RESEARCH PAPER

Development and validation of a simple, rapid and sensitive LC-MS/MS method for the measurement of urinary neurotransmitters and their metabolites

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Received: 26 July 2017 / Revised: 13 September 2017 / Accepted: 27 September 2017

Abstract Neurotransmitters play crucial roles in physiological functions and their imbalances have demonstrated association in the pathology of several diseases. The measurement of neurotransmitters possesses a great potential as a significant clinical tool. This study presents the development and validation of an LC-MS/MS method for simultaneous quantification of multi-class neurotransmitters associated with dopamine, tryptophan and glutamate-γ-aminobutyric acid pathways. A total of ten neurotransmitters and their metabolites (dopamine, epinephrine, metanephrine, tryptophan, serotonin, kynurenic acid, kynurenine, anthranilic acid, GABA, glutamic acid) were determined based on a simple and rapid 'dilute and shoot' method using minimal urine volume. The chromatographic separation was achieved using a Poroshell 120 Bonus-RP LC Column in combination with a gradient elution within an 8.5-min time frame. The method exhibited good sensitivity as the limits of quantification ranged between 0.025 and 0.075 μg/mL with acceptable matrix effects (< ± 14.5%), no carryover and good linearity (r^2 > 0.98). The accuracy and precision for all analytes were within tolerances, at < ± 9.9% mean relative error (MRE) and < 8.6% relative standard deviation (RSD), respectively. The method was successfully applied in measuring the neurotransmitter concentrations in urine of healthy donors. Furthermore, the undertaken stability experiments indicated that acidified urine specimens allowed the analytes to be stable for prolonged durations in comparison to those untreated. The study also reveals the performance of the method is unaffected by the absence of expensive deuterated reference standards under the experimental conditions employed which further simplifies the analytical procedures and provides a significant cost saving for running the assay.

Keywords Neurotransmitter · Liquid chromatography · Mass spectrometry · Urine

Introduction

Neurotransmitters are endogenous substances which act as primary chemical messengers, released from neurons to target cells by relaying, amplifying and modulating signals. The major neurotransmitters that function at central and peripheral levels are classified into two categories: monoamine and amino acid neurotransmitters [1]. Monoamine neurotransmitters

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include dopamine, serotonin, epinephrine and norepinephrine [2]. Amino acid neurotransmitters consist of glutamic acid, tryptophan, γ-aminobutyric acid (GABA), alanine, aspartic acid and taurine [3].

Neurotransmitters play essential roles in regulating the body's nervous, immune and cardiovascular system [4]. These neuroactive metabolites have been found to be impli- cated with a wide range of diseases including cancer tumours, neurodegenerative, psychiatric and psychological disorders [5]. Recent research shows that the imbalance of tryptophan metabolism is closely linked with the development of lung

Published online: 13 October 2017

and breast cancer [6]. Studies which examined urinary neurotransmitter concentrations in psychological disorders indicated that depressive symptoms were in correlation with the increase of catecholamines in the body [7–9]. The imbalances of glutamate and GABA metabolism have been closely associated with the occurrence of seizures such as epilepsy [10]. The crucial involvement of neurotransmitters in neurological, endocrinological and immunological functions has directed great attention to researchers and clinicians towards these molecules and metabolites as diagnostic indicators for monitoring disease states and therapeutic interventions [11, 12]. Hence, there are high demands for the development of validated analytical methods for the quantification of neurotransmitter concentrations in biological matrices.

Neurotransmitters have been measured in several biological matrixes, including urine, cerebral spinal fluid (CSF), plasma, saliva, serum and platelets [13]. The CSF matrix provides the best representation of CNS neurotransmitter levels. However, measuring neuroactive compounds in the CSF reflects major drawbacks in the sample collection and preparation stages causing interpretation difficulties [14]. In literature, blood and urine are the most common matrices employed in neurotransmitter measurements [15]. Urine is described as non-invasive in nature, enabling inexpensive methods, being largely available and simple compared to other matrices such as plasma and blood [16]. For this study, urine was selected based on the advantages and simplicity of this matrix. It is known that an analyte concentration in urine is susceptible to variation due to hydration status; this variation, however, can be corrected by normalising the analyte concentration to the creatinine level in urine if necessary [17].

Traditionally, quantitative analysis of neurotransmitters in biological samples is performed by radioenzymatic or immunological assays, fluorimetry and gas chromatography [18]. Developments and research throughout the past two decades have resulted in these procedures to be superseded by greatly selective and sensitive chromatographic methods coupled to mass spectrometry detection systems [19]. GC-MS is characterised as a potent technique in the identification of neuroactive compounds whereby analytes are derivatised to attain volatility and stability. The rise of simple and economical LC techniques coupled with tandem mass spectrometry significantly decreases run times, improves detection limits, analytical sensitivity and reproducibility of biological preparations [20].

One of the most challenging aspects to account for in the quantitative measurement of neurotransmitters is sample preparation. The determination of neurotransmitters in biological samples is challenging due to the high possibility of interferences, chemical instability issues and low concentrations of analytes present in biological matrices [3]. Generally, common sample preparation requires lengthy separation and extraction procedures prior to instrumental analysis. Currently, solid-phase extraction (SPE) is the most frequently employed extraction technique as it offers high selectivity, precision and extraction yields of above 90% [21]. Liquid-phase micro-extraction (LPME) is a relatively newly developed extraction process described to have low solvent consumption [22]. As the extraction of hydrophilic compounds in the organic phase is more difficult, most studies focus on extracting hydrophobic compounds [23]. To overcome the challenges involved in LPME, Jiang et al. [22] developed a novel method to solve the collection of acceptor solutions through the solidification of floating organic drop. Another established sample preparation method for biological samples is sample dilution. The advantages of this technique are the removal of extensive clean-up procedures, ability to prevent sample deterioration, time efficiency and cost-effectiveness [3, 24, 25]. Sample dilution is mainly used in the sample preparation of CSF or dialysates [26]. Although this technique has not been widely adapted, it possesses potential as an alternative to existing sample preparation methods.

This study aimed to develop and validate an analytical LC-MS/MS method that can simultaneously detect and quantify several neurotransmitters and their metabolites with minimal sample preparation steps and reduced running costs. There has been minimal literature on multiclass neurotransmitter analysis and quantification, particularly for metabolites of the tryptophan pathway. The uniqueness of this analytical method is demonstrated in the optimization of an original sample preparation procedure which involves sample dilution avoiding traditional extensive extraction clean-up and derivatisation procedures and the removal of the use of expensive deuterated internal standard (IS) reference materials. It is expected that the innovations of this methodology have not only great research potential but also enable the practicality of neurotransmitter quantifications within a clinical setting.

Materials and method

Chemicals and reagents

Anthranilic acid, dopamine hydrochloride, epinephrine hydrochloride, L-glutamic acid, GABA, kynurenic acid, L-kynurenine, metanephrine, serotonin hydrochloride and L-tryptophan were purchased as powders from Sigma Aldrich (Sydney, Australia). D_3-Dopamine, D_5-glutamic acid, D_6-GABA, D_5-kynurenic acid and D_3-metanephrine were supplied by CDN Isotopes (Quebec, Canada). HPLC grade acetonitrile was purchased from Honeywell (Sydney, Australia). Formic acid and acetic acid were supplied by RCI Labscan (Bangkok, Thailand). Three millilitre syringes were purchased from Beckton Dickinson (Sydney, Australia) and 0.22 μm hydrophilic syringe filters were supplied by Micro-Analytix Pty Ltd. (Taren Point, NSW, Australia).

Urine collections

Surine, commercial synthetic urine devoid of endogenous neurotransmitters, was purchased from Sigma Aldrich (Sydney, Australia). Authentic urine specimens were obtained from ten healthy donors of Asian and Caucasian origin including six male and four female participants in the age group of 20−40 years. Participants were each provided with a sterile urine specimen container during mid-day for urine collection. The collected urine samples were immediately frozen in a −20 °C freezer and analysed within a week.

Liquid chromatography

The chromatographic separation of analytes was achieved on an Agilent Poroshell 120 Bonus-RP LC column (2.1 mm × 100 mm 2.7 μm particle size) using the Agilent 1290 infinity liquid chromatography system coupled to the Agilent 6490 triple quadrupole mass spectrometer. A gradient elution was performed at a flow rate of 0.18 mL/min with two mobile phases. Mobile phase A was composed of water containing 0.2% (*v*/*v*) formic acid and mobile phase B comprised of acetonitrile with 0.1% (*v*/*v*) formic acid. The gradient elution are as follows: 100% A (0 min) ➔ 100% A (hold 2 min) ➔ 95% B (linear increase in 0.5 min) ➔ 95% B (hold 2.5 min) ➔ 100% A (linear decrease in 0.25 min) ➔ 100% A (hold 3 min).

Mass spectrometry

The Agilent 6490 triple quadrupole (QQQ) LC/MS detector was operated in the multiple reaction monitoring (MRM) mode using positive electrospray ionisation (ESI+) mode. The source conditions were as follows: gas temperature was 290 °C, flow rate of 660 L/h, fragmentor voltage 120 Vand capillary voltage of 3500 V. Initially, the MS was acquired in the targeted MS/MS full scan data mode. The precursor ion was selected in the first quadrupole and subsequently fragmented in the high-energy collisional dissociation cell. Following fragmentation, the product ions were obtained in subsequent quadrupoles. When the precursor ions and the collision energy of their most abundant product ions were identified for each analyte, the QQQ parameters were set to MRM mode. A total of two MRM transitions were used for each analyte.

Preparation of standard solutions

Standard stock solutions (500 μg/mL) of all ten analytes were individually prepared in 0.2 M acetic acid. A 25 μg/mL mixed standard solution containing the analytes was generated by obtaining aliquots of each individual stock solution. The solutions were stored in −20 °C freezer prior to usage. The mixed standard solution was diluted appropriately with 0.2 M acetic acid to prepare the working solution series. A 5 μg/mL mixed IS solution in 0.2 M acetic acid was prepared from 100 μg/mL individual stock solutions of the five internal standards (D_3-dopamine, D_5-glutamic acid, D_6-GABA, D_5-kynurenic acid, D_3-metanephrine).

Sample preparation

Six calibration points were prepared by spiking, into 2 mL micro-centrifuge tubes, 1 mL of synthetic blank urine with the mixed standard solution at concentrations of 0.50, 1.25, 2.50, 3.75, 6.00 and 7.50 μg/mL, respectively. Three quality control (QC) samples were similarly prepared at 0.20 (low QC), 2.75 (medium QC) and 5.00 μg/mL (high QC). These samples were made into 2 mL by diluting with acetonitrile, vortexed for 60 s, precipitated in ice for 10 min followed by centrifugation for 10 min at a temperature of 2 °C and velocity of 5600 g. Subsequently, 1 mL of the mixture was pipetted from the micro-centrifuge tube and diluted with 0.25 mL of the mixed IS solution and 1.25 mL of 0.2 M acetic acid. The solution was filtered through a 0.22 μm hydrophilic syringe filter. The filtrate was subjected to LC-MS/MS analysis. Authentic urine was similarly prepared resulting in an overall five-fold dilution into 0.2 M acetic acid for LC-MS/MS analysis.

For method validation with the use of the internal standards, peak area ratios of analyte to its deuterated IS counterpart were used. For the analytes without their corresponding deuterated standards, D_5-kynurenic acid was used to quantify anthranilic acid, tryptophan and kynurenine. D_3-Dopamine and D_3-metanephrine were used for epinephrine and serotonin, respectively. For method validation without the use of the internal standards, the absolute peak areas of analytes were used.

Method validation

Method validation was performed in accordance with published procedures [27]. Linearity was assessed using the linear coefficient of determination statistical measure known as the R-squared value. A linear association is established when the detected signal and concentration of an analyte are proportional ($r^2 > 0.98$). The limit of detection (LOD) and limit of quantification (LOQ) were determined by spiking decreasing concentrations of the mixed stock solution into blank synthetic urine. The LOD is defined as the lowest concentration point at which the instrument exhibits signal-to-noise (S/N) ratio equal to 3. LOQ concentration of an analyte is defined as the lowest concentration reliably quantified and fulfils the criteria of not exceeding ± 20% mean relative error (MRE) and < 20% relative standard deviation (RSD).

Accuracy and precision were evaluated simultaneously using different concentration levels (QC samples) with five replicates on five different days. Accuracy was measured using the %MRE. Precision was measured by the %RSD.

For the method to be deemed accurate and precise, the %MRE and %RSD values must fall within the maximum acceptable range of ± 20 and < 20 respectively.

Matrix effects were examined through the assessment of analyte enhancement or suppression. As a 'dilute and shoot' approach was used and no extractions were involved, standard addition method was considered appropriate [28] and was applied, whereby authentic human urine specimens were spiked at 2.75 and 5.00 μg/mL of analytes. Primarily, both the spiked and the original samples of individual authentic urine sets were prepared using the dilution procedure described in 'Sample preparation' section and analysed using the method without IS. The differences in concentration between the spiked and the original sample represent the measured concentration of the spiked analytes. The following equation was used for the determination of the percentage matrix effect (%ME) where target concentration was the spiked concentration:

$$\%ME = \frac{|Measured\ concentration - target\ concentration|}{Target\ concentration} \times 100$$

In relation to %ME, a value of 0 indicates there are no matrix effects involved, > 0 means that ion enhancement is present and < 0 implies ion suppression. The acceptable range of ion suppression or enhancement is within ± 15%ME.

Stability experiments were conducted to assess the stability of the ten analytes under different experimental conditions. The comparison of absolute peak areas of freshly prepared working solutions with samples stored under various conditions was of interest. Post-preparative stability was performed by re-analysing the QC samples after being left in the autosampler at a maintained constant temperature of 4 °C for 48 h. The freeze-thaw stability was conducted by assessing authentic urine specimens over three cycles. The urine specimens were frozen at −20 °C and thawed at room temperature. Long-term stability was performed by testing untreated and acidified authentic urine stored at −20 °C over 90 days. Stability for all experiments was measured by re-analysing the specimens stored in their designated conditions. The absolute peak areas must not exhibit a difference greater than 20% to the initial peak area to be regarded as stable.

For the assessment of carryover, a high concentration, 10 μg/mL mixed standard solution and 0.2 M acetic acid were prepared. The 0.2 M acetic acid was injected immediately after a high concentration and this process was performed in replicates. A method is free from carryover when the acetic acid sample does not give a peak area higher than the LOD and a signal to noise (S/N) ratio larger than 3.

Results and discussion

Method optimisation

The qualitative and quantitative analysis of neurotransmitters were achieved through determination of retention time, precursor ion and a minimum of two product fragment ions of each compound with acceptable ion ratios within ± 30% of those for the reference standards. The MRM transition data acquired in the positive ion mode are recorded in Table 1. Individual 1 μg/mL standards were analysed to obtain the retention times of each analyte. The analytes all eluted within

Table 1 MRM transitions of ten analytes and five deuterated internal standards including precursor and product ions. The first product ion transitions were used as the quantifying ions

Analyte	Precursor ion $[M + H]^+$	1st product ion		2nd product ion		Ratio of 2nd/1st product ions
	m/z	*m/z*	Collision energy (eV)	*m/z*	Collision energy (eV)	
Tryptophan	205	188	5	118	29	0.35
Kynurenine	209	192	5	94	17	0.39
Kynurenic acid	190	144	25	116	37	0.44
Anthranilic acid	138	120	9	92	21	0.26
Glutamic acid	148	130	5	84	17	0.27
GABA	104	87	9	69	13	0.31
Dopamine	154	137	25	119	9	0.36
Epinephrine	184	166	9	107	21	0.25
Metanephrine	198	180	9	148	21	0.22
Serotonin	177	160	9	115	33	0.29
D_5-Kynurenic acid	195	149	17	121	37	0.35
D_5-Glutamic acid	153	135	9	88	13	0.39
D_6-GABA	110	93	9	92	9	0.29
D_3-Dopamine	157	139	9	93	25	0.41

D_3-Metanephrine	201	183	9	151	13	0.28

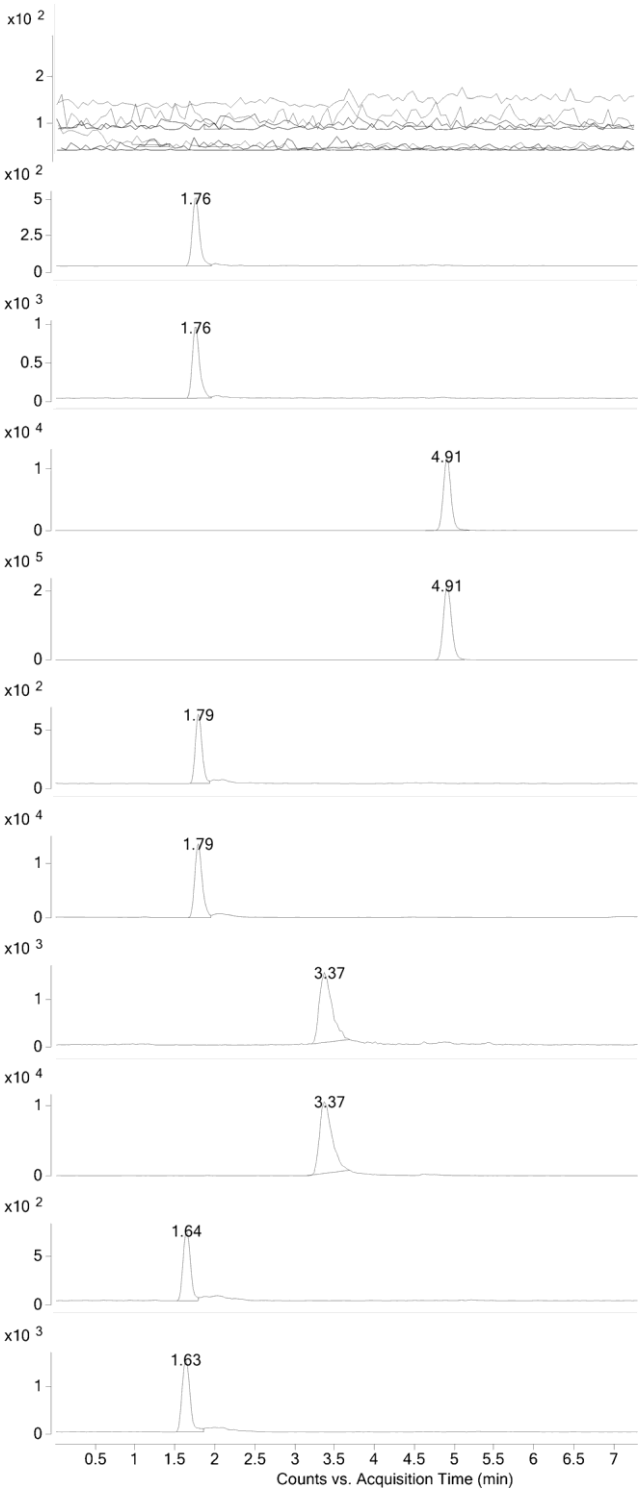

Fig. 1 Representative extracted ion chromatograms of the LOQ and authentic urine for the amine analytes (from top to bottom): (1) synthetic urine blank, (2) dopamine in surine, (3) dopamine in human urine, (4) kynurenine in surine, (5) kynurenine in human urine, (6) epinephrine in surine, (7) epinephrine in human urine, (8) serotonin in surine, (9) serotonin in human urine, (10) metanephrine in surine and (11) metanephrine in human urine

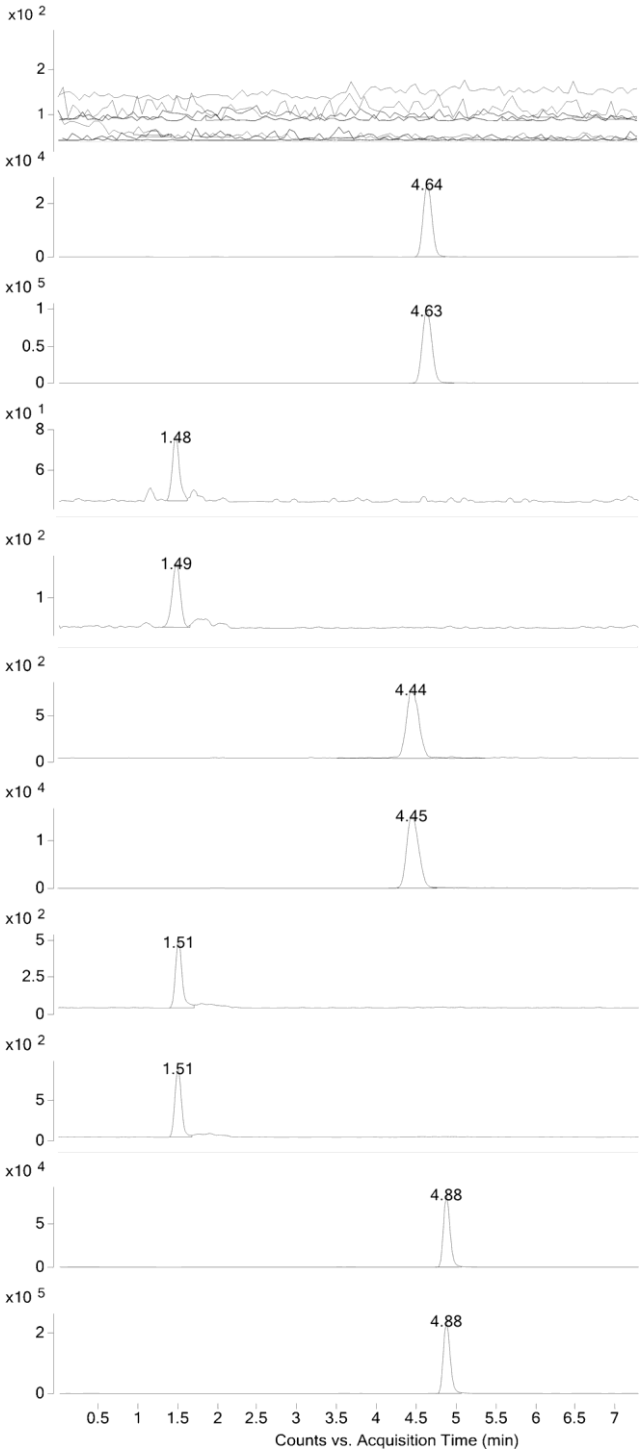

Fig. 2 Representative extracted ion chromatograms of the LOQ and authentic urine for the amino acid analytes (from top to bottom): (1) synthetic urine blank, (2) anthranilic acid in surine, (3) anthranilic acid in human urine, (4) glutamic acid in surine, (5) glutamic acid in human urine, (6) tryptophan in surine, (7) tryptophan in human urine, (8) GABA in surine, (9) GABA in human urine, (10) Kynurenic acid in surine and (11) kynurenic acid in human urine

Table 2 Linearity, LOD and LOQ of the validated method

Analyte	Correlation coefficient (r^2) with IS	Correlation coefficient (r^2) without IS	LOD (μg/mL)	LOQ (μg/mL)
Tryptophan	0.998	0.997	0.005	0.025
Kynurenine	0.990	0.986	0.005	0.025
Kynurenic acid	0.997	0.985	0.025	0.075
Anthranilic acid	0.994	0.989	0.025	0.050
Glutamic acid	0.999	0.998	0.050	0.075
GABA	0.996	0.988	0.050	0.075
Dopamine	0.993	0.981	0.050	0.075
Epinephrine	0.995	0.992	0.050	0.075
Metanephrine	0.998	0.997	0.050	0.075
Serotonin	0.997	0.995	0.050	0.075

an 8.5-min window with glutamic acid as the first to elute and kynurenic acid eluting last. The chromatograms of the ten analytes at their LOQ concentrations are illustrated in Figs. 1 and 2.

The simple dilution combined with filtration sample preparation method possesses advantages in the LC-MS/MS analysis of neurotransmitters and their metabolites. The simplicity of the procedure does not require prolonged preparation durations. The nature of 'dilute and shoot' preparation procedures allows minimal volumes of urine to be used due to the absence of extraction processes involved. Although a sample volume of 1 mL was used in this study for the convenience, it is expected that the urine volume could go much lower in theory if other reagents and solutions are proportionally reduced at the same time during sample preparation. These are extremely beneficial for fast-paced laboratory settings with high demands in the analysis of samples.

Method validation

The linearity of the method was from the LOQ to 7.50 μg/mL for each analyte. The six selected calibration points of the working range generated correlation coefficients (r^2) higher than 0.98 using linear regression (Table 2). The linearity results obtained from both approaches, i.e. with and without the use of internal standards, were comparable.

The LOD and LOQ of the analytes were in the range of 0.005−0.050 and 0.025−0.075 μg/mL respectively (Table 2). This indicated a highly sensitive method was developed for the simultaneous quantification of ten neurotransmitters and their metabolites.

The results for accuracy and precision for the QC samples with and without IS are compared in Tables 3 and 4 respectively. There are no noticeable differences between the two approaches; both approaches afforded accurate and precise analytical methods. For the method without IS, the accuracy ranged from −9.9% to +8.1% MRE, and the precision ranged from 1.7% to 8.3% RSD. These values had met the acceptable method validation criteria, indicating that the method performance was not compromised by the omission of the expensive internal standards.

The validation of linearity, accuracy and precision showed that the method maintained high analytical performance in the absence of internal standards; this is extremely advantageous

Table 3 Accuracy results at different QC concentration levels with and without internal standards (n = 5)

Analyte	Accuracy (%MRE) With IS			Accuracy (%MRE) Without IS		
	0.20 μg/mL	2.75 μg/mL	5.00 μg/mL	0.20 μg/mL	2.75 μg/mL	5.00 μg/mL
Tryptophan	−3.7	3.9	−5.1	4.9	5.8	6.3
Kynurenine	4.6	2.8	3.3	−5.3	−3.4	4.3
Kynurenic acid	5.5	−3.1	−1.7	−7.2	4.7	−2.4
Anthranilic acid	−4.1	−5.4	2.2	3.7	7.3	1.8
Glutamic acid	5.7	7.5	−2.8	6.6	−8.2	−3.3
GABA	−6.4	6.3	−4.9	8.1	−7.1	−6.1
Dopamine	−6.8	−5.7	−3.0	−7.8	−7.6	5.4

Epinephrine	3.9	−3.6	5.9	4.3	3.4	6.8
Metanephrine	7.0	3.9	7.5	−8.5	−5.3	−9.9
Serotonin	−5.2	−3.0	4.2	5.7	4.0	−4.9

Table 4 Precision results at different QC concentration levels with and without internal standards (n = 5)

Analyte	Inter-day precision (%RSD) With IS			Inter-day precision (%RSD) Without IS		
	0.20 μg/mL	2.75 μg/mL	5.00 μg/mL	0.20 μg/mL	2.75 μg/mL	5.00 μg/mL
Tryptophan	5.8	6.0	2.4	7.3	3.9	2.5
Kynurenine	5.1	3.7	6.3	4.8	2.2	4.0
Kynurenic acid	3.4	4.2	3.8	4.6	3.4	4.9
Anthranilic acid	6.2	4.9	5.9	6.9	2.1	5.2
Glutamic acid	7.0	3.6	6.1	8.3	1.7	3.0
GABA	4.7	5.6	4.4	5.4	5.2	3.7
Dopamine	3.3	3.5	5.2	6.7	2.6	6.8
Epinephrine	3.5	5.7	7.8	3.2	5.0	4.3
Metanephrine	3.9	4.3	5.4	3.5	6.2	2.7
Serotonin	4.6	2.7	8.6	5.1	2.8	5.4

as it significantly reduces the running cost in the operation of this method.

As the developed sample preparation procedure does not involve an extraction process, ion suppression or enhancement were assessed through the standard addition method. The results for the matrix effects investigated at 2.75 and 5.00 μg/mL of the method are outlined in Table 5. All analytes were found to be within tolerances (± 15% ME); therefore, the method is not affected by ionisation enhancement or suppression due to matrix influence. Additionally, this indicates the selectivity of the method as target analytes were not subject to interference of other endogenous substances in human urine matrix.

The chemical instability of neurotransmitters and their metabolites is one of the most challenging fields in quantitative analysis. The main causes of sample degradation due to chemical instabilities are daylight, temperature and dissolved oxygen. In order to prevent degradation of neuroactive metabolites, stabilising precautions are required to be conducted during storage, sampling and analysis [29]. The degradation of metabolites was minimised through the addition of acetic acid and storage of samples on ice during the preparation process.

The ten analytes in working solutions were found to maintain stability for 48 h in the autosampler at a maintained constant temperature of 4 °C. As laboratories or clinical workplaces may require the use of stock solutions over an extended period, it is useful to understand the duration and conditions under which each analyte is able to maintain stability when stored for a lengthy period. For this reason, freeze-thaw and long-term stability experiments were subjected to extensive analysis. A summary of the results for the assessed analytes in these two studies are outlined in Table 6. The metabolites were found to maintain stability for at least three freeze-thaw cycles when stored at −20 °C. Long-term storage of urine showed analytes were stable for at least 90 days in treated authentic urine with acetic acid. On the other hand, the majority of the analytes in untreated authentic urine were found to remain stable for up to 30 days upon excretion and immediate storage in a −20 °C freezer. Lu et al. [30], in a study of

Table 5 Matrix effect results at 2.75 and 5.00 μg/mL concentration levels (n = 10)

Analyte	%ME spiked at 2.75 μg/mL	%ME spiked at 5.00 μg/mL
Tryptophan	5.6	8.3
Kynurenine	−13.1	−9.2
Kynurenic acid	11.5	−9.9
Anthranilic acid	−10.2	7.8
Glutamic acid	−7.8	−5.2
γ-Aminobutyric acid	−9.1	−8.5
Dopamine	4.5	−7.7
Epinephrine	−8.4	8.9
Metanephrine	−12.2	−6.5

Table 6 Freeze-thaw and long-term stability of acidified authentic urine (n = 5)

Analyte	Freeze-thaw stability (% difference) after 3 cycles	Long-term stability (% difference) after 90 days
Tryptophan	7.4	8.7
Kynurenine	−10.5	13.3
Kynurenic acid	−8.1	−14.9
Anthranilic acid	19.2	−18.6
Glutamic acid	−13.8	12.1
GABA	12.7	10.3
Dopamine	−9.5	13.7
Epinephrine	18.1	−19.8
Metanephrine	16.3	11.9

Serotonin	−14.5	−10.4	Serotonin	−11.6	−12.6

Table 7 Average concentrations of all analytes in freshly prepared authentic urine (n = 10)

Analyte	Average concentration ± SD (μg/mL)
Tryptophan	6.310 ± 0.390
Kynurenine	0.790 ± 0.048
Kynurenic acid	0.887 ± 0.042
Anthranilic acid	1.580 ± 0.051
Glutamic acid	3.050 ± 0.150
GABA	0.543 ± 0.036
Dopamine	0.468 ± 0.028
Epinephrine	0.095 ± 0.008
Metanephrine	0.181 ± 0.012
Serotonin	0.210 ± 0.014

tryptophan pathway metabolites, reported that freshly prepared authentic urine with 0.1% ascorbic acid was stable for three freeze-thaw cycles and at least 30 days in a −20 °C freezer.

The stability findings obtained in this study were found to be consistent with those published in the literature. In addition, it has also been reported that amine neuroactive molecules are stable for a year in acidified urine and storage at −20 °C [31]. Hence, it is highly recommended for urine to be treated with acidification upon collection in order to maintain the stability of neuroactive metabolites for prolonged durations.

The gradient elution profile used was found to be efficient in flushing out any remaining analytes between runs as blank responses were zero or significantly less than LOD peak areas, supporting the method being free of carryover.

Application of the validated method in the determination of neurotransmitters and their metabolites in human urine

The physiological concentrations of the neurotransmitters assessed in the method are outlined in Table 7 with representative chromatograms presented in Figs. 1 and 2. For all analytes, these values were found to be within the reported reference ranges of various studies [32, 33]. This demonstrates that the method is able to produce reliable quantitative data when compared with values that have been reported using different methods.

Conclusion

An analytical method using liquid chromatography triple quadrupole mass spectrometry was developed and validated for the detection and quantification of ten neurotransmitters associated with three neurotransmitter pathways in human urine. Sample preparation of neurotransmitters and their metabolites was achieved by a simple dilution method in an acidic medium followed by filtration. The analytes of interest were determined in an 8.5-min window. The method was validated in accordance with standard laboratory protocols and guidelines. This included the assessment of an analytical criterion investigating the linearity range, LOD, LOQ, accuracy, precision, matrix effect, carryover and stability. The method was further validated by performing analysis of neurotransmitters in authentic human urine. Another notable finding is that the method performance was not compromised in the absence of deuterated internal standards when both approaches (with and without internal standards) were compared under the experimental conditions employed. The simple sample preparation procedures together with a short chromatographic run time offer a significant advantage over many other published methods when lengthy sample preparation and derivatisation steps are involved for a busy clinical laboratory to handle volume samples and to shorten turn-around time. The omission of expensive deuterated internal standards in the method will provide a significant cost-saving for any laboratory involved in performing the analysis and will be particularly beneficial for those laboratories under financial constraint. It is important to note that a laboratory should conduct careful matrix effect assessment using its specific specimen sets especially when IS are not used. The developed method presents a useful addition to the analytical tools available for investigating the links between neurotransmitter imbalance and a range of health conditions. The study further emphasises the importance of storing samples under acidic conditions due to instability of these neurotransmitter analytes under normal physiological pH and provides basis to guide researchers in experimental design and particularly on how samples ought to be collected, stored and analysed.

Compliance with ethical standards Ethics approval for collecting human urine samples was obtained from the UTS Human Research Ethics Committee (Ethics Approval No. UTS HREC 2010268A). Informed consent was obtained from all donors who provided urine samples.

Conflict of interest The authors declare that they have no conflict of interest.

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