Journal of Chromatography B, 868 (2008) 28–33

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Urinary 5-HIAA measurement using automated on-line solid-phase extraction–high-performance liquid chromatography–tandem mass spectrometry

Wilhelmina H.A. de Jong[a], Kendon S. Graham[b], Elisabeth G.E. de Vries[c], Ido P. Kema[a,*]

[a] Department of Laboratory Medicine, University Medical Center, Groningen, University of Groningen, P.O. Box 30001, 9700 RB Groningen, The Netherlands
[b] Waters Corporation, Manchester, UK
[c] Department of Medical Oncology, University Medical Center, University of Groningen, Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 2 November 2007
Accepted 9 April 2008
Available online 15 April 2008

Keywords:
5-HIAA
Automation
On-line
Mass spectrometry
Urine
Solid-phase extraction
Analysis

ABSTRACT

Quantification of 5-hydroxyindole-3-acetic acid (5-HIAA) in urine is useful for diagnosis and follow-up of patients with carcinoid tumors and for monitoring serotonin (5-hydroxytryptamine) metabolism in various disorders. We describe an automated method (XLC–MS/MS) that incorporates on-line solid-phase extraction (SPE), high-performance liquid chromatography (HPLC) and tandem mass spectrometric (MS/MS) detection to measure urinary 5-HIAA. Automated pre-purification of urine was carried out with HySphere-Resin GP® SPE cartridges containing strong hydrophobic polystyrene resin. The analyte (5-HIAA) and internal standard (isotope-labelled 5-HIAA-d_2) were, after elution from the cartridge, separated by reversed-phase HPLC and detected with tandem MS. Total cycle time was 5 min. 5-HIAA and its deuterated internal standard (5-HIAA-d_2) were retained on and eluted from the SPE cartridges in high yields (81.5–98.0%). Absolute recovery was 96.5–99.6%. Intra-assay (n = 20) and inter-assay (n = 20) CVs for the measurement of 5-HIAA in urine in three concentration levels ranged from 0.8 to 1.4% and 1.7 to 4.2%, respectively. For urine samples from patients (n = 78) with known or suspected metastatic carcinoid tumors, results obtained by XLC–MS/MS were highly correlated (R^2 = 0.99) with the routinely used fluorometric method. This XLC–MS/MS method demonstrated lower imprecision and time per analysis (high-throughput) than manual solvent extraction methods and higher sensitivity and specificity than non-mass spectrometric detection techniques.

1. Introduction

5-Hydroxyindole-3-acetic acid (5-HIAA) is the most abundant metabolite of serotonin (5-hydroxytryptamine: 5-HT) [1]. The neurotransmitter/neurohormone serotonin is synthesized from the essential amino acid tryptophan in the enterochromaffin cells of the gut and in serotonergic neurons in the central nervous system [2,3]. Peripheral serotonin is metabolized mainly in the lung and the liver through enzymatic conversion by monoamine oxidase-A (MAO-A; EC 1.4.3.4), resulting in urinary excretion of 5-HIAA. Serotonin plays an important role in carcinoid syndrome [4,5]. Therefore, quantification of urinary 5-HIAA is especially important in the diagnosis and follow-up of carcinoid patients.

Furthermore serotonin is hypothesized to be involved in schizophrenia, depression, migraine, and autism [4,6,7], while 5-HIAA is increased in Whipple disease, celiac disease and tropical spruce. Additionally, urinary 5-HIAA can be influenced by the diet serotonin content [8].

Today, analytical methods have been described to measure 5-HIAA in urine, including immunoassays, gas chromatography and liquid chromatography coupled to several detection techniques [9]. These methods may suffer from interferences and are time consuming, because of necessary sample clean-up.

We developed an automated on-line solid-phase extraction-liquid chromatographic method with tandem mass spectrometric detection (XLC–MS/MS) for the measurement of urinary 5-HIAA. This method combines the best of two previously described methods: selective and specific liquid chromatographic–tandem mass spectrometry [10,11] and on-line solid-phase extraction (SPE) cou-

Abbreviations: 5-HIAA, 5-Hydroxyindole-3-acetic acid; XLC–MS/MS, on-line solid-phase extraction–high-performance liquid chromatography–tandem mass spectrometry; SPE, solid-phase extraction; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; 5-HIAA-d_2, deuterium-labelled 5-hydroxyindole-3-acetic acid.

* Corresponding author. Tel.: +31 503613779; fax: +31 503612290.
E-mail address: i.p.kema@lc.umcg.nl (I.P. Kema).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.04.009

pled to high-performance liquid chromatography (HPLC) with fluorometric detection [12]. XLC–MS/MS is in our laboratory considered as a promising method for several applications [13].

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland); formic acid 98–100% ultrapure from BDH Laboratory Supplies (Poole, UK) and ammonium formate 99.995+% from Sigma–Aldrich Ltd. (Steinheim, Germany). 5-HIAA was purchased from Sigma–Aldrich Ltd. (Steinheim, Germany) and 5-HIAA-d_2 from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada); urine preservatives ascorbic acid and EDTA were from Merck KGaA (Darmstadt, Germany). Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure. All chemicals and solvents were of analytical reagent grade.

2.2. Instruments

A Spark Holland Symbiosis® on-line SPE system (Spark Holland, Emmen, the Netherlands) was used for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger or ACE), a solvent delivery unit (two high-pressure dispensers), and an HPLC pump, as shown in Fig. 1. The ACE module contains two connectable six-way valves and a SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and clean-up. The integrated HPLC pump was a binary high-pressure gradient pump. Column temperature was controlled with a Mistral Column Oven (Spark, Holland). Detection was performed with a Quattro® Premier tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters, Milford, MA). All aspects of system operation and data acquisition were controlled using MassLynx V4.1 software with automated data processing using the QuanLynx Application Manager (Waters).

2.3. Sample preparation

Stock solutions of 5-HIAA and 5-HIAA-d_2 (1 g/L) were prepared in aqueous formic acid (0.1 mol/L). Working solutions were water diluted from the stock solutions. Calibrators were prepared in water by addition of working solution corresponding to concentrations of 0.0, 6.6, 13.2, 26.5, 66.2, 198.5, 529.3 and 1191.0 μmol/L 5-HIAA.

Fifty microliters of urine (acidified to pH 4 and containing the conservatives ascorbic acid and EDTA, added prior to collection) was mixed directly in an autosampler vial with 100 μL internal standard solution (1 μg). After dilution with 850 μL water, 50 μL of each sample was injected into the XLC–MS/MS system. This injection volume was equivalent to 2.5 μL of urine.

For method-comparison studies, urine samples were used from 78 patients with suspected or known metastatic carcinoid tumors of the mid-gut which already had been measured with the routinely used HPLC-fluorometric method [12].

2.4. On-line SPE

Sample clean-up took place by on-line SPE, following a similar procedure as described previously [12,13]. HySphere-Resin GP® 10 mm × 2 mm cartridges (Spark, Holland) were used for sample extraction. The Symbiosis® system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted by solvents delivered by two high pressure dispensers (HPD1 and HPD2). Both HPD's have several ports connected to different solvents. The analytes were eluted directly on the analytical column, as is schematically shown in Fig. 1. The cartridges, located in the left clamp, were conditioned and equilibrated with acetonitrile and water (Fig. 1A), respectively. Subsequently, the sample was loaded on the cartridge with water (B) and wash steps were performed with water and 10 mM ammoniumformate pH 3 (C). After cartridge transfer to the right clamp, the analytes were eluted from the cartridge by LC gradient elution (200 μL of mobile phase; D). After elution, and during chromatography, the cartridge was regenerated in the right clamp by clamp flushes with water and acetonitrile (E).

Processing of subsequent samples was carried out in parallel.

Table 1
LC gradient parameters

Time	Flow (mL/min)	0.2% formic acid (A)	Acetonitrile (B)
0:00	0.40	70	30
1:00	0.40	70	30
4:00	0.40	95	5
4:30	0.40	70	30
5:00	0.40	70	30

2.5. Liquid chromatography

Chromatographic separation was achieved by using a reversed-phase Atlantis dC18 HPLC column (3 mm × 100 mm I.D.; 3 μm; Waters). A gradient flow of 0.2% aqueous formic acid (A) and acetonitrile (B) (flow rate 0.40 mL/min) was applied to the chromatographic column as shown in Table 1. Total cycle time per sample was 5 min. 5-HIAA and its deuterated internal standard co-eluted after 2.5 min.

Gradients applied were linear. Column temperature was kept at 25 °C.

2.6. Mass spectrometry

The mass spectrometer was directly coupled to the chromatographic column. In positive electrospray ionization mode 5-HIAA and its deuterated internal standard were protonated to produce ions at the form $[M+H]^+$, with m/z 192 and m/z 194, respectively. Upon collision-induced dissociation (CID) with Argon gas, these precursor ions produced characteristic product ions of m/z 146 [M–COOH] and 117 [M–C_2COOH] for 5-HIAA and m/z 148 and 119 for the deuterated internal standard. A multiple reaction-monitoring mode (MRM) was developed for the specific m/z

Table 2
Main working parameters for tandem mass spectrometry

MS part	Parameter	Value
Source	Ion mode	ESI+
	Capillary voltage	4 kV
	Cone voltage	17 V
	Extractor voltage	4 V
	RF lens voltage	0.2 V
	Source temperature	150 °C
	Desolvation temperature	450 °C
	Desolvation gas flow	1000 L/h
	Cone gas flow	50 L/h
Collision cell	Argon flow	0.4 mL/min
	Collision energy	17 eV

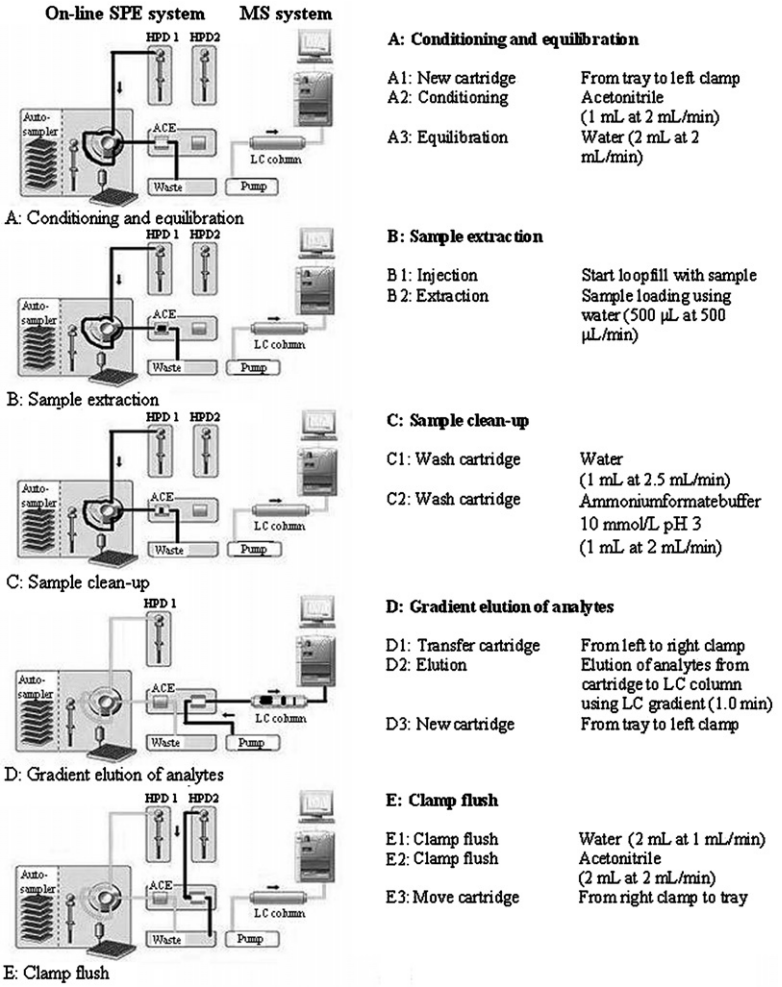

Fig. 1. Schematic representation of the Symbiosis® on-line SPE system coupled to HPLC with mass spectrometric detection (XLC–MS/MS). (A) Conditioning and activation of the SPE cartridge in the left clamp. (B) Sample extraction after filling of the autosampler loop. (C) Sample clean-up. (D) Right clamp gradient elution of analytes with LC mobile phase, directly followed by chromatographic separation and mass spectrometric detection. (E) Cartridge and clamp clean-up. HPD: high-pressure dispenser.

transitions 192 → 146 and 192 → 117 (5-HIAA) and 194 → 148 and 194 → 119 (internal standard) using a dwell time of 0.1 s and an interchannel delay of 10 ms. The second mass transition was used as qualifier, following EU directive 2002/657/EC. Main mass spectrometric parameters are shown in Table 2. Ion suppression was not observed when 5-HIAA and its deuterated internal standard were infused constantly during injection of blank samples and urine samples.

3. Results

3.1. Chromatography

Total sample analysis time, including extraction, was 5 min. A deuterated internal standard was used, since the mass spectrometer monitored precursor as well as product ions with high analytical specificity. The identity of the compound was confirmed by the specific mass spectrum. In Fig. 2 mass chromatograms are shown of a standard (A), a patient sample with normal 5-HIAA concentration (B) and a carcinoid patient sample with elevated 5-HIAA concentration (C). An additional peak from an unknown compound can appear in chromatograms near to the 5-HIAA peaks. This peak is present in most patients and healthy controls. In some samples, the extra peak is not visible because of the significant difference in concentration with respect to 5-HIAA (C).

3.2. Detection limits

The lower limit of detection (LLOD), defined as the minimum signal-to-noise ratio of at least 3:1 was <0.10 µmol/L. The limit of quantification (S/N 10:1) was 0.13 µmol/L with a CV of 9.5% (n = 20). Chromatograms of a blank sample (A) and of LLOD (B) and LLOQ (C) are shown in Fig. 3. LLOQ and LLOD are both diluted urine samples (healthy controls), which explains the fact that in these samples the additional peak of the unknown compound occurs.

3.3. Linearity and precision

The inter-assay linearity (n = 8) obtained over a concentration range from 0 to 1200 µmol/L 5-HIAA was excellent. The mean slope was 0.0102, intercept was 0.004 µmol/L and correlation coefficient was 0.9996. Spiked urine calibration curve gave a comparable slope (0.0098), but a different intercept (0.17 µmol/L), because of the endogen basal 5-HIAA concentration present in pooled urine, which confirms the selectivity of the method.

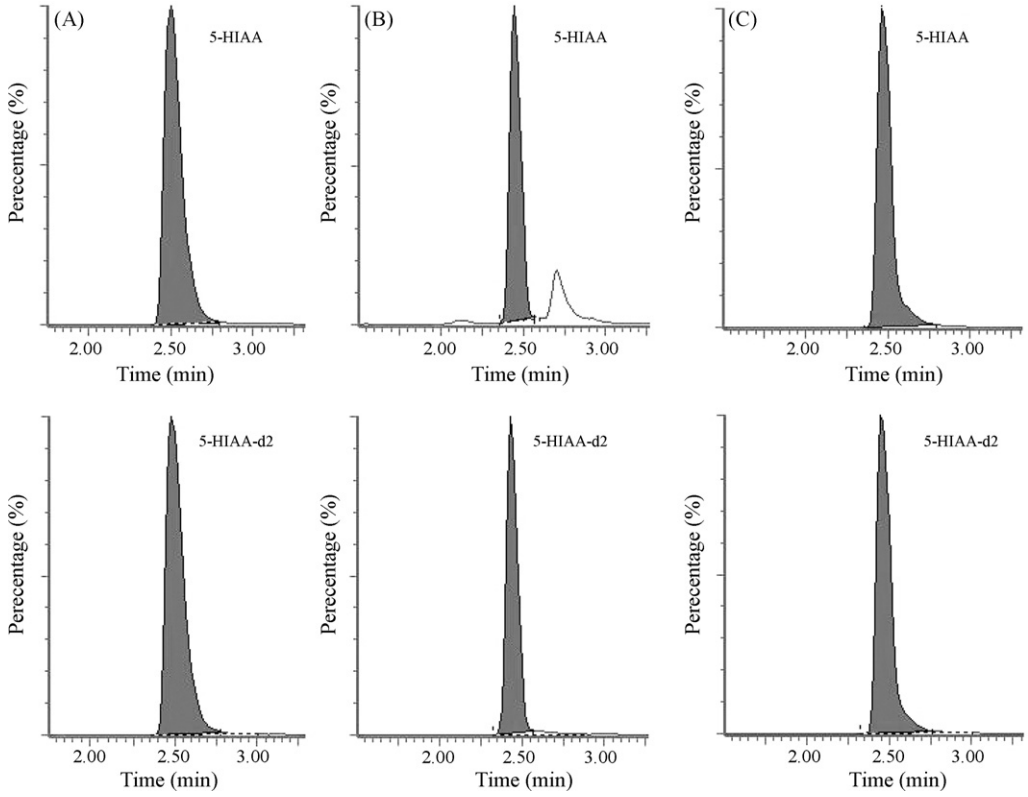

Fig. 2. Chromatograms of 5-HIAA in an aqueous calibrator and two urine samples, obtained by XLC–MS/MS analysis. Retention time of 5-HIAA is 2.5 min. (A) Aqueous standard containing 66.17 μmol/L 5-HIAA, d0 area 373034, d2 area 474911. (B) Urinary patient sample with low concentration of 5-HIAA (6.53 μmol/L), d0 area 31921, d2 area 390900. (C) Urinary patient sample with high concentration of 5-HIAA (125.09 μmol/L), d0 area 495205, d2 area 351582.

Intra-assay precision was determined by replicate analyses in a single run at three concentrations ($n = 20$). Inter-assay was determined by analysis of three concentrations over 8 weeks ($n = 20$). For all concentrations CV was found to be <5%. Intra-assay CV ($n = 20$) was 0.8–1.4%. Inter-assay CV ($n = 20$) was 2.2–4.9%. Precision data are shown in Table 3. Plasma samples with high 5-HIAA concentrations that exceed the calibration range can be diluted up to 100 times.

3.4. Recovery and stability

Recovery experiments were conducted with spiked standard addition in three concentrations (low, medium and high) measured with and without solid-phase extraction. Recoveries ranged from 96.5 to 99.6%. Absolute recovery on the SPE cartridge was measured with spiked urine samples in the same concentration levels. These recoveries ranged from 81.5 to 98.0%.

Samples, containing conservatives as described above, were found to be stable during three freeze–thaw cycles and for 48 h at 4 °C, 10 °C (autosampler) and at room temperature. Stability results are shown in Table 4. Consistent results were obtained of repeated sample measurement on the same cartridges ($n = 30$), without occurrence of carry-over (<0.1%).

3.5. Method comparison

For method comparison 78 patient samples, routinely analyzed in our laboratory for 5-HIAA by on-line SPE coupled to

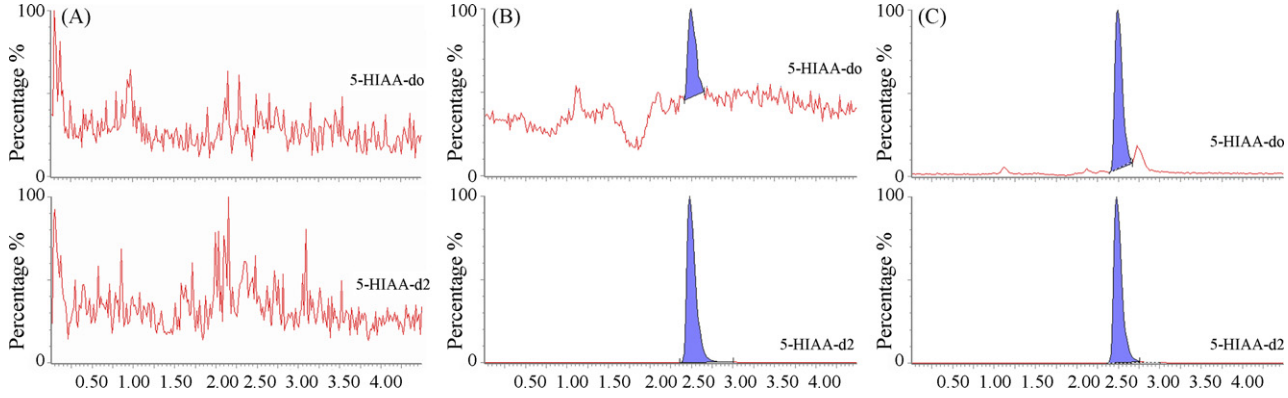

Fig. 3. Chromatograms of an aqueous blank sample and of urine samples at LLOD and LLOQ. Retention time of 5-HIAA is 2.5 min. Precursor product transitions of 191.90 → 146.15 (d0) and 193.90 → 148.15 (d2) are shown. (A) Aqueous blank sample. (B) Urine sample at LLOD (<0.10 μmol/L), d0 area 445, d2 area 799745. (C) Urine sample at LLOQ (0.13 μmol/L), d0 area 6257, d2 area 754968.

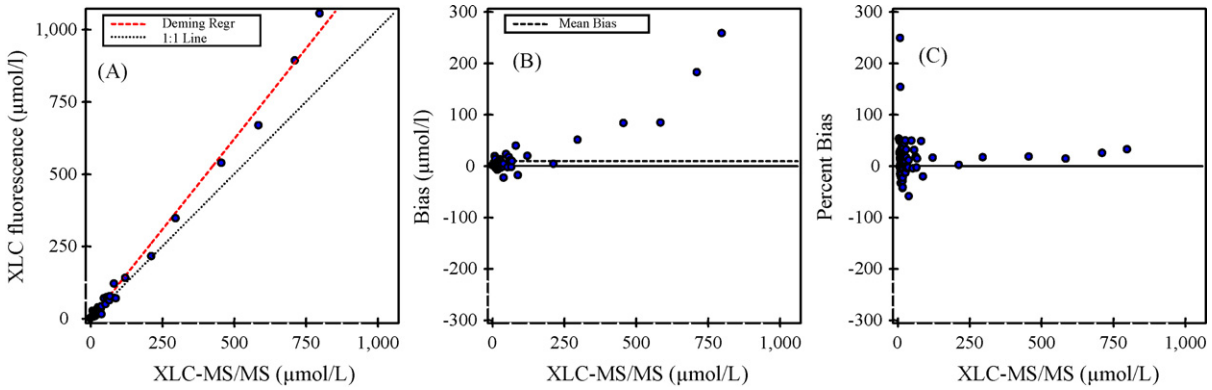

Fig. 4. Correlation between the new (x: XLC–MS/MS) and the old (y: HPLC-fluorometric detection) method for the measurement of 5-HIAA in urine. (A) Deming regression scatter plot; equation $y = 1.251x - 4.458$. (B) Bias plot. (C) Percentage bias plot.

Table 3
Precision of XLC–MS/MS method

	Concentration (SD) (µmol/L)	CV (%)
LOD	<0.10	
LOQ ($n = 20$)	0.13	9.48
Intra-assay ($n = 20$)		
Low	13.7 (0.1)	0.91
Medium	257.2 (3.6)	1.39
High	989.1 (7.7)	0.78
Inter-assay ($n = 20$)		
Low	13.6 (0.7)	4.93
Medium	252.2 (9.2)	3.63
High	968.6 (21.5)	2.22

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation; CV, variation coefficient.

HPLC with fluorometric detection with a concentration range up to 60 mmol/mol creatinine, were reanalyzed with the new XLC–MS/MS method. The regression equation (according to Deming regression analysis) for the XLC–MS/MS method (x) and the HPLC method (y) had a slope of 1.251 and an intercept of −4.458 µmol with a correlation coefficient of 0.99. Graphs are shown in Fig. 4.

Table 4
Stability of 5-HIAA in urine

Concentration (µmol/L)			
Freeze–thaw ($n = 3$)	0×	3×	
Low	14.1	14.2	
Medium	253.8	266.2	
High	997.5	1003.2	
	0 h	24 h	48 h
Stability 4 °C			
Low	14.1	14.0	13.6
Medium	253.8	254.9	259.2
High	997.5	989.3	983.4
Stability 10 °C			
Low	14.1	14.3	13.6
Medium	253.8	260.6	254.5
High	997.5	980.1	982.6
Stability RT			
Low	14.1	14.1	13.6
Medium	253.8	260.2	256.9
High	997.5	982.4	997.9

Abbreviation: RT, room temperature.

4. Discussion

An automated on-line solid-phase extraction-liquid chromatographic method with tandem mass spectrometric detection (XLC–MS/MS) for the measurement of urinary 5-HIAA was developed by combining previously described methods [10–12]. The current method uses the best characteristics of these methods: selective and specific detection with mass tandem mass spectrometry and automated on-line sample clean-up.

Routinely, we already used an automated on-line sample procedure with fluorometric detection [12] for the quantification of 5-HIAA in urine. Automated sample preparation reduces sample time and intra- and inter-assay variation, which increases laboratory efficiency and accuracy. However, major limitations of this HPLC method include the long analysis time (19 min per sample), the use of a non-isotope labelled internal standard (5-HICA) and the bias results for high concentrated samples. In addition, the detection method could be improved by the use of mass spectrometry. MS/MS detection is more specific because of the selection of the precursor to product mass transition, which enables 'simple' identification of the analyte. Furthermore MS/MS allows the use of a stable isotope-labelled internal standard, while time for chromatographic separation can be reduced.

SPE was directly coupled to the HPLC, as described before [12]. Retention of 5-HIAA on SPE cartridges was based on hydrophobic interaction between the analytes and the sorbent. By increasing the organic phase in the elution step, analytes were released from the cartridge. The use of anion exchange cartridges did not improve analyte extraction at different pH, possibly due to the amphoteric nature of 5-HIAA, as became clear after research for the best cartridge material.

Urine was acidified after collection for conservation by preventing ionization of the carboxyl group and to facilitate retention of 5-HIAA on the reversed-phase SPE stationary matrix. Furthermore, manual sample preparation consisted of a 20-fold dilution in order to reduce ion suppression during the mass spectrometric analysis and addition of the deuterated internal standard.

During method development the additional peak of the unknown compound in the chromatogram was not an issue, since it was well separated from the 5-HIAA peak. However, after 2 months of column use for routine sample measurement, column characteristics changed. The peak from the unknown compound came nearer to the 5-HIAA peak, which made it more difficult to quantify 5-HIAA. To improve resolution between the two compounds, the column was replaced by a longer column (Atlantis dC18; 3.0 mm × 150 mm; particle size 3 µm) to improve chromatographic separation of the two compounds. Retention time is thereby prolonged by 2 min.

5. Conclusion

We have developed a method for the routine determination of urinary 5-HIAA that overcomes the limitations of an existing on-line HPLC procedure. In particular, when using XLC–MS/MS, sample analysis time is considerably shorter (5 min versus 19 min), chance of chromatographic interferences is reduced and dilution of concentrated samples is not necessary because of the broad linear calibration range. Furthermore the method is more reliable because of the use of an isotope-labelled internal standard and the 5-HIAA-specific mass transitions measured. Finally, XLC–MS/MS is a promising method that enables automated, high-throughput, accurate quantification of several other clinical important biomarkers.

Financial disclosures

Waters and the Department of Pathology and Laboratory Medicine of the University Medical Centre Groningen have no mutual financial or commercial obligations originating from the presented work. The authors I.P.K. and K.S.G. do not regard this participation in the study as a conflict of interest.

References

[1] D.G. Grahame-Smith, Q. J. Med. 67 (1988) 459.
[2] G.M. Tyce, J. Cardiovasc. Pharmacol. 16 (Suppl. 3) (1990) S1.
[3] S.N. Young, K.L. Teff, Prog. Neuropsychopharmacol. Biol. Psychiatry 13 (1989) 373.
[4] I.P. Kema, E.G. De Vries, F.A. Muskiet, J. Chromatogr. B Biomed. Sci. Appl. 747 (2000) 33.
[5] W.G. Meijer, I.P. Kema, M. Volmer, P.H. Willemse, E.G. De Vries, Clin. Chem. 46 (2000) 1588.
[6] M. Naughton, J.B. Mulrooney, B.E. Leonard, Hum. Psychopharmacol. 15 (2000) 397.
[7] G.M. Anderson, J. Am. Acad. Child Adolesc. Psychiatry 41 (2002) 1513.
[8] I.P. Kema, A.M. Schellings, G. Meiborg, C.J. Hoppenbrouwers, F.A. Muskiet, Clin. Chem. 38 (1992) 1730.
[9] A.C. Deacon, Ann. Clin. Biochem. 31 (Pt 3) (1994) 215.
[10] C.A. Kroll, M.J. Magera, J.K. Helgeson, D. Matern, P. Rinaldo, Clin. Chem. 48 (2002) 2049.
[11] P. Manini, R. Andreoli, S. Cavazzini, E. Bergamaschi, A. Mutti, W.M. Niessen, J. Chromatogr. B Biomed. Sci. Appl. 744 (2000) 423.
[12] E.J. Mulder, A. Oosterloo-Duinkerken, G.M. Anderson, E.G. De Vries, R.B. Minderaa, I.P. Kema, Clin. Chem. 51 (2005) 1698.
[13] W.H. de Jong, K.S. Graham, J.C. van der Molen, T.P. Links, M.R. Morris, H.A. Ross, E.G. De Vries, I.P. Kema, Clin. Chem. 53 (2007) 1684.