Comparison of serum and urinary 5-hydroxyindoleacetic acid as biomarker for neuroendocrine neoplasms

Becker Anna¹, Schalin-Jäntti Camilla² Itkonen Outi¹

1. HUSLAB, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.
   Electronic annabecker@hus.fi

2. Endocrinology, Abdominal Center, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.

© The Author(s) 2021. Published by Oxford University Press on behalf of the Endocrine Society.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
Corresponding author’s contact information:

Anna Becker
Helsinki University Hospital
Diagnostic Center and Department of Endocrinology
PO Box 720
FI-00029 HUS
Finland
Telephone: +358 50 4271459
E-mail: anna.becker@hus.fi

Grants

This work was supported by grants from the Helsinki University Hospital Research Funds (TYH2018223 and TYH2019254, to CS-J) and Finska Lääkäresällskapet (to CS-J).

Disclosure summary

No disclosures to declare.
Abstract:

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 two different LC-MS/MS assays with samples prepared either by protein precipitation (PP) or solid phase extraction (SPE). 24-h 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 5-10 000 nmol/l linearity and ≤2.5% variation with our new serum 5HIAA assay. In ROC analysis the area under curve (AUC) was 85% by serum assays (URL value 123 nmol/l) and 88% by the 24-h urine 5HIAA assay (URL value of 47.1 µmol), 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.

Keywords:

Neuroendocrine neoplasms, 5-hydroxyindoleacetic acid, LC-MS/MS, serum, urine
Introduction

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 (1,2,3). NENs derive from chromaffin cells and comprise neuroendocrine tumours (NET) and neuroendocrine carcinomas (NEC) 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, 5-HT) 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 24h-urine (7,8,9) or serum (10,11,12) 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 NEN 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 fluorimetric assay (18), immunoassay (19), gas chromatography (GC) (20,21,22) or liquid chromatography (LC), using different detection modalities (8,23). It is generally known that colorimetric and fluorimetric 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, i.e. in patients on proton-pump inhibitors, 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, liquid chromatography tandem mass spectrometry (LC-MS/MS) assays for 5HIAA in urine (9,26,27) and serum or plasma (10,11,12) 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), mean age 47 yrs (range 24-74 yrs). A serotonin-deficient diet avoiding red wine, avocado, pineapple, banana, kiwi, plums, blue cheese, tomato and nuts was followed for three 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 tumour, radiological and biochemical findings, current medication) were retrieved from the electronic patient files of the Helsinki University Hospital and reviewed by an endocrinologist (CS-J) with expertise in treating patients with neuroendocrine tumours. Patients were classified as having small intestine NEN (Si-NEN), appendiceal NEN, pancreatic NEN or pulmonary carcinoid tumours (pulmonary carcinoids), or unknown primary tumour (Table 1). Active disease was denoted as NEN patients with a visible tumour on
imaging (CT, MRI, somatostatin-receptor PET/CT) and/or elevated biochemical tumour markers, ie. CgA or 5HIAA concentrations. Radiologic remission was defined as no visible tumours on imaging (CT, MRI, somatostatin-receptor PET/CT), and biochemical remission as normal CgA or 5HIAA concentrations. 24-h 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 two level controls (Chromsystems Gmph, Germany) (100 μL) were pipetted into a 96-deep well plate along with 100 μL of stable isotope-labeled 5HIAA-13C6 internal standard (IS) (Medical Isotopes Inc., USA). The plate was shaken for 30 secs (1300 rpm) and left for two mins. Serum proteins were then precipitated with 400 μL of acetonitrile (ACN). The plate was shaken for five mins as above followed by centrifugation for ten mins in +4 °C at 2890 RCF. 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 x 100 mm, Waters, USA) and 8.5 min linear gradient of ACN and 0.1% acetic acid with a flow rate 450 μl/min. Total runtime was 10 mins, 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 → 146 for 5HIAA and m/z 194 → 147.9 for the IS. In conclusion, on 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-labelled IS and applied into the wells of Oasis® WAX µElution plates (Waters). After two washing steps, 5HIAA was eluted from the µElution plates and analyzed by LC-MS/MS. Plasma CgA was measured by a subcontracted (Synlab Finland Ltd.) radioimmunoassay. 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 determined according to Marchi et al. (Table 2) (32). 5HIAA assays were compared by Passing Bablok correlation, Bland-Altman regression, receiver operating characteristics (ROC) 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, i.e. -7 nmol/L (95% CI -14 – 1 nmol/L) or -2%, (95% CI -3% – 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
of 66 (42%) active NEN patients, serum 5HIAA concentration was under the URL. Of these, 13 had Si-NEN, nine pancreatic NEN, one appendiceal NEN, one breast NEN, one kidney NEN, one pulmonary NEN, and two had NEN of unknown origin. In 38 out of 66 (58%) active NEN patients, serum 5HIAA concentration was above the URL. In ROC 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 four of 35 (11%) samples from healthy volunteers 24-h urine 5HIAA was above the URL of 47.1 µmol, and in six 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, sensitivity 92% and specificity 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 eight individuals. In five of these, urinary but not serum 5HIAA was elevated, and for three 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 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 work-up 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 0.88, with 95% and 92% sensitivities, respectively. The AUC for our previous SPE-LC-MS/MS assay was 0.86 and sensitivity 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 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 three 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 serotonin-restricted diet for one 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 one day only for 5HIAA monitoring.

We at 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 mo) 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, i.e. 123 nmol/l (10), 118 nmol/l (11) and 115 nmol/l (27), and for plasma 5HIAA 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 (10,11,12). 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 NEN.

Conclusion

In conclusion, we have developed a simple PP-LC-MS/MS assay for serum 5HIAA that performs equally well for the diagnosis of NEN 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.
Acknowledgments

The authors thank all laboratory staff of HUSLAB for expert technical assistance and healthy volunteers for sample donation.

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.
References

1. Rindi G, Inzani F. Neuroendocrine neoplasm update: Toward universal nomenclature. *Endocr Relat Cancer*. 2020;27(6):R211-R218. doi: 10.1530/ERC-20-0036 [doi].

2. Dasari A, Shen C, Halperin D, *et al*. Trends in the incidence, prevalence, and survival outcomes in patients with neuroendocrine tumors in the united states. *JAMA Oncol*. 2017;3(10):1335-1342. doi: 10.1001/jamaoncol.2017.0589 [doi].

3. Del Arco CD, Sastre J, Peinado P, Diaz A, Medina LO, Fernandez Acenero MJ. Neuroendocrine neoplasms in rare locations: Clinicopathological features and review of the literature. *Indian J Endocrinol Metab*. 2018;22(3):308-315. doi: 10.4103/ijem.IJEM_446_17 [doi].

4. Nagtegaal ID, Odze RD, Klimstra D, *et al*. The 2019 WHO classification of tumours of the digestive system. *Histopathology*. 2020;76(2):182-188. 
   https://pubmed.ncbi.nlm.nih.gov/31433515
   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7003895/. doi: 10.1111/his.13975.

5. Halperin DM, Shen C, Dasari A, *et al*. Frequency of carcinoid syndrome at neuroendocrine tumour diagnosis: A population-based study. *Lancet Oncol*. 2017;18(4):525-534. doi: S1470-2045(17)30110-9 [pii].

6. Karppinen N, Linden R, Sintonen H, *et al*. Health-related quality of life in patients with small intestine neuroendocrine tumors. *Neuroendocrinology*. 2018;107(4):366-374. doi: 10.1159/000494293 [doi].

7. Shihabi ZK, Scaro J. Liquid-chromatographic assay of urinary 5-hydroxy-3-indoleacetic acid, with electrochemical detection. *Clin Chem*. 1980;26(7):907-909.
8. Corcuff J, Chardon L, El HR, Brossaud J. Urinary sampling for 5HIAA and metanephrines determination: Revisiting the recommendations. Endocrine Connections. 2017;6(6):R87-R98. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5527357/. doi: 10.1530/EC-17-0071.

9. de Jong WH, Graham KS, de Vries EG, Kema IP. 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(1-2):28-33. doi: 10.1016/j.jchromb.2008.04.009 [doi].

10. Tohmola N, Itkonen O, Sane T, et al. Analytical and preanalytical validation of a new mass spectrometric serum 5-hydroxyindoleacetic acid assay as neuroendocrine tumor marker. Clinica Chimica Acta. 2014;428:38-43.

11. Adaway JE, Dobson R, Walsh J, et al. Serum and plasma 5-hydroxyindoleacetic acid as an alternative to 24-h urine 5-hydroxyindoleacetic acid measurement. Ann Clin Biochem. 2016;53(Pt 5):554-560. doi: 10.1177/0004563215613109 [doi].

12. Miller AG, Brown H, Degg T, Allen K, Keevil BG. Measurement of plasma 5-hydroxyindole acetic acid by liquid chromatography tandem mass spectrometry—Comparison with HPLC methodology. Journal of Chromatography B. 2010;878(7):695-699.

13. Scott AT, Howe JR. Evaluation and management of neuroendocrine tumors of the pancreas. Surg Clin North Am. 2019;99(4):793-814. doi: S0039-6109(19)30040-4 [pii].

14. Oberg K, Modlin IM, De Herder W, et al. Consensus on biomarkers for neuroendocrine tumour disease. Lancet Oncol. 2015;16(9):e435-e446. doi: S1470-2045(15)00186-2 [pii].

15. Marotta V, Zatelli MC, Sciammarella C, et al. Chromogranin A as circulating marker for diagnosis and management of neuroendocrine neoplasms: More flaws than fame. Endocr Relat Cancer. 2018;25(1):R11-R29. doi: 10.1530/ERC-17-0269 [doi].
16. UDENFRIEND S, TITUS E, WEISSBACH H. The identification of 5-hydroxy-3-indoleacetic acid in normal urine and a method for its assay. *J Biol Chem*. 1955;216(2):499-505.

17. MACFARLANE PS, DALGLIESH CE, DUTTON RW, LENNOX B, NYHUS LM, SMITH AN. Endocrine aspects of argentaffinoma, with special reference to the use of urinary 5-hydroxyindoleacetic acid estimations in diagnosis. *Scott Med J*. 1956;1(4):148-155. doi: 10.1177/003693305600100402 [doi].

18. Korf J, Valkenburgh-Sikkema T. Fluorimetric determination of 5-hydroxyindoleacetic acid in human urine and cerebrospinal fluid. *Clin Chim Acta*. 1969;26(2):301-306. doi: 0009-8981(69)90383-0 [pii].

19. Brashear J, Zeitvogel C, Jackson J, *et al.* Fluorescence polarization immunoassay of urinary 5-hydroxy-3-indoleacetic acid. *Clin Chem*. 1989;35(3):355-359.

20. de Jong EB, Horsten BP, Goldschmidt HM. Determination of nine catecholamine metabolites and 5-hydroxyindolacetic acid in urine by capillary gas chromatography. *J Chromatogr*. 1983;279:563-572. doi: 10.1016/s0021-9673(01)93658-1 [doi].

21. Goodwin BL, Ruthven CR, Weg MW, Sandler M. A specific assay for urinary 5-hydroxyindole-3-acetic acid by gas chromatography. *Clin Chim Acta*. 1975;62(3):439-442. doi: 0009-8981(75)90097-2 [pii].

22. Tanaka K, Hine DG, West-Dull A, Lynn TB. Gas-chromatographic method of analysis for urinary organic acids. I. retention indices of 155 metabolically important compounds. *Clin Chem*. 1980;26(13):1839-1846.

23. Fornstedt N. Determination of 5-hydroxyindole-3-acetic acid in urine by high performance liquid chromatography. *Anal Chem*. 1978;50(9):1342-1346. https://doi.org/10.1021/ac50031a038. doi: 10.1021/ac50031a038.
24. Pedersen L, Nybo M. Preanalytical factors of importance for measurement of chromogranin A. *Clin Chim Acta*. 2014;436:41-44. doi: 10.1016/j.cca.2014.04.026 [doi].

25. Hofland J, Kaltsas G, de Herder WW. Advances in the diagnosis and management of well-differentiated neuroendocrine neoplasms. *Endocr Rev*. 2019. doi: bnz004 [pii].

26. 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(11):2049-2051.

27. Perry H, Keevil B. Online extraction of 5-hydroxyindole acetic acid from urine for analysis by liquid chromatography-tandem mass spectrometry. *Ann Clin Biochem*. 2008;45(Pt 2):149-152. doi: 10.1258/acb.2007.007067 [doi].

28. Tellez MR, Mamikunian G, O'Dorisio TM, Vinik AI, Woltering EA. A single fasting plasma 5-HIAA value correlates with 24-hour urinary 5-HIAA values and other biomarkers in midgut neuroendocrine tumors (NETs). *Pancreas*. 2013;42(3):405-410. doi: 10.1097/MPA.0b013e318271c0d5 [doi].

29. RRID: SCR_016771

30. Lindstrom M, Tohmola N, Renkonen R, Hamalainen E, Schalin-Jantti C, Itkonen O. Comparison of serum serotonin and serum 5-HIAA LC-MS/MS assays in the diagnosis of serotonin producing neuroendocrine neoplasms: A pilot study. *Clin Chim Acta*. 2018;482:78-83. doi: S0009-8981(18)30146-3 [pii].

31. Odink J, Korthals H, Knijff JH. Simultaneous determination of the major acidic metabolites of catecholamines and serotonin in urine by liquid chromatography with electrochemical detection after a one-step sample clean-up on sephadex G-10; influence of vanilla and banana ingestion. *J Chromatogr*. 1988;424(2):273-283. doi: 10.1016/s0021-9673(88)80215-7 [pii].
32. Marchi I, Viette V, Badoud F, et al. Characterization and classification of matrix effects in biological samples analyses. *J Chromatogr A*. 2010;1217(25):4071-4078. doi: 10.1016/j.chroma.2009.08.061 [doi].

33. Oberg K, Couvelard A, Delle Fave G, et al. ENETS consensus guidelines for standard of care in neuroendocrine tumours: Biochemical markers. *Neuroendocrinology*. 2017;105(3):201-211. doi: 10.1159/000472254 [doi].

34. Calanchini M, Tadman M, Krogh J, Fabbri A, Grossman A, Shine B. Measurement of urinary 5-HIAA: Correlation between spot versus 24-h urine collection. *Endocr Connect*. 2019;8(8):1082-1088. doi: 10.1530/EC-19-0269 [doi].

35. Zuutelenhorst J, Korse C, Bonfrer J, Peter E, Lamers C, Taal B. Daily Cyclic Changes in the Urinary Excretion of 5-Hydroxyindoleacetic Acid in Patients with Carcinoid Tumors, Clinical Chemistry, 2004;50(9):1634 – 1639, https://doi.org/10.1373/clinchem.2004.032151

36. Gedde-Dahl M, Thiis-Evensen E, Tjølsen AM, Mordal KS, Vatn M, Bergestuen DS. Comparison of 24-h and overnight samples of urinary 5-hydroxyindoleacetic acid in patients with intestinal neuroendocrine tumors. *Endocr Connect*. 2013;2(1):50-54. doi:10.1530/EC-12-0077

37. Tohmola N, Johansson A, Sane T, Renkonen R, Hamalainen E, Itkonen O. Transient elevation of serum 5-HIAA by dietary serotonin and distribution of 5-HIAA in serum protein fractions. *Ann Clin Biochem*. 2015;52(Pt 4):428-433.
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 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* (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 tumour     | 5                 | -                | -                | -               | -           |
| Remission (radiological/biochemical) | -             | -                | 36 (19/31)       | 14 (7/14)       | -           |

*These 14 subjects represent individuals for whom 5HIAA was used as a screening marker but further diagnostic work-up confirmed that none of them had an underlying serotonin secreting NEN. Further diagnostic work up indicated five healthy, three diabetes mellitus, one paraganglioma, one burn, one hepatic cirrhosis, one schwannoma, one renal hypertension and one spondylarthritis.
Table 2. Analytical parameters of the newly developed serum 5HIAA LC-MS/MS assay

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

*Limit of detection/quantitation
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                      |

**Abbreviations**

5HIAA, 5-hydroxyindoleacetic acid

5-HT, 5-hydroxytryptamine, serotonin

CgA, chromogranin A

NEN, Neuroendocrine neoplasms

NET, Neuroendocrine tumors

NEC, Neuroendocrine carcinomas

LC-MS/MS, liquid chromatography tandem mass spectrometry

HPLC, high performance liquid chromatography

LC, liquid chromatography

PP, protein precipitation
SPE, solid phase extraction
AUC, area under the curve
ROC, receiver operating characteristics
ACN, acetonitrile
IS, internal standard
CV, coefficient of variation
LOD, lower limit of detection
LOQ, lower limit of quantitation
CI, confidence interval
URL, upper reference limit
Figure 1

- Serum PP (nmol/L) with p < 0.001
- Serum SPE (nmol/L) with p < 0.001
- Urine (μmol) with p < 0.001

Comparison between Active NEN (N=66) and No NEN (n=85) groups for Serum PP and SPE, as well as for Urine levels.