81], electrochemical [82] and mass spectrometric detection [83], they aim at measurement of either single indoles or a selection of tryptophan-derived indoles (profiling methods). Among these methods, HPLC outnumbers the others by far.

The chromatography of urinary indole derivatives has previously been reviewed by Van Haard and Pavel [84]. More recently, Deacon published an extensive review on the measurement of 5-HIAA in urine [85]. From these publications it is obvious that

HPLC with either fluorometric or electrochemical detection gradually replaces less specific colorimetric methods in clinical chemical laboratories. HPLC-based methods combine selectivity, sensitivity and high precision, and enable the simultaneous investigation of several metabolically related indoles. Compared with other chromatographic techniques they necessitate only minimal sample prepurification. Although analytical innovation is gradually introducing techniques like capillary zone electrophoresis [86–88] and liquid chromatography–mass spectrometry in specialised clinical chemical laboratories [89], their application in the analysis of indoles has been limited up to now. Automation of HPLC analyses, using direct injection techniques in combination with column switching, is an emerging technique that potentially offers the possibility of combining sample prepurification and analysis, and thereby reduce the analytical turnaround time [82,90–94].

Analyses of serotonin and other 5-hydroxyindoles, such as its precursor 5-HTP and major metabolite 5-HIAA, are indispensable for the elucidation of their (patho)physiological roles. In clinical chemistry attention is mainly focused on the diagnosis and follow-up of carcinoid tumours. For this most laboratories routinely measure urinary 5-HIAA [43,57]. More recently, measurements of serotonin in platelets and urine have been advocated [12,36]. Platelet serotonin may be the most sensitive parameter for the detection of carcinoid tumours that secrete only small amounts of serotonin and/or its precursor 5-HTP [36]. Table 1 summarises the concentrations of some clinically relevant indoles as previously reported in the literature.

5.1. Preanalytical considerations

The analysis of indoles in biological samples is confounded by their sensitivity to light, oxygen and high or low pH. Consequently, special precautions have to be taken during sample collection, sample prepurification and analysis [7,84,85]. For their circumvention, the following preventive measures have been advised: filtering or exclusion of (sun-)light, immediate refrigeration after sample collection, prevention of repeated thawing and freezing, acidification ($pH>2$), and addition of antioxidants such as sodium metabisulfite, ascorbic acid, EDTA or L-cysteine.

Several antioxidant mixtures have been investigated for their ability to stabilise serotonin and its metabolites. Thorre et al. [95] found that the stability of serotonin and 5-HIAA was poor in acidic medium that merely contains Na_2EDTA. Significant improvement was noticed upon the addition of L-cysteine and ascorbic acid. It was previously noticed that the ability of Na_2EDTA to complex metal traces diminishes at pH values below 5. Also the ability of ascorbic acid to act as an antioxidant reduces at lower pH, since protonation diminishes its ability to become oxidised. Indolamines are less stable at high or very low pH. Thorre et al. considered pH 3.2 in combination with the above-mentioned antioxidants optimal. Conversion of oxyhaemoglobin to carboxyhaemoglobin prior to acid protein precipitation prevents serotonin oxidation [96]. An important factor that has to be taken into account in the choice of antioxidants for analyte preservation is their compatibility with the subsequent analytical procedure. Several antioxidants interfere with electro-

Table 1
Concentrations of tryptophan-related indoles in several biological matrices[a]

	Tryptophan	5-HTP	5-HT	5-HIAA
Urine	73–330 μmol/24 h [112]	<0.7 μmol/24 h [12]	340–950 nmol/24 h [112]	2–50 μmol/24 h [138]
Platelet-rich plasma			3.81±0.87 nmol/10^9 platelets [100]	
Platelet-poor plasma	61±5 μmol/l (total) 6.1±0.7 μmol/l (free) [115]		2.8±1.4 nmol/l [101]	11.0±0.5 nmol/l [116]
CSF	1257–2757 nmol/l [137]	5.3–10.8 nmol/l [137]	5.7–12.0 nmol/l [137]	80.8–143.2 nmol/l [137]

[a] Abbreviations: CSF: cerebrospinal fluid; 5-HTP: 5-hydroxytryptophan; 5-HIAA: 5-hydroxyindoleacetic acid. Unless otherwise stated, levels of non-conjugated compounds are given. Data are given as ranges or as mean±SD, references in parentheses.

chemical detection. The presence of haemoglobin and oxygen in whole blood alters the recovery of serotonin during deproteinisation with concentrated perchloric acid. Addition of ascorbic acid prior to deproteinisation resulted in an improved recovery in methods using fluorometric detection, but caused interference in amperometric methods [97]. Pussard et al. noted that addition of sodium borohydride to whole blood prior to perchloric acid protein precipitation prevents oxidation of serotonin, without causing subsequent chromatographic interferences using amperometric detection [97].

There has been some confusion regarding the nomenclature of the different serotonin pools [81]. Notably the difference between whole-blood, plasma, platelet-rich plasma and platelet-poor plasma should be taken into account when data are reported [98,99]. The vast majority (>95%) of whole blood serotonin is confined to platelets, but a distinct extracellular pool of freely circulating serotonin has also been noted. Values have been reported for whole blood, platelet-rich plasma, platelet pellets, platelet-poor plasma and blood (micro)dialysate and the data have been expressed in pmol/mg plasma protein, nmol/l, and nmol/10^9 platelets [12,97,100]. It was noted [97] that in hypertensive patients, whole blood serotonin concentrations, expressed in nmol/l, were significantly higher while standing compared with the supine position. In contrast, platelet serotonin concentrations were similar in both positions. The difference appeared to be due to the observation that platelet counts increase substantially from the supine to standing position. Correction for platelet numbers gave rise to similar whole blood concentrations. It is obvious that results of whole blood serotonin and platelet-rich plasma serotonin when expressed in terms of protein concentration or volume do not allow correction for variation in platelet counts and are therefore less meaningful for intra- or inter-individual comparisons.

5.2. Preparation of platelet-poor plasma

There has been controversy regarding an accurate manner to assess serotonin concentrations in platelet-poor plasma. The existence of a genuine extracellular serotonin pool was questioned, since the measured concentrations might as well derive from activation of platelets during the preanalytical procedure [98]. Marshall and Leitch noted that for this two factors have to be taken into consideration: at no stage before obtaining cell-free plasma should the sample be frozen, and also the length and strength of centrifugation of whole blood or platelet-rich plasma should be such that it is rendered cell free [98]. Middelkoop et al. [101] investigated the influence of several types of anticoagulants, the influence of the sampling method, and the influence of collecting the first or second 10 ml volume. Serotonin concentrations in platelet-poor plasma prepared from blood that was anticoagulated with citrate or with citrate plus aggregation inhibitors were about two times higher compared with platelet-poor plasma prepared from K_3-EDTA anticoagulated blood. Collection by an evacuated and non-evacuated blood sampling technique, or taking the first or second 10 ml blood sample had no effect. They found that K_3-EDTA gave the best results. Picard et al. [102] noticed a significant effect of centrifugation on the concentration of serotonin in platelet-poor plasma. They centrifuged platelet-rich plasma for 15 min at 4°C and speeds ranging from 1000 up to 70 000 *g*. The concentration of serotonin in the supernatant was found to be lowest at 6000 *g*. Reducing or increasing the centrifugal force resulted in higher platelet-poor plasma serotonin values due to incomplete platelet sedimentation or platelet damage, respectively. Takkenberg et al. [93] also noted that the concentration of serotonin in plasma and the number of platelets decreased with increasing centrifugal speed, albeit at lower centrifugal forces than reported by Picard et al. A stable platelet-poor plasma serotonin concentration and no detectable platelet counts were found at a centrifugal force above 500 *g* and a centrifugal force of 900 *g* was considered optimal to prevent brake effects after centrifugation [93]. A relatively new approach to determine the circulating extraplatelet serotonin pool has been illustrated by Paez and co-workers [103,104]. They measured circulating free plasma serotonin with the technique of venous microdialysis to reduce the probability of platelet rupture and the concomitant release of serotonin during blood collection. Their results proved in the lower range of previously reported data obtained by venous puncture. It was shown that microdialysis is

an efficient alternative method to monitor platelet-free plasma serotonin.

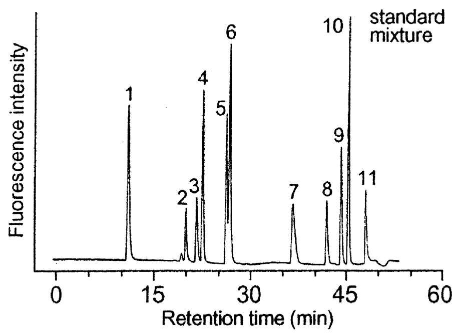
5.3. Extraction and deproteinisation

Protein-containing matrices such as whole blood, plasma and tissue homogenates require prepurification prior to analysis. Tryptophan-related indoles differ considerably with respect to functional groups and consequently different strategies such as solvent partition and ion-exchange have been applied. Solvent extraction was one of the first methods that was used for the extraction of serotonin. In this procedure tissue was homogenised in 0.1 mol/l hydrochloric acid and centrifuged. The pH of the supernatant was adjusted to 10 and the solution was saturated with sodium chloride. Extraction and back-extraction of serotonin was done with *n*-butanol and dilute acid, respectively [105]. Picard et al. [102] described a similar procedure for the extraction of serotonin from platelet-poor and platelet-rich plasma. Samples that were stored in a solution containing antioxidants, were adjusted to pH 11 using a buffer of sodium hydroxide, glycine, and sodium chloride. Extraction and back-extraction were performed in chloroform–1-pentanol (60:20, v/v) and 0.1 mol/l hydrochloric acid. For organic solvent extraction several other fluids such as ethyl acetate, hexane and chloroform were investigated with extraction recoveries ranging from 0 to 90%. The extraction procedure was compatible with subsequent analysis by HPLC with electrochemical detection.

Serotonin is also isolated by the use of weak cation-exchange resins such as BioRex 70 and Amberlite CG-50, or strong cation-exchange resins such as Dowex 50 [7,82]. Kwarts et al. [106] described a method using Amberlite CG-50 as cation-exchange resin. In this procedure, either platelet-rich plasma, platelet-poor plasma, serum, urine or cerebrospinal fluid were mixed with an ammonium acetate buffer, pH 7.5. Samples were subsequently applied to Amberlite CG-50 columns, and washed with the ammonium acetate buffer and 0.01 mol/l acetic acid. Elution was done with 1.0 mol/l acetic acid that contained ascorbic acid, followed by HPLC with fluorometric detection. This method has in our hands over a period of 20 years found to be a reproducible and robust procedure for serotonin extraction from different protein-containing matrices. This prepurification procedure has also been employed in combination with electrochemical detection [107]. Solutions of high ionic strength may cause poor absorption of serotonin due to poor performance of Amberlite CG-50 at high salt concentrations. Selective elution of the strong cation-exchange resin Dowex 50 can be used for the simultaneous isolation of 5-HTP, serotonin and 5-HIAA [7,82]. For this application several other solid-phase extraction procedures have been developed, employing porous polystyrene polymer [108], Sep-Pak C_{18} [109,110] or protein-coated ODS [111]. We used C_{18} reversed-phase extraction columns in combination with octane sulfonic acid for the extraction of indoles from protein-containing matrices such as plasma and tissue homogenate (Fig. 2) [112]. Protein elimination via precipitation, followed by filtration, centrifugation, or on-line enrichment is one of the most frequently employed methods for the purification of samples prior to indole profiling. Precipitation is usually done by perchloric acid or trichloroacetic acid [97,113–115]. Others have used ultrafiltration to remove proteins [116]. The use of antioxidants is necessary to prevent oxidation of indoles at low pH during purification, as outlined before. A more contemporary version of the gravity fed solid-phase extraction is in-line solid-phase extraction. This technique makes use of column switching for automated analyte extraction and concentration. Several applications have been described for the automated analysis of serotonin or related indoles [82,90–94]. The choice of extraction procedures depends on factors such as sample matrix, analyte concentrations, the ability to combine it with the HPLC and detection systems, and whether one or more components are to be analysed.

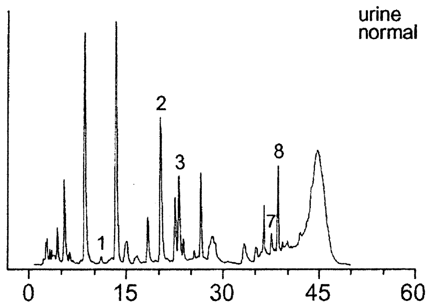
5.4. HPLC separation and detection

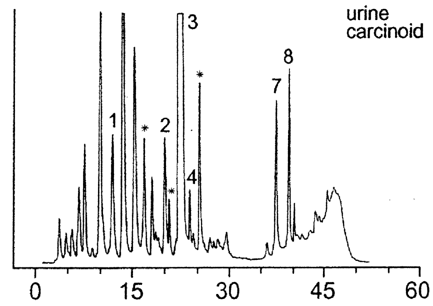
When screening the literature for HPLC analyses of indole derivatives, its becomes apparent that in broad outlines a single stationary phase, a limited number of mobile phase solvents and two detection systems are being used [84]. Already one of the first publications dealt with optimisation of the chromatographic conditions for the simultaneous determination of six indoles, including three hydroxyindoles,

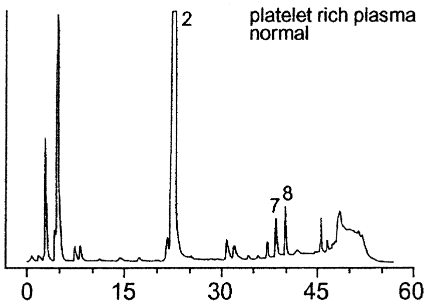


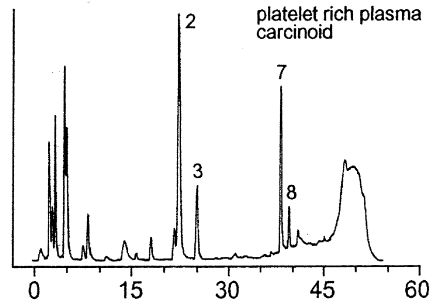
Peak identity:

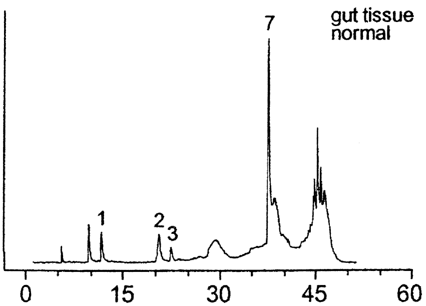
1: 5-hydroxytryptophan (5HTP)
2: tryptophan (TRP)
3: 5-hydroxyindole-3-acetic acid (5-HIAA)
4: 5-hydroxytryptophol (5-HTOL)
5: N-acetyl-5-hydroxytryptamine (NAS)
6: 5-methoxytryptophan (5-MTP)
7: 5-hydroxytryptamine (serotonin, 5-HT)
8: indole-3-acetic acid (IAA)
9: tryptophol (TOL)
10: melatonin (N-acetyl-5-methoxytryptamine, MEL)
11: tryptamine (TAM)

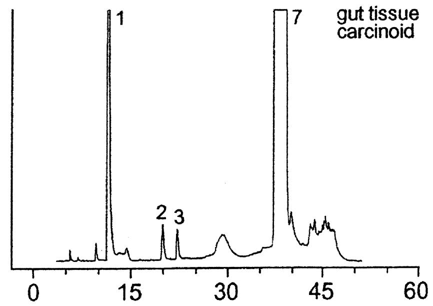












in deproteinised urine samples [117]. Since then many publications have reported optimisation of chromatographic conditions as adapting to specific requirements. The physiochemical characteristics of the four tryptophan-related indoles allow predictions regarding their chromatographic behaviours under different chromatographic conditions. Important factors for the modification of chromatographic behaviour are stationary phase and composition of the mobile phase. Mailman and Kilts [118] investigated the influence of the mobile phase composition, such as ion pair reagent, organic modifier content and pH. Experiments were performed with an octadecyl (C_{18}) reversed-phase column, which is the most commonly used packing material for these applications. As was to be expected, the effects of mobile phase pH on the capacity factors for 5-HTP, serotonin and 5-HIAA were dependent on their degree of ionisation at a given pH. An increase of mobile phase pH relative to the pK_a of 5-HIAA caused increasing deprotonisation and a decrease in its capacity factor. The capacity factor of serotonin was not particularly affected in the tested pH range of 3 to 6, since its pK_a value is well above this range. Analogously, deprotonisation of the 5-HTP carboxylic function at higher pH resulted in the formation of a more polar zwitterion and thus reduction of the capacity factor in a reversed-phase system. Similar experiments have been described for other tryptophan-related indoles and other biogenic amines under different chromatographic conditions [113,114,119–121].

The concentrations of tryptophan-related indoles in biological matrices range between pmol/ml to nmol/ml (Table 1). Consequently, simultaneous determination of all components requires a wide linear dynamic range of the employed detector. Sensitive and specific detection methods are required for the lower concentration ranges. Characteristic features of indoles are their easy oxidisibility (with the exception of tryptophan) and their native fluorescence properties. It is therefore not surprising that electrochemical and fluorometric detection have become the detection modes of choice. Most applications make use of electrochemical detection and either apply voltammetry or (array) coulometry. This detection mode combines high sensitivity and good precision even at very low solute concentrations. Selectivity can be influenced by variation of the electrochemical detection potential, reducing the demands on the sample prepurification. Since the working electrode is in direct contact with the test solution, it is difficult to maintain the electrode surface in a constantly activated state. Therefore suitable conditions for electrochemical measurement must be found for each application. This requires basic knowledge of electrochemistry and some experience [122]. Electrochemical detection requires a constant electrically conductive mobile phase. The choice of mobile phase composition is therefore limited, whereas gradient elution may result in variation of the background current. The potential regions of tryptophan and its metabolites are given in Fig. 3. For the major plasma 5-hydroxyindoles the oxidation potential is below 0.6 V. Tryptophan

Fig. 2. Chromatograms of body fluids and tissues from healthy persons and patients with carcinoid tumours, as obtained by HPLC with quaternary gradient elution and fluorescence detection (excitation wavelength 295 nm, emission wavelength 340 nm). Panel A: separation of 11 standard indoles [injected amounts: 8.3 ng each, except for NAS (6.9 ng), 5-HT (3.6 ng) and TAM (6.8 ng)]. Panels B: urinary indole profiles of a healthy adult (B1) and a patient with a midgut carcinoid (B2) obtained by direct injection of 20-μl aliquots of filtered urine samples that had been diluted to a creatinine concentration of 1 mmol/l. Calculated concentrations (for panels B1 and B2, respectively) in the urine samples were: 5-HTP: (16.2, 499.5) μmol/mol cr; TRP: (1.0, 1.5) mmol/mol cr; 5-HIAA: (0.5, 52.0) mmol/mol cr; 5-HTOL: (not detectable, 293.6) μmol/mol cr; 5-HT: (65.44, 871.2) μmol/mol cr. Panels C: indole profiles of C_{18} prepurified platelet-rich plasma samples of a healthy adult (C1), and a patient with a midgut carcinoid (C2). Calculated concentrations (for panels C1 and C2, respectively) in the platelet-rich plasma samples were: TRP: (34.1, 8.2) μmol/l; 5-HIAA: (not detectable, 1.8) μmol/l; 5-HT: (0.7, 2.5) μmol/l. Panels D: Chromatograms of C_{18} prepurified tissue homogenates from normal enterochromaffin tissue (D1) and carcinoid tissue of midgut origin (D2). On a wet weight basis the calculated levels of compounds (for panels D1 and D2, respectively) were: 5-HTP: (3.8, 2079.0) nmol/g; TRP: (11.6, 334.9) nmol/g; 5-HIAA: (5.0, 251.3) nmol/g; and 5-HT: (39.7, 6993.1) nmol/g. Peak identity numbers and abbreviations: 1=5-hydroxytryptophan (5-HTP), 2=tryptophan (TRP), 3=5-hydroxyindole-3-acetic acid (5-HIAA), 4=5-hydroxytryptophol (5-HTOL), 5=*N*-acetyl-5-hydroxytryptamine (NAS), 6=5-methoxytryptophan (5-MTP), 7=5-hydroxytryptamine (serotonin, 5-HT), 8=indole-3-acetic acid (IAA), 9=tryptophol (TOL), 10=melatonin (*N*-acetyl-5-methoxytryptamine, MEL), 11=tryptamine (TAM), cr=creatinine. (Reprinted from Ref. [112] with permission).

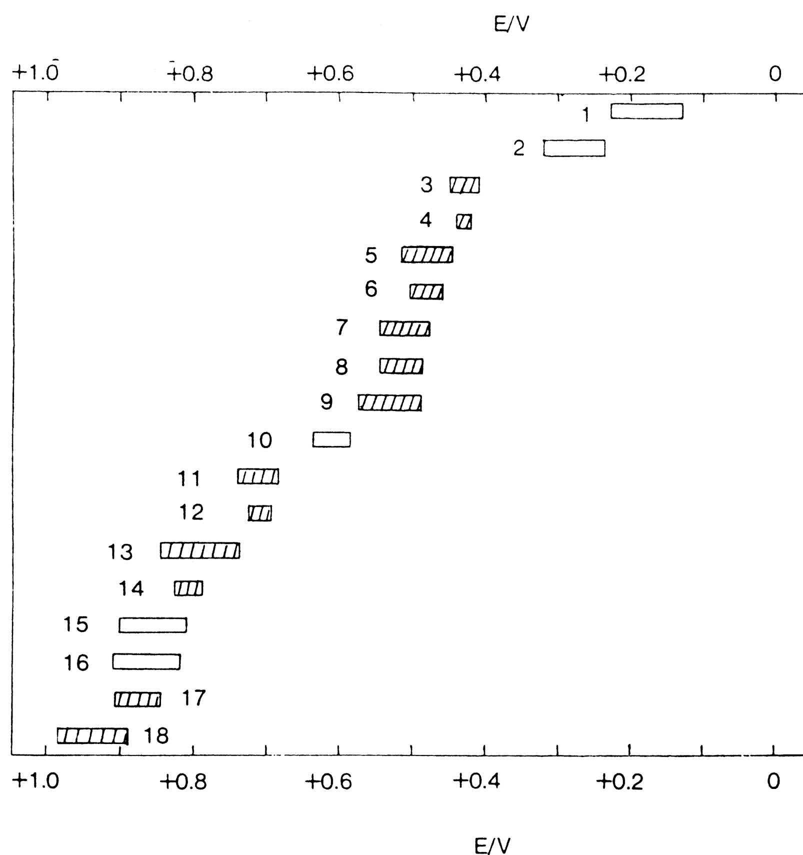


Fig. 3. Potential regions of the oxidation of tryptophan and its metabolites. Empty boxes: irreversible oxidation; shaded boxes: a reversible product is produced, which is not the principle oxidation product. Carbon-paste electrode, medium 90% 0.1 *M* citrate (pH 4.0) and 10% ethanol. Potential scan rate, 200 mV/s. 1=Indolepyruvic acid; 2=5,6-dihydroxytryptamine; 3=3-hydroxyanthranilic acid; 4=5-hydroxytryptophol; 5=3-hydroxykynurenine; 6=5-hydroxyindoleacetic acid; 7=*N*-acetyl-5-hydroxytryptamine; 8=5-hydroxytryptamine; 9=5-hydroxytryptophan; 10=indoxyl; 11=melatonin; 12=xanthurenic acid; 13=indoleacetic acid; 14=anthranilic acid; 15=*N*,*N*-dimethyltryptamine; 16=tryptamine; 17=tryptophan; 18=kynurenine. (Reprinted from Ref. [139] with permission of Bioanalytical Systems, West Lafayette, IN, USA).

oxidises at much higher potential of approximately 0.8 V. Combined electrochemical detection of TRP, 5-HT, serotonin and 5-HIAA may thus require a relatively high oxidation potential, which augments the risk of interference by other electrochemically active compounds. Combining two or more coulometric electrochemical cells into an array enables stepwise oxidation thus reducing the risk of interference and enabling compound identification and more sensitive detection [123,124]. For the electrochemical detection of indoles detection limit are in the picogram range, which is sufficient for detection of the most important plasma indoles.

Comparisons of detection limits of electrochemical and fluorometric methods do not always show consistent results in favour of one of these [125]. Nevertheless most users of electrochemical detection methods motivate their choice for this detection technique with the argument of sensitivity. An explanation for this discrepancy could be that the delineation of suitable conditions for electrochemical measurement requires specific knowledge of its principles [122]. Fluorometric detection is less demanding in this respect [84]. Indole derivatives can be detected fluorometrically using an excitation wavelength in the range of 280–320 nm and an

emission between 300 and 370 nm [68,126]. Fluorescence may be affected by pH. For example, the emission wavelength of 5-HIAA at pH 7 is at 330 nm but amounts to 550 nm in strong acid without a change in excitation wavelength. The discrepancy is due to differences in degree of ionisation and a pH should preferably be selected at which virtually 100% of the analyte exists as a single species [126]. Methods combining fluorometric and electrochemical detection have been described [125], but seem unnecessarily complicated for the aim. Kai et al. [127] described a procedure, which employs fluorescence detection of indoles after derivatisation with 1,2-diphenylethylendiamine. This HPLC method permits the simultaneous quantification of serotonin and 5-HIAA at detection limits at sub-femtomole levels.

5.5. Biological variation, interpretation of data

Several factors can influence levels of indoles in plasma and other biological matrices. Platelet serotonin content was found to be age, but not gender dependent. Significant differences were observed between newborns and children or adults. Moreover platelet serotonin concentration in elderly subjects was significantly lower than in adults and children and significantly higher than in newborns [100]. No significant variation in platelet serotonin content was observed over a period of 24-h, nor was any difference found between levels established in different seasons [115]. Both free and total plasma tryptophan showed a circadian rhythm, with maximum values observed in the afternoon and minimum values at night [115].

Platelet serotonin content, plasma 5-HTP and 5-HIAA concentrations, and urinary serotonin and 5-HIAA concentrations are increased in the presence of serotonin producing carcinoid tumours [12,36]. Platelet serotonin proved a more sensitive marker for increased serotonin production by carcinoid tumours than urinary 5-HIAA. In cases with high serotonin secretion rate platelet serotonin reaches a maximum at approximately 50 nmol/10^9 platelets, whereas urinary 5-HIAA does not [36]. Other neuroendocrine tumours and coeliac disease have been reported to give moderately increased platelet and plasma serotonin content, urinary serotonin and 5-HIAA excretion [12,128,129]. Increased platelet-poor plasma serotonin concentrations have been found in several disease states such as preeclampsia [101] and type 1 diabetes [130]. Menstrual cycle dependency was found in a study of 20 healthy women [131]. A higher periovulatory and premenstrual concentration of serotonin was found in platelet-poor plasma. We investigated the relation between platelet serotonin contents and corresponding platelet-poor plasma serotonin in both healthy persons and carcinoid patients. Increased platelet serotonin content in these patients was accompanied by increased concentrations of serotonin in platelet-poor plasma [36]. Short-term dietary intake of serotonin-containing foods did not influence platelet serotonin content, but increased the urinary excretion of both serotonin and 5-HIAA [27]. Increased platelet serotonin concentrations were found after prolonged intake of dietary serotonin [132] or ingestion of the pure amine for several days [37].

Significantly reduced platelet serotonin can be found in subjects using selective serotonin reuptake inhibitors [133]. Plasma tryptophan levels are dependent on dietary intake and have been found reduced in malabsorption syndromes [134], in several psychiatric disease states [135] and carcinoid disease. Cerebrospinal fluid (CSF) levels of indoles are dependent on dietary intake of tryptophan and are reduced in several neurodegenerative and psychiatric disease states [136]. Differences in concentrations were found between CSF from lumbar and ventricular origin, with higher concentrations in ventricular CSF [137].

6. Concluding remarks

The field of serotonin research is vast and rapidly expanding. The aim of the present review was to place the analysis of indoles in biological matrices in a biochemical, physiological and clinical context and to highlight several important steps in its chromatographic analysis and quantification. It can be concluded that developments in chromatographic analyses have moved the analysis of indoles away from the point at which they were considered complicated, cumbersome and sensitive to interference, to a position were they have become accessible to clinical

chemical laboratories that are equipped with modern chromatographic facilities.

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