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Short communication

Measurement of plasma 5-hydroxyindole acetic acid by liquid chromatography tandem mass spectrometry—Comparison with HPLC methodology

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ABSTRACT

In patients with carcinoid disease, urinary concentration of the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) is currently used to monitor disease progression or response to treatment as it is the metabolic end-product resulting from free and stored serotonin turnover. However, due to the undignified, cumbersome and error-prone nature of 24-h urine collections, there is constant pressure to replace them. It has been demonstrated using high performance liquid chromatography (HPLC) with fluorescence detection technology that plasma can achieve this, with the added advantage that it can be used for diagnostic purposes also. Here we describe a much simpler method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) that is twice as fast as a HPLC method currently in routine use. The sample preparation protocol requires 50 μL of plasma and a simple protein precipitation step facilitated by acetonitrile. Chromatography was performed on a Phenomenex C18 Security Guard™ column coupled to a SIELC Primesep B reversed-phase, anion-exchange dual chemistry column and methanolic mobile phase gradient elution. Eluant was directly connected to a Waters® Quattro Premier™ XE tandem mass spectrometer operating in positive ion mode. We detected multiple reaction monitoring transitions *m*/*z* 191.9 > 145.6 and 193.9 > 147.6 for 5-HIAA and d2-5-HIAA respectively, which co-eluted at 2.1 min. Ion suppression was negligible, recovery from spiked plasma was 103% (range 97–113%) and the method showed good linearity to 10,000 nmol/L (r^2 = 0.999). Within-batch and between-batch imprecision was <10% and bias <15% at 3 concentrations, the limit of detection was 5 nmol/L and lower limit of quantitation 15 nmol/L. No interference was observed with L-tryptophan or 5-hydroxytryptamine. Comparison of LC–MS/MS and HPLC showed good agreement between the two methods but this LC–MS/MS assay displays several advantages; it requires 10-fold less sample, has a simpler extraction procedure and extended linearity, thus increasing laboratory throughput, lowering reagent costs and removing the need to dilute samples in patients with established carcinoid disease being monitored for therapeutic efficacy.

1. Introduction

Midgut carcinoid tumours are derived predominantly from enterochromaffin cells of the intestine and appendix and secrete a plethora of bioactive substances including serotonin, chromogranins and vasoactive mediators [1]. In patients with established carcinoid disease, urine concentration of the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) is used to monitor disease progression or response to treatment as it is the end-product resulting from enzymatic metabolism (monoamine oxidase; EC 1.4.3.4) of free and stored serotonin turnover [2].

A wide-range of urine 5-HIAA analytical techniques has been reported over the past few decades including spectrophotometry [3], immunoassay [4] and HPLC with varying detection modalities [5]. More recently, LC–MS/MS employing pre-analytical preparation steps such as on-line solid-phase extraction [6] and our own simple dilution procedure [7] have been utilised although a urine 5-HIAA method reported by Johnson et al. found that derivatisation of 5-HIAA with trimethylsilane (TMS) was essential to achieve chromatographic resolution and analytical sensitivity [8]. Though these techniques have proved useful, the diurnal variation of 5-HIAA production dictates that urine must be collected over a 24-h period [9] and these collections are cumbersome, error-prone and not favoured by patients or laboratory staff alike [10], which has led the drive for a replacement analytical matrix. This has been compounded by the finding that fasting plasma samples are less susceptible to interference from certain foods such as tomatoes,

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bananas and walnuts etc. that are known to falsely elevate urine 5-HIAA concentrations [10].

While urinalysis of 5-HIAA has been the cornerstone of carcinoid disease monitoring for quite some time, in 2000 it was demonstrated that platelet serotonin can act as a highly discriminating marker for diagnosis of carcinoid tumours [11]. Other studies have since shown that, employing a cut-off of 118 nmol/L, plasma 5-HIAA has a diagnostic specificity and sensitivity of 97 and 89%, respectively, superior to both urinalysis of 5-HIAA and measurement of serotonin in platelets [10], enabling plasma 5-HIAA to be employed for both diagnostic and monitoring purposes.

The plasma 5-HIAA method described by Degg et al. [12] and currently in routine use at Leeds Teaching Hospitals, U.K. employs HPLC with native fluorescence detection and although the method shows good reliability and precision, the sample preparation procedure requires relatively large sample volumes, uses hazardous and volatile chemicals and has a run time of 17 min [12]. By employing LC–MS/MS and a simpler sample preparation procedure, we aimed to cut both the sample preparation and run time considerably, while improving on some analytical attributes of the HPLC method.

2. Experimental

2.1. Materials

HPLC grade methanol, HPLC grade acetonitrile, ammonium acetate, 5-HIAA, L-tryptophan, serotonin (5-HT), phosphate buffered saline tablets and bovine serum albumin were purchased from Sigma (Poole, Dorset, UK). Deuterated internal standard (5-hydroxyindole-3-acetic-2,2-D2) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Formic acid (AnalaR grade) and hydrochloric acid were purchased from VWR International (Lutterworth, Leicestershire, UK). Polypropylene 1.2 mL tubes and 96-well shallow plates were purchased from Thermo Scientific (Epsom, Surrey, UK).

2.2. Calibration standards and quality control material

A 1 mmol/L stock solution of 5-HIAA was prepared in 0.1 M hydrochloric acid. Having demonstrated similar detector response gradients of calibrators prepared in spiked plasma, water and phosphate buffered saline pH 7.4, 0.1% (w/v) bovine serum albumin (PBS/BSA), working calibrators were prepared by dilution of the stock in PBS/BSA to concentrations of 0, 25, 50, 100, 500, 1000 and 10,000 nmol/L. In-house quality controls (QC) were prepared by diluting a separate 1 mmol/L stock of 5-HIAA in PBS/BSA to concentrations of 20, 100 and 500 nmol/L.

Stock solutions of 250 μg/L (1.3 μmol/L) deuterated internal standard were prepared in 0.1 M hydrochloric acid.

2.3. Sample preparation

After the addition of 10 μL d2-5-HIAA internal standard (concentration in reaction tube 50 nmol/L), protein was precipitated from 50 μL plasma, calibrator or QC material by addition of 200 μL of acetonitrile. Samples were vortexed for 1 min, centrifuged at 14,000 × *g* for 2 min and the supernatant transferred to a shallow-well plate. The plate was sealed with thermoseal film prior to analysis to prevent evaporation.

2.4. Liquid chromatography

High pressure liquid chromatography was carried out on a Waters® ACQUITY™ UPLC platform. 40 μL of extracted sample was injected onto a C18 reversed-phase Phenomenex Security Guard™ column (Phenomenex, Macclesfiled, UK) coupled to a Primesep B (SIELC Inc., Prospect Heights, IL, USA) 3.2 mm × 50 mm, 5 μm mixed-mode (reversed-phase plus anion exchange) column.

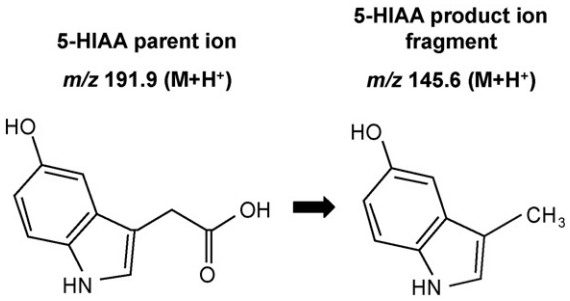

Fig. 1. The fragmentation pattern of 5-HIAA in our LC–MS/MS method. The ion fragment (right panel, *m*/*z* 145.6) has been utilised in previous methods [13,14].

Mobile phase A contained 10 mmol/L ammonium acetate and 0.1% (v/v) formic acid in water. Mobile phase B contained 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid in methanol. Initial conditions were 10% mobile phase B for 1.5 min and elution was performed by a single step gradient to 100% mobile phase B, held for 1.5 min before returning to 10% B for 3.9 min to allow the columns to re-equilibrate. Mobile phase flow rate was maintained at 0.7 mL/min and the inter-injection time was 7.9 min.

2.5. Tandem mass spectrometry

Electro-spray ionisation (ESI) tandem mass spectrometry was carried out on a Waters® Quattro Premier™ XE triple quadrupole (Waters, Watford, UK) instrument operating in positive ion mode. The instrument conditions were as follows: electrospray capillary voltage 0.5 kV, sample cone voltage 21 V and collision energy 20 eV. Desolvation gas flow and temperature were maintained at 700 L/h and 350 °C respectively and the source temperature was 140 °C. Both 5-HIAA and internal standard were detected in multiple reaction monitoring (MRM) mode with a dwell time of 0.4 seconds per channel. Ion transitions were *m*/*z* 191.9 > 145.6 and *m*/*z* 193.9 > 147.6 for 5-HIAA and d2-5-HIAA respectively (Fig. 1). Although no qualifier transitions were employed to confirm specificity, these ion transitions have been previously utilised for urine [13] and whole blood [14] 5HIAA LC–MS/MS methods.

The extractor voltage was 5 V and RF lens voltage 0.1 V. Resolution was 14.5 for MS1 and 14.0 for MS2, the photomultiplier energy was 645 V.

2.6. HPLC methodology

The plasma 5-HIAA HPLC method in routine use at Leeds Teaching Hospitals has been described in detail previously [12]. Briefly, 100 μL of internal standard (5-hydroxyindole-2-carboxylic acid) was added to 500 μL of heparinised plasma and diluted with 1 mL of water. Following acidification with 25 μL glacial acetic acid, 1 g sodium chloride and 5 mL diethyl ether was added and mixed by shaking for 2 min. After centrifugation at 2000 × *g* for 5 min, the ether layer was transferred to a glass tube, dried under nitrogen at 39 °C and re-suspended in 350 μL mobile phase (0.1 mol/L ammonium acetate, pH 4.55 containing 7% (v/v) acetonitrile). Chromatography was performed using isocratic separation (1 mL/min) on a 4.6 mm × 250 mm C18, 5 μm Symmetry column (Waters, Watford, UK) and peaks detected using a PerkinElmer LS5 (PerkinElmer, Beaconsfield, UK) spectrofluorimeter ($\lambda_{excitation}$—280 nm, $\lambda_{emission}$—345 nm).

2.7. LC–MS/MS method validation

2.7.1. Ion suppression

Ion suppression experiments were performed by continuous post-column infusion of 5-HIAA (500 nmol/L in 50:50 (v/v) water:methanol) directly into the mass spectrometer (T-piece) at a flow rate of 10 μL/min. Plasma samples ($n = 6$) and a water blank sample were prepared as described and injected via the autosampler; ion suppression/enhancement was interpreted by any drop or increase in baseline ion count at the retention time of the analyte.

2.7.2. Linearity of the assay

Linearity of the assay was assessed by repeat ($n = 6$) analysis of calibrators with concentrations ranging from 0 to 10,000 nmol/L. Reproducibility of the calibrator replicates was assessed as a function of variation (CV). LC–MS/MS response was plotted against nominal concentration values by use of QuanLynx™ software (Waters, Watford, UK). Linearity of the assay was confirmed by weighted linear regression with a correlation coefficient $r^2 > 0.99$.

2.7.3. Recovery

The recovery of 5-HIAA was determined by comparing the amount of 5-HIAA measured both before and after plasma samples were spiked with a known amount of 5-HIAA (50, 100, 500 and 1000 nmol/L, $n = 6$). Recovery was calculated as a percentage using the formula: (detector response of spiked plasma − detector response of unspiked plasma)/amount of 5-HIAA spiked into plasma) × 100.

2.7.4. Precision and accuracy

The imprecision of the method was assessed by the repeated preparation and analysis of three QC samples at concentrations of 20, 100 and 500 nmol/L. These samples were analysed ten times within a single run to determine within-batch imprecision, and also analysed in duplicate in separate batches ($n = 15$) over a 3-week period to calculate between-batch precision, both of which were assessed as a function of the variation (CV). Accuracy was assessed as a function of deviation from theoretical values. According to validation guidelines issued by the US Food and Drug Administration [15], precision and accuracy were deemed acceptable if <15%.

2.7.5. Lower limit of detection and quantitation (LOD and LLOQ)

The lower limit of detection (LOD) was determined as the smallest detectable peak in plasma, above baseline noise (signal:noise ratio >3:1, peak to peak). The LLOQ was determined by measuring ever-decreasing concentrations of 5-HIAA ten times and calculating the CV and percentage deviation from theoretical values. The LLOQ was assigned to the lowest concentration with a CV <20% and mean value <20% from the theoretical target value.

2.7.6. Stability

Plasma samples ($n = 15$) were subjected to 1, 2 or 3 freeze–thaw cycles by allowing plasma to thaw at ambient temperature for 1 h and re-freezing at −30 °C for at least 2 h prior to analysis.

Post-extraction stability of samples was assessed by preparing a batch ($n = 40$) of plasma samples in duplicate. One batch was injected immediately following preparation and the duplicate batch run again 72 h later following incubation in a sealed 96-well plate at ambient temperature. The mean percentage change in measured concentration was then calculated. The detector stability was determined by repeat injection of a pooled plasma sample every 7.9 min over a 14-h period. The assay was deemed stable if no systematic loss in mass spectrometer response was observed.

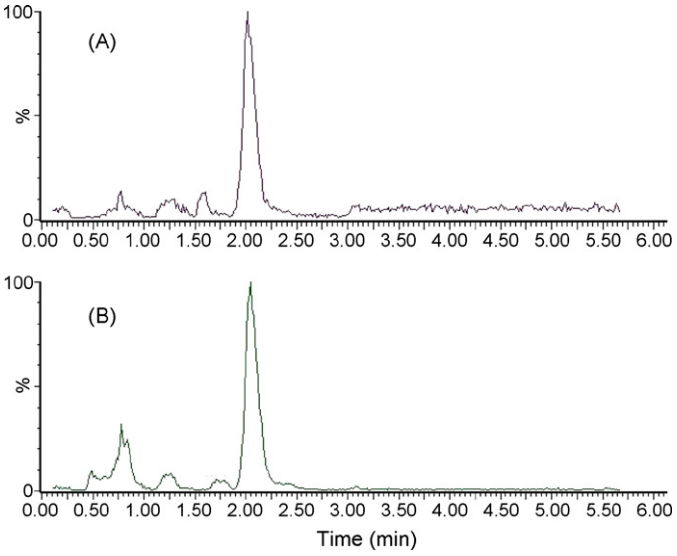

Fig. 2. Typical chromatograms produced by the new LC–MS/MS method. (A) Chromatogram of the internal standard with a detector response of 1.01×10^4 cps (50 nmol/L). (B) chromatogram of a plasma sample with a detector response of 2.61×10^4 cps, giving a 5-HIAA concentration of 120.44 nmol/L. Both chromatograms illustrate negligible interference in the immediate region of elution and both 5-HIAA and d2-5-HIAA have similar retention times of 2.1 min.

2.7.7. Interference

The ability of the 5-HIAA precursors L-tryptophan and 5-hydroxytryptamine to interfere with the 5-HIAA assay were assessed by injecting separate 1 μmol/L concentrations prepared in mobile phase A. They were considered unobtrusive to the function of the assay if no peak was observed in the chromatogram at the elution time corresponding to 5-HIAA.

2.7.8. HPLC and LC–MS/MS method comparison

To compare HPLC and LC–MS/MS methodologies, plasma 5-HIAA concentrations in 72 patients with suspected or established carcinoid disease were determined by HPLC fluorescence detection at Leeds Teaching Hospitals, UK. Samples were frozen at −20 °C, transported on ice and stored at −30 °C prior to analysis by LC–MS/MS. Results were compared using Analyse-It™ statistics software package (Analyse-It Software Ltd., Leeds, UK).

3. Results and discussion

3.1. Sample preparation and chromatography conditions

The chromatographic retention time for both 5-HIAA and d2-5-HIAA was 2.1 min within a total run time of 6.9 min. MRM ion chromatograms highlighted the specificity of the assay with clean elution peaks and no observed interference in the region of elution (Fig. 2).

Ion suppression experiments showed minimal signal interference with the simultaneous injection of extracted plasma samples and water into the mass spectrometer. Although acetonitrile has been used previously to precipitate protein from CSF prior to 5-HIAA analysis by HPLC [16], ion suppression had posed a major challenge in preliminary experiments of the method work-up, despite investigating many mobile phase gradients and chromatographic column composition. Employing a mixed-mode column that utilises anion-exchange and reversed-phase chromatography, these suppression effects were overcome. We believe it is the ability of the column to mediate resolution of 5-HIAA from potentially suppressive compounds that facilitates quantitation of plasma 5-HIAA by such a simple sample preparation method.

Table 1
Method validation data.

Imprecision and accuracy		
5-HIAA target value (nmol/L)	CV (%)	Deviation from theoretical target value (%)
Intra-assay (n = 10)		
20	4.7	0.5
100	2.8	3
500	1.9	3.1
Inter-assay (n = 15)		
20	9.8	5.7
100	2.6	8.3
500	2.9	3.4
Recovery		
Mean	Range	
103%	97–113%	
Limit of detection (LOD) 5 nmol/L		
Lower limit of quantitation (LLOQ) 15 nmol/L		

A run time of 6.9 min was necessary to allow re-equilibration of columns to baseline conditions. Despite this, our method is twice as fast as the HPLC method (17 min) and more than three times faster than a LC–MS/MS method for analysing 5-HIAA in whole blood (23 min) [14].

3.2. Method validation

Validation of the method was performed according to US FDA guidelines for industry [15] and validation data is summarised in Table 1. The method was linear to a concentration of 10,000 nmol/L (r^2 = 0.9998; y = 0.0014x + 0.0016), which is in contrast to the HPLC method employed routinely at Leeds Teaching Hospitals, UK. The HPLC method is linear to 1000 nmol/L and therefore plasma with concentrations exceeding this, a common finding in patients with established carcinoid disease, need to be diluted and re-analysed causing a duplication of workload and potential source of error. Variability of each calibrator was <8% (n = 12) and due to the extended wash and equilibration period following elution from the column, carry-over from the top calibrator to the zero calibrator was 0.037%.

Recovery from six plasma samples spiked with four different concentrations of 5-HIAA showed a mean recovery of 103% (range 97–113%), which is acceptable according to US FDA guidelines.

The assay displayed acceptable levels of inter- and intra-assay precision and accuracy, coefficients of variation (CV) were 9.8, 2.8 and 2.6% at 20, 100 and 500 nmol/L, respectively between-batch and 4.7, 2.8 and 2%, respectively within-batch, with a mean positive bias of 5.7% from theoretical levels.

The LOD was 5 nmol/L and the lower limit of quantitation (LLOQ) was 15 nmol/L. This LLOQ is comparable with the method reported by Danaceau et al. (10.5 nmol/L), though their extraction protocol is also protracted and requires long (23 min) chromatographic run times [14].

Non-extracted 5-HIAA stability following 1, 2 or 3 freeze–thaw cycles (n = 15) showed a net decrease in detector response of 6.96%, while extracted samples incubated for a 72 h period at ambient temperature showed a mean decrease in detector response of 3.1%. The stability of a single extract repeatedly injected over a 14 h period was also assessed and no systematic loss in sensitivity was observed over this period for both 5-HIAA and internal standard peak areas; analyte responses had CVs of 7.8 and 8.1% for 5-HIAA and d2-5-HIAA respectively and a detector response CV of 3.0%. This confirms the feasibility of analysing large batches of samples in a single run without compromising data quality.

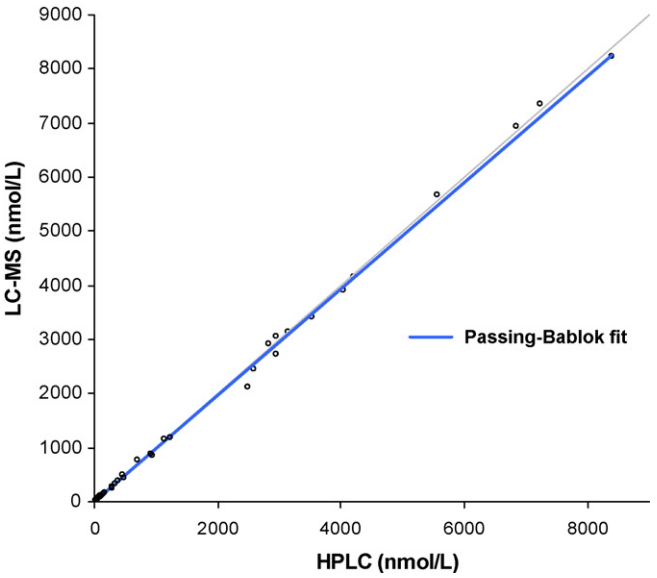

Fig. 3. Passing–Bablok analysis between plasma analysed by HPLC or LC–MS/MS gave the correlation LC–MS/MS = 0.98 (HPLC) + 2.38, r^2 = 0.99 (n = 72).

No interference peaks were observed at the time 5-HIAA eluted from the column by two structurally related compounds; the amino acid precursor L-tryptophan and the bioamine neurotransmitter 5-hydroxytryptamine, confirming the specificity of the assay.

Taken together, these data confirm the utility of the LC–MS/MS assay to precisely, accurately and consistently measure plasma concentrations of 5-HIAA.

3.3. Method comparison

Plasma samples previously analysed by HPLC fluorescence detection method were compared with our LC–MS/MS method. Passing and Bablok analysis showed good correlation between the two plasma 5-HIAA methods giving the equation LC–MS/MS = 0.98 (HPLC fluorescence) + 2.38, r^2 = 0.99, n = 72 (Fig. 3). Bland–Altman

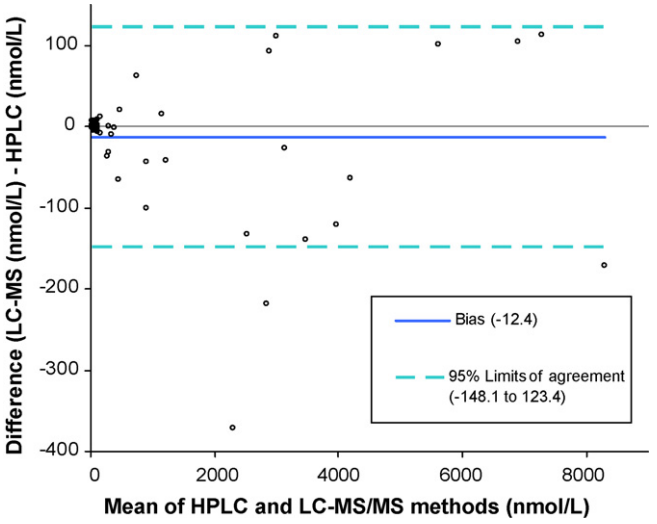

Fig. 4. Bland–Altman plot showing agreement between the LC–MS/MS method and an existing, routine method employing HPLC with fluorescence detection. Agreement showed a mean bias of −12.4 (LC–MS/MS–HPLC), 95% limits of agreement: −148.1 to 123.4.

also showed good agreement between the methods with a bias of −12.4 nmol/L (95% CI limits of agreement −148.1–123.4 nmol/L (Fig. 4).

4. Conclusion

To our knowledge, this is the first report describing LC–MS/MS quantification of 5-HIAA in plasma prepared by a simple protein precipitation step. The novelty of the method is conferred by a mixed-mode (anion-exchange plus reversed-phase) chromatography column that mediates partition of 5-HIAA from potentially ionisation-suppressing molecular entities.

Employing our LC–MS/MS method allows replacement of the protracted and time-consuming preparation step required to analyse plasma 5-HIAA by HPLC and other LC–MS/MS methods, thus lowering costs and removing the use of hazardous and volatile reagents. Sample volume requirements are decreased 10-fold, run times halved and the analytical range expanded 10-fold, with no loss in analytical sensitivity. Despite the simplicity of the sample preparation, the method retains clinical effectiveness, as confirmed by an LLOQ of 15 nmol/L, which is well below the 118 nmol/L cut-off required for high diagnostic sensitivity and specificity. Altogether, our method serves to increase laboratory throughput and removes the need for workload duplicity, further improving productivity and reducing costs. It will also help to eradicate the necessity for troublesome and unpleasant urine collections thus proving beneficial to both patients and laboratory staff alike.

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