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

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Introduction

5-Hydroxyindole-3-acetic acid (5-HIAA) is the most abundant metabolite of serotonin (5-hydroxytryptamine: 5-HT). 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 (1). 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 (2, 3). We previously showed that platelet serotonin is the most discriminating marker for this disease (3). However, quantification of urinary 5-HIAA proves to be the best marker for follow-up of carcinoid patients, since platelets can be saturated with serotonin. Urinary 5-HIAA is increased in Whipple disease, celiac disease and tropical sprue. Additionally, urinary 5-HIAA can be influenced by the diet serotonin content (4).

Today, analytical methods have been described to measure 5-HIAA in urine, including immunoassays, gas chromatography and liquid chromatography coupled to several detection techniques (5). 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 (6). This method combines two previously described methods: liquid chromatographic–tandem mass spectrometry (7;8) and on-line solid-phase extraction (SPE) coupled to high performance liquid chromatography (HPLC) with fluorometric detection (9). In our laboratory XLC-MS/MS is considered to be a promising method for several applications (10).

Methods

HPLC-grade acetonitrile was obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland); 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). All chemicals and solvents were of analytical reagent grade.

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 0.00, 6.62, 13.23, 26.47, 66.17, 198.50, 529.34 and 1191.01 µmol/L 5-HIAA.

50 µL 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 on-line SPE, Hysphere Resin GP® cartridges (Spark Holland, Emmen, The Netherlands) were used. The on-line SPE technique was described previously (10). The cartridges were conditioned and equilibrated with acetonitrile and water, respectively. The sample was loaded on the cartridge with water and wash steps were performed with water and 10 mM ammoniumformate pH 3. The analytes were eluted by LC gradient elution (200 µL of mobile phase). Chromatographic separation was achieved by using a reversed phase Atlantis dC18 HPLC column (3 x 150 mm I.D.; 3 µm). A gradient flow (400 µL/min) of 0.2% aqueous formic acid (A) and acetonitrile (B) was applied to the chromatographic column: isocratic 70/30 (0-1 min), linear gradient to 95/5 (1-4 min), linear gradi ent to 70/30 (4-4.5 min), isocratic 70/30 (4.5-5 min). For method-comparison studies, we used urine samples from 78 patients with suspected or known metastatic carcinoid tumors of the midgut which already had been measured with the routinely used HPLC-fluorometric method (9).

Detection was performed with a Quattro® Premier tandem mass spectrometer equipped with a Z Spray® ion source operated in positive elektrospray ionisation mode (Waters Corporation, Milford, MA). All aspects of system operation and data acquisition were controlled using MassLynx v4.1 software (Waters). A multiple reaction monitoring mode was optimized for specific *m/z* transitions 192→146 and 192→117 for 5-HIAA and 194→148 and 194→119 for 5-HIAA-d_2.

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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

Results

Total cycle time per sample was 4.5 min. 5-HIAA and its deuterated internal standard co-eluted after 2.5 min. The chromatogram belonging to a sample from a healthy subject (concentration 14.1 µmol/L) is shown in Figure 1A. An additional peak from an unknown compound can appear in chromatograms near to the 5-HIAA peak. 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. The inter-assay linearity (n = 8) obtained over a concentration range from 0-1200 µmol/L 5-HIAA was excellent. The mean slope was 0.0102 (range 0.0100-0.0105), intercept was 0.004 µmol/L (range 0.003-0.007) 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 endogenous basal 5-HIAA concentration present in pooled urine, which confirms the selectivity of the method. The lower limit of detection, 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).

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

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, CVs were found to be < 5%. Precision data are shown in table 1.

Samples, containing conservatives as described above, were found to be stable for 48 hr at 4 ºC, 10 ºC (autosampler) and at room temperature. Consistent results were obtained of repeated sample measurement on the same cartridges (n=30), without occurrence of carry-over (< 0.1%).

For method comparison 78 patient samples, routinely analyzed in our laboratory for 5-HIAA by on-line SPE coupled to 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 (range 1.230-1.273) and an intercept of -4.458 (range -7.573 - -1.343) µmol with a correlation coefficient of 0.99.

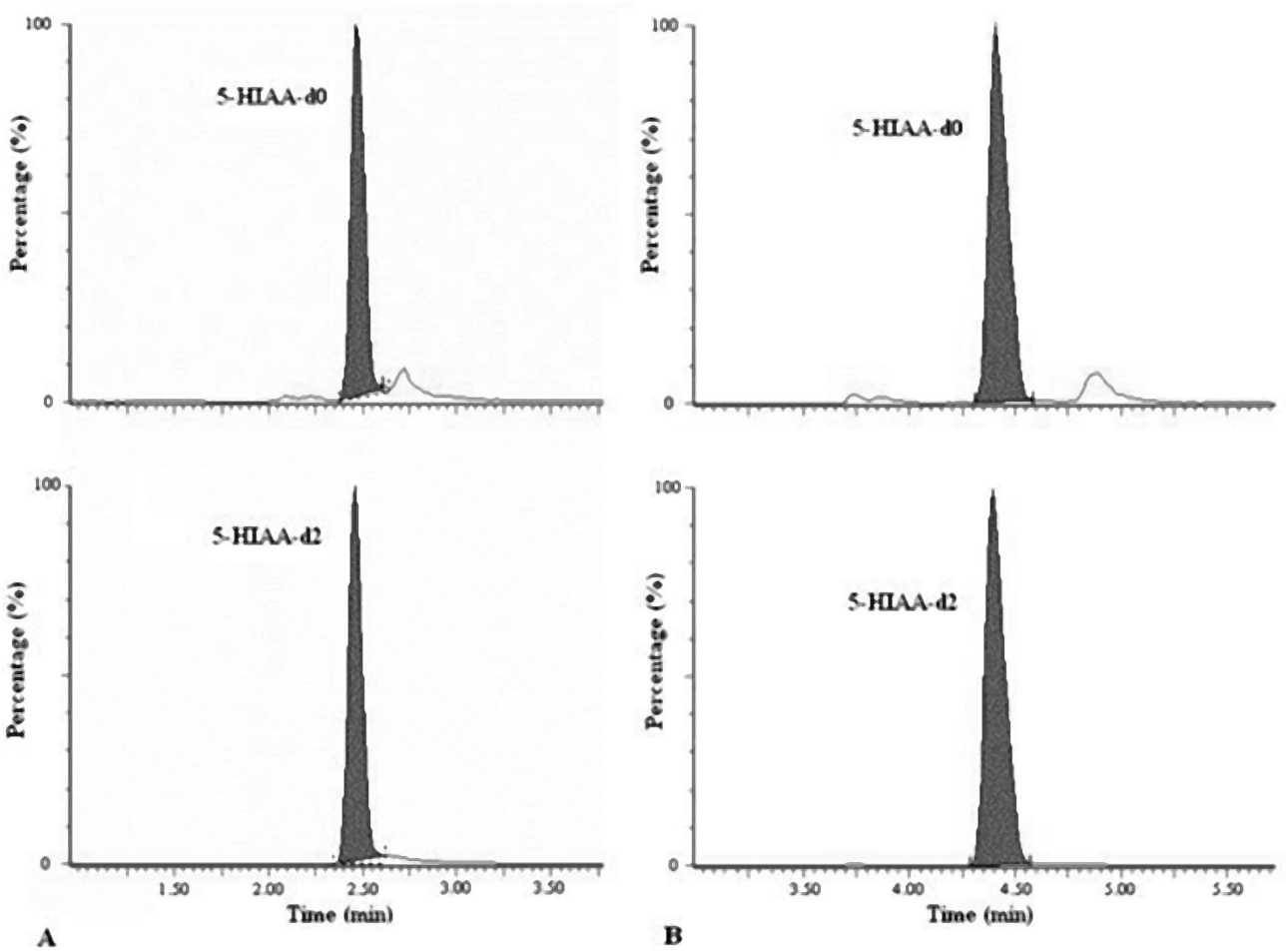

Figure 1. Chromatograms of 5-HIAA and its internal standard (5-HIAA-d2) in urine of healthy subjects. A: Chromatogram using the initial column length. An additional peak occurring in the chromatogram is not completely separated from the 5-HIAA peak. Retention time of 5-HIAA is 2.5 min, sample concentration is 14.1 µmol/L. B: Chromatogram using the longer column. 5-HIAA and the additional peak are well separated. Retention time of 5-HIAA is 4.4 min, concentration is 12.3 µmol/L.

Table 1. Precision of XLC-MS/MS method versus XLC-fluorescence method

	XLC-MS/MS Concentration (SD) (µmol/L)	CV (%)	XLC-fluorescence Concentration (SD) (µmol/L)	CV (%)
LOD	< 0.10		0.8	
LOQ (n=20)	0.13	9.5		
Intra-assay (n=20)				
Low	13.7 (0.1)	0.9	2.3 (0.1)	3.9
Medium	257.2 (3.6)	1.4	25.0 (0.4)	1.6
High	989.1 (7.7)	0.8	147.9 (1.7)	1.2
Inter-assay (n=20)				
Low	13.6 (0.7)	4.9	2.05 (0.2)	7.6
Medium	252.2 (9.2)	3.6	26.3 (1.0)	3.8
High	968.6 (21.5)	2.2	154.8 (3.2)	3.2

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

Discussion

Major limitations of the former 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. Automated sample preparation reduces sample time and intra- and inter-assay variation, which increases laboratory efficiency and accuracy. Routinely, we already used an automated on-line sample procedure with fluorometric detection (9). However, mass spectrometric detection is more specific because of the selection of the parent to daughter 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 (9). Retention 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 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 two 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. When the column was replaced by a longer column (Atlantis dC18; 3.0 x 150 mm; particle size 3 µm) the chromatographic separation of the two compounds improved. Retention time was thereby prolonged by two minutes. In Figure 1B a chromatogram is shown of sample from a healthy subject (concentration: 12.3 µmol/L) using the longer column. Method comparison showed a slope of 1.25. This deviation form 1 was expected since the previously used method resulted in lower 5-HIAA concentrations than accurate, as was shown by the measurement of high concentrated control samples (SKML).

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 (6 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. XLC-MS/MS is a promising method that enables automated, high-throughput, accurate quantification of several other clinical important biomarkers.

References

1. Tyce GM. Origin and metabolism of serotonin. J Cardiovasc Pharmacol 1990; 16 Suppl 3: S1-S7.
2. Kema IP, Vries EG de, Muskiet FA. Clinical chemistry of serotonin and metabolites. J Chromatogr B Biomed Sci Appl 2000; 747: 33-48.
3. Meijer WG, Kema IP, Volmer M, Willemse PH, Vries EG de. Discriminating capacity of indole markers in the diagnosis of carcinoid tumors. Clin Chem 2000; 46: 1588-1596.
4. Kema IP, Schellings AM, Meiborg G, Hoppenbrouwers CJ, Muskiet FA. Influence of a serotonin- and dopamine-rich diet on platelet serotonin content and urinary excretion of biogenic amines and their metabolites. Clin Chem 1992; 38: 1730-1736.
5. Deacon AC. The measurement of 5-hydroxyindoleacetic acid in urine. Ann Clin Biochem 1994; 31 (Pt 3): 215-232.
6. Jong WHA de, Graham KS, Vries EGE de, Kema I.P. Urinary 5-HIAA measurement using automated on-line solid-phase extraction-high performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2008; 868: 28-33.
7. Kroll CA, Magera MJ, Helgeson JK, Matern D, Rinaldo P. Liquid chromatographic-tandem mass spectrometric method for the determination of 5-hydroxyindole-3-acetic acid in urine. Clin Chem 2002; 48: 2049-2051.
8. Manini P, Andreoli R, Cavazzini S, Bergamaschi E, Mutti A, Niessen WM. Liquid chromatography-elektrospray tandem mass spectrometry of acidic monoamine metabolites. J Chromatogr B Biomed Sci Appl 2000; 744: 423-431.
9. Mulder EJ, Oosterloo-Duinkerken A, Anderson GM, Vries EG de, Minderaa RB, Kema IP. Automated on-line solid-phase extraction coupled with HPLC for measurement of 5-hydroxyindole-3-acetic acid in urine. Clin Chem 2005; 51: 1698-1703.
10. Jong WH de, Graham KS, Molen JC van der, Links TP, Morris MR, Ross HA et al. Plasma free metanephrine measurement using automated online solid-phase extraction HPLC tandem mass spectrometry. Clin Chem 2007; 53: 1684-1693.