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Targeted analysis of seven selected tryptophan-melatonin metabolites: Simultaneous quantification of plasma analytes using fast and sensitive UHPLC–MS/MS

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ABSTRACT

Tryptophan-derived metabolites, a group of neurotransmitters essential for various brain functions, play key roles in regulating mood, movement, sleep, and cognition. However, the comprehensive characterisation of tryptophan-melatonin pathway metabolites is challenging due to factors such as their structural diversity, chemical complexity, low concentrations, and instability of these metabolites. In this study, we developed and validated an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) methodology with electrospray ionisation for the simultaneous separation and quantification of tryptophan metabolites in human plasma. The analytical calibration ranges in plasma were 0.50–200 ng/mL for serotonin, 0.01–5 ng/mL for *N*-acetylserotonin, 0.01–20 ng/mL for tryptamine, 0.01–20 ng/mL for 6-sulfatoxymelatonin, 0.01–20 ng/mL for 6-hydroxymelatonin, 0.01–100 ng/mL for melatonin, and 0.10–20 ng/mL for *N*-acetyltryptamine, with correlation coefficients ranging from 0.954 for *N*-acetyltryptamine to 0.997 for tryptamine. The intraday and interday precision remained consistently below 15 % for all analytes. Most analytes met the accuracy criteria, except for *N*-acetyltryptamine at the lowest quality control level (0.2 ng/mL), where the intraday and interday accuracy were 22.4 % and 17.4 %, respectively. In conclusion, this novel method allows for rapid identification of tryptophan-melatonin pathway intermediates in less than ten minutes, including seven distinct melatonin-related analytes. This suggests that it may find use in everyday clinical and scientific endeavours.

1. Introduction

L-Tryptophan (TRP) metabolism yields a range of bioactive compounds with significant implications for human physiology [1,2]. These effects extend far beyond the neurological domain, exerting regulatory influence over many physiological processes. Therefore, low molecular weight potential biomarkers are becoming recognised as important parts of the complex molecular landscape linked to different diseases [1,3,4]. TRP is subject to enzymatic processes such as decarboxylation, hydroxylation, and methylation, leading to the formation of TRP derivatives in the nervous system of humans [1,2,5,6]. Notably, hepatic metabolism contributes significantly to the biotransformation of melatonin (MEL), wherein MEL is hydroxylated to form 6-hydroxymelatonin (6-HMEL) [7]. Subsequently, renal processes lead to the conjugation of 6-HMEL with sulfate, resulting in the formation of 6-sulfatoxymelatonin (6-SMEL), which is excreted in urine. Monitoring urinary levels of 6-SMEL, a major hepatic metabolite of MEL, serves as a valuable indirect method for assessing pineal gland MEL production [7]. A simplified overview of selected parts of TRP metabolism in this study is shown in Fig. 1.

Over time, scientists have created many different methods, each with its own strengths and weaknesses. Chromatographic techniques such as gas chromatography (GC) and liquid chromatography (LC), in combination with mass spectrometry (MS), play a crucial role in the development of analytical chemistry methods owing to their ability to selectively and sensitively analyse substances [8,9]. However, the

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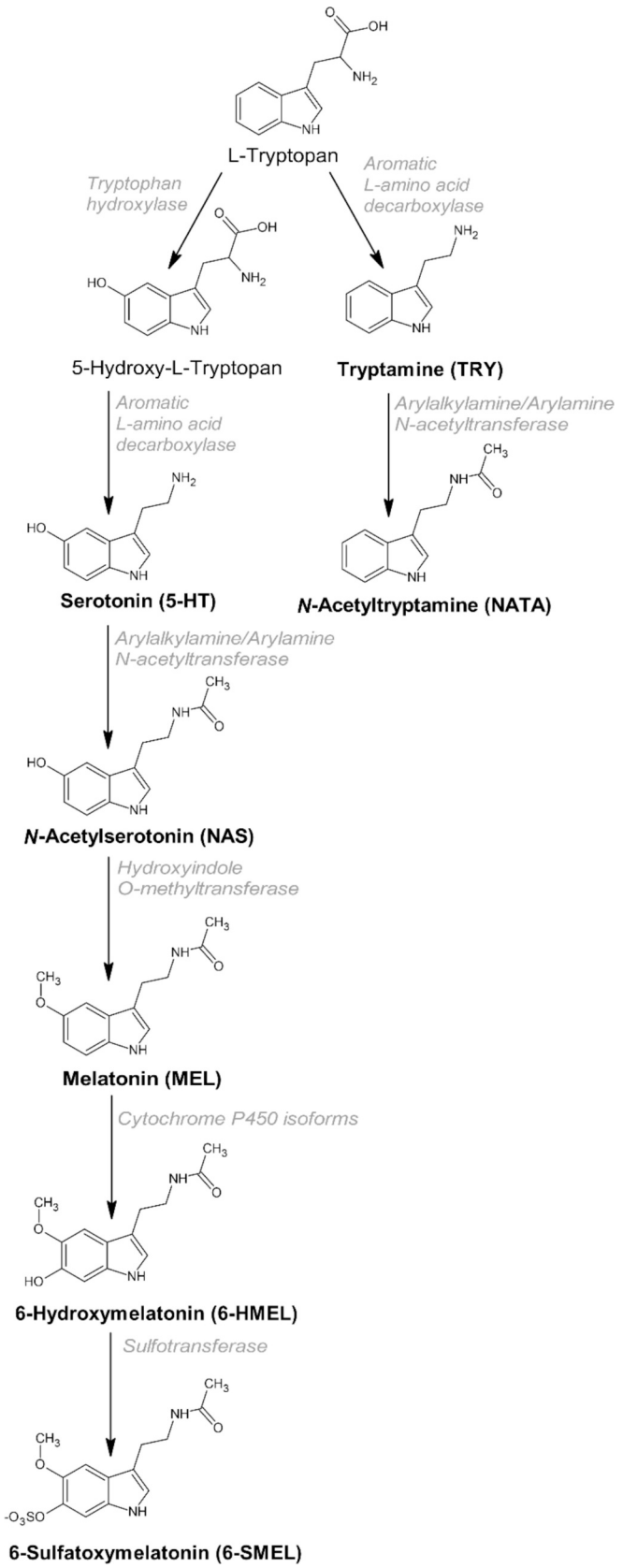

Fig. 1. An overview of melatonin and *N*-acetyltryptamine synthesis and metabolism. Selected analytes are in bold.

utilisation of GC–MS in TRP metabolite analysis is less common, especially in clinical settings, largely due to the requirement for chemical derivatisation [9,10]. In recent years, methods combining liquid chromatography–tandem mass spectrometry (LC–MS/MS) with positive electrospray ionisation (ESI^+) have attracted great interest. LC–MS/MS-based methods have been successfully used for the determination of TRP metabolites in various biological samples using different chemistry of reverse stationary phases [8,9,11–16]. One of the most notable advantages of LC–MS/MS methods is that they do not require chemical derivatisation. In summary, recent developments in LC–MS/MS technology have ushered in a new era of TRP-related compound analysis, offering researchers higher sensitivity, improved specificity, faster results, and greater analytical efficiency [8].

The primary objective of this study was to develop and validate a highly sensitive ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method capable of simultaneously detecting and quantifying specific TRP-related metabolites in human plasma Fig. 1. Following successful validation, the method was applied to analyse these target analytes in the body fluids of a few volunteers.

2. Material and methods

2.1. Chemicals and reagents

Standards serotonin (5-HT), *N*-acetylserotonin (NAS), tryptamine (TRY), 6-SMEL, 6-HMEL, MEL, *N*-acetyltryptamine (NATA) from Merck (Stockholm, Sweden), and the deuterated internal standards serotonin-d_4 (5-HT-d_4), *N*-acetylserotonin-d_3 (NAS-d_3), tryptamine-d_4 (TRY-d_4), 6-sulfatoxymelatonin-d_4 (6-SMEL-d_4), 6-hydroxymelatonin-d_4 (6-HMEL-d_4), and melatonin-d_4 (MEL-d_4) were purchased from (TRC, Toronto, Canada). All stock and working solutions of both internal and unlabelled standards were prepared in 100 % methanol and stored at −80 °C in the dark until required for experimental use. Acetonitrile, methanol, formic acid, and ascorbic acid of the highest purity were obtained from Fisher Scientific (Göteborg, Sweden). Ultra-pure water was produced using the Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Participants and sample collection

Healthy human plasma samples were provided by Academic Hospital, Uppsala, Sweden. The research was approved by the National ethical review board. Informed consent was obtained from all participants involved in the study. Peripheral blood was collected by venepuncture under standardised and sterile conditions at around 8:00 a.m. into EDTA vacutainer tubes, then centrifuged at 3500 *g* for 15 min. The obtained plasma was aliquoted and stored at −80 °C in the dark until analysis.

2.3. Sample preparation

In brief, 250 µL of human plasma samples were mixed with 50 µL of corresponding deuterated internal standard mixtures. To measure the amount of each analyte in the sample, 5-HT-d_4 (20 ng/mL), NAS-d_3 (3 ng/mL), TRY-d_4 (0.5 ng/mL), 6-SMEL-d_4 (10 ng/mL), 6-HMEL-d_4 (10 ng/mL), and MEL-d_4 (20 ng/mL) were added as internal standards. The concentrations given in brackets correspond to the final concentration of each internal standard in the processed samples. The inclusion of these internal standards facilitated analyte quantification via the isotope dilution method. The simple protein precipitation and liquid extraction method was carried out with 750 µL of 0.1 % formic acid in acetonitrile at 4 °C. The extraction solvent mixture also contains 0.01 % ascorbic acid as an antioxidant. After vortexing for 5 min, the samples underwent a 15-min incubation at −80 °C, followed by centrifugation at 13.000 rpm for 20 min at 4 °C. The supernatant was transferred to a new tube and gently evaporated under a nitrogen stream. Before analysis, the dry sample residues were reconstituted in 50 µL of a solution containing 0.01 % formic acid in methanol. Throughout the sample processing,

light exposure was minimised to prevent analyte degradation.

The quantitative analysis relied on matrix-matched calibration curves constructed using healthy human plasma obtained from Uppsala Academic Hospital in Sweden. The plasma was treated by charcoal stripping to produce analyte-free plasma [17,18]. A new aliquot of the analyte-free plasma was used for each experiment and stored in the dark at −80 °C until use. Calibrants were prepared in triplicate, following the same procedure as for the tested plasma samples. To each aliquot of analyte-free matrix (250 µL), 50 µL of a mixture of unlabelled standards at varying concentrations was added, along with 50 µL of a mixture of internal standards at fixed concentrations (the final concentrations of internal standards in the processed samples are listed above). The addition of the extraction reagent (750 µL, 0.1 % formic acid, and 0.01 % ascorbic acid in acetonitrile) was followed by vortexing, incubation, centrifugation, evaporation of the supernatant, and reconstitution. The final concentrations of unlabelled standards in each calibration sample are provided in the Supplementary Material in **Table S1**.

2.4. UHPLC–MS/MS analysis

Selected TRP metabolites were analysed by UHPLC–MS/MS using an Acquity™ UPLC system (Waters, Milford, MA, USA) coupled with Xevo® TQ-S MS (Waters, Milford, MA, USA). The samples were placed in an autosampler maintained at 8 °C during UHPLC–MS/MS analysis. The best separation efficiency was achieved using an Acquity UPLC premier HSS T3 column (1.8 µm, 2.1 × 100 mm, Waters). This chromatography column was equipped with the corresponding Acquity pre-column (1.8 µm, 2.1 × 5 mm, Waters). The temperature of the column system was set at 40 °C. The flow rate of the mobile phase containing 0.1 % formic acid in water (mobile phase A) and methanol (mobile phase B) was maintained at 0.3 mL/min. The injection volume was 10 µL. Analytes were eluted using a 10-min gradient that included a washing and equilibration step. The gradient elution was set to: 0.0–0.10 min, 2 % B; 0.1–7.0 min, 2–60 % B; 7.0–8.0 min, 60–99 % B; 8.0–9.0 min, 99–2 % B; 9.0–10.0 min, 2 % B. The total run time was 10 min per sample including the washing step.

The triple quadrupole MS with ESI^+ was operated in the multiple-reaction monitoring (MRM) mode. The desolvation temperature was set at 500 °C. The desolvation gas flow (nitrogen) was set to at 1000 L/h, and cone gas flow (nitrogen) was maintained to 150 L/h. The capillary voltage was 3 kV and the source offset was 50 V. The optimised MS parameters such as mass MRM transitions and collision energies (CE) are listed in Table 1. MassLynx™ 4.1 software (Waters, Milford, MA, USA) was used for system control and data acquisition and processing.

2.5. Method validation

Validation of the developed method was based on guidelines and recommendations issued by the European Medicines Agency (EMA) [19,20]. The validation included the evaluation of parameters such as the limit of quantification (LOQ), the linear range of the method for each analyte, carry-over, precision, accuracy, matrix effect (ME), recovery (RE), and stability.

LOQ represents the lowest concentration point of the calibration curve that can be reliably quantified, i.e. with certain precision (coefficient of variation, CV <20 %) and accuracy (relative error within ±20 %) [19,20]. The linearity of the method was tested using matrix-matched calibration curves prepared in analyte-free human plasma in triplicate. Regression analysis was used to determine linearity. The concentrations of the calibrators varied based on the analytes as shown in **Table S1**. In addition, blank samples (processed matrix without analyte and internal standards) and zero samples (processed matrix spiked with internal standards) were also included. The carry-over for the developed method was determined by analysing a pure solvent (100 % methanol) immediately after injection of the highest calibration point.

Analyte-free plasma prepared by charcoal stripping was spiked with standards at known different concentrations to prepare replicate quality control samples (QC1–QC4). These quality control samples were used to determine accuracy, precision, RE, and ME. The selected quality control levels (ng/mL) for each analyte are given in Table 3. The prepared set of validation samples was measured on one day (intraday precision and accuracy) and in three different days (interday precision and accuracy). In the case of accuracy, the mean measured concentrations should be within ±15 % of the nominal value of the quality control samples (±20 % for LOQ) [19,20]. Precision is expressed as the CV, which should not exceed 15 % [19,20]. Post-extraction spiked plasma samples were compared with a spiked neat solution to determine the ME. The resulting ME was expressed as a percentage. An ME value lower or higher than 100 % indicates ionisation suppression or enhancement, respectively. Method RE was obtained by comparing the results of the analysis of plasma samples spiked to three quality control concentration levels before extraction and plasma samples spiked after extraction (represents 100 % RE).

The two-day stability in the autosampler was assessed for all analytes in processed plasma samples spiked at the QC2 level ($n = 3$, concentration of standards shown in **Table 3**) during storage at 8 °C for 48 h against freshly prepared calibrators. The bench-top stability of all analytes in the human plasma matrix was assessed by processing and analysing the endogenous QC2 after storage at room temperature for 4 h ($n = 3$). Freeze-thaw stability at −80 °C was investigated by subjecting three replicates of the endogenous QC2 samples to a total of five freeze-

Table 1
Optimised multiple-reaction monitoring transitions and collision energy for individual analytes and corresponding internal standards.

Analyte/ Internal standard	Internal standard	Molecular weight (g/mol)	Confirmation transition (m/z)	Quantification transition (m/z)	Collision energy (eV)
5-HT	5-HT-d_4	176.2	177.2>117.1	177.2.0>160.1	28/12
NAS	NAS-d_3	218.3	219.1>202.1	219.1>159.9	5/15
TRY	TRY-d_4	160.2	161.1>117.0	161.1>143.9	22/5
6-SMEL	6-SMEL-d_4	328.3	329.0>158.0	329.0>190.0	33/21
6-HMEL	6-HMEL-d_4	248.3	249.0>158.1	249.0>190.1	25/15
MEL	MEL-d_4	232.3	233.0>158.9	233.0>174.1	31/14
NATA	TRY-d_4	202.3	203.1>127.1	203.1>117.1	30/30
5-HT-d_4	-	180.2	181.1>121.1	181.1>164.2	28/12
NAS-d_3	-	221.3	**222.1>162.9**	**222.1>205.1**	**12/6**
TRY-d_4	-	164.2	165.1>121.1	165.1>147.9	22/5
6-SMEL-d_4	-	332.4	333.0>161.0	333.0>194.0	35/23
6-HMEL-d_4	-	252.3	253.0>161.1	253.0>193.1	27/15
MEL-d_4	-	236.3	237.0>162.9	237.0>178.0	31/15

5-HT, serotonin; NAS, *N*-acetylserotonin; TRY, tryptamine; 6-SMEL, 6-sulfatoxymelatonin; 6-HMEL, 6-hydroxymelatonin; MEL, melatonin; NATA, *N*-acetyltryptamine; 5-HT-d_4, serotonin-d_4; NAS-d_3, *N*-acetylserotonin-d_3; TRY-d_4, tryptamine-d_4; 6-SMEL-d_4, 6-sulfatoxymelatonin-d_4; 6-HMEL-d_4, 6-hydroxymelatonin-d_4; MEL-d_4, melatonin-d_4.

thaw cycles. Each freeze–thaw cycle consisted of thawing the quality control samples at room temperature and refreezing for a minimum of 12 h between each cycle. The previously unthawed zero-cycle quality control samples and fifth-cycle quality control samples were then processed and analysed.

3. Results and discussion

A simple and fast UHPLC–MS/MS method was developed and validated for the simultaneous analysis of seven selected TRP-related metabolites (5-HT, NAS, TRY, 6-SMEL, 6-HMEL, MEL, and NATA) in human plasma. The plasma comprises a diverse array of metabolites, which collectively create a complex mixture and make significant challenges in accurately detecting low-abundance metabolites within the sample. The developed method is anticipated to have considerable research potential and facilitate the quantification of the above-mentioned compounds. Finally, the results of the validation experiments are summarised in Tables 2, 3, 4, 5, and 6 and were based on recommendations issued by the EMA guidelines [19,20].

3.1. Sample preparation

The comprehensive profiling of low molecular weight metabolites in biological samples requires meticulous clean-up procedures to minimise the presence of interfering substances and to concentrate samples, especially when the analyte of interest is present at low levels. Plasma and serum samples inherently contain not only high concentrations of proteins but also lipids, salts, and small metabolites. These components can increase sample complexity, leading to matrix effects that interfere with accurate analyte quantification [21–23]. Additionally, they may cause damage to sensitive components of analytical instrumentation, particularly chromatographic systems, manifesting as reduced separation efficiency or column clogging. Furthermore, it is important to account for the fact that certain analytes, particularly steroid hormones, may be bound to plasma carrier proteins; thus, disrupting these interactions is crucial for the accurate measurement of total metabolite content. Sample preparation of low-abundance endogenous metabolites in complex biological matrices usually involves protein precipitation (e.g. with acetonitrile, methanol, or acetone/methanol) [6,14,24,25] and/or solid-phase extraction (SPE; offline or online solutions) to purify and concentrate the samples [15,24,26,27]. Protein precipitation was evaluated with the efficacy of four different extraction solvents, comparing their performance at different volumes in 250 µL of human plasma. This comparative analysis aimed to identify the optimal precipitation solvent and volume ratio to achieve efficient protein removal for subsequent analysis, and the selected optimal extraction method was stated in the methodology section (750 µL of 0.1 % formic acid in acetonitrile, 4 °C).

Table 2
Linear ranges, quantification limits, and regression parameters of calibration curves (prepared in analyte-free plasma, $n = 3$).

Analyte	Linear range (ng/mL)	LOQ[a] (ng/mL)	Regression equation	r^2
5-HT	0.50–200	0.50	y = 0.033x + 0.025	0.987
NAS	0.01–5	0.01	y = 0.027x + 0.031	0.991
TRY	0.01–20	0.01	y = 0.024x + 0.069	0.997
6-SMEL	0.01–20	0.01	y = 0.051x + 0.047	0.995
6-HMEL	0.01–20	0.01	y = 0.039x + 0.172	0.993
MEL	0.01–100	0.01	y = 0.041x + 0.052	0.985
NATA	0.10–20	0.10	y = 0.285x + 0.259	0.954

LOQ, limit of quantification; r^2, correlation coefficient; 5-HT, serotonin; NAS, *N*-acetylserotonin; TRY, tryptamine; 6-SMEL, 6-sulfatoxymelatonin; 6-HMEL, 6-hydroxymelatonin; MEL, melatonin; NATA, *N*-acetyltryptamine.

[a] **LOQ is defined as the lowest level of an analyte that can be reliably quantified, i.e. with certain precision (coefficient of variation < 20 %) and accuracy (relative error within ± 20 %).**

Table 3
Accuracy and precision results for spiked plasma at four quality control levels.

Analyte	Spiked amount (ng/mL)		Intraday ($n = 6$)		Interday ($n = 18$)	
			Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
5-HT	QC1	2	10.04	5.37	4.36	12.44
	QC2	5	5.22	10.22	2.55	10.25
	QC3	10	2.52	6.37	2.14	5.26
	QC4	50	3.66	8.36	1.53	5.24
NAS	QC1	0.02	7.35	2.64	12.03	5.35
	QC2	0.5	9.33	1.38	8.33	5.64
	QC3	2	3.48	2.64	4.23	3.16
	QC4	10	4.14	1.62	4.01	5.55
TRY	QC1	0.2	5.27	7.35	6.43	6.37
	QC2	0.5	4.02	4.02	7.10	4.75
	QC3	2	4.77	4.16	5.01	2.42
	QC4	30	3.42	6.93	5.23	3.73
6-SMEL	QC1	0.02	9.14	5.29	13.11	10.11
	QC2	0.05	6.03	1.73	9.53	5.26
	QC3	0.5	1.47	-0.72	6.36	5.01
	QC4	5	5.03	6.77	2.12	4.04
6-HMEL	QC1	0.02	2.73	5.35	10.42	8.84
	QC2	0.05	3.84	5.15	10.02	5.37
	QC3	0.5	2.53	3.55	5.48	5.88
	QC4	5	0.46	1.78	4.52	6.35
MEL	QC1	0.02	2.41	1.34	12.21	6.31
	QC2	0.05	1.74	2.47	10.93	5.14
	QC3	0.5	3.16	1.84	6.20	5.60
	QC4	10	8.55	13.46	7.74	11.52
NATA	QC1	0.2	10.36	22.41	8.06	17.37
	QC2	2	9.11	11.75	6.27	8.92
	QC3	10	9.06	0.99	2.94	2.77
	QC4	30	4.37	3.58	2.04	1.52

5-HT, serotonin; NAS, *N*-acetylserotonin; TRY, tryptamine; 6-SMEL, 6-sulfatoxymelatonin; 6-HMEL, 6-hydroxymelatonin; MEL, melatonin; NATA, *N*-acetyltryptamine.

Although protein precipitation alone usually does not yield extracts as pure as those obtained by a sample processing involving various modifications of SPE and liquid-liquid extraction [27,28], our proposed sample preparation, which utilises only simple precipitation, appears to be sufficient. This is supported by the ME test results, which did not exceed ±15 % for most analytes (see section 3.4 Validation results for further details).

3.2. Separation based on liquid chromatography

The reversed-phase LC is the most used separation technique for the study of this type of analytes [6,8,15,29,30]. The choice of a chromatographic column allowing satisfactory separation was therefore based on this assumption. Several chromatographic columns with different stationary phases were tested to achieve the best separation of target analytes: Kinetex C_{18} (2.6 µm, 100 × 2.1 mm, Phenomenex), Acquity UPLC premier HSS T3 (1.8 µm, 2.1 × 100 mm, Waters), Acquity UPLC BEH C_{18} (1.7 µm, 2.1 × 50 mm, Waters), Acquity UPLC BEH Amide (1.7 µm, 2.1 × 100 mm, Waters), and Atlantis Premier BEH C_{18} AX Column (1.7 µm, 2.1 × 100 mm). The best separation efficiency was achieved with Acquity UPLC premier HSS T3 column based on C_{18} chemistry. In the case of reverse stationary phases based on varying lengths of hydrocarbons, analyte retention is mainly determined by hydrophobic and van der Waals interactions [31–33]. The indole or phenyl ring of TRP metabolites exhibits hydrophobic properties, while the addition of functional groups, such as hydroxy, amine, and carboxyl, increases the hydrophilicity of the molecules. A representative UHPLC–MS/MS chromatogram of the seven target analytes is shown in Fig. 2. Under optimised separation conditions, the retention times for the analytes were as follows: 2.50 min for 5-HT, 4.05 min for NAS, 4.12 min for TRY, 4.70 min for 6-SMEL, 5.57 min for 6-HMEL, 7.03 min for MEL, and 7.27 min for NATA (see Fig. 2). The drift between the

Table 4
Matrix effect and recovery from spiked plasma at different concentrations of tryptophan metabolites ($n = 6$).

Analyte	Spiked amount (ng/mL)		Matrix effect (%)	Coefficient of variation (%)	Recovery (%)	Coefficient of variation (%)
	QC2	5	83.23	1.53	90.15	2.07
5-HT	QC3	10	90.64	1.25	91.52	3.73
	QC4	50	97.37	0.52	101.03	1.85
	QC2	0.5	91.02	1.50	95.39	5.22
NAS	QC3	2	93.55	0.83	90.22	4.61
	QC4	10	97.41	2.94	103.16	5.37
	QC2	0.5	87.43	1.70	102.60	5.71
TRY	QC3	2	95.52	3.07	102.28	2.50
	QC4	30	88.73	2.51	90.38	14.26
	QC2	0.05	88.44	5.40	87.29	3.10
6-SMEL	QC3	0.5	94.29	2.71	94.55	2.25
	QC4	5	107.28	0.45	101.04	7.34
	QC2	0.05	102.30	2.63	85.28	4.25
6-HMEL	QC3	0.5	90.31	1.73	94.33	3.14
	QC4	5	110.46	3.24	87.38	2.55
	QC2	0.05	91.22	3.73	96.44	1.80
MEL	QC3	0.5	84.57	3.61	90.31	2.63
	QC4	10	89.34	4.40	90.45	2.53
	QC2	2	81.57	7.04	79.76	15.91
NATA	QC3	10	89.53	2.55	90.22	3.46
	QC4	30	85.72	1.83	93.72	2.74

5-HT, serotonin; NAS, *N*-acetylserotonin; TRY, tryptamine; 6-SMEL, 6-sulfatoxymelatonin; 6-HMEL, 6-hydroxymelatonin; MEL, melatonin; NATA, *N*-acetyltryptamine.

Table 5
The autosampler, bench-top, and freeze-thaw stability (bias %; $n = 3$).

Analyte	Stability (%)		
	Autosampler stability[a]	Bench-top stability[b]	Freeze-thaw stability[c]
	48 h	4 h	-80 °C
5-HT	3.05	9.76	-5.72
NAS	2.52	3.52	-2.64
TRY	2.39	1.45	-2.37
6-SMEL	-1.28	-1.29	-1.59
6-HMEL	1.73	1.74	-1.63
MEL	2.74	4.22	-2.37
NATA	3.14	-3.62	-5.41

5-HT, serotonin; NAS, *N*-acetylserotonin; TRY, tryptamine; 6-SMEL, 6-sulfatoxymelatonin; 6-HMEL, 6-hydroxymelatonin; MEL, melatonin; NATA, *N*-acetyltryptamine.

[a] Autosampler stability of analytes in plasma spiked to QC2 level at 8 °C for 48 h.

[b] Bench-top stability of analytes in plasma spiked to QC2 level at room temperature for 4 hours.

[c] Stability of analytes in plasma spiked to QC2 level during five freeze-thaw cycles from -80 °C to room temperature.

retention time of the analytes and their internal standards was within approximately less than 0.2 min. All analytes tested were successfully separated at baseline. Retention times achieved high stability between injections ($n = 5$), with the highest standard deviation (SD) value of ±2 %. Analytical time was only 8 min per sample.

3.3. Mass spectrometry detection

A triple quadrupole MS with ESI^+ was used in MRM mode for the analysis. Due to the highest selectivity and great S/N ratio of ion scanning methods, this mode is most commonly used to quantify low-abundance analytes [34,35]. The qualitative and quantitative analysis of analytes was achieved through the determination of retention time, precursor ion and a minimum of two product fragment ions of each compound with acceptable ion ratios. Fragmentation via MS/MS plays a crucial role in both the identification and quantification of metabolites. These metabolites typically exhibit a positive charge during ESI due to their high pK_a (> 7.0) in acidic conditions. 6-SMEL has an acidic sulphate group (SO_3H), which promotes protonation in positive ESI together with other experimental conditions although its pK_a value is low (−2.1) [36]. Therefore, the initial stages of method development involve identifying the most suitable product ions and its corresponding highly intense precursor ion for identification purposes. This is achieved through direct infusion of 1 µg/mL of each analyte/internal standard combination, with ESI conducted in positive mode. The tandem MS continuously scans between 50 and 600 *m/z*, changing source parameters such as cone voltage and CE to optimise S/N ratios/intensity of

Table 6
Endogenous levels of target analytes in participant's plasma.

Individuals	Endogenous levels (ng/mL)						
	5-HT	NAS	TRY	6-SMEL	6-HMEL	MEL	NATA
Subject 1	2.11	0.0150	0.510	0.0210	0.0120	Tr	ND
Subject 2	5.74	0.0120	3.31	0.0270	0.0160	0.0130	ND
Subject 3	10.7	0.0160	1.23	0.0160	Tr	Tr	ND
Subject 4	2.58	0.0130	4.33	0.0530	0.0230	0.0170	ND
Subject 5	4.85	0.0110	2.65	0.0110	Tr	Tr	ND
Subject 6	2.56	Tr	1.44	0.0260	0.0120	0.0150	ND
Subject 7	3.58	0.0150	6.33	0.0170	Tr	Tr	ND
Subject 8	3.06	0.0130	5.35	0.0220	0.0140	ND	ND
Subject 9	2.76	0.0120	4.69	0.0300	0.0150	Tr	ND
Subject 10	4.58	Tr	4.45	0.0420	0.0210	0.0110	ND

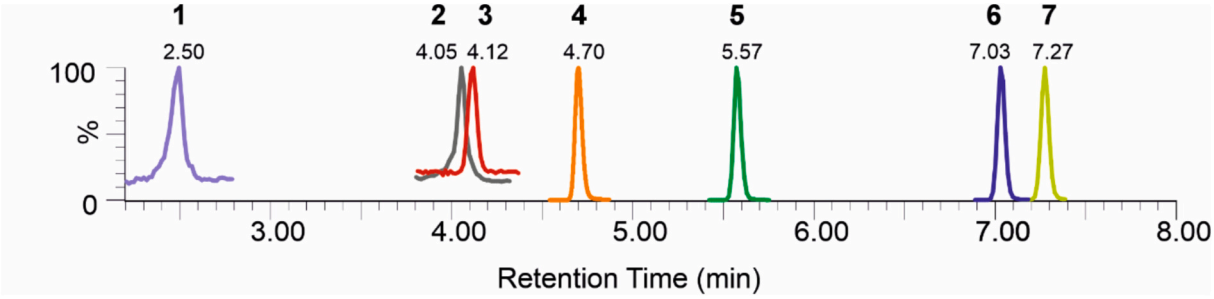

Fig. 2. Visualization of chromatographic separation of selected tryptophan metabolites dissolved in 100% methanol (10 ng/mL, injection 10 µL): serotonin (1; 5-HT), *N*-acetylserotonin (2; NAS), tryptamine (3; TRY), 6-sulfatoxymelatonin (4; 6-SMEL), 6-hydroxymelatonin (5; 6-HMEL), melatonin (6; MEL), *N*-acetyltryptamine (7; NATA).

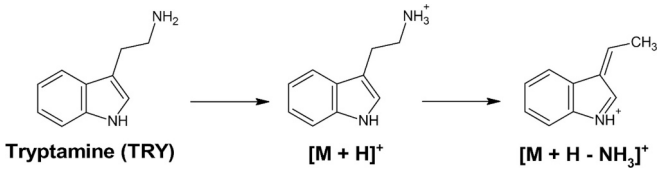

Fig. 3. Formation of precursor and product ions of tryptamine.

product ion fragments while ensuring peak signal stability. In this study, all analysed compounds possess a common phenethylamine structure (e.g. TRY), as shown in Fig. 3. However, it is notable that phenethylamine derivatives undergo a process of protonation during ESI^+, resulting in the subsequent loss of NH_3. The quantitative determination of these analytes relies on highly intense product ions, which enhance the S/N ratio for each compound. This transformation involves converting the precursor ion $[M+H]^+$ into the product ion $[M+H - NH_3]^+$ (Fig. 3). Consequently, the utilisation of a highly intense quantifier ion facilitates precise and sensitive quantitative analysis of phenethylamine derivatives. The precursor and product ions, together with their respective CE values were selected for the quantification of each analyte, are shown in Table 1. The selection of MRM transitions is consistent with previously published methods [6,14,15,37]. In addition, the dwell time values were optimised so that the number of scan points across the full width of the chromatographic peak was optimally 12–15 [38]. Optimal dwell time values were determined by repeated analysis of standards, assessing the impact of dwell time on the number of scan points across the chromatographic peak width. This setting ensures a reproducible and precise quantitative analysis.

3.4. Validation results

Information on the quantification limits of the developed method and the regression parameters of matrix-matched calibration are given in Table 2. The LOQ for the selected analytes in the plasma matrix investigated falls within the range of 0.01 to 0.5 ng/mL with appropriate accuracy (relative error within ±20 %) and precision (CV <20 %). The lowest LOQs (0.01 ng/mL) were obtained for NAS, 6-SMEL, 6-HMEL, MEL, and TRY. The LOQs were determined to be in the low ng/mL range, ensuring the method's ability to detect even trace amounts of the analytes in the samples. Since the target endogenous substances are naturally present in human plasma, matrix-matched calibration curves, specifically spiked analyte-free plasma prepared by charcoal stripping, were used to determine the calibration parameters (Table 2). The analytical ranges were from 0.5 to 200 ng/mL for 5-HT, from 0.01 to 5 ng/mL for NAS, from 0.01 to 20 ng/mL for TRY, from 0.01 to 20 ng/mL for 6-SMEL, from 0.01 to 20 ng/mL for 6-HMEL, from 0.01 to 100 ng/mL for MEL, and from 0.10 to 20 ng/mL for NATA. Selected calibration concentration points were in the linear range. Importantly, the LOQs achieve similar or better values than previously published methods [8,14,24,39–41]. Moreover, the method shows a high degree of linearity with correlation coefficient (r^2) values greater than 0.954. No significant carry-over in analyte retention times was observed when several samples of pure solvent (100 % methanol) were injected after the highest calibration point at the upper limit of quantification. This ensures that there is no interference from previous samples.

The method precision and accuracy values for individual analytes in human plasma ($n = 6$) spiked at four quality control levels (QC1–QC4) are given in Table 3. Intraday and interday (repeated on three separate days) were controlled to evaluate key validation parameters. The accuracy results, expressed as the difference between measured and nominal values, ranged from −0.72 % (for 6-SMEL at QC3) to 22.41 % (for NATA at QC1). Except for the QC1 level of NATA, the intraday and interday accuracy for all other analytes fell within the recommended EMA guidelines (±15 % for higher concentrations and ± 20 % for the LOQ level). The results for precision, shown as the CV, results were consistent with the EMA threshold at all four quality control levels (<15 or < 20 % for the LOQ level) for all analytes. The intraday precision ranged from 0.46 % (for 6-HMEL) to 10.36 % (for NATA) and the interday precision ranged from 1.53 % (for 5-HT) to 13.11 % (for 6-SMEL). Overall, the developed method demonstrates robust, accurate, and precise quantification of a selected group of TRP metabolite in plasma samples, with the exception of the QC1 level for NATA, as previously noted.

The RE results indicate that there is no significant loss of analytes during sample processing and that the chosen preparation procedure, one-step precipitation and extraction, followed by evaporation with inert nitrogen is highly efficient (Table 4). The analytical RE values were generally high, with most analytes exhibiting REs of no less than 90 % across the quality control levels tested. However, the overall reliability of the method is maintained by the use of corresponding deuterated internal standards (Table 1), which effectively account for potential analyte losses during processing. In the case of NATA, due to the unavailability of a specific deuterated standard, TRY-d4 was used as a substitute internal standard. Internal standards compensate for analyte losses during sample preparation (e.g., due to adsorption, transfer, or evaporation), ensuring the method accurately reflects analyte performance throughout the analytical process [42,43]. The CV values for all quality control levels varied from 1.80 to 15.91 %, further confirming the method's precision.

In addition to the target analytes, other endogenous and exogenous substances such as metabolites, analogues, carbohydrates, salts, pigments, and plastic residues are present in the sample injections. These substances can negatively impact the analytical performance by interfering with the ionisation efficiency of the analytes, either suppressing or enhancing the ionisation process [42,44,45]. A particularly important matrix component in blood plasma is phospholipids, which are known to cause such alterations. As a result, ME was evaluated to assess the potential negative impact of co-eluting components during the validation process. The results of ME testing for all analytes at the three quality

control levels (QC2, QC3, and QC4) in human plasma are shown in Table 4. The absolute ME values ranged from 81.57 % to 110.46 %, indicating that the analytes were not significantly impacted by endogenous or exogenous interferents in the sample. For most of the analytes, ionisation efficiency was slightly reduced, with the most pronounced suppression observed for NATA (81.57 % at QC2 level). However, there are effective strategies to reduce or eliminate the influence of interfering matrix components, such as the use of appropriate internal standards and matrix-matched calibration, as confirmed by the accuracy and precision results (see Table 3). Notably, during the ME evaluation, none of the CV exceeded the 15 % threshold, with values ranging from 0.45 % to 7.04 % across all quality control levels.

Since analyte degradation can occur at any stage of sample handling due to factors such as cellular metabolism, exposure to light, air, or temperature fluctuations, assessing the stability of analytes under various conditions is a critical validation parameter [46]. The stability of analytes depends on their chemical nature and the type of biological sample, necessitating careful design of sample processing techniques to minimise degradation. Strategies to mitigate degradation include reducing the time between sample collection and analysis, using antioxidants, minimising light exposure, maintaining low temperatures during handling, adjusting pH to enhance analyte stability, or employing internal standards. Several studies have tested the stability of TRP metabolites under different storage conditions [6,8,14,29,47]. For example, the neurotransmitter 5-HT remains stable in plasma samples through three freeze-thaw cycles, after 4 h of storage at −80 °C or in an autosampler at 4 °C for 12 h [47]. It also shows no degradation after a month of storage at −80 °C. In contrast, degradation of 6-HMEL and an increase in NAS were observed after 4 months of plasma storage at −80 °C [14]. 5-HT and MEL remained stable. Based on these findings, it is recommended that samples be processed and analysed as quickly as possible to prevent degradation. Instability was observed for 6-HMEL in plasma and 5-HT in whole blood when stored at room temperature (25 °C) for 4 h [14] emphasising the importance of maintaining low temperatures during sample processing. The stability study results are summarised in Table 5. All tested analytes showed excellent stability (ranging from −5.72 % to 9.76 %) in spiked plasma samples stored in an autosampler at 8 °C for 48 h. They also remained stable for at least 4 h at room temperature and after undergoing five freeze-thaw cycles (−80 °C to room temperature). A limitation of the stability experiments is that they were conducted at only one concentration level (QC2), which precludes a comprehensive assessment of analyte stability across the entire calibration range.

The developed method was applied for metabolic profiling of endogenous levels of target TRP metabolites in plasma samples from ten participants. The volunteer cohort comprised of healthy females aged 24–37 years, with no ongoing medical treatments. Plasma samples were collected at 8:00 a.m. following an overnight fasting period (with no food intake after 10:00 p.m. the previous night). Only 250 µL of plasma was required for the analysis. Table 6 presents the experiment's results. The measured levels of most endogenous TRP metabolites were found to be lower than those reported in previous publications (5-HT: 70–270 ng/mL in human serum [48]; NAS: 2.44 ± 0.45 ng/mL in human serum [49]; TRY: *6–7 ng/mL in blood some mammalian* [50]*; 6-HMEL: usual concentration in human plasma or serum not reported*; MEL: 0.01–0.02 ng/mL during the daylight phase in human serum [51]; NATA: 0.006 ± 0.002 ng/mL in human daytime plasma [24]). This variance may be attributed to various factors, the selection criteria for volunteers (e.g., sample size, health status), differences in the sampling method (e.g., time of sample collection, fasting state), variations in sample processing techniques, and differences in analytical methodologies (e.g., immunoassay vs. mass spectrometry). Notably, the level of 6-SMEL in human plasma can vary significantly based on factors such as age, sex, circadian rhythm, and individual differences. Typically, 6-SMEL levels in human plasma range from 100 to 300 pg/mL in healthy individuals [40,52], but these levels fluctuate considerably throughout the day and night due to circadian influences and light exposure [53]. The inherent variability is exemplified by MEL concentration, which peak at 80–120 pg/mL during the dark part of the day and drop nearly tenfold to 10–20 pg/mL during the daytime [54]. Seasonal variations in MEL levels have also been reported [53]. Given these significant sources of variability, it is crucial to account for such factors when designing and conducting larger, long-term targeted metabolomics studies. The findings from this study may offer valuable insight into the metabolic profiles of TRP-related compounds and highlight potential variations within the studied cohort, which may serve as a foundation for future research.

4. Conclusion

We have developed and validated a novel method for the robust, high-throughput, and specific quantitative analysis of seven endogenous TRP metabolites (5-HT, NAS, TRY, 6-SMEL, 6-HMEL, MEL, and NATA) in human plasma samples within a single acquisition run and a short analytical time frame. The method's reliability has been rigorously confirmed through compliance with EMA validation guidelines, with all evaluated parameters yielding satisfactory results. The approach is very adaptable and widely applicable since it involves a simplified sample preparation procedure that involves one-step protein precipitation and extraction, followed by rapid UHPLC–MS/MS analysis, making it highly versatile and broadly applicable. Large-scale epidemiological studies that seek to comprehend human metabolic status during normal physiological processes, assess therapeutic interventions (e.g., dose optimisation, treatment efficacy monitoring), and identify changes in TRP metabolism linked to different pathological conditions could benefit greatly from the application of this analytical approach. Furthermore, this method has the potential to reveal specific metabolic profiles with clinical significance, including the discovery of novel biomarkers that could enhance patient care and contribute to the overall health system. To the best of our knowledge, no other UHPLC–MS/MS method is currently available that profiles this comprehensive set of analytes, particularly MEL metabolites, in human plasma.

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Credit authorship contribution statement

Michal Kaleta: Writing – original draft, Methodology, Investigation, Formal analysis. **Bhagya S. Kolitha:** Writing – original draft, Methodology, Formal analysis. **Ondřej Novák:** Writing – review & editing, Data curation. **Farshid Mashayekhy Rad:** Validation, Data curation. **Jonas Bergquist:** Supervision, Resources, Funding acquisition, Conceptualization. **S.J. Kumari A. Ubhayasekera:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

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

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2025.124520.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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