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Quantification of serotonin and eight of its metabolites in plasma of healthy volunteers by mass spectrometry

Philippe J. Eugster [*], Marielle Dunand, Baptiste Grund, Anton Ivanyuk, Nathalie Fogarasi Szabo, Carine Bardinet, Karim Abid, Thierry Buclin, Eric Grouzmann [1], Haithem Chtioui [1]

Service of Clinical Pharmacology, Lausanne University Hospital and University of Lausanne, Switzerland

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ABSTRACT

Serotonin is transformed into melatonin under the control of the light/dark cycle, representing a cornerstone of circadian rhythmicity. Serotonin also undergoes extensive metabolism to produce 5-hydroxyindoleacetic acid (5-HIAA), a biomarker for the diagnosis and monitoring of serotonin secreting neuroendocrine tumors (NETs). While serotonin, melatonin and their metabolites are part of an integrated comprehensive system, human observations about their respective plasma concentrations are still limited.

We report here for the first time a multiplex UHPLC-MS/MS assay for the quantification of serotonin, 5-HIAA, 5-hydroxytryptophol (5-HTPL), *N*-acetyl-serotonin (NAS), Mel, 6-OH-Mel, 5-methoxytryptamine (5-MT), 5-methoxytryptophol (5-MTPL), and 5-methoxyindoleacetic acid (5-MIAA) in human plasma. Analytes were extracted by protein precipitation and solid phase extraction. Plasma concentrations for these analytes were determined in 102 healthy volunteers.

The LLOQ of the assay ranges from 2.2 nM for serotonin to 1.0 pM for 6-OH-Mel. This sensitivity enables the quantification of circulating serotonin, 5-HIAA, NAS, Mel, and 5-MIAA, even at their lowest diurnal concentrations.

This assay will enable specific, precise and accurate measurement of serotonin, Mel and their metabolites to draw a detailed picture of this complex pineal metabolism, allowing a dynamic understanding of these pathways and providing promising biomarkers and a metabolic signature for serotonin-secreting NETs.

1. Introduction

Serotonin (5-HT) and melatonin (Mel) are two indolic signaling molecules deriving from tryptophan, an essential amino acid provided by food (Fig. 1). They are both widely distributed in animal and plant species as well as unicellular organisms [1,2]. In humans, 5-HT is found in high concentrations in enterochromaffin cells located in the gastrointestinal tract and in storage granules of platelets [1]. Mel is a pineal gland hormone, notably regulated by the light/dark cycle, but other organs also have an ability to produce it [3]. Both neurohormones are involved in several regulations including circadian rhythmicity, chronobiology and further adaptations to the environment [1,2,4–6].

Several works highlighted the clinical importance of 5-HT, Mel, and their metabolites. Some of them contribute in mediating physiologic functions, while others represent useful biomarkers for altered health conditions [7,8]. For example, while serotonin syndrome (considered to result from 5-HT overexposure) is a recognized adverse effect of several drugs acting on the 5-HT system, we still lack diagnostic tools for this syndrome. 5-hydroxyindoleacetic acid (5-HIAA), a main 5-HT metabolite, is a biomarker for the diagnosis and treatment monitoring of serotonin-secreting neuroendocrine tumors (NETs) and is currently measured in urine [9]. The 5-hydroxytryptophol (5-HTPL)/5-HIAA ratio in urine has been proposed as a marker of recent ethanol consumption [10]. *N*-acetyl-serotonin (NAS) is an important intermediate in the two-step interconversion of 5-HT into melatonin [5]. NAS has been thoroughly investigated in sleep research and may be of interest for the study of aging associated cognitive impairment, depression or oxidative stress [11]. Most methoxyindoles are documented in animal studies to play a

* Corresponding author.
E-mail address: philippe.eugster@chuv.ch (P.J. Eugster).
[1] Both senior authors contributed equally to this work.

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role in a daily rhythmicity [12]. Although high concentrations of 5-methoxyindoleacetic acid (5-MIAA) are reported in animal pineal gland, its plasma concentration is still poorly documented in humans [13].

Serotonin, melatonin and their metabolites should be considered as an integrated comprehensive system, and measurement methods providing a complete picture of this complex metabolism are best suited to understand the ongoing physiologic processes and the interplay between metabolic intermediates. However, studying the serotonin-melatonin system and measuring plasma concentrations represents a technical and methodological challenge, as the pineal gland has poor accessibility and several molecules of interest exhibit a large range of concentrations and often circulate at very low concentrations in the plasma. Analytical assays must thus provide sufficient sensitivity and specificity in order to accurately assess and quantify small variations over time, allowing for example the accurate determination of Dim Light Melatonin Onset (DLMO) [14].

Immunoassays were developed mainly for the quantification of melatonin [15]. Later, liquid chromatography (LC) methods were applied to the quantification of 5-HIAA, 5-HTPL and Mel based on the fluorimetric detection of the indole nucleus [16,17], or using electrochemistry [18]. However, though sensitive, fluorimetric and electrochemical detections suffered from many artifacts originating from the matrix. On the other hand, mass spectrometry (MS) has successfully been used for the quantification of endogenous 5-HT [19–21], 5-HIAA [19,22,23], NAS [24], and Mel [24–29] in plasma and urine. MS-based methods combine both high sensitivity and specificity, lacking in the aforementioned techniques, together with the ability to quantify many metabolites simultaneously. However, no method has been yet reported to quantify 5-HT metabolites that circulate with four orders of magnitude concentration differences in a single analysis run and with sufficient sensitivity to measure diurnal NAS, Mel or 6-

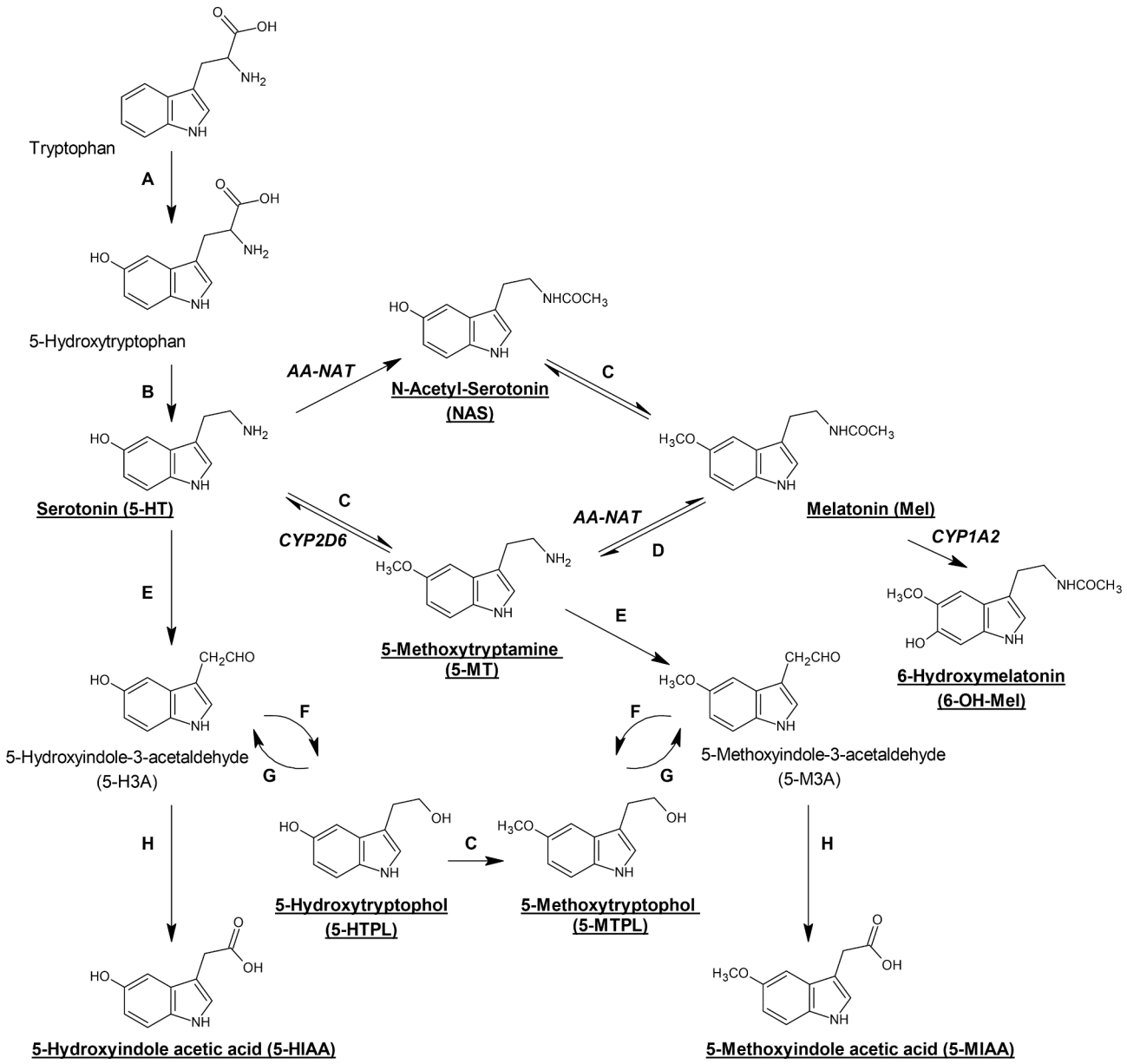

Fig. 1. Metabolic pathways from serotonin and its derivatives. **A**: tyrosine hydroxylase (EC 1.14.16.4); **B**: aromatic-l-amino acid decarboxylase (AADC; EC 4.1.1.28); **C**: hydroxyindole O-methyltransferase (HIOMT; EC 2.1.1.4); **D**: arylacylamidase (AAA; EC 3.5.1.13); **E**: monoamine oxidase type A (MAO-A; EC 1.4.3.4); **F**: aldehyde reductase (ALDR; EC 1.1.1.2); **G**: alcohol dehydrogenase (ALH; EC 1.1.1.1); **H**: aldehyde dehydrogenase (ALDH; EC 1.2.1.3); **AA-NAT**: Arylalkylamine *N*-acetyl transferase (AANAT; EC 2.3.1.87). Metabolites measured by our method are underlined. Figure modified from [30].

hydroxymelatonin (6-OH-Mel) at their lower concentration during the circadian rhythm. In this regard, proper reference intervals in plasma are established only for 5-HT and 5-HIAA [19,20,23].

The first aim of this work was to develop and fully validate the first multiplex ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) assay for the simultaneous quantification of 5-HT, 5-HIAA, 5-HTPL, NAS, Mel, 6-OH-Mel, 5-methoxytryptamine (5-MT), 5-methoxytryptophol (5-MTPL), and 5-MIAA in human plasma. The second aim was to describe the physiological ranges of corresponding concentrations at a given time-point in healthy volunteers.

2. Materials and methods

2.1. Chemicals and reagents

Charcoal-stripped plasma was obtained from Seracare (Milford, MA, USA). Water, acetonitrile (ACN), isopropyl alcohol (IPA), methanol (MeOH) and formic acid (FA) ULC/MS grade were purchased from Biosolve (Dieuze, France). Ascorbic acid, acetic acid, and ammonium formate were from Fluka (Buchs, Switzerland), ammonium acetate was obtained from Sigma-Aldrich (Saint-Louis, USA), and EDTA titriplex was purchased from Merck (Darmstadt, Germany). 5-HT, D_4-5-HT, 5-HIAA, 6-OH-Mel, and 5-MT were purchased from Sigma. D_5-5-HIAA, D_8-5-HTPL, D_4-NAS, Mel, D_4-Mel, D_4-6-OH-Mel, D_5-5-MT, 5-MTPL, D_8-5-MTPL, 5-MIAA, and D_6-5-MIAA, were purchased from Alsachim (Illkirch-Graffenstaden, France). 5-HTPL was purchased from LGC standards (Teddington, UK) and NAS was purchased from Interchim (Montluçon, France). Two ammonium acetate 12.5 mM buffers were prepared: buffer A containing 100 μM ascorbic acid set at pH 4 and buffer B set at pH 7.

2.2. QC and calibrants preparation

Calibration curves and quality control (QC) samples were prepared at the concentrations listed in Tables S1 and S2 in charcoal-stripped plasma, previously spiked with EDTA 0.34 M and ascorbic acid 10 mM (12 and 10 μL per mL of plasma, respectively). The calibration curve was prepared by serial dilutions and QCs were spiked separately and stored at −80 °C.

2.3. Sample preparation

The plasma samples (400 μL) were added to 1200 μL of ACN and 20 μL of internal standard (IS) solution in a Protein Crash plate (2 mL, pore size 0.2 μm, Interchim) and vortex mixed. The IS solution contained the deuterated analytes at the following concentrations in buffer A: D_4-5-HT, 250 ng/mL; D_5-5-HIAA, 2′000 ng/mL; D_8-5-HTPL, 5 ng/mL; D_4-NAS, 2.0 ng/mL; D_4-Mel, 0.125 ng/mL; D_4-6-OH-Mel, 12.8 ng/mL; D_4-5-MT, 1.28 ng/mL; D_8-5-MTPL, 2.4 ng/mL; and D_6-5-MIAA, 10 ng/mL. The supernatant was filtered by positive pressure into a 2 mL square collection plate (Waters, Milford, MA, USA). The filtrate was evaporated to dryness under nitrogen at 45 °C and the solid extract residue was reconstituted in 150 μL of a mix of buffer A:MeOH (95:5) and shaken for 10 min. The solution was mixed with 500 μL of buffer A and loaded on an Oasis HLB μElution Plate (Waters) previously conditioned with 200 μL ACN and 200 μL H_2O. The phase was washed with 400 μL of buffer B and the analytes eluted with 2x25 μL 90 % ACN in H_2O in a 700 μL round 96-well plate (Waters) containing 5 μL ascorbic acid 1 mM per well. The wells were evaporated to dryness under a steam of nitrogen at 45 °C, the residue was reconstituted in 20 μL of a solution of buffer B:MeOH (95:5), shaken for 10 min and centrifuged at 2500 RCF for 10 min prior to injection.

2.4. UHPLC-MS/MS analysis

Separations were carried out on a Waters Acquity UPLC Acquity I-class (FTN) system coupled to a Xevo TQ-S equipped with its standard electrospray ionization (ESI) source. The auto-sampler and column temperatures were set to 10 and 35 °C, respectively, and the injection volume to 10 μL. Compounds were separated on an Acquity BEH C18, 130 Å, 1.7 μm, 2.1 × 100 mm column (Waters) protected by a VanGuard BEH C18, 130 Å, 1.7 μm, 2.1 × 5 mm pre-column (Waters). Analytes were separated using the gradient described in Table S3, with a mobile phase composed of (A) ammonium formate 7.5 mM in water set to pH 2.65, and (B) 0.1 % FA in ACN. After injection, the needle was washed with ACN:MeOH:water:IPA (1:1:1:1) and 5 % ACN in water as strong and weak wash solutions, respectively. Compounds of interest were detected in multiple reaction monitoring (MRM) mode using positive ionization, with the following parameters: the capillary voltage was set at 0.75 kV, the desolvation temperature was set at 650 °C, the desolvation, cone and nebulizer gas flow were set at 600 l/h, 150 l/h and 7.0 bar respectively. The transitions, cone and collision voltages and dwell times are listed in Table S4.

2.5. Method validation

Intra-assay imprecision and bias were evaluated by extracting and analyzing independently quintuplicates of the three validation samples in one single series. Inter-assay values were determined by analyzing the three samples in 14 different series. The imprecision was expressed as the relative standard deviation (CV) calculated on the concentrations measured for the replicates, and the bias as the difference between the mean measured value and the target value. Limit of detection (LOD) and lower limit of quantification (LLOQ) were assessed by measuring the analytes in quintuplicates of samples obtained by dilutions of the calibration curve in charcoal-stripped plasma. LOD was defined as the lowest concentration providing a discrete peak with a signal over noise ratio (S/N) > 3 and LLOQ was determined as the lowest concentration providing CV and deviation from theoretical concentration lower than 20 % and S/N > 5. Matrix effects and recovery were evaluated using the method described by Matuszewski [31] at concentrations corresponding to the three levels. Carry-over was monitored by injecting a blank plasma after the highest calibrant. The area measured in the blank should be$<$20 % of the peak area measured in the lower calibrant. Selectivity was assessed by searching for interferences in blank plasma samples and in the healthy volunteers' samples (see below).

2.6. Stability study of the analytes

Freeze and thaw stability of the analytes in plasma was tested for three cycles, from −80 °C to 25 °C, using triplicates of low and high QC samples. The short-term stability in blood was studied at 25 °C for 2 and 4 h, and in charcoal-stripped and native plasma at 25 °C for 2 and 4 h, as well as at 4 °C for 4 and 24 h, all of them at low and high QC levels (only high level for charcoal-stripped plasma). Post-preparative stability was assessed by storing extracted samples from volunteers in the autosampler at + 10 °C for 72 h before injection in the UHPLC-MS/MS system. Stability of the analytes in stock solutions was studied with 20 ng/mL in buffer B:MeOH (95:5) solutions placed on ice for 6 h. Long-term stability was studied on 14 samples from volunteers that were analyzed before and after storage at −80 °C for 4 months. The stability values were expressed as a relative recovery, i.e. ratios of post-experiment over pre-experiment peak areas.

2.7. Statistical analyses

The non-parametric calculation of the percentiles 2.5 and 97.5 (with corresponding confidence intervals) of physiological concentrations was performed for each analyte.

All descriptive and statistical calculations (including Wilcoxon's rank-sum and Spearman's nonparametric correlation analyses from volunteer's data) were performed using the Stata® software (version 17 StataCorp, 4905 Lakeway Drive, College Station, Texas 77,845 USA; https://www.stata.com) and Prism 8.3.0 (GraphPad Software, San Diego, CA). By convention, all values below LLOQ were replaced by LLOQ/2 in statistical descriptions [32].

2.8. Observational study and plasma sample collection

We conducted an observational study in healthy subjects in order to determine plasma concentrations of serotonin, melatonin and seven metabolites. The study was approved by the ethical committee of the Etat de Vaud, Switzerland (ref:470/13), and was conducted according to good clinical practice and the international conference on harmonization (ICH) requirements. All subjects gave their written informed consent. Subjects aged between 18 and 80 years, with body mass index (BMI) between 18 and 29.9 kg/m^2, who had no history of any significant disease, no acute illness, no sleep disorders, all non-smokers and not taking any medication during the last week, were eligible for the study (contraception was tolerated in women). Subjects were asked to refrain from taking any food rich in serotonin or possibly interfering with its metabolism (chocolate, bananas, nuts, coffee, tea, alcohol, etc…) for at least 24 h before the study visit. Subjects were instructed to fast overnight.

Blood was collected between 8 and 10 am, in 9 mL EDTA Monovettes tubes (Sarstedt, Nümbrecht, Germany) containing 108 μL of a 0.34 M EDTA titriplex solution. Blood was centrifuged at 4 °C, 2753 RCF for 10 min, within 15 min after collection. Plasma underwent quick-freeze procedure before being stored in Eppendorf tubes at −80 °C until analysis.

3. Results

3.1. Method development

A two-steps sample preparation based on protein precipitation by acetonitrile and a reversed-phase solid phase extraction was required to obtain the desired sensitivity. The chromatographic separation on a C18 column was optimized to separate the nine metabolites without co-elution over 12.6 min. Deuterated IS for each of the analytes were used to decrease imprecision and bias. A charcoal-stripped commercial blank plasma was used to prepare calibration and quality control (QC) samples as it contains no or low concentration of naturally occurring analytes. However, traces of endogenous Mel (2.5 pM) and 5-MIAA (30 pM) were still detectable in this plasma, and were taken into account for the preparation of QC and calibrants (Table S1). Fig. 2 shows chromatograms of the analysis of a healthy volunteer sample.

3.2. Method validation

The intra-assay imprecision (CV on quintuplicates) on the three QC levels ranged from 0.7 % to 20.9 % (Table 1). The inter-assay imprecision and bias (calculated on 14 series) ranged from 1.7 % to 18.4 % and from −17.8 % to 20.0 %, respectively, except for NAS at the lowest level (36.8 and 52.9 %). The high CV and bias on the lowest QC for NAS were

Table 1
Intra- and inter-assay imprecision and bias.

Analyte	Target concentration	Intra-assay imprecision (CV, n = 5)	Inter-assay imprecision (CV, n = 14)	Bias (n = 14)
5-HT (nM)	5.68	1.7 %	6.0 %	13.2 %
	177	2.0 %	2.3 %	1.0 %
	426	1.4 %	2.8 %	−17.8 %
5-HIAA (nM)	1.05	6.0 %	16.4 %	3.2 %
	65.4	1.6 %	2.9 %	2.0 %
	157	1.3 %	3.5 %	4.7 %
5-HTPL (nM)	0.451	20.4 %	18.3 %	−11.6 %
	14.1	2.7 %	9.5 %	−1.5 %
	33.9	2.5 %	9.9 %	−4.4 %
NAS (pM)	0.458[a]	19.5 %	36.8 %	52.9 %
	2.75[a]	20.9 %	14.5 %	23.3 %
	143	3.2 %	7.3 %	13.2 %
	344	2.4 %	7.0 %	11.4 %
Mel (pM)	6.83	1.3 %	1.7 %	7.3 %
	272	1.2 %	3.0 %	19.4 %
	648	0.7 %	1.8 %	17.5 %
6-OH-Mel (pM)	2.82	8.3 %	8.7 %	9.1 %
	644	1.6 %	3.1 %	−13.9 %
	1547	2.2 %	2.2 %	6.7 %
5-MT (pM)	52.6	3.3 %	7.4 %	14.7 %
	841	1.9 %	6.0 %	20.0 %
	2019	2.4 %	4.7 %	18.7 %
5-MTPL (pM)	26.1	3.5 %	5.3 %	12.2 %
	784	2.4 %	3.9 %	14.5 %
	1883	1.0 %	4.4 %	13.6 %
5-MIAA (pM)	73.6	7.9 %	18.4 %	−1.4 %
	5729	3.7 %	6.0 %	16.6 %
	13,711	3.9 %	6.5 %	16.6 %

[a] Low concentration samples for NAS were modified form 0.46 to 2.7 pM to lower imprecision and bias (see section 3.2).

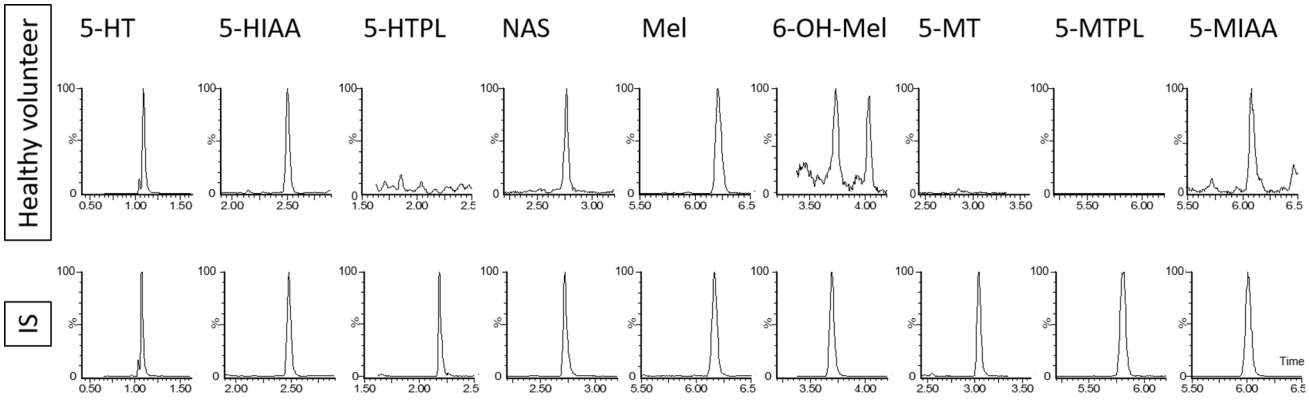

Fig. 2. UHPLC-MS/MS chromatograms of the analysis of the plasma from a healthy volunteer with the following concentrations: 5-HT: 134.8 nM; 5-HIAA: 32 nM; NAS: 18.8 pM; Mel: 85.7 pM; 6-OH-Mel 5.2 pM; 5-MIAA: 162 pM. The three other metabolites, 5-HTPL, 5-MT, and 5-MTPL were never detected. The upper chromatograms show the analytes, the lower chromatograms the corresponding internal standards.

explained by the *in vitro* production of NAS (see section 3.3. Stability of the analytes and Fig. S1). For this reason, its concentration was increased to 2.7 pM, providing inter-assay imprecision and bias of 14.5 % and 23.3 %. The limit of detection (LOD) and lower limit of quantification (LLOQ) are detailed in Table 2. 6-OH-Mel provided the lowest LLOQ (1.0 pM). Recovery (RE) and matrix effects (ME) were assessed using the method described by Matuszewski [31] and ranged from 0.9 % to 90.4 %, and from 59.6 % to 104.7 %, respectively, while process efficiency (PE) ranged from 0.9 % to 87.2 % (Table S5). The low CVs (<15 %) on the values measured for RE, ME, and PE confirm the good reproducibility of the extraction procedure and the efficient correction by the IS. No carry-over was detected, except for 5-HT (12.2 %) and 5-MT (9.7 %); endogenous concentrations prevented the proper measure of carry-over for Mel.

3.3. Stability of the analytes

The stability of all analytes was investigated in each step of the assay. In summary, the analytes appeared stable during freeze and thaw cycles (Table S6), as well as 4 h in whole blood (except 5-HT, Table S7) and plasma (except 6-OH-Mel, Table S7), 6 h in stock solutions placed on ice, and 72 h in the autosampler after sample preparation.

Long-term stability of the analytes in plasma was investigated on 14 samples from volunteers stored at −80 °C during 4 months (see Table S8). Among the six detected endogenous analytes, 5-HT, 5-HIAA, and Mel were stable in the studied conditions. Conversely, 5-MIAA showed degradation (-26.1 %) and 6-OH-Mel was not detectable in 10 out of the 14 samples after 4 months. Surprisingly, NAS increased (+82 %) because of *in vitro* production from 5-HT (Fig. S1). Based on these observations, we recommend analyzing the samples shortly after collection for NAS measurements. All results are detailed in Supporting Information.

3.4. Observational study

We included 111 healthy volunteers who fulfilled the study criteria, but due to a technical pre-analytical problem during centrifugation, only 106 samples were assessable. Four subjects were also excluded from the analysis because blood was sampled after 10 am. The remaining 102 volunteers were included in the analysis (50 males and 52 females); their median age was 28 years (range 18 to 70). Mean body weight was 66.8 kg (SD 12.0) and mean body mass index was 22.6 kg/m^2 (SD 2.2). Among these subjects, four women were taking oral contraceptives and two were carrying a hormonal intrauterine device.

The concentrations of the analytes observed in the samples from the 102 volunteers are presented in Table 3, Fig. 3, and detailed in Table S9. The overall distribution of values was skewed and much closer from a log-normal than a normal one. The highest concentrations of analytes were found as expected for 5-HT followed by 5-HIAA, whereas 1′000-10′000 lower concentrations were determined for 5-MIAA > Mel = NAS ≫ 6-OH-Mel. Endogenous 5-HTPL, 5-MT, and 5-MTPL were not detected in any samples.

Table 2
Limit of detection (LOD) and lower limit of quantification (LLOQ) of the UHPLC-MS/MS assay obtained for the nine analytes in plasma.

Analyte	LOD	LLOQ
5-HT (nM)	1.1	2.2
5-HIAA (nM)	0.41	0.41
5-HTPL (nM)	0.18	0.18
NAS (pM)	0.46	0.46 / 2.7[a]
Mel (pM)	1.7	1.7
6-OH-Mel (pM)	1.0	1.0
5-MT (pM)	2.6	21
5-MTPL (pM)	2.5	9.8
5-MIAA (pM)	38	38

[a] LLOQ for NAS was initially measured at 0.46 pM, but was set at 2.7 pM to cope with inter-assay imprecision (see section 3.2 Method validation).

Table 3
Concentrations of serotonin and its metabolites observed in a cohort of 102 healthy volunteers with 95% confidence interval between brackets. Plasma was collected between 8 and 10 am. All exogenous sources of 5-HT and its derivatives were banned during the 24 h preceding blood collection, as described in the Materials and methods section.

	Percentile 2.5	Percentile 50	Percentile 97.5
5-HT (nM)	69.8 (47.3–82.1)	192.7 (173.3–203.8)	389 (327.8–463.6)
5-HIAA (nM)	20.7 (16.9–22.1)	31.7 (29.4–33.7)	52.5 (49.4–54.5)
5-HTPL (nM)	N/A[a]	N/A[a]	N/A[a]
NAS (pM)	12.4 (11.0–14.8)	28.5 (24.9–32.7)	150 (78–248)
Mel (pM)	9.0 (7.8–11.1)	30.5 (24.9–34.8)	196 (117–362)
6-OH-Mel (pM)	N/A[b]	2.0 (1.7–2.3)	9.8 (5.9–18)
5-MT (pM)	N/A[a]	N/A[a]	N/A[a]
5-MTPL (pM)	N/A[a]	N/A[a]	N/A[a]
5-MIAA (pM)	118 (112–148)	264 (230–294)	578 (491–860)

[a] undetectable metabolites, [b] below LLOQ.

We did not observe any significant difference in the measured plasma concentrations associated to gender, except for 6-OH-Mel (median: 1.73 pM (range: 0.5–17.9) in males, 2.29 pM (range: 0.5–10.9) in females, $p = 0.03$). Only melatonin concentrations exhibited a poor but significant inverse-correlation with age (Spearman's correlation coefficient $r_s = -0.35$, $p = 0.02$), the highest concentrations being observed in the youngest subjects.

A clear correlation between metabolites was observed only for Mel and 6-OH-Mel ($r_s = 0.60$, $p < 0.0001$). Other correlations, as e.g. between 5-HIAA and NAS ($r_s = 0.44$, $p < 0.0001$), were not considered as relevant as r_s was lower than 0.5 (Table S10).

4. Discussion

Method validation

We describe for the first time an efficient UHPLC-MS/MS method that enables the simultaneous quantification of 5-HT and eight of its metabolites in human plasma. We succeeded in measuring analytes that circulate in a wide range of concentration. We optimized the sensitivity of the method for analytes present in low concentrations, without seriously compromising the analytical conditions for compounds circulating in high concentration. This assay allowed the specific and multiplex measurement of endogenous concentrations of six of the nine targeted metabolites from 400 µL of plasma. Only 5-HTPL, 5-MT, and 5-MTPL could not be detected in healthy volunteers, despite the remarkably low LOD of our method (<3 pM).

The cost of consumables per result for this assay and for immunoassays is similar, around USD 10. The sample preparation time for the MS assay is clearly longer than for the immunoassay (3 h vs some minutes), and the machine time is also longer, but fits well with overnight use of the MS instrument. However, this MS assay provides a unique multiplex capability, e.g. replacing two immunoassays for 5-HT and 5-HIAA that clinicians often require together, and an excellent specificity that immunoassays lack. The selection of MS or immunoassay must be carefully evaluated according to the needs of the laboratory.

Preanalytical conditions were optimized to prevent analytes degradation by adding ascorbic acid to the collection tubes to prevent 6-OH-Mel oxidation. However, some degradation of the analytes- cannot be completely avoided. First, 5-HT, but not its metabolites, degrades in whole blood at 25 °C. We therefore recommend centrifuging whole blood samples immediately after collection. Second, 6-OH-Mel degrades in plasma at 25 °C, but not at 4 °C. Storage of thawed plasma on ice is therefore recommended. Finally, 6-OH-Mel, and to a lesser extent 5-MIAA, degrade in plasma stored at −80 °C, but not during freezing nor thawing. Moreover, we observed, in plasma kept over a few hours at

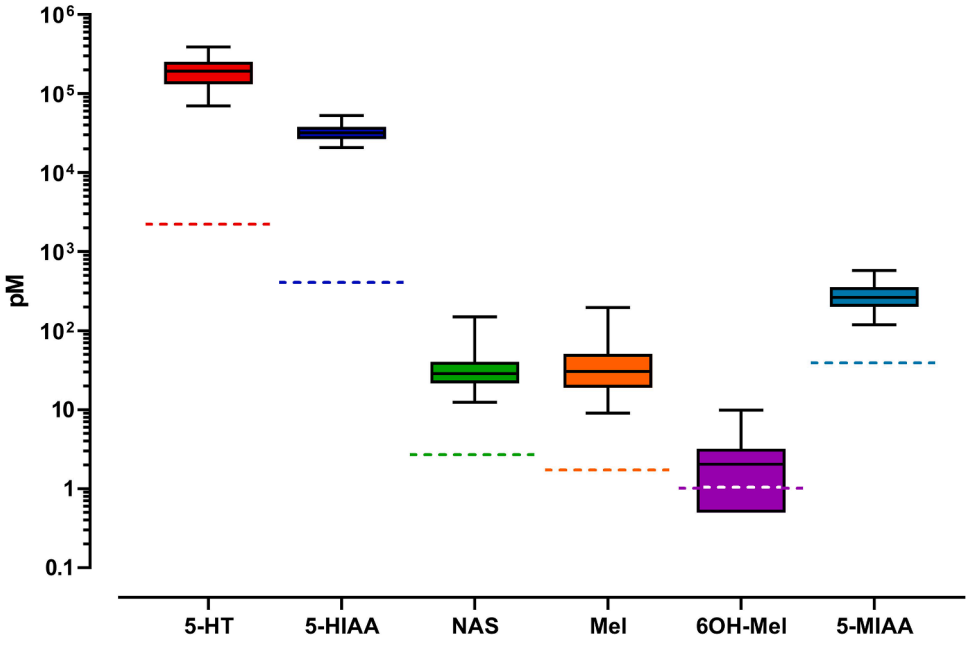

Fig. 3. Concentrations of serotonin and its metabolites observed in the plasma of a sample of 102 healthy volunteers collected between 8 and 10 am. The center line of each box represents the median, the box height the interquartile range, the whiskers the percentile 2.5 and 97.5 (log scale) and the dashed lines the LLOQ.

25 °C, an increase in NAS concentrations, possibly resulting from interconversion from other analytes (e.g. 5-HT) however without significant impact on other metabolite concentrations. We thus strongly recommend analyzing NAS and 6-OH-Mel rapidly after sample collection.

This development delivers a cutting-edge measurement tool that will enable further detailed investigation of circadian physiology of the serotonin-melatonin system. Our study provides a snapshot of analyte concentrations between 8 and 10 am, illustrating the dynamic range of this assay and its ability to describe physiological concentration ranges of analytes known for circadian variations, many of which have never before been quantified in human plasma. Fig. 3 represents an instant picture of analyte concentrations between 8 and 10 am in our 102 fasting subjects, capturing a wide range of concentrations (especially low concentrations) and emphasizing the suitability of our method for a routine clinical utilization. This tool will be especially useful in circadian rhythms and sleep research, for an accurate determination of daytime fluctuations and DLMO, where small variations of very low concentrations (<4 pM) need to be detected. Our assay, with an LLOQ for melatonin at 1.7 pM (0.4 pg/ml), is the first fully validated MS assay that allows to accurately quantitate melatonin at low picomolar physiological concentrations, with a specificity that lacks to immunoassay methods [14].

The inter-individual variability (expressed as CV) for the six detectable metabolites was relatively low (CV ≤ 43 %, see Table S9) except for Mel (CV 107 %), its precursor NAS (CV 86 %) and its direct metabolite 6-OH-Mel (CV 96 %), possibly as a consequence of individual circadian phase shifts for these analytes.

5-HT

5-HT is the most abundant indolic compound in plasma (69.8–389 nM). Physiological concentration values reported in the literature are heterogeneous. The 5-HT concentrations we observe are higher than those measured in earlier studies by LC-MS/MS (4.6 nM, 5–30 nM) [20,21], LC-ECD (3.5 nM) [18], or fluorescence detection (16.7 nM) [16]. Differences in preanalytical procedures may partly explain this variability, as it is necessary to avoid both spontaneous 5-HT degradation in the sample and contamination by 5-HT released from platelets. Several studies opted for platelet-poor plasma (PPP), but strategies for sample centrifugation proved to be both time consuming and highly variable (multi-step centrifugation, modified speeds and temperatures). Several authors suggested centrifugation at 22–25 °C to prevent platelet activation [33], resulting in PPP with low 5-HT concentrations. Our concentrations are however lower than those measured in platelets-rich plasma (1021 ± 233 nM) [34]. In our study, we avoided extreme centrifugation speeds or prolonged complex procedures. Preanalytical conditions were adjusted to ensure the stability of other analytes (including those for which concentrations have not previously been established) and to facilitate widespread use of the method in routine clinical settings.

5-HIAA

5-HT is mainly metabolized by MAO-A into an unstable intermediate 5-H3A, which is further transformed by ALDH to produce 5-HIAA. The measured concentration range (20.7–52.5 nM) is in line with the recently reported interquartile ranges of 37–56 nM (n = 68) in PPP [19] and 35–123 nM (n = 111) in serum [23]. The high nanomolar concentration of 5-HIAA show how efficient and predominant is the MAO-A/ALDH pathway at the expense of the other putative pathways.

Plasma 5-HIAA being less sensitive than 5-HT to preanalytical conditions, it appears of particular interest for routine clinical practice and its measurement in plasma would allow avoiding cumbersome 24-hour urine collections. Our method represents a valuable tool for the evaluation of this and possibly other candidate biomarkers for neuroendocrine tumors, providing a broader picture than a single analyte and establishing a metabolic profile of the secreting tumor, useful for both the diagnosis and the post-treatment monitoring of these patients. This deserves further investigations in dedicated studies.

5-HTPL is not detected in human platelet poor plasma

An alternative metabolic pathway from 5-H3A involving ALDR/ALH leads to 5-HTPL. However, 5-HTPL is not detected in our healthy volunteers' samples (despite our LLOQ at 0.18 nM), suggesting either that this metabolism is not efficient or that 5-HTPL is efficiently recycled into 5-H3A to produce 5-HIAA. In previous studies, 5-HTPL has been

measured by HPLC in human platelet-rich plasma [35] and in human plasma and brain by GC–MS [36] with reported concentrations of plasma free HTPL at 0.9 nM, as well as in urine by GC–MS (>96 % conjugated forms) [10]. Yet, our assay detected plasma 5-HTPL in relatively high amounts in a patient treated for serotonin secreting NET, suggesting a specific metabolic signature (data not shown).

NAS, Mel and 6-OH-Mel

The classical melatonin synthesis pathway occurs in the pineal gland by the acetylation of 5-HT by arylalkylamine *N*-acetyltransferase (AANAT) into NAS (Fig. 1) [37]. This rate-limiting step is under the control of the circadian light/dark cycle. We quantified NAS in all volunteers (12.4–150 pM). Previous studies reported slightly lower NAS ranges (7–29 pM) in PPP at 8 am [24]. Melatonin was also quantified in all 102 volunteers (9–196 pM), at concentrations in line with previous reports [17,24]. Our very low LLOQ at 1.7 pM (0.4 pg/ml) outperformed any available immunoassay required for its quantification during the light period [14]. Mel is further hydroxylated by CYP1A2 into 6-OH-Mel. Despite our excellent sensitivity (LLOQ = 1.0 pM), we quantified 6-OH-Mel in only 75 % of the samples (<1.0–9.8 pM) taken at a standard sampling time in the morning. To the best of our knowledge, ranges for 6-OH-Mel plasma concentrations have not been reported in the literature. 6-OH-Mel concentrations were clearly correlated to Mel ($r_s = 0.60$, $p < 0.0001$), confirming their tight metabolic relationship (see Table S10).

5-MT and 5-MTPL are not detected in human blood

Another Mel production route has been postulated for certain organisms, in which 5-HT is first O-methylated into 5-MT and then *N*-acetylated into melatonin. This may emphasize an interfacing role for 5-MT in the metabolism from Mel to 5-MIAA [38]. To add another level of complexity, Mel is also able to regenerate NAS (involving cytochrome enzymes) [39]. However, Axelrod found in pioneer studies with HIOMT purified from bovine pituitary glands that in contrast to NAS, 5-HT is a poor substrate of this enzyme, though it is still debated whether it is also the case with the human HIOMT [37]. We believe this alternative hypothesis represents a minor pathway in man, since 5-HT circulates at high concentrations and 5-MT has never been detected in any of our 102 samples (LOD 2.6 pM). Only few references [40,41] reported the detection of 5-MT in a limited number of human plasma samples, suggesting its rapid transformation, whereas others failed to detect it in hundreds of human plasma samples [42]. However, we cannot exclude that the synthesis of 5-MT is restricted to some tissues like the pineal gland with poor diffusion in plasma, or that the absence of 5-MT would be due to a high turnover, since it can be both converted back into 5-HT by CYP2D6 [30], and rapidly degraded into 5-M3A by MAO-A [43]. In this respect, the non-detection of 5-MTPL in plasma parallels what is observed for 5-HTPL. 5-MTPL is thought to derive from 5-MT after oxidation by MAO-A into 5-M3A and reduction by ALDR. Interestingly, 5-MTPL was measured in plasma from children aged 6–14 years using a RIA (52–105 pM, or 10–20 pg/mL), which actually questions the specificity of this immunoassay [44].

What could be the origin of 5-MIAA?

5-MIAA that has been found in relatively high concentrations (118–578 pM) is expected to derive from 5-M3A. This questions the origin of this end-product metabolite in the absence of detectable precursor substrate (5-MT), unless this pathway could be restricted to specific tissues without being completely reflected in plasma measurements, or unless a yet unknown alternative pathway would be responsible for the metabolism of Mel into 5-MIAA. The transformation of hydroxyindoles into methoxyindoles by HIOMT is also reported but seems here to occur with a relatively low efficiency since 5-MIAA concentration is only 1 % of 5-HIAA concentration, without correlation between concentrations of both metabolites (see Table S10).

In our subjects, a significant difference between women and men was only found for 6-OH-Mel. A statistically significant correlation, but with poor r_s was observed between Mel and age. The relation between Mel and age is widely discussed in the literature, the latter essentially affecting the amplitude of Mel peaks during the night [45]. The poor relevance of the correlation observed here could be explained by the circadian shift between subjects and the absence of extreme ages within our volunteers.

In conclusion, this is the first multiplex LC-MS/MS method enabling the quantification of 5-HT and its metabolites in human plasma with sufficient sensitivity to quantify simultaneously 5-HT, 5-HIAA, NAS, Mel, 6-OH-Mel, and 5-MIAA. This assay is suitable for routine analysis and provides high throughput with the use of 96-wells plates and a total chromatographic run time of 12.6 min. It provides a broad picture of serotonin-melatonin metabolism, promoting the perspective of a better integrated understanding of the system. The high sensitivity and the low imprecision of this analytical assay will enable its use in investigating pineal physiology and for the determination of parameters as sensitive as DLMO. It will offer a dynamic insight into this metabolism through repeated measurements over 24 h. This assay also opens new perspectives for looking at the measured analytes as potential biomarkers in a vast range of health and disease conditions extending from oncology to psychiatry. It offers also a valuable tool for the investigation of drug metabolizing enzymes using the measured substances as endogenous probe substrates. In particular, recent studies are evaluating plasma 5-HIAA as an emerging accurate biomarker for the diagnosis and management of NETs. Moreover, combining 5-HIAA with other analytes may be of interest in establishing a metabolic signature for serotonin secreting NETs.

CRediT authorship contribution statement

Philippe J. Eugster: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Supervision, Writing – original draft, Writing – review & editing. **Marielle Dunand:** Data curation, Formal analysis, Methodology, Validation, Visualization. **Baptiste Grund:** Data curation, Formal analysis, Methodology, Writing – original draft. **Anton Ivanyuk:** Resources. **Nathalie Fogarasi Szabo:** Resources. **Carine Bardinet:** Resources. **Karim Abid:** Resources. **Thierry Buclin:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing. **Eric Grouzmann:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. **Haithem Chtioui:** Conceptualization, Funding acquisition, Resources, Data curation, Formal analysis, Investigation, Visualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2022.08.012.

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