

Research Article

Comparison of Serum and Urinary 5-Hydroxyindoleacetic Acid as Biomarker for Neuroendocrine Neoplasms

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Abbreviations: 5HIAA, 5-hydroxyindoleacetic acid; AUC, area under the curve; CgA, chromogranin A; IS, internal standard; LC, liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; NEN, neuroendocrine neoplasm; PP, protein precipitation; SPE, solid phase extraction; URL, upper reference limit

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Abstract

Context: Patients with serotonin-secreting neuroendocrine neoplasms (NENs) have increased serum 5-hydroxyindoleacetic acid (5HIAA) concentrations. Serum 5HIAA thus serves as a biomarker in NEN.

Objective: To evaluate an improved tandem mass spectrometric serum 5HIAA assay for diagnosis and follow-up of NEN in a clinical cohort.

Design: A retrospective study during 2016–2018 at the Diagnostic Center and Department of Endocrinology at Helsinki University Hospital, Finland.

Methods: Detailed patient data was obtained from 116 patients. Serum 5HIAA was analyzed by 2 different liquid chromatography with tandem mass spectrometry (LC-MS/MS) assays with samples prepared either by protein precipitation or solid phase extraction. Twenty-four-hour urine 5HIAA samples (n = 33) were analyzed by amperometric LC, and the results were compared. Specificity and sensitivity were calculated by receiver operating characteristic (ROC) analysis.

Results: We achieved 5 to 10 000 nmol/L linearity and $\leq 2.5\%$ variation with our new serum 5HIAA assay. In ROC analysis, the area under curve was 85% by serum assays [upper reference limit (URL) value 123 nmol/L] and 88% by the 24-h urine 5HIAA assay (URL value of 47.1 μ mol), respectively. A difference ($P < 0.001$) between patients with active NEN and patients in remission was found by all 5HIAA assays.

Conclusion: Serum 5HIAA by LC-MS/MS after protein precipitation performs equally well for the diagnosis of NEN as urinary 5HIAA LC assay. The outcome and sensitivity for serum and 24-h urine assays are convergent. Due to much more reliable and convenient sampling, we recommend serum instead of 24-h urine 5HIAA for diagnosis and follow-up of NEN patients.

Key Words: neuroendocrine neoplasms, 5-hydroxyindoleacetic acid, LC-MS/MS, serum, urine

Neuroendocrine neoplasms (NENs) derive from neuroendocrine cells in pure endocrine organs, nerve structures, and the diffuse neuroendocrine cell system, and they are commonly found in the small intestine, pancreas, or lungs [1-3]. NENs derive from chromaffin cells and comprise neuroendocrine tumors and neuroendocrine carcinomas and display characteristic histomorphological features and immunohistochemical profiles [1,4]. Patients suffering from carcinoid syndrome display very high serum and urine 5-hydroxyindoleacetic acid (5HIAA) concentrations, which often cause profound diarrhea and flushing and also may lead to carcinoid heart syndrome [5,6].

Serotonin (5-hydroxytryptamine) is produced and secreted by chromaffin cells of the bronchial walls and intestine and modulates bowel function by controlling smooth muscle contraction. 5HIAA is the major metabolite of serotonin. Therefore, 5HIAA in 24-h urine [7-9] or serum [10-12] can be used to monitor and support the diagnosis, treatment, and follow-up of NEN in combination with imaging techniques and immunohistochemistry [13,14].

Traditionally, biochemical diagnosis and monitoring of NENs is based on circulating chromogranin A (CgA) and urinary 5HIAA measurements [15]. The former is analyzed by immunoassay [15]; the latter, by colorimetric [16,17] or fluorometric assay [18], immunoassay [19], gas chromatography [20-22], or liquid chromatography (LC), using different detection modalities [8,23]. It is generally known that colorimetric and fluorometric assays may lack specificity and therefore be prone to interference [8]. Furthermore, CgA is a relatively labile molecule [24], and sample handling and storage require special attention. Circulating CgA is also rather nonspecific, as increased concentrations are encountered in many other conditions (ie, in patients on proton-pump inhibitors or in atrophic gastritis and subjects with impaired kidney function) [25]. Traditional urine 5HIAA LC assays are time-consuming. Urine samples need to be hydrolyzed to release 5HIAA from conjugates, and LC assays require long run times. Therefore, LC tandem mass spectrometry (LC-MS/MS) assays for 5HIAA in urine [9,26,27] and serum or plasma [10-12] have been developed to overcome these issues. Reliable sampling of 24-h urine is a challenge. Previously, Tohmola et al [10] and Tellez et al [28] compared newly developed serum 5HIAA LC-MS/MS assays to 24-h urine 5HIAA LC assay. In this study, we describe analytical and clinical validation of a simplified LC-MS/MS assay for serum 5HIAA employing a large clinical sample. For comparison, we also analyzed 24-h urine samples from 33 patients and 35 healthy volunteers by an LC assay.

Materials and Methods

Subjects

We obtained serum and 24-h urine samples from healthy volunteers among laboratory and hospital staff ($n = 35$, 24 women and 11 men) with a mean age of 47 years (range 24-74 years). A serotonin-deficient diet avoiding red wine, avocado, pineapple, banana, kiwi, plums, blue cheese, tomato, and nuts was followed for 3 days before and during urine sampling. Informed consent was obtained from all volunteers. Serum samples from patients ($n = 247$) suspected to suffer from or treated and under follow-up because of confirmed NEN were analyzed for 5HIAA by LC-MS/MS assays as part of the clinical workup and assay validation. Detailed patient characteristics were obtained from 116 patients. Of these, 66 had active NEN, 36 had NEN that currently was in remission (radiological or biochemical remission, or both), and 14 had other diagnoses or were considered healthy (Table 1). Patient data (age, gender, primary tumor, radiological and biochemical findings, current medication) were retrieved from the electronic patient files of the Helsinki University Hospital and reviewed by an endocrinologist (C.S.-J.) with expertise in treating patients with neuroendocrine tumors. Patients were classified as having small intestine NEN, appendiceal NEN, pancreatic NEN, pulmonary carcinoid tumors (pulmonary carcinoids), or unknown primary tumor (Table 1). Active disease was denoted as NEN patients with a visible tumor on imaging [computed tomography (CT), magnetic resonance imaging, somatostatin-receptor positron emission tomography/CT] and/or elevated biochemical tumor markers (ie, CgA or 5HIAA concentrations). Radiologic remission was defined as no visible tumors on imaging (CT, magnetic resonance imaging, somatostatin-receptor positron emission tomography/CT), and biochemical remission as normal CgA or 5HIAA concentrations. Twenty-four-hour urine samples were collected from 33 out of 116 patients and analyzed by LC. Of these, 19 had active disease, and 14 patients with NEN were currently in remission (radiological or biochemical remission, or both). This study was approved by the ethical committee of Helsinki University Central Hospital, Finland.

The Simplified LC-MS/MS Assay for Serum 5HIAA

We used a liquid handling robot (Tecan, Switzerland) for sample preparation [29]. Serum samples, calibrators (catecholamine metabolites mix, Cerilliant®, Sigma-Aldrich Co, USA) and 2 level controls (100 μ L; Chromsystems GmH, Germany) were pipetted into a 96-deep well plate

Table 1. Subject characteristics

Character	Active NEN, serum	Active NEN, urine	Remission, serum	Remission, urine	Other, serum
n (women/men)	66 (28/38)	19 (9/10)	36 (20/16)	14 (9/5)	14 ^a (8/6)
Mean age, years (range)	66 (32-89)	68 (54-82)	64 (42-88)	64 (50-79)	63 (32-88)
Si-NEN	46	19	30	13	—
Appendiceal NEN	1	—	1	1	—
Pancreatic NEN	11	—	4	—	—
Pulmonary carcinoids	3	—	1	—	—
Unknown/other primary tumor	5	—	—	—	—
Remission (radiological/biochemical)	—	—	36 (19/31)	14 (7/14)	—

Abbreviation: Si-NEN, small intestine NEN.

^aThese 14 subjects represent individuals for whom 5HIAA was used as a screening marker, but further diagnostic workup confirmed that none of them had an underlying serotonin secreting NEN. Further diagnostic workup indicated 5 healthy, 3 diabetes mellitus, 1 paraganglioma, 1 burn, 1 hepatic cirrhosis, 1 schwannoma, 1 renal hypertension, and 1 spondylarthritis.

along with 100 μL of stable isotope-labeled 5HIAA-¹³C₆ internal standard (IS; Medical Isotopes Inc, USA). The plate was shaken for 30 sec (1300 rpm) and left for 2 mins. Serum proteins were then precipitated with 400 μL of acetonitrile. The plate was shaken for 5 mins as previously described, followed by centrifugation for 10 mins in 4°C at 2890 relative centrifugal force. The supernatant was used for analysis. Our LC-MS/MS instrumentation was recently described by Lindström et al [30]. For chromatographic separation, we used XTERRA® MS C18 column (3.5 μm , 3.9 \times 100 mm, Waters, USA) and 8.5-min linear gradient of acetonitrile, and 0.1% acetic acid with a flow rate 450 $\mu\text{L}/\text{min}$. Total runtime was 10 min; column temperature, 35°C; and injection volume, 5 μL . A bypass valve was used to divert the first 5.3 mins and the last 3.7 mins of the flow to waste and only 60 sec was directed to mass detection. Ionization was carried out in positive ion mode, and the followed MS/MS transitions were m/z 192.1 \rightarrow 146 for 5HIAA and m/z 194 \rightarrow 147.9 for the IS. In conclusion, contrary to our previous serum 5HIAA assay [10] with solid phase extraction for sample preparation (SPE-LC-MS/MS), we now use simple protein precipitation (PP-LC-MS/MS).

Other Methods

Urine 5HIAA results were measured by a subcontracted (Synlab Finland Ltd.) LC assay [31]. The results of serum 5HIAA by the new PP-LC-MS/MS were also compared to those obtained by our previous SPE-LC-MS/MS assay as described in (10). Briefly, serum samples (100 μL) were mixed with isotope-labeled IS and applied into the wells of Oasis® WAX $\mu\text{Elution}$ plates (Waters). After 2 washing steps, 5HIAA was eluted from the $\mu\text{Elution}$ plates and analyzed by LC-MS/MS. Plasma CgA was measured by a subcontracted (Synlab Finland Ltd) radioimmunoassay.

Table 2. Analytical parameters of the newly developed serum 5HIAA LC-MS/MS assay

Parameters	Result
Retention time of 5HIAA, min	5.9
Linear range, nmol/L	5-10 000
Intra-assay variation (CV, n = 14), %	1.0-1.2
Inter-assay variation (CV, n = 21) %	2.4-2.5
LOD/LOQ (n = 14), nmol/L	1.3/2.4
Process efficiency (n = 4), %	96.9-102.6
Matrix effect (n = 5), %	102

Abbreviation: CV, coefficient of variation; LOD/LOQ, limit of detection/limit of quantitation.

The CgA results were used only by the clinician for setting diagnoses.

Statistical Analysis

Our new PP-LC-MS/MS assay for serum 5HIAA was fully validated analytically as previously described [30], and process efficiency was determined according to Marchi et al (Table 2) [32]. 5HIAA assays were compared by Passing Bablok correlation, Bland-Altman regression, receiver operating characteristics analysis and box plot using Analyse-it for Microsoft Excel 3.76.1. (Analyse-it Software Ltd, <http://www.analyse-it.com>). A *P*-value < 0.05 was considered statistically significant.

Results

Comparison of 5HIAA Assays

Serum 5HIAA concentration by the PP-LC-MS/MS and SPE-LC-MS/MS assay [8] was compared employing samples from patients and healthy volunteers (n = 282). The mean concentrations by the assays were 305 nmol/L and 312 nmol/L,

respectively, and the mean difference was nonsignificant [ie, -7 nmol/L (95% CI -14 to 1 nmol/L) or -2% , (95% CI -3% to 1%)]. Therefore, we apply the same upper reference limit (URL) of 123 nmol/L [10] to both assays. By the PP-LC-MS/MS, mean 5HIAA concentration was 53 nmol/L (range 31 - 96 nmol/L, $n = 35$) and 485 nmol/L (range 37 - 4860 nmol/L, $n = 66$) in serum from healthy volunteers and from NEN patients, respectively. In 28 out of 66 (42%) active NEN patients, serum 5HIAA concentration was under the URL. Of these, 13 had small intestine NEN; 9, pancreatic NEN; 1, appendiceal NEN; 1, breast NEN; 1, kidney NEN; 1, pulmonary NEN; and 2, NEN of unknown origin. In 38 out of 66 (58%) active NEN patients, serum 5HIAA concentration was above the URL. In receiver operating characteristics analysis comparing healthy individuals and patients in remission to patients with active disease, the area under the curve (AUC) was 0.86 for both PP- and SPE-LC-MS/MS assays for serum 5HIAA (Table 3). With a URL of 123 nmol/L for serum 5HIAA, we achieved over 90% sensitivity and 58% specificity with both assays.

In samples from healthy volunteers, the mean 24-h urine 5HIAA concentration was 28 μmol (range 10 - 64 μmol , $n = 35$) and in patients with NEN, 206 μmol (range 20 - 1354 μmol , $n = 33$). In 4 of 35 (11%) samples from healthy volunteers 24-h urine 5HIAA was above the URL of 47.1 μmol , and in 6 of 19 (32%) samples from patients with active NEN concentration was less than 47.1 μmol . With a URL value of 47.1 μmol for 24-h urine 5HIAA LC assay, the AUC was 0.88 , with sensitivity of 92% and specificity of 63%. The significant difference ($P < 0.001$) between patients with active NEN vs healthy individuals and

patients with NEN in remission combined is illustrated in Figure 1.

There was discrepancy between 24-h urine and serum 5HIAA results in samples from 8 individuals. In 5 of these, urinary but not serum 5HIAA was elevated, and for 3 individuals, serum but not urinary 5HIAA was elevated. No discrepancy was found between PP-LC-MS/MS and SPE-LC-MS/MS assays.

Discussion

This study confirms that serum 5HIAA is a sensitive and specific marker for serotonin secreting NENs. Adaway et al reported that the diagnostic concordance between paired patient serum and urine tests give identical results in 95.5% of cases ($n = 95$) by LC-MS/MS assays [11]. The results of simultaneous urine and plasma 5HIAA assays from the

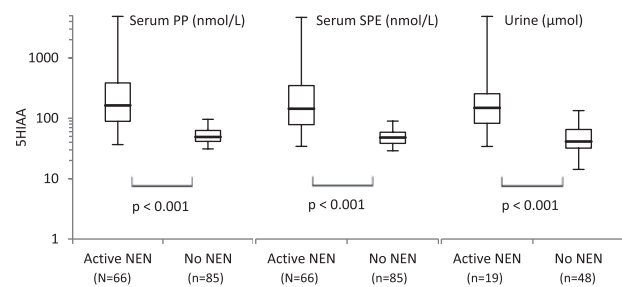


Figure 1. Box plot comparison of serum and urine 5HIAA by different assays. No NEN includes samples from healthy individuals and from NEN patients in remission. Serum PP, protein precipitation LC-MS/MS assay; serum SPE, solid phase extraction LC-MS/MS assay; urine, LC assay with amperometric detection.

Table 3. Comparison of serum and urinary 5HIAA assays by ROC analysis

	S-5HIAA (PP) (URL 123 nmol/L)	S-5HIAA (SPE) (URL 123 nmol/L)	dU-5HIAA (URL 47.1 μmol)
Healthy volunteers vs. patients with active NEN			
n, healthy/active NEN	35/66	35/66	35/19
AUC	0.92	0.92	0.88
Sensitivity (%)	100	100	88
Specificity (%)	61	61	63
Healthy individuals and patients in remission vs. patients with active NEN			
n, healthy/remission/active NEN	35/36/66	35/36/66	35/14/19
AUC	0.86	0.86	0.88
Sensitivity (%)	95	94	92
Specificity (%)	58	60	63
Patients in remission vs. patients with active NEN			
n, remission/active NEN	36/66	36/66	14/19
AUC	0.81	0.80	0.89
Sensitivity (%)	92	89	100
Specificity (%)	58	61	68

Abbreviation: ROC, receiver operating characteristics.

same individuals ($n = 115$) were proportional in a study by Tellez et al using gas chromatography coupled to MS/MS [28]. We reported earlier [10] that serum 5HIAA assay by SPE-LC-MS/MS is comparable with urine 5HIAA LC assay in the clinical diagnosis of NEN ($n = 88$). Here we show that our simplified and fast PP-LC-MS/MS assay for serum 5HIAA performs equally well as 24-h urine 5HIAA LC assay and our previous SPE-LC-MS/MS assay in the workup of patients suspected to suffer from and treated for NENs.

In this study we compared our previous 5HIAA assay with samples treated by SPE to our newly developed PP-LC-MS/MS assay. Sample preparation by PP using a liquid handling robot instead of manual SPE saves time, physical stress to laboratory personnel, and consumable expenses. Our results show that simple PP is as effective as SPE for assessment of 5HIAA in serum.

When comparing 5HIAA in serum and urine samples from NEN patients, we found that the AUC for serum 5HIAA by our new PP-LC-MS/MS was 0.86 and that for urine LC assay was 0.88, with 95% and 92% sensitivities, respectively. The AUC for our previous SPE-LC-MS/MS assay was 0.86 and sensitivity was 94% [10]. In the study of Adaway et al, the AUC for plasma 5HIAA ($n = 112$) and urine 5HIAA ($n = 108$) assays were 0.92 and 0.92 and sensitivities were 80% and 74%, respectively [11]. The slight differences in the AUCs may be explained by the fact that Adaway et al [11] used as the URL 118 nmol/L and 140 nmol/l for plasma and serum 5HIAA, respectively, whereas ours is 123 nmol/l for serum 5HIAA [10].

There is a slight discrepancy between 5HIAA results from urinary and serum samples. One reason may be the less reliable 24-h urinary sampling as compared to serum sampling. Our serum PP-LC-MS/MS seems slightly less specific than the urine LC assay. Assay sensitivity and specificity are decision-limit dependent. The URL of our previous 5HIAA assay by SPE-LC-MS/MS had worked well clinically, and there was not a significant difference between 5HIAA concentrations by the SPE- and PP-LC-MS/MS assays. Therefore, we left the URL unchanged upon assay change. However, this finding encourages reviewing the URL in the future, despite very good performance feedback from local endocrinologists.

Urinary 5HIAA collection guidelines [33] recommend serotonin-restricted diet for 3 days before sampling. All urine excreted during the 24-h sampling time should be collected. In addition, the sample should be kept refrigerated and acidified to ensure stability. This is challenging and cumbersome for the patient. Calanchini et al reported good correlation between 5HIAA in spot and 24-h urine samples

($n = 136$) [34], confirming previous studies [35,36]. Thus, spot urine samples could also be used for 5HIAA assessment. Serum sampling is far more precise and convenient than collection of 24-h urine. We found earlier [37] that a serotonin-restricted diet for 1 day is enough for serum 5HIAA sampling. To minimize preanalytical issues and to allow faster sampling, we suggest using serum sample after diet restriction for 1 day only for 5HIAA monitoring.

We at the Diagnostic Center, Helsinki University Hospital, have routinely analyzed serum 5HIAA by SPE-LC-MS/MS since 2011 and by PP-LC-MS/MS since February 2018. The latter assay is very robust with <3% variation in validation (Table 2) and <4% long-term (3 month) variation. Preparation of one 96-well plateful of samples takes only about 30 mins. The chromatographic runs are left overnight, and results are reported the next morning. Easy and reliable sampling together with good correlation to urine 5HIAA LC assay has encouraged clinicians in Finland to use this test instead of urinary test. This has led to a yearly increase of 20% and the handling of about 100 samples per week in our laboratory. Roughly 50% of samples analyzed in our laboratory are sent to us from other parts of Finland. Simultaneously, the amount of urine 5HIAA tests has decreased to approximately 200 tests per year, mainly requested due to ongoing research projects.

Laboratories have reported relatively equal URLs for serum 5HIAA assays (ie, 123 nmol/L [10], 118 nmol/L [11], and 115 nmol/l [27]) and for plasma 5HIAA assays (140 nmol/L [11]). Commercial quality assurance plasma samples for internal use are now available, and we found them suitable for serum assay as well. Several studies have now shown that serum and plasma assays for 5HIAA perform equally well as urine tests for diagnosis of NEN [10-12]. Furthermore, preanalytical issues are fewer with serum and plasma than with 24-h urine samples. Thus, it may soon be time for the endocrinology societies to review their recommendations of the primary sample type in 5HIAA test for diagnosis and monitoring of NENs.

Conclusion

In conclusion, we have developed a simple PP-LC-MS/MS assay for serum 5HIAA that performs equally well for the diagnosis of NENs as urinary 5HIAA LC assay. Due to fast, reliable, and convenient sampling for the patient, together with our straightforward and robust LC-MS/MS assay procedure, we recommend using serum or plasma instead of 24-h urine 5HIAA for diagnosis and follow-up of NEN patients.

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Additional Information

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Data availability: Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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