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Short communication

Validation of the measurement of low concentrations of 5-hydroxytryptamine in plasma using high performance liquid chromatography

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Abstract

A sensitive and rapid assay is described for the measurement of low concentrations of 5-hydroxytryptamine (5-HT) present in human platelet-depleted plasma (PDP) using reverse-phase high performance liquid chromatography (HPLC) with fluorimetric detection. With an analysis time of 12 min, this method is particularly useful for large-scale clinical trials investigating small differences in PDP 5-HT concentrations in conditions such as functional gastrointestinal disorders (FGID). The limit of detection and quantification were 1 and 3 nmol/l, respectively, and the calibration curve linear between 1 and 1000 nmol/l. The within-day and between-day precision were 4.3 and <13.6%, respectively.

Keywords: Validation; Plasma; 5-HT; HPLC

1. Introduction

The majority of 5-hydroxytryptamine (5-HT) in the peripheral blood is derived from the gastrointestinal tract, where it is stored predominantly within the mucosal enterochromaffin cells [1]. Following its release, 5-HT is either rapidly taken up and stored within the platelets [2], or metabolised by the liver and kidney to its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) [3]. The concentration of 5-HT in platelets, which is approximately equivalent to that of whole blood, is estimated to be $\leq$10 μmol/l [4] and is consequently of the magnitude of 100 times greater than that found in platelet-depleted plasma (PDP), where any change is small and of short duration making detection and measurement difficult.

5-HT acts via a variety of receptors to modulate the motor, sensory and secretory functions of the gut [5–7]. Disorders of gastrointestinal sensorimotor function, such as irritable bowel syndrome (IBS) and functional dyspepsia (FD), have recently been shown to be associated with significantly higher postprandial concentrations of PDP 5-HT compared with those found in healthy volunteers suggesting a role for 5-HT in the pathogenesis of these conditions [8–10].

Many of the previously described HPLC assays coupled with either electrochemical [11–13] or fluorimetric detection [14,15], are intended for the measurement of high concentrations of 5-HT found in the whole blood of patients, for example with carcinoid tumours [16] or those receiving cisplatin treatment for cancer [17,18]. Previously published HPLC techniques using fluorimetric detection for measuring the low concentrations of 5-HT found in platelet-depleted plasma are limited with most using ion-pair separation [14,15]. In addition many lack the methodological detail required for their reproduction and if validated, were done so at relatively high concentrations of 5-HT, leading to uncertainty regarding their accuracy and sensitivity.

Our aim was to develop a rapid and precise HPLC based assay using fluorimetric detection capable of measuring low concentrations of 5-HT found in platelet-depleted plasma. In addition, we have measured the physiological increase in PDP 5-HT concentration following meal ingestion [19] in a group of healthy volunteers, demonstrating one potential application of this assay.

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2. Experimental

2.1. Chemicals and reagents

5-Hydroxytryptamine hydrochloride was purchased from Sigma (Sigma–Aldrich Co., USA). Methanol was of HPLC grade and all other reagents used were of analytical grade.

2.2. Equipment and chromatographic conditions

The HPLC system consisted of a Shimadzu Liquid Chromatograph LC-10AT/VP (Shimadzu Scientific Instruments, Kyoto, Japan), a UV–vis fluorescence detector (RF-10A, Shimadzu), an autoinjector (Model SIL-10AD, Shimadzu) and data processing software (Shimadzu class VP version 6.10 software programme).

For the measurement of 5-HT, a 20 μl aliquot of the supernatant (in the case of plasma samples) was injected onto the Synergi 4 μm Hydro-RP separation column, (internal diameter: 75 mm × 3.0 mm; Phenomenex, Macclesfield, UK), fitted with a SecurityGuard C18 cartridge column (internal diameter: 4 mm × 3.0 mm, Phenomenex, Macclesfield, UK). The mobile phase consisted of 100 mmol/l ammonium acetate:methanol (250:20, v/v) and the aqueous phase adjusted to pH 4.5 using concentrated acetic acid. The mixture was degassed by filtration through a nylon membrane filter (0.2 μm pore) (Whatman, Maidstone, UK). The column eluted at a flow rate of 0.8 ml/min at ambient temperature. UV detection (UV–vis fluorescence detector, model RF-10A, Shimadzu) with a 150 watt xenon lamp set at excitation wavelength of 290 nm, emission wavelength of 337 nm, with a gain 16×, sampling frequency of 2 Hz and standard response was used.

2.3. Preparation of standard solutions

A stock solution of 5-HT (100 nmol/l) was diluted in 10% perchloric acid to prepare working standards. Quality control (QC) samples were prepared at concentrations of 120, 52 and 27 nmol/l for 5-HT by spiking stock solutions into plasma. The samples were stored at −20 °C and analysed with each batch of clinical samples to monitor the performance of the method during use and assess the stability of 5-HT.

2.4. Collection and preparation of platelet-depleted plasma samples

Venous blood samples (5 ml) were collected in EDTA vacutainer tubes, transferred to tubes containing 0.5 ml of 3.12% trisodium citrate and centrifuged (room temperature); initially at 2500 rpm for 10 min and then at 4000 rpm for a further 10 min. Platelet-depleted plasma was aspirated and a 200 μl aliquot pipetted into a 1.5 ml Eppendorf tube. Plasma proteins were precipitated by the addition of 200 μl of 6% perchloric acid (v/v), the mixture was vortexed and separated by microcentrifugation at 11000 × *g* for 3 min. Finally, 200 μl of the supernatant was transferred to an autoinjector vial for HPLC analysis.

2.5. Validation of the method

The linearity of the method was determined from a calibration curve constructed by plotting the peak height against the concentration of 5-HT over the working range (1–1000 nmol/l; $n = 10$) of the calibration standards. Precision was evaluated by calibration curves and the coefficient of variation (C.V.) calculated between 12 replicate analyses of quality-control samples performed in different batches over a 1-month period (inter-assay precision) and 10 replicate analyses of quality-control samples performed within a single batch (intra-assay). Accuracy was determined as the difference between the determined and recovered concentrations of pooled plasma samples spiked at four different concentration levels of 5-HT (200 μl, 100 μl, 50 μl and 0 μl; $n = 6$). Recovery was analysed by comparing the observed peak heights of six replicate plasma samples spiked at each concentration, with the expected values at these concentrations determined from a standard calibration curve. Sensitivity was determined by the detection limit (three times the value of the background noise signal) and the quantification limit (lowest concentration of standard measurable at over 10 times the value of the background noise signal, with a C.V. < 20%). The stability of 5-HT in the supernatant was assessed by repeated injections of the extract from a pooled sample (30 nmol/l), with 20 μl injections being made every 12 min over a 15-h period.

3. Results

3.1. Chromatography

5-HT eluted as a small, well-defined peak with a mean retention time of 2.5 min prior to a large tryptophan peak at 4 min (Fig. 1). The duration of the chromatogram was 12 min, permitting the analysis of a large number of samples in a short period of time.

3.2. Assay validation

3.2.1. Linearity, precision and recovery

The standard calibration curve was linear between 1 and 1000 nmol/l, with a mean correlation coefficient (r^2) of 0.999 ($n = 10$). The within-day and between-day precision for 5-HT

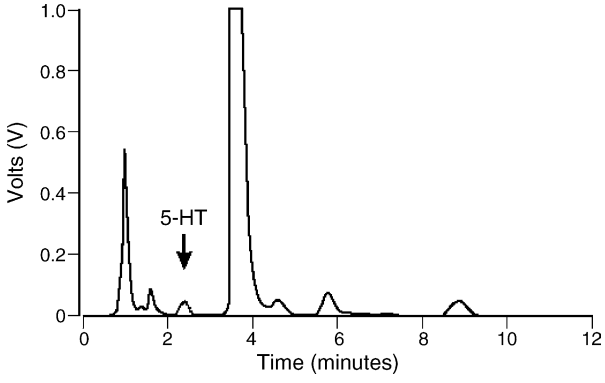

Fig. 1. Chromatogram of 5-HT in human plasma (5 nmol/l).

Table 1
Precision of the 5-HT assay

	n	Mean concentration (nmol/l) (S.D.)	CV (%)
Inter-assay			
High	12	119.6 (6.6)	5.5
Mid-high	12	51.7 (3.5)	6.8
Mid-low	12	27.4 (2.8)	10.3
Low	12	7.8 (1.2)	13.6
Intra-assay			
Pool	10	15.7 (0.7)	4.3

analysis is shown in Table 1. Within-day precision (C.V. = 4.3%) and between-day precision (C.V. < 13.6%) were both acceptable, indicating a low variability between the values obtained for each concentration. Recovery of 5-HT from plasma at 200, 100, 50 and 25 nmol/l concentrations was 94.0, 95.1, 93.5 and 92.6%, respectively, with C.V.s lower than 1.7%.

3.2.2. Sensitivity

The limit of detection for the 5-HT assay was 1 nmol/l (based on a 20 μl injection volume). The limit of quantification of the HPLC system for the detection of 5-HT (based on 20 μl injection volume) was at least 3 nmol/l, with a signal-to-noise ratio of greater than 10 and a coefficient of variation <20%.

3.2.3. Stability

No systemic loss in sensitivity for 5-HT was observed in the peak heights measured on these analytes over a 1-month period. Furthermore, the coefficient of variation for the peak height ratio following repeated injections (every 12 min) from a pooled sample (30 nmol/l) over a 15-h period was 4.3% (Fig. 2).

3.3. Analytical application

To demonstrate the suitability of this method for use in clinical studies, this assay was used to determine the 5-HT concentration of PDP samples obtained from a group of healthy volunteers ($n = 15$) under fasting conditions (two-time points, 60-min intervals) and following ingestion of a standard carbohydrate-rich meal [630 kcal; 12 g protein, 65.5 g carbohydrate and 46 g fat (fed conditions: eight-time points, 30-min intervals)]. The difference in mean 5-HT concentration between the fasted and fed state was analysed statistically using the paired Wilcoxon test for non-parametric data. In the fasting state, the median plasma concentration of 5-HT was 13.1 nmol/l [interquartile range; (8.3, 19.9) nmol/l], which increased significantly to 17.2 nmol/l (9.9, 32.3) nmol/l, following meal ingestion ($p = 0.027$). A similar increase in the peak concentration of 5-HT was also recorded, which rose from 14.7 nmol/l (2.2, 32.0) nmol/l under fasting conditions, to reach a maximum of 50.8 nmol/l (10.9, 159.4) nmol/l between 150- and 180-min post-meal ingestion ($p = 0.007$).

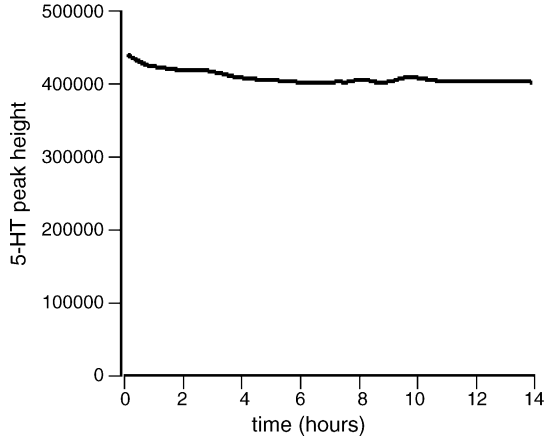

Fig. 2. Stability of 5-HT in human plasma over a 15-h recording period (30 nmol/l).

4. Discussion

A sensitive, rapid method for measuring low concentrations of 5-HT in platelet-depleted plasma using reverse-phase high performance liquid chromatography (HPLC) with fluorimetric detection was described. This assay is simple and easy to perform, not requiring the use of ion-pair technology commonly employed in previous techniques [14,15] and provides a method with increased sensitivity and specificity for the measurement of low platelet-depleted plasma concentrations of 5-HT. We have validated and demonstrated the suitability of this method for use in a clinical setting, along with its ability to measure subtle changes in PDP 5-HT concentrations under different physiological conditions (fed and fasted states) in a group of healthy volunteers.

Plasma concentrations of 5-HT have previously been shown to be increased in patients with functional gastrointestinal disorders (FGID) compared with healthy individuals following meal ingestion [8–10] and may be associated with the postprandial exacerbation of their symptomatology [9,10]. Potentially, the measurement of PDP 5-HT concentration could act as a biomarker of these conditions, aiding diagnosis and helping to predict the efficacy of 5-HT receptor modulating drugs, currently indicated for the treatment of some patients with FGIDs. Thus, an accurate, reliable, yet easy to run method for the determination of 5-HT concentration in human platelet-depleted plasma is essential.

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