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Determination of serotonin and its precursors in human plasma by capillary electrophoresis–electrospray ionization–time-of-flight mass spectrometry

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Abstract

A specific capillary electrophoresis–time-of-flight mass spectrometry (CE–TOFMS) method for the determination of serotonin (5HT) and its precursors tryptophan (Trp) and 5-hydroxytryptophan (5HTP) in human platelet rich plasma is described. The analytes were removed from the plasma samples and preconcentrated by solid phase extraction (SPE) on mixed mode cation-exchange sorbents. The SPE recoveries were 71.6 ± 3.1 for 5HT, 91.0 ± 2.8 for Trp, and $95.3 \pm 5.9\%$ for 5HTP. Deuterated analogues of 5HT and Trp were used as internal standards for quantitation purposes. Submicromolar detection limits were obtained for standard mixtures of all compounds and their deuterated isotopes, except 5HTP, which had detection limits in the low micromolar range. The potential usefulness of this method in the clinical setting was demonstrated by analyzing plasma extracts from healthy volunteers as well as from pathological samples. While 5HTP was not present in any of the analyzed samples, the levels of 5HT and Trp in both normal and pathological plasma were determined.
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1. Introduction

The bioactive indolamine serotonin (5-hydroxytryptamine, 5HT), is an established neurotransmitter and vasoconstrictor. It is formed by decarboxylation of 5-hydroxytryptophan (5HTP), its immediate precursor, in one of tryptophan's (Trp) metabolic pathways [1–3]. Serotonin has been implicated in a variety of physiological processes, including smooth muscle contraction, blood pressure regulation, and both peripheral and central nervous system transmission [1–3]. The involvement of 5HT in many pathological conditions, such as depression, schizophrenia, anorexia nervosa, dementia, and carcinoid syndrome has been established [1–6]. Carcinoid syndrome, characterized by flushing, diarrhea, valvular heart disease, and bronchoconstriction, arises from excessive synthesis and secretion of 5HT (and sometimes 5HTP) by a carcinoid tumor [1–3, 7–10].

The most commonly used laboratory test for the determination of a serotonin-secreting carcinoid tumor is the measurement of urinary 5-hydroxyindoleacetic acid (5HIAA), a major serotonin metabolite [3,4,7,9,11]. However, in the case of tumors producing only small amounts of serotonin, the predictive value of an increased urinary 5HIAA is low [10]. In addition, the concentration of 5HIAA in urine may be increased by consumption of certain 5HT-rich foods [1,12]. It has been shown that platelet serotonin is the most sensitive indole marker for the diagnosis of carcinoid tumors, especially those with low serotonin production [3,4, 7,10].

A variety of methods such as spectrophotometry, fluorometry, enzyme immunoassay, radioimmunoassay, GC–MS, have been used to measure serotonin [1,13–17]. In clinical practice, however, serotonin is usually assayed by HPLC with either electrochemical [6–8,11,18,19] or fluorometric

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detection [4,5,9,10,20–22]. Although some of these methods have substantial sensitivity, they cannot match the identification power and selectivity of a separation technique coupled to mass spectrometric detection [23]. The mass spectrometer gives another dimension to the analysis by identifying the analytes based on their mass-to-charge ratios and thus provides the detection specificity that most other types of detection methods lack. While a few accounts describing an HPLC separation of indole compounds from biological matrices followed by mass spectrometric detection have been published [23–25], such approaches have only recently been applied to clinical determination of serotonin and related indoles in human blood [26].

In recent years, capillary electrophoresis (CE) has been growing in popularity as a separation technique complementary to HPLC. Although HPLC is a robust, well established separation technique, CE does have certain advantages over HPLC: the solvent consumption (particularly for organic solvents) is less, which makes this technique both more cost effective and environmentally friendly. Also, CE capillaries are significantly cheaper than HPLC columns. Several authors have reported the use of CE in the analysis of one or more indolamines in biological matrices including urine, serum, brain tissue, and individual cells [27–34]. As with chromatographic separations, the predominant modes of detection have been electrochemical [33,34] and laser-induced fluorescence [28–32].

The aim of the present study was to evaluate the capabilities of capillary electrophoretic separation coupled with highly specific time-of-flight mass spectrometric (TOFMS) detection for the analysis of indole markers in human plasma as an alternative to HPLC methods. The major advantage of the TOFMS instrument is its ability to detect ions of different mass-to-charge ratios (m/z) simultaneously and, therefore, resolve incompletely separated or co-eluting peaks, unless the eluting compounds form ions of identical m/z ratios [35,36]. To our knowledge, such an approach has not yet been accomplished for serotonin and related compounds in complex biological specimens such as serum or plasma.

The developed CE–TOFMS method was applied to determination of serotonin and its precursors Trp and 5HTP extracted from platelet-rich plasma using mixed cation exchange solid phase extraction columns. Deuterated 5HT and Trp were used as internal standards in order to increase the reliability of quantitation [17,23]. Plasma specimens from healthy individuals as well as pathological samples were assayed.

2. Experimental

2.1. Materials and chemicals

Serotonin (5-hydroxytryptamine hydrochloride, 5HT), L-tryptophan (Trp), and 5-hydroxy-L-tryptophan (5HTP) were purchased from Sigma (St. Louis, MO, USA). Deuterated internal standards (serotonin-α,α,β,β-d_4 creatinine sulfate complex, d5HT, L-tryptophan-2′,4′,5′,6′,7′-d_5, dTrp) were purchased form C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Polyvinyl alcohol (PVA, >99%, average MW 89,000–98,000 g/mol) was purchased from Aldrich (Milwaukee, WI, USA). HPLC-grade methanol, HPLC-grade water, hydrochloric acid, acetic acid, and formic acid were from Mallinckrodt (Paris, KY, USA). Ammonium hydroxide was from EM Science (a division of EM Industries, Gibbstown, NJ, USA) and ammonium acetate and ammonium formate from Fisher Scientific (Fair Lawn, NJ, USA). SPE columns, Oasis MCX 1 cc (30 mg), were purchased from Waters (Milford, MA, USA). Centricon® centrifugal filter devices with regenerated cellulose 50,000 molecular weight cut-off membranes were from Millipore (Bedford, MA, USA).

2.2. Buffers and standard solutions

Ammonium formate separation buffers were prepared at 25 mM concentration and adjusted to a final pH of 3.0 or 3.5 with formic acid. Formic acid solutions (0.5, 1, and 1.5%) were prepared by mixing concentrated formic acid with water in ratios of 0.5:99.5, 1:99, and 1.5:98.5 (v/v), respectively. Ammonium acetate SPE buffer was prepared at 20 mM concentration and adjusted to pH 7.6 with acetic acid. All buffers were filtered using Acrodisc® syringe filters with 0.2 μm HT Tuffryn® membranes (Pall Gelman Laboratory, Ann Arbor, MI, USA) and degassed by sonication. The concentration of hydrochloric acid SPE wash solution was 0.1 M. SPE eluents were prepared by mixing 30% ammonium hydroxide with 100% methanol in ratios of 5:95, 7.5:92.5, and 10:90 (v/v) to produce 5, 7.5, and 10% solutions, respectively. Stock solutions of 10 mM 5HT, d5HT, Trp, dTrp, and 5HTP were prepared in 0.2% acetic acid and were kept in the dark at −20 °C. Under these conditions, the solutions were stable for at least one month. The stock solutions were thawed and further diluted to the desired concentrations with water immediately prior to use.

2.3. Instrumentation

A Crystal CE Model 300 (UNICAM, Madison, WI, USA) capillary electrophoresis apparatus was used for all separations. The temperature during runs was set at 25 °C. For CE with UV–vis detection, a Model 759 A UV absorbance detector (Applied Biosystems, Foster City, CA, USA) was used. CE–UV data collection and processing was accomplished with ChromPerfect 3.54 Data Acquisition software (Justice Laboratory Software, Palo Alto, CA, USA). A Jaguar™ time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA) was used as the detector for all CE–TOFMS separations. Spectra were collected at a rate of 5000 Hz, with a sum rate of 3200, which resulted in 1.6 spectra s^{-1}. The nozzle board was heated to 90 °C. A commercial electrospray interface (LECO, St. Joseph, MI, USA) was used for all direct infusion experiments.

2.4. CE–MS electrospray interface

The liquid sheath electrospray interface used here was the same as described in detail in earlier publications [35,36], except that a 500-μL glass syringe was employed for the liquid sheath instead of 250-μL syringe. When the larger syringe was used, the liquid sheath solution did not need to be replenished as often. This in turn extended the period of continuous operation of the interface. Also, the end of the separation capillary was tapered using 9 μm diamond grit paper to aid in the production of a Taylor cone.

2.5. Preparation of CE capillaries

CE capillaries (75 μm i.d. × 365 μm o.d. and 50 μm i.d. × 365 μm o.d., Polymicro Technologies, Phoenix, AZ, USA) were coated with PVA prior to use to suppress the electroosmotic flow. The procedure was similar to that used by Clarke et al. [37]. Briefly, a solution of 6% PVA in water was thoroughly degassed using sonication and then centrifuged (1500 × *g*, 5 min). Then, the fused silica capillary (2–3 m in length) was attached to a small in-house made pressure vessel containing a vial with the 6% PVA solution. The PVA solution was passed through the capillary column at 100 psi for 1 h. The column was then emptied at 30 psi and dried under a flow of nitrogen for 1 h. Finally, the column was placed in a GC oven (HP 5890, Agilent, Palo Alto, CA, USA) under a stream of nitrogen (20 psi) and subjected to a temperature program of 40 °C ramped to 145 °C at 5 °C min^{-1}, followed by holding at 145 °C for 5 h. The entire process was repeated with the capillary being filled from the opposite end to ensure sufficient and uniform coating.

2.6. Sample collection, preparation, and solid phase extraction

Fresh blood samples for the determination of normal 5HT levels were collected from healthy volunteers in 4-mL Vacutainer® tubes containing 7.2 mg K_2EDTA, and chilled on ice immediately. Within 30 min of collection, the blood was centrifuged at 500 × *g* for 30 min and the resulting platelet rich plasma (PRP) was either used immediately or stored frozen at −80 °C. All plasma was analyzed within one week of collection. One- to three-day-old, non-pathological, discarded, EDTA-blood was obtained at the Brigham Young University Health Center, processed identically to fresh blood samples, and used for SPE optimization and recovery studies.

Discarded pathological blood samples were kindly donated by ARUP Laboratories, Salt Lake City, UT. These specimens were drawn into EDTA Vacutainer® tubes, transferred into ARUP serotonin transport tubes (STT) containing ascorbic acid, mixed, frozen immediately, and shipped to ARUP as such. We obtained these samples frozen in the STTs after they were used for an HPLC whole blood serotonin assay. The frozen samples were thawed and centrifuged in the same way as fresh blood samples. However, because the red blood cells had lysed and released hemoglobin as a consequence of freezing, additional ultrafiltration using Centricon® centrifugal filter devices was performed to eliminate hemoglobin, other large proteins and residual cell debris before subjecting the samples to SPE.

A modified Waters Generic Oasis MCX method was used for extraction of basic compounds. Oasis MCX columns were conditioned with 1 mL of methanol and 1 mL of 0.5% (v/v) formic acid, pH 2.5. For recovery studies and analysis of healthy plasma, 1 mL of platelet rich plasma was mixed with 4 mL of 0.5% (v/v) formic acid, pH 2.5. For CE–TOFMS, the samples were spiked with d5HT and dTrp which served as internal standards. After sample application, the columns were washed with 1 mL of 0.1 M HCl and 1 mL of methanol. The analytes were eluted with 1.3 mL of 7.5% ammonium hydroxide in methanol (v/v) and collected into Eppendorf vials. The eluates were dried using vacuum centrifugation and reconstituted in 100 μL of water.

The plasma volumes obtained from pathological samples were less than 1 mL. Therefore, these samples were diluted with 0.5% formic acid to a volume equal to four times the plasma ultrafiltrate volume. The volumes of water used for reconstitution of the dried sample were also adjusted to provide samples with a concentration factor of four or five. The rest of the procedure was the same as for normal plasma.

2.7. Recovery studies

In order to evaluate extraction recoveries, discarded non-pathological plasma samples were divided into two parts. One portion of the samples was supplemented with analyte standards to final concentrations of 2 μM for 5HT, d5HT, and 5HTP, and 20 μM for Trp and dTrp. The second portion (blank) was used without addition of standards. The spiked samples and their corresponding blanks were extracted by SPE and analyzed by CE–UV the same day. The average analyte signal (peak area) from the blank samples was subtracted from the analyte signal from the supplemented samples, and recovery was calculated as the ratio of this difference (between the spiked and blank samples) to the peak areas of a mixture of standards of corresponding concentrations.

2.8. Linearity, detection limits, and quantitation for CE–TOFMS

In order to evaluate linearity and detection limits of the CE–TOFMS method, we analyzed mixtures of indolamines and their deuterated standards at several different concentration levels. The most concentrated mixture consisted of 1.34×10^{-3} M 5HT, 1.01×10^{-3} M d5HT, 1.32×10^{-3} M Trp, 1.08×10^{-3} M dTrp, and 1.02×10^{-3} M 5HTP. This mixture was diluted 10, 20, 100, 200, 1000, 2000, and 10,000 times to generate the calibration solutions. The limit of detection (LOD) was calculated from the calibration curve as the concentration corresponding to the value of the *y*-intercept plus three standard deviations of the *y*-intercept. The assay

linearity was determined as the correlation coefficient values (R) for each individual analyte.

The concentrations of the analytes, C_a, were calculated using the following equation:

$$C_a = C_{dis} \times \left[\frac{A_a}{A_{dis}}\right] \times f \qquad (1)$$

where A_a and A_{dis} are the peak areas of the analyte and the deuterated internal standard, respectively, C_{dis} is the concentration of the internal standard, and f is a correction factor that accounts for the fact that signal intensities for the analyte and its internal standard at the same concentration levels are not equal. The factor f is calculated from CE–TOFMS analysis of standard mixtures of analytes and their deuterated standards at known concentrations:

$$f = \left[\frac{A_{ds}/C_{ds}}{A_{as}/C_{as}}\right] \qquad (2)$$

where A_{ds} and A_{as} are deuterated standard and analyte standard peak areas, and C_{ds} and C_{as} are their respective concentrations.

2.9. Within-series and between-series reproducibility

To assess the assay reproducibility (calculated as coefficient of variation, CV), standard solutions of serotonin and deuterated serotonin, used as an internal standard were run. Three concentration levels were analyzed several times in one day (for within series reproducibility) and throughout the period of one month (for between series reproducibility) using the developed CE–TOFMS method.

3. Results and discussion

3.1. CE separation of indolamines

Although 5HT, Trp, and 5HTP have similar structures, their acid–base characteristics and, therefore, the dependence of their electromigration as a function of pH are somewhat different. Trp and 5HTP are amino acids and, therefore, they contain two ionizable moieties: a carboxylic acid group (pK_a 2.4 and 2.7, respectively) and an amine group (pK_a 9.4 and 9.6, respectively). Serotonin is an amine with an amino group pK_a of 9.8. From the comparison of the pK_a's of these compounds, it is clear that while 5HT is positively charged over a wide range of pH, Trp and 5HTP carry a net positive charge only below pH ∼3–3.5. Serotonin and 5HTP also contain an ionizable hydroxyl group on the indole ring, but the pK_a's (11.1 and 10.7, respectively) are such that these groups do not contribute to the net charge of the analytes under most working conditions and, therefore, are not considered here.

Most published methods for the separation of all or some of the indolamines, use high pH, nonvolatile buffers, uncoated capillaries [28–30,32–34] and often micelle-forming additives [28–30]. Such approaches may work well with UV absorption, laser induced fluorescence, or electrochemical detectors, nevertheless, they are not suitable for ESI-MS detection. Although some studies have demonstrated that nonvolatile buffers and/or buffer additives can, under some circumstances, be used with liquid sheath ESI-MS [38–40], it is generally accepted that volatile buffers free of non-volatile additives provide the best ESI-MS sensitivity and overall performance [40–42].

We chose to work in the lower pH range where there is a wider selection of volatile buffers. Using an uncoated silica capillary, we evaluated 0.5% formic acid, pH 2.5, and 25 mM ammonium formate, pH 3.0 and 3.5, for the separation of 5HT, Trp, and 5HTP. While 5HT was well separated from the other two analytes, it was not possible to separate Trp from 5HTP under these conditions. At higher pH, Trp and 5HTP had a net zero charge and thus migrated with the electroosmotic flow (EOF) marker.

When a PVA coated capillary was used in combination with formic acid as a buffer, we obtained baseline separation of 5HT, Trp, and 5HTP. Increasing the concentration of formic acid from 0.5 to 1.5%, and thus decreasing the buffer pH, reduced the migration times and also produced sharper peaks for Trp and 5HTP. Fig. 1 shows a CE–UV separation of a standard mixture of 5HT and its precursors in 1.5% formic acid, pH 2.07. The PVA coating was stable under these conditions for several weeks.

3.2. CE–TOFMS analysis of standard compounds

By coating the CE capillary, the EOF was eliminated, which in turn necessitated the use of a liquid sheath ESI interface. Several mixtures of methanol/water and acetonitrile/water containing 0.2% formic acid were tested for their suitability as liquid sheath solutions for CE–TOFMS of serotonin and its precursors. Standards of the analytes were

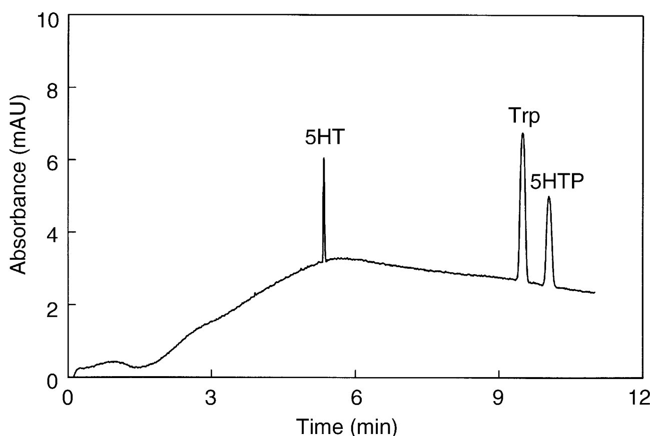

Fig. 1. CE separation of indolamine standards with UV detection. Sample: 6.70 μM 5HT, 26.4 μM Trp, and 20.4 μM 5HTP. Conditions: buffer, 1.5% formic acid (pH 2.07); capillary, 52/63 cm (L_{det}/L_{tot}), 50 μm i.d., 360 μm o.d., PVA coated; injection, 100 mbar/0.2 min; run voltage, 30 kV; current, 40 μA; λ = 215 nm. Peak assignments: 5HT, serotonin; Trp, tryptophan; 5HTP, 5-hydroxytryptophan.

diluted in 60:40 and 80:20 (v/v) acetonitrile/water, and 50:50, 60:40 and 80:20 (v/v) methanol/water mixtures, all containing 0.2% formic acid. Formic acid was chosen as the liquid sheath electrolyte in order to minimize the formation of moving ionic boundaries, a phenomenon that occurs when background electrolyte contains different counter ions than the liquid sheath [43]. The mixtures were analyzed by direct infusion using a commercial ESI interface. The compounds exhibited a much higher signal in solutions containing methanol than in acetonitrile mixtures, consistent with the observations reported by Artigas and Gelpí [23]. A mixture of methanol and water, 60:40 (v/v), gave the highest signal.

Nevertheless, when methanol/water solutions containing 40 or 50% water were used as liquid sheaths for CE-MS separation, it was difficult to maintain a well shaped, stable Taylor cone. Therefore, the methanol content was increased to 70%. Using this less viscous solution, the electrospray was more stable, while still providing very good analyte signal.

Another parameter that played a role in signal sensitivity was the concentration of the separation buffer [44,45]. We found that decreasing the concentration of formic acid in the CE electrolyte from 1.5 to 0.5%, increased the signal intensity of the analytes. It is well known that the buffer electrolytes compete for charges with the analytes, thus decreasing detection sensitivity [45]. To compensate for the longer separation times resulting from the higher pH and lower ionic strength of the buffer and to suppress occasional formation of bubbles in the capillary, a small positive pressure (10 mBar) was applied to the injection end of the separation capillary during the CE run. This pressure did not have a significant influence on the peak shape and separation efficiency, however, it greatly enhanced the electrospray stability and overall performance of the analysis.

To allow for better focusing of the electrospray voltage, thus forming higher electric field on the tip and ultimately aiding the formation of ions, the capillary outlet end was ground to a sharp tip.

The mass spectrometer voltage settings and nitrogen counter flow were initially adjusted for highest analyte signal while directly infusing standards of indolamines in methanol/water/formic acid liquid sheath solutions using the commercial interface. However, the voltage necessary for the formation of a stable electrospray was about 1000 V higher for CE–TOFMS liquid sheath interface than the voltage used with the microspray continuous infusion commercial interface. Such a large voltage change can be expected to influence the rest of the MS voltages on the instrument's front end. Therefore, the voltages and curtain gas flow were adjusted to optimize signal while spraying an approximately 100 μM mixture of indolamines directly through the CE separation capillary using the CE–TOFMS liquid sheath interface.

The commercial ESI interface was also used for the determination of the types of ions produced while electrospraying the analytes. The analytes were diluted in methanolic sheath liquid and the resulting ions were determined from summed mass spectra. Two types of ions were observed: less intense ($M + 1$) molecular ions and more intense product ions that resulted from a 17-Da fragment leaving the main ($M + 1$) structure. The mass of the fragment corresponds to either an OH group or an NH_3 group. Both are a possibility for 5HT, d5HT and 5HTP because of their ring hydroxyl group. However, Trp and dTrp, which omit the hydroxyl, still formed product ions with *m*/*z* of 17 Da less than the molecular ions, suggesting that the NH_3 group was lost. This is supported by the work of McClean et al. [25], Danaceau et al. [26], and Numan and Danielson [46].

When standards of the analytes were subjected to CE–TOFMS analysis, we found that there were additional product ions present, apparently resulting from further fragmentation of the molecules [25,26,46]. The molecular and product ions of the analytes are listed in Table 1 along with their relative abundances. The relative ion abundances were calculated using the CE–TOFMS peak areas and normalized to the signal of the most intense product ions (loss of a 17-Da fragment). These major product ions were assigned relative signal intensity of 100%. We found one inconsistency in the ionization pattern: dTrp was expected to have its major ionization product at *m*/*z* 193, consistent with the loss of an ammonium group. Instead, we found that the most intense ion had *m*/*z* 192. One explanation might be an exchange of a hydrogen for a deuterium.

Comparing the relative ion abundances of the 5HT and d5HT ions with the Trp, dTrp, and 5HTP ions, it can be seen that while 5HT and d5HT ionize almost exclusively as the ($M + 1 - 17$) ions, the ionization pattern for Trp, dTrp, and 5HTP produced intense molecular ions and other fragments. This observation, along with CE peak broadening for later eluting Trp, dTrp and 5HTP, may explain why the signal of

Table 1
Mass-to-charge (*m*/*z*) ratios and relative abundances (RA) of protonated molecular ions and fragment ions of the analytes and internal standards

	5HT		d5HT		Trp		dTrp		5HTP	
	m/*z*	RA (%)	*m*/*z*	RA (%)	*m*/*z*	RA (%)	*m*/*z*	RA (%)	*m*/*z*	RA (%)
($M + 1$) ions	177	4.3	181	6.3	205	31.2	210	37.9	221	28.7
Major products	160	100	164	100	188	100	192	100	204	100
Minor products	132	1.3	136	1.3	160	3.2	193	65.2	162	17.7
					146	35.1	164	8.3	160	4.4
					132	7.4	150	26.2		
							136	8.6		

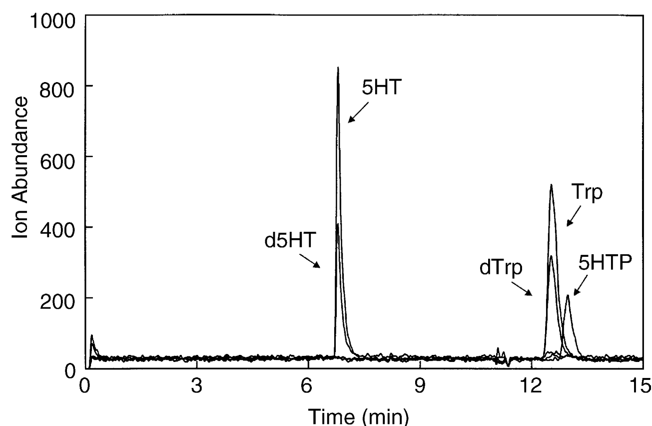

Fig. 2. CE–TOFMS analysis of indolamine standards. Sample: 6.70 μM 5HT, 5.05 μM d5HT, 26.4 μM Trp, 21.6 μM dTrp, and 20.4 μM 5HTP. CE conditions: buffer, 0.5% formic acid (pH 2.5); capillary, 60 cm length, 50 μm i.d., 360 μm o.d., PVA coated; injection, 80 mbar/0.2 min; run voltage/pressure, 30 kV/10 mbar; current, 19 μA. MS conditions: ESI voltage, 3400 V; curtain gas flow, 600 mL/min; liquid sheath flow and composition, 2 μL/min, 70:30:0.2 methanol/water/formic acid (v/v/v). Peak identification: 5HT, serotonin; d5HT, deuterated serotonin; Trp, tryptophan; dTrp, deuterated tryptophan; 5HTP, 5-hydroxytryptophan.

Trp, dTrp, and 5HTP appeared less intense than the signal of 5HT and d5HT using TOFMS detection.

Fig. 2 shows the separation of a standard mixture of indolamines and their deuterated internal standards under optimized CE–TOFMS conditions. The analyte signal is presented as the *m/z* traces of the most intense ions. Having optimized the separation and detection parameters, we determined the detection limits (LOD) and assay linearity using standard solutions of indolamines as described in the Section 2. The LOD values, correlation coefficients and regression equations are summarized in Table 2.

3.3. Sample cleanup

Due to the complexity of biological matrices, sample cleanup is usually a necessary part of current clinical assays. Both CE separation and ESI–TOFMS detection are sensitive to high salt concentrations; thus, the primary goal of our sample pretreatment was to minimize the salt content. In addition, our sample workup allowed for a 4- to 10-fold concentration.

The SPE procedure described by Kwarts et al. [5], utilizing the weak cation exchanger Amberlite CG-50, was tested for use on Supelclean LC-WCX (100 mg, 1 mL) columns using 1 M acetic acid (containing 4% ascorbic acid) for analyte elution. However, in our hands, very low recoveries were achieved and the analytes were also present in the acetic acid wash. When these eluates were dried in order to obtain more concentrated samples, substantial quantities of white solid (most likely ascorbic acid), insoluble in the volumes of water intended for sample reconstitution, remained. As expected, such high concentrations of charged species (acetic and ascorbic acids) in the eluent had a detrimental effect on the analyte peak shapes. We have, therefore, concluded that this procedure was not suitable for use with CE separation.

In our previous paper [35], we reported successful solid phase extraction of catecholamines and metanephrines on Oasis MXC cation exchange columns. Since indolamines have structures similar to the above-mentioned compounds, we tested the Oasis MXC mixed mode cation exchange columns for SPE of indolamines. Several SPE parameters were investigated: sample matrix, wash solvent, elution solvent concentration and volume, and the addition of a neutralizing agent. The manufacturer's extraction method suggested loading an acidified sample, washing the cartridge with 0.1 M HCl and methanol and eluting the analytes with 5% ammonium hydroxide in methanol.

Preliminary experiments were performed with indolamine standards in various matrices, namely ammonium acetate (20 mM, pH 7.6), PBS (pH 7.46), water, or formic acid (0.1, 0.5, and 1.5%). Ammonium acetate and PBS yielded marginal recoveries of 5HT (∼60%) when either 0.1 M HCl wash or water wash were used. Recoveries of Trp and 5HTP were very low (a few percent) with a water wash, but increased dramatically for Trp (above 90%) and marginally for 5HTP (∼40–50%) with HCl wash. The results were similar for plasma samples diluted with ammonium acetate.

In other experiments, analyte standards diluted in 0.5% formic acid or water gave recoveries of 70% or higher for 5HT and in the high 80 to 90% for Trp and 5HTP. The recoveries obtained from 0.1 and 1.5% formic acid were slightly lower than those from 0.5% formic acid. However, when these matrices were used to dilute spiked plasma samples, good overall recoveries were obtained only from 0.5% formic acid in combination with an HCl wash. The recoveries of 5HT were influenced the least by the matrix and wash, whereas Trp and 5HTP yielded zero to very low recoveries when water dilution of plasma and/or water wash were used. Therefore, the dilution of plasma with 0.5% formic acid (1:4, v/v) and an HCl wash were used in all subsequent experiments.

The analytes captured on the SPE MCX sorbent were eluted with a mixture of 30% ammonium hydroxide and 100% methanol. Solutions of ammonium hydroxide in methanol (2.5, 5.0, 7.5, and 10.0%, v/v) were tested. The recoveries were very low using 2.5% ammonium hydroxide, increased markedly when 5.0 and 7.5% ammonium hydroxide eluents were used (with slightly higher recoveries at 7.5%) and decreased again with 10% ammonium hydroxide in methanol. Therefore, 7.5% ammonium hydroxide in

Table 2
Regression equations, correlation coefficients (*R*), and detection limits (LOD) for the indolamine analytes and internal standards obtained with CE–TOFMS

Compound	Regression equation	*R*	LOD (μM)
5HT	$y = 4.03E + 07x + 0.89$	0.9998	0.15
d5HT	$y = 2.56E + 07x + 1.13$	0.9998	0.13
Trp	$y = 1.13E + 07x + 4.96$	0.9998	0.66
dTrp	$y = 7.87E + 06x + 1.76$	0.9995	0.52
5HTP	$y = 5.25E + 06x + 3.07$	0.9994	3.23

methanol was chosen for the elution of serotonin and its precursors from the SPE columns.

The volume of solvent necessary to elute the analytes was also determined. The eluent was applied on columns in four 0.5-mL portions and each fraction was collected, dried and analyzed separately. It was found that serotonin eluted in the first two fractions with approximately equal recoveries of ∼35% in each, with the third fraction containing another ∼3–6%. Trp and 5HTP eluted almost quantitatively in the first fraction. It was therefore decided that eluent volumes of 1.3 mL were sufficient for quantitative recovery of all three compounds. Because 1.5-mL Eppendorf vials were used for eluate collection and drying, using 1.3 mL rather than 1.5 mL also decreased the risk of spills during sample handling. Water and 0.5% formic acid were tested as column conditioning solvents. When the columns were conditioned with formic acid, slightly better recoveries were obtained.

In our previously reported SPE of catecholamines and metanephrines [35], we used a small amount of concentrated acetic acid to neutralize the ammonium hydroxide, and thus limit the exposure of the analytes to high pH. Because indolamines are also sensitive to pH extremes, we used the same precaution in their SPE analysis. However, after neutralization, an unknown peak was found migrating immediately following the serotonin peak. In order to investigate the origin of that peak, 1.3-mL portions of the ammonium hydroxide eluent were placed in Eppendorf vials, spiked with the analyte standards, and small amounts of acetic acid (20 and 90 μL) or formic acid (60 μL) were placed in the bottoms of some of the vials. These solutions were then dried in a vacuum centrifuge. Upon CE analysis, it was found that the extra peak was a degradation product of 5HT. The signal of the 5HT degradation product was at most 4% of the serotonin signal for samples with formic acid or samples with no acid added, while samples containing acetic acid exhibited degradation product peaks from ∼10 to 50% of the 5HT signal. The samples with no acid added had the highest recoveries of all analytes and dried in just 1.5–2.0 h, while the samples with acid addition took several hours for complete evaporation, formic acid samples being the slowest to dry. Because ammonium hydroxide is quite volatile, it likely evaporated very quickly from the samples containing no acid additive and, thus, the exposure of the analytes to high pH was limited anyway.

Nevertheless, it is possible that at lower analyte concentrations, e.g. in platelet-poor plasma, the influence of the high pH environment would be more pronounced. Under such circumstances, it would be anticipated that non-ionizable antioxidants such as mercaptoethanol [21] or dithiothreitol [47], which do not increase the ionic strength of the eluate and therefore are not likely to interfere with the CE separation and/or electrospray ionization processes, would be of value.

In summary, based on the above results, it was determined that the following SPE conditions provided the best recoveries for 5HT, Trp and 5HTP: column conditioning with 1 mL of methanol and 1 mL of 0.5% formic acid, application of plasma sample acidified with 0.5% formic acid 1:4 (v/v), washing with 1 mL of 0.1 M HCl, elution with 1.3 mL of 7.5% ammonium acetate, and drying in a vacuum centrifuge for ∼1.5–2 h. The dried eluates were reconstituted in 100 μL of water, except when the ultrafiltrates of the pathologic samples were analyzed. In the latter case, the reconstitution volumes were adjusted to yield a four-fold concentration of the analytes.

Table 3
SPE recoveries of indolamines from plasma samples spiked with 2 μM 5HT, d5HT and 5HTP, and 20 μM Trp and dTrp

Compound	Recovery (%)
5HT	71.6 ± 3.1
d5HT	68.9 ± 2.5
Trp	91.0 ± 2.8
dTrp	88.2 ± 2.6
5HTP	95.3 ± 5.9

Recoveries are given as the mean ± S.D. for 12 sets of experiments.

As described in Section 2, the SPE recoveries of indolamines were determined using non-pathological plasma samples enriched with the analyte standards at concentrations of 2 μM for 5HT, d5HT, and 5HTP and 20 μM for Trp and dTrp. As predicted, good agreement between the recoveries of 5HT and d5HT and between Trp and dTrp were obtained. The SPE recoveries are summarized in Table 3.

3.4. Determination of serotonin and its precursors in SPE extracts of human platelet rich plasma

Following the optimization of the SPE conditions, we demonstrated the usefulness of the developed method by analyzing extracts of both healthy and pathological human plasma using both CE–UV and CE–TOFMS. Figs. 3 and 4 show representative examples of CE–UV analysis of SPE extracts of plasma with normal indolamine levels and pathological plasma with elevated indolamine levels, respectively.

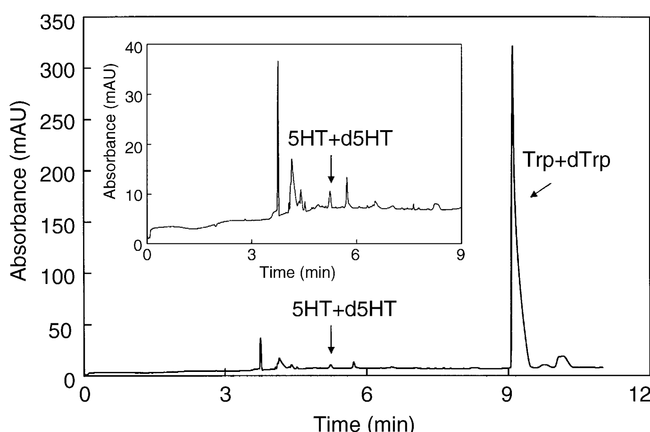

Fig. 3. CE–UV analysis of an SPE extract of 1 mL of healthy human plasma spiked with deuterated standards (2.02 μM d5HT, 108.0 μM dTrp). The extract was reconstituted in 100 μL of water. Conditions are as in Fig. 1, peak identification is as in Fig. 2.

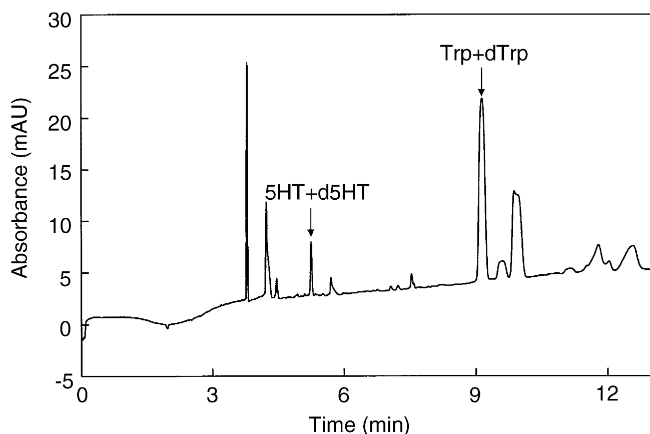

Fig. 4. CE–UV analysis of an SPE extract of 150 μL of pathological plasma spiked with deuterated standards (4.04 μM d5HT, 21.6 μM dTrp). The extract was reconstituted in 60 μL of water. Conditions are as in Fig. 1, peak identification is as in Fig. 2.

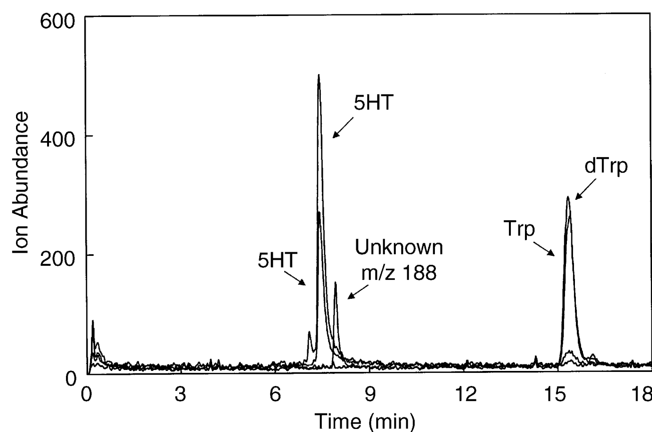

Fig. 6. CE–TOFMS analysis of an Oasis MCX extract of 150 μL of pathological plasma spiked with deuterated standards (4.04 μM d5HT, 21.6 μM dTrp) and reconstituted in 60 μL of water (same sample as in Fig. 4). CE and MS conditions same as in Fig. 5 except ESI voltage was 3380 V. Peak identification is as in Fig. 2.

CE–TOFMS analysis of the same samples is presented in Figs. 5 and 6.

In CE with non-selective UV detection, the position of the peak in the electropherogram is the only factor specific for the compounds of interest. If two compounds were to coelute, the UV detector would not distinguish them. On the other hand, as can be seen from Figs. 5 and 6, using TOFMS, the analytes are identified on the basis of both their position in the electropherogram and their characteristic m/z ratios. Although the TOFMS detector collects, with each pulsed extraction, a complete spectrum of the sample that is being electrosprayed, the instrument software allows for the construction of a selected ion plot, which shows only the desired m/z traces. This simplifies the CE–TOFMS electropherograms compared to the ones produced by CE–UV, as can be seen by comparing Figs. 5 and 6 with Figs. 3 and 4.

To accomplish analyte quantitation, we used deuterated isotopes of serotonin and tryptophan [17,23]. In order to evaluate the linearity of the dependence of the analyte to

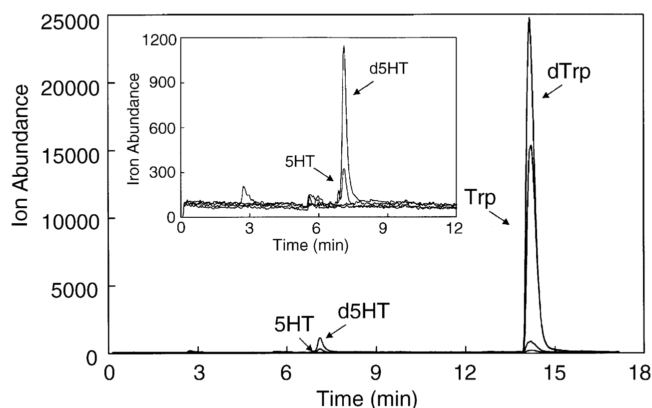

Fig. 5. CE–TOFMS analysis of an Oasis MCX extract of 1 mL of healthy human plasma spiked with deuterated standards reconstituted in 100 μL of water (same sample as in Fig. 3). CE and MS conditions same as in Fig. 3 except CE injection was 100 mbar/0.2 min and ESI voltage was 3550 V. Peak identification is as in Fig. 2.

deuterated standard signal ratio on analyte concentration in real samples, we have analyzed several aliquots of low-serotonin plasma, each spiked with d5HT as an internal standard (2 μM). The ratio of 5HT to d5HT signals was plotted against the concentration of 5HT added to the sample aliquots. The relationship was found to be linear over the given concentration range, with a regression equation $y = 0.7353x + 0.1734$ and a correlation coefficient $R = 0.9994$.

Because of the high degree of linearity of the internal standard calibration curve, it is reasonable to assume that one point calibration gives sufficient quantitative accuracy. Therefore, the concentrations of 5HT and Trp in plasma samples were calculated using Eqs. (1) and (2) as described in Section 2. With the one point calibration, it was necessary to use a factor correcting for the fact that the analyte and its deuterated standard at equal concentrations did not give the same signal intensities. The correction factor was calculated based on data obtained from CE–TOFMS analysis of a mixture of standards of both deuterated and non-deuterated compounds. Using this approach, it was found sufficient to analyze only one standard mixture when assaying several biological samples instead of running a set and constructing a calibration curve. Although no deuterated isotope was readily available for 5HTP, the quantitation of 5HTP in samples with increased concentrations of this compound could be performed using deuterated tryptophan, which migrated close to 5HTP.

Analyte concentrations determined for the healthy plasma sample shown in Fig. 5 were 0.35 μM for 5HT and 56.39 μM for Trp, consistent with levels of these compounds reported for normal specimens [4,26]. The pathological samples showed increased levels of 5HT and a decrease in the concentration of Trp, consistent with the data obtained by Kema et al. [3,4]. The concentrations of 5HT and Trp in the pathological sample shown in Fig. 6 were determined to be 5.25 and 13.32 μM, respectively. 5HTP was not detected

Table 4
Within-series and between-series assay reproducibility for CE–TOFMS measurement of serotonin

Concentration (μM)	CV (%)	
	Within-series	Between-series
1.34	6.23 ($n = 4$)	8.47 ($n = 8$)
6.70	5.68 ($n = 6$)	5.80 ($n = 15$)
13.4	2.57 ($n = 7$)	2.24 ($n = 12$)

The number of separate analyses (n) in each series is given in parentheses next to the CV value.

in healthy or pathological human plasma, also in agreement with Kema et al. [3,4]. The within- and between-series reproducibilities of the developed assay were estimated as described in Section 2 and are summarized in Table 4.

The concentration detection limits for serotonin and Trp we obtained with CE are about 2–10 times higher than with HPLC [4,26]. Nevertheless, the detection limits appear adequate for the analyte concentrations found in platelet rich plasma as demonstrated by our results. While sample volumes required in HPLC are usually 20–30 μL, the injection volumes in CE with simple pressure injection are about 1000-fold lower, thus allowing for repeated injections from even a small sample volume. Moreover, on-line preconcentration techniques, such as ITP or field amplified injection, could also be implemented to increase sensitivity for the analysis of samples with lower levels of serotonin were it necessary.

4. Conclusions

The specificity of mass spectrometric detection remains unsurpassed by other detection modes commonly used in analytical laboratories. In this paper, we have presented the successful use of this highly specific and selective detection method coupled to a capillary electrophoretic separation for the determination of serotonin and its precursors tryptophan and 5-hydroxytryptophan in complex biological matrices such as human plasma. Moreover, the analytes were efficiently removed from the plasma samples and preconcentrated by solid phase extraction. All analytes and their internal standards extracted from plasma were well resolved from other peaks by CE and could be determined both by location in the electropherogram (confirmed by the co-migration of its deuterated isotope) and by their characteristic m/z ratios. Submicromolar detection limits were achieved for standard mixtures of all compounds and their deuterated analogues with the exception 5HTP where the detection limit was in the low micromolar range. While 5HTP was undetectable, the concentrations of 5HT and Trp in both healthy and pathological plasma were readily determined.

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