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Short communication

Measurement of serotonin in platelet depleted plasma by liquid chromatography tandem mass spectrometry

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ABSTRACT

5-Hydroxytryptamine (5-HT) in human platelet depleted plasma (PDP) is a biomarker in functional gastrointestinal disorders (FGID), with levels reflecting acute changes in circulating 5-HT concentration. PDP 5-HT is currently measured by reversed phase high performance liquid chromatography (HPLC) fluorimetric detection. We have developed a simple and rapid liquid chromatography tandem mass spectrometry (LC–MS/MS) method that is two times more rapid than the current HPLC methodology. Our method employs a simple protein precipitation requiring no further downstream sample preparation. 10 μL of extract was injected directly onto a SecurityGuard SCX cation exchange column followed by isocratic elution onto an Onyx Monolithic C18 analytical column and methanolic gradient elution. Eluant was connected directly to a Quattro Premier XE tandem mass spectrometer operating in ES+ mode. We detected multiple reaction monitoring transitions m/z 160 > 114.9 and m/z 164.1 > 118.9 for 5-HT and d_4-5-HT, respectively. 5-HT and d_4-5-HT co-eluted at 2.79 min and cycle time between injections was 6 min. Mean recovery was 98%, limit of detection 1.5 nmol/L, lower limit of quantification 5 nmol/L, linearity to 1000 nmol/L (r^2 = 0.999), imprecision <10% and bias <13.4%. 5-HT eluted with no ion suppression. No interference was found with L-tryptophan or 5-hydroxyindoleacetic acid (5-HIAA). This assay was compared to a previously published HPLC method. Passing-Bablok regression analysis showed LC–MS/MS = 0.91 (HPLC)–0.83, r^2 = 0.97, n = 80. Bland Altman analysis showed general agreement, with a mean bias of 3.3 nmol/L. We have developed a simple and robust assay for PDP 5-HT that will increase throughput for clinical trials.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) functions both as a neurotransmitter in the central nervous system (CNS) and as a hormone in the peripheral vascular system of the gastrointestinal (GI) tract [1]. 95% of serotonin in the body is located in the gut with 90% within enterochromaffin cells and 10% in serotonergic neurons of the myenteric plexus. Following the release of 5-HT, it is rapidly sequestered by platelets [2] or otherwise metabolised by the liver and kidneys to its metabolite 5-hydroxyindoleacetic acid (5-HIAA) by the catalytic action of the mitochondrial flavoprotein monoamine oxidase (MAO; EC 1.4.3.4) and aldehyde dehydrogenase (EC 1.2.1.3) [3].

The concentration of 5-HT in platelet depleted plasma (PDP) is approximately 100 times lower than compared to whole blood concentration, making the measurement of 5-HT in PDP more demanding. PDP 5-HT levels could act as a biomarker in conditions such as functional gastrointestinal disorders (FGID) [4,5]. A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the measurement of 5-HT in whole blood has been developed and validated [6]. However, this method has not been validated for the measurement of 5-HT in PDP matrix, and based on the lower limit of quantification (LLOQ) for the whole blood assay of 5 ng/mL (28.4 nmol/L), this method lacks the analytical sensitivity required for the measurement of 5-HT at the physiological concentrations found in PDP. The validation of a HPLC with amperometric detection method for the measurement of 5-HT in platelet poor plasma has been described [7]. With this method 5-HT has a retention time of 12.3 min. More recently, a second method based on HPLC with fluorescence detection for the measurement of PDP 5-HT has also been described [8]. This current HPLC method has an analysis time of 12 min. Our aim was to therefore develop and validate a robust

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LC–MS/MS PDP 5-HT assay with a faster analysis time to permit high-throughput analysis for clinical trials.

2. Experimental

2.1. Materials

HPLC grade methanol, HPLC grade acetonitrile, ammonium acetate, 5-HT, L-tryptophan, 5-HIAA, phosphate buffered saline tablets and bovine serum albumin were purchased from Sigma (Dorset, UK). Deuterated internal standard (α,α,β,β-d_4 serotonin creatinine sulphate complex, d_4-5-HT) was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Formic acid (AnalaR grade) and hydrochloric acid were purchased from VWR International (Leicestershire, UK). Polypropylene 1.2 mL, 96-deep well plates were purchased from Thermo Scientific (Surrey, UK).

2.2. Calibration standards and QC material

A 10-mg/mL stock solution of serotonin hydrochloride was prepared in 0.1 M hydrochloric acid. Working calibrators were prepared by dilution of the stock calibrator into phosphate buffered saline (pH 7.4) containing bovine serum albumin 0.1% (w/v) (PBS-BSA) to give calibrators with concentrations corrected for the molecular weight of pure serotonin of 0, 12.5, 25, 50, 100, 500 and 1000 nmol/L. Quality controls (QCs) were prepared by dilution of an independent 10 mg/mL stock of 5-HT with PBS-BSA to yield QC of 20, 40 and 120 nmol/L. Dilutions of QC stock solution were prepared to determine the LLOQ for the assay. A 10 mg/mL internal standard stock solution of α,α,β,β-d_4 serotonin creatinine sulphate complex (d_4-5-HT) was prepared in 0.1 M hydrochloric acid and a working solution of 1 mg/L was prepared by two sequential 1:100 dilutions in deionised water.

2.3. Sample preparation

50 μL of PDP samples, calibrators or QC were added to 10 μL of internal standard and 100 μL of HPLC grade acetonitrile to precipitate protein in a 96-deep well plate. The plate was sealed with thermosealing film, vortex mixed and centrifuged at 3500 rpm/10 min prior to analysis.

2.4. Liquid chromatography

Chromatography was performed on a Waters Acquity™ UPLC system. 10 μL of extracted sample was injected onto a Phenomenex Security Guard™ strong cation exchange (SCX), 4.0 mm × 3.0 mm, guard column (Phenomenex, Macclesfield, UK). 5-HT eluted isocratically from the SCX guard column in aqueous 10 mM ammonium acetate pH 3 for 2 min onto the Phenomenex Onyx Monolithic C18, 25 mm × 4.8 mm, analytical column (Phenomenex, Macclesfield, UK). A 20–100% methanol gradient containing 2 mM ammonium acetate and 0.1% (v/v) formic acid was introduced for 1 min. The columns were washed with methanolic mobile phase for 1.5 min, after which the columns were re-equilibrated back to initial conditions with aqueous mobile phase for 1.5 min prior to the next sample injection. Column flow rate was maintained at 0.8 mL/min and chromatography was performed at ambient temperature. The eluant from the SCX guard column was diverted to waste for the first 0.7 min to prevent instrument contamination.

2.5. Tandem mass spectrometry

The analytical column eluate was injected directly into a Waters Quattro Premier XE tandem mass spectrometer operated in the positive electrospray ionisation mode (Waters, Hertfordshire, UK). The electrospray capillary voltage was 1.0 kV, the source temperature was 140 °C, the sample cone voltage was 28 V and the collision energy was 22 eV at a collision gas pressure 0.52 Pa. The desolvation gas flow was 600 L/h. Multiple reaction monitoring mode was utilised for analysis with a dwell time of 0.1 s per channel and transitions m/z 160 > 114.9 and m/z 164.1 > 118.9 for 5-HT and d_4-5-HT, respectively. In positive electrospray ionisation mode, the serotonin parent ion is 177 m/z. However, increasing the sample cone voltage to 28 V caused fragmentation of 5-HT at the cone, resulting in a more abundant parent ion 160 m/z. The extractor voltage was 3 V and RF lens voltage 0.6 V. Resolution was 14 for MS1 and 13 for MS2 and the photomultiplier energy was 645 V.

2.6. Method validation

2.6.1. Ion suppression

Ion suppression experiments were performed by continuous postcolumn infusion of serotonin (300 nmol/L deionised water) directly into the mass spectrometer via a Tee-piece at a flow rate of 10 μL/min. PDP samples (n = 6) and a water blank sample were prepared as described and simultaneously injected in the mass spectrometer via the autosampler and ion suppression/enhancement was interpreted by any drop or increase in baseline ion count at the retention time of the analyte.

2.6.2. Linearity

We determined the linearity of the method by analysing a set of calibrators with concentrations ranging from 0 to 1000 nmol/L. Nominal values were then plotted against the LC–MS/MS response (5-HT:d_4-5-HT peak-area ratio) by use of QuanLynx™ software (Waters, Hertfordshire, UK). Linearity of the calibrators was confirmed if the correlation coefficient as calculated by weighted linear regression was >0.99.

2.6.3. Recovery

The recovery of 5-HT was determined by comparing the amount of 5-HT measured both before and after PDP samples were spiked with a known amount of pure 5-HT (20, 100 and 500 nmol/L, n = 6). Recovery was calculated using the formula: ((detector response of spiked PDP − detector response of unspiked PDP)/amount of 5-HT spiked into PDP) × 100.

2.6.4. Precision and accuracy

The imprecision of the method was assessed by the analysis of three QC samples at concentrations of 20, 100 and 300 nmol/L. These samples were analysed 10 times within a single run to determine within-batch precision, and also analysed in separate batches (n = 15) over a 2-week period to calculate between-run precision. Both CV and percentage deviation from target value were calculated. Precision and accuracy were defined as acceptable if <15%.

2.6.5. Lower limit of quantification

The LLOQ was determined by measuring low concentrations of 5-HT (10, 5 and 2.5 nmol/L) 10 times each and calculating the CV and percentage deviation from the target values. The LLOQ was assigned to the lowest concentration with a CV <20% and mean value <20% from the target value.

2.6.6. Stability

The post-extraction stability of samples was assessed by running a batch of samples immediately following sample preparation, and then again 48 h later after incubation in a sealed 96-deep well plate at ambient temperature. The mean percentage change in measured concentration was then calculated. The detector stability was determined by repeat analysis of a 100 nmol/L standard every 6 min over a 14-h period. The assay was deemed stable if no

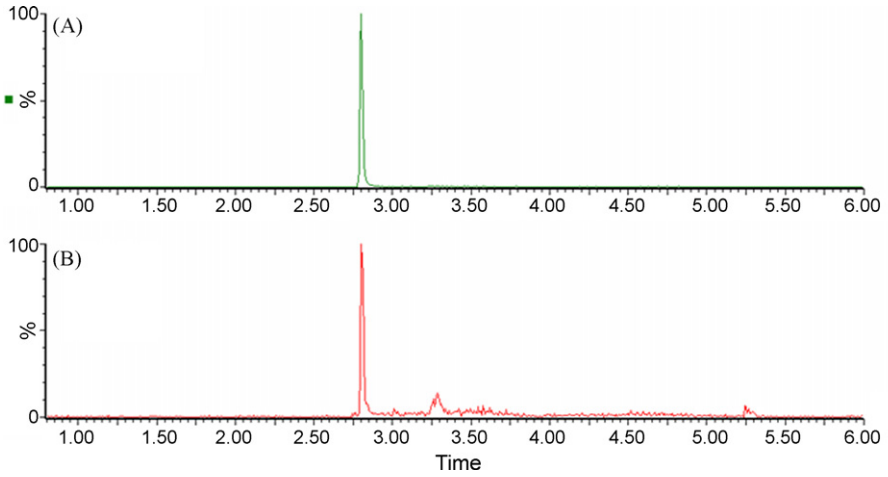

Fig. 1. Typical chromatograms produced by the new LC–MS/MS method. (A) Chromatogram of the internal standard with a detector response of 2.58×10^5 cps (1 mg/L); (B) chromatogram of a platelet depleted plasma sample with a detector response of 3.23×10^4 cps, giving an endogenous 5-HT concentration of 23.0 nmol/L. The chromatograms both show that there is negligible interference in the region of elution and that 5-HT and d_4-5-HT have an identical retention time of 2.79 min.

systematic loss in mass spectrometer response was observed. To assess the storage stability of 5-HT, we added low, medium and high concentrations of 5-HT (24, 300 and 500 nmol/L) to three plasma samples and incubated these samples at 20 °C for 1–5 days prior to analysis. Additionally, we subjected 5-HT samples ($n = 12$) to 3 freeze–thaw cycles by allowing samples to thaw at room temperature for 1 h before returning them to −20 °C for 23 h prior to analysis by LC–MS/MS.

2.6.7. Interference

The 5-HT precursor amino acid L-tryptophan and the major 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) both a 1 μmol/L concentration in aqueous 10 mmol/L ammonium acetate pH 3, were prepared for analysis as described in Section 2.3. These compounds were considered not to interfere in the 5-HT assay if they did not produce a signal in the chromatogram at the time point where 5-HT elutes. The effect of L-tryptophan and 5-HIAA on the ionisation efficiency of 5-HT was also assessed by comparison of 5-HT ion counts before and after the addition of these compounds to 1 μmol/L final concentration. A difference in ion counts of <10% was judged to indicate no effect.

2.6.8. Method comparison

Patient blood samples used during the method comparison were collected in tubes containing 0.5 mL of 3.12% trisodium citrate and centrifuged to obtain PDP using the protocol described previously [9]. PDP samples were stored at −80 °C until analysis by HPLC and LC–MS/MS methods. Approval to use these samples was granted by the local ethics committee. PDP samples ($n = 80$) were analysed using the new LC–MS/MS method and the HPLC fluorescence detection method published by Atkinson et al. [8]. Results were compared using Analyse-It™ statistics software package (Analyse-It Software Ltd., Leeds, UK).

3. Results and discussion

3.1. Sample preparation and liquid chromatography

The chromatographic retention time for both 5-HT and d_4-5-HT was 2.79 min within a total run time of 6 min. The resulting chromatograms highlighted the specificity of the assay with clean 5-HT and d_4-5-HT elution peaks and no observed interference in this region (Fig. 1).

Ion suppression experiments showed minimal signal interference on the simultaneous injection of extracted plasma samples and water into the mass spectrometer, indicating that there is negligible ion suppression observed under the conditions used. The postcolumn infusion experiments did highlight that a run time of 6 min was necessary to allow re-equilibration of columns to baseline conditions. The method was then validated according to published acceptance criteria [10].

3.2. Linearity

The assay was linear up to at least 1000 nmol/L ($r^2 = 0.9998$; $y = 0.0058x + 0.007$ nmol/L). Carryover between the top and zero calibrators was 0.3%. All calibrators were prepared in PBS-BSA artificial matrix because it was difficult to obtain enough 5-HT-stripped PDP for routine use. However, during the analysis of a batch of patient samples, one of the samples assayed had a very low concentration of 5-HT that was below the LLOQ of the method, we therefore used this PDP to prepare calibrators to compare to our existing calibrators prepared in PBS-BSA. Both sets of calibrators gave very similar mass spectrometer detector responses (5-HT peak areas; Fig. 2). Linear regression analysis gave an r^2 value for the plotted line of 0.998 and the equation of the line was: detector response of calibrators prepared in 5-HT-free PDP = 0.95 × detector response of calibrators prepared in PBS-BSA + 45.42. These results confirmed the validity of using calibrators prepared in PBS-BSA for routine use in this assay.

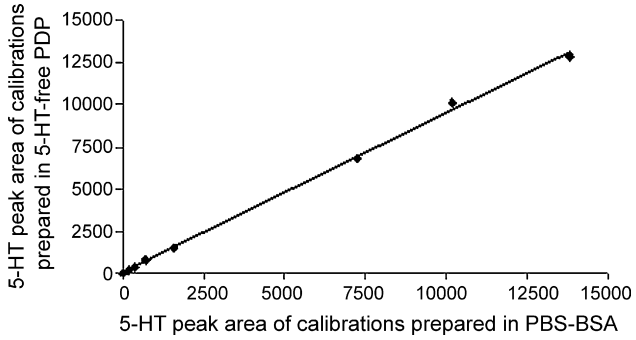

Fig. 2. A comparison of the mass spectrometer 5-HT peak areas from the measurement of one set of calibrators prepared in PBS-BSA and a separate set of calibrators of the same concentration prepared in 5-HT-free PDP.

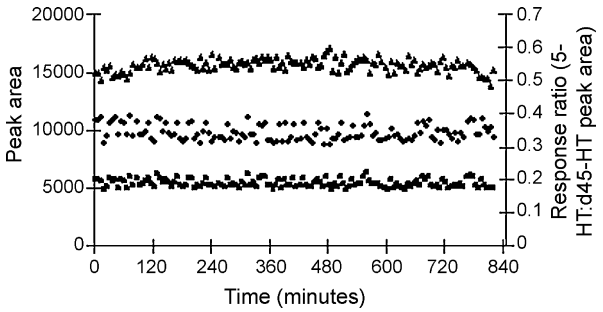

Fig. 3. The detector stability of the assay. 140 × 10 μL injections were performed over a 14 h period. The primary *y*-axis refers to the peak area of 5-HT (squares) and internal standard (diamonds). The secondary *y*-axis refers to the response ratio (5-HT:d_4-5-HT peak-area ratio; triangles) and is plotted for each injection.

3.3. Recovery and stability

The mean recovery calculated from the measurement of PDP samples before and after the addition of pure 5-HT was 98%, with a range of 89.5–115.5 nmol/L (n = 18). Extracted samples were stable over a 48 h period at room temperature with a mean percentage decrease in concentration of 1.6%. The stability of a single extract over 14 h was also assessed. We observed no systematic loss in sensitivity over this period for both 5-HT and internal standard peak areas, and the CV of the peak-area ratio was 3.6% (Fig. 3). This confirms that it is possible to analyse a large batch of samples in one run over an extended period of time using this assay without compromising sample results. Storage of 5-HT samples at 20 °C for 5 days resulted in a mean decrease in 5-HT concentration of 2.8% when compared to fresh samples (n = 12). We therefore considered plasma samples to be stable at 20 °C for at least 5 days. Freeze–thaw experiments were performed over 3 cycles prior to analysis and the mean difference in 5-HT concentration was 3.8% at all sample concentrations (n = 12). Plasma samples were therefore deemed stable for 5-HT measurement.

3.4. Imprecision and bias

Imprecision and accuracy were calculated for both within-batch and between-batch analyses at three concentrations of 5-HT (20, 100 and 300 nmol/L). Within-batch CV were all <10% across the range of concentrations measured, with mean values within 14% of the target concentration. Between-batch CV were all <12% using the same concentrations of 5-HT, with mean values all within 2% of the target concentration (Table 1).

The LLOQ, designated as the lowest concentration with a CV <20% and a mean value within 20% of the theoretical value and determined by replicate analysis of QC samples (n = 10) was 5 nmol/L. This value is below the mean value reported by Atkinson et al. [11], for PDP 5-HT concentrations in healthy volunteers of 21.31 nmol/L (n = 35) and thus confirms the new assay has sufficient analytical sensitivity. The limit of detection (LOD), calculated as the smallest detectable peak above baseline noise (signal-to-noise ratio >3:1), was 1.5 nmol/L.

Table 1
Validation results.

5-HT target value (nmol/L)	CV (%)	Deviation from theoretical target value (%)
Within-batch (n = 10)		
20	9.9	11.8
100	4.3	12.5
300	3.7	13.3
Between-batch (n = 15)		
20	11.7	1.6
100	6.3	0.3
300	5.1	1.5

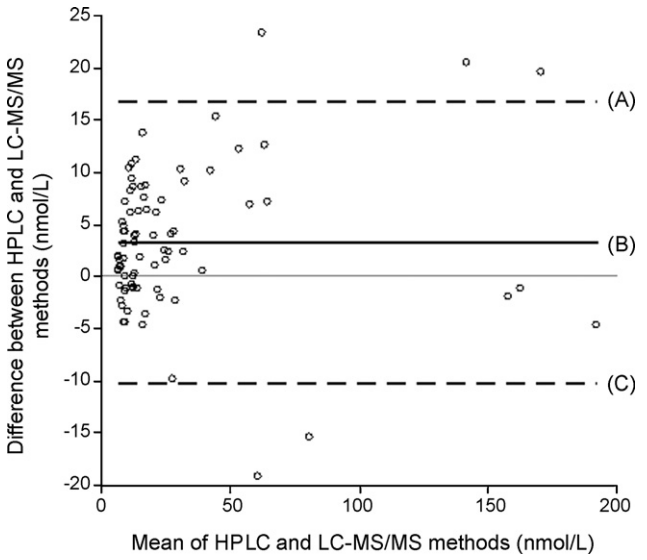

Fig. 4. Bland Altman difference plot comparing the new LC–MS/MS method to the existing HPLC method. Lines A and C represent the 95% limits of agreement for the mean difference between the two methods. Line B indicates the mean difference of 3.3 nmol/L.

3.5. Interference

We observed no peaks at the region of 5-HT elution from the 5-HT precursor amino acid L-tryptophan and the major physiological 5-HT metabolite 5-HIAA when injected into the mass spectrometer at 1 μmol/L. The difference in ionisation efficiency of 5-HT in the presence of 1 μmol/L L-tryptophan and 5-HIAA was <9% indicating that these compounds had no effect on the ionisation efficiency of 5-HT.

3.6. Method comparison

Passing-Bablok regression analysis comparing the new LC–MS/MS method with the existing HPLC method showed LC–MS/MS = 0.91 (HPLC)–0.83, r^2 = 0.97, n = 80. Bland Altman analysis showed good general agreement between LC–MS/MS and HPLC methods with a mean bias of 3.3 nmol/L (95% limits of agreement = −10.2 to 16.7 nmol/L) (Fig. 4).

4. Conclusions

This novel LC–MS/MS method for the quantification of 5-HT in human PDP uses a simple protein crash extraction and exploits a two-dimensional chromatography strategy with a cation exchange online clean up step to elute 5-HT away from regions of ion suppression. 5-HT is isocratically eluted from the SCX guard column by use of low pH aqueous phase (pH 3) over a total of 36 column volumes to remove major contaminating matrix components. The efficient extraction procedure requires no derivatization or sample transfer into separate vials and as such reduces staff burden. The sample analysis time of 6 min is half that of the current HPLC method and requires only 50 μL PDP, a quarter of the volume that is needed for analysis by HPLC. Additionally, the HPLC fluorescence detection hardware has a working deuterium lamp life of only 500 h which poses another methodological disadvantage of HPLC in comparison to the novel LC–MS/MS method described.

Here we have described for the first time the measurement of 5-HT in human PDP by LC–MS/MS employing a two-dimensional chromatography approach. This novel assay provides a high-throughput, robust methodology for the measurement of PDP 5-HT concentration, toward the diagnosis of FGIDs. The assay may also have a potential utility in predicting the efficacy of drug treatment for patients being prescribed 5-HT receptor modulating drugs.

References

[1] M.D. Gershon, A.B. Drakontides, L.L. Ross, Science 149 (1965) 197–199.
[2] M. Da Prada, J.P. Tranzer, A. Pletscher, Experientia 28 (1972) 1328–1329.
[3] V. Erspamer, A. Testini, J. Pharm. Pharmacol. 11 (1959) 618–623.
[4] S.P. Dunlop, N.S. Coleman, E. Blackshaw, A.C. Perkins, G. Singh, C.A. Marsden, R.C. Spiller, Clin. Gastroenterol. Hepatol. 3 (2005) 349–357.
[5] L.A. Houghton, W. Atkinson, R.P. Whitaker, P.J. Whorwell, M.J. Rimmer, Gut 52 (2003) 663–670.
[6] J.P. Danaceau, G.M. Anderson, W.M. McMahon, D.J. Crouch, J. Anal. Toxicol. 27 (2003) 440–444.
[7] E. Pussard, N. Guigueno, O. Adam, J.-F. Giudicelli, Clin. Chem. 42 (1996) 1086–1091.
[8] W. Atkinson, S.J. Lockhart, L.A. Houghton, B.G. Keevil, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 832 (2006) 173–176.
[9] L.A. Houghton, W. Atkinson, C. Lockhart, P.J. Whorwell, B. Keevil, Neurogastroenterol. Motil. 19 (2007) 724–731.
[10] Food and Drug Administration, Department of Health and Human Services, Guidance for Industry: Bioanalytical Method Validation, 2001, http://www.fda.gov/cder/Guidance/4252fnl.pdf.
[11] W. Atkinson, S. Lockhart, P.J. Whorwell, B. Keevil, L.A. Houghton, Gastroenterology 130 (2006) 34–43.