New Developed UHPLC Method for Selected Urine Metabolites

Valko-Rokytovská M*, Očenáš P, Salayová A and Kostecká Z
Department of Chemistry, Biochemistry and Biophysics, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovak Republic

Abstract

An ultra-high-performance chromatography for simultaneous separation and determination of the phenylalanine, tyrosine and their metabolites (vanillylmandelic acid, homovanillic acid, homogentisic acid), tryptophan and its metabolites (5-hydroxyindolacetic acid, kynurenic acid, indoxylsulphate, 3-indolacetic acid) was developed. The separation was carried out on Thermo Scientific Acclaim™ 120 C18 column with a mobile phase composed of methanol and water (containing 0.1% formic acid) in gradient mode at a flow rate of 0.6 mL/min. The limit of detection of 10-15 ng/mL and limit of quantitation of 29-45 ng/mL were determined. The method was evaluated in terms of recovery, precision, linearity, limit of detection, limit of quantification. The determined parameters are in the commonly acceptable ranges for that kind of analysis. The developed chromatographic method allows the rapid screening of urine metabolites, which can serve as potential markers of various tumor diseases as well as for the monitoring of treatment processes.

Keywords: UHPLC; Urine; Metabolites

Abbreviations: UHPLC: Ultra-High-Performance Liquid Chromatography; HPLC: High Performance Liquid Chromatography; Phe: Phenylalanine; Tyr: Tyrosine; NE: Norepinephrine; E: Epinephrine; DA: Dopamine; VMA: Vanillylmandelic Acid; HVA: Homovanillic Acid; HGA: Homogentisic Acid; Trp: Tryptophan; KNYA: Kynurenic Acid; DAD: Diode Array Detector; LOD: Limit of Detection; LOQ: Limit of Quantitation; FLD: Fluorescence detector; IS: 3-Indoxyl Sulphate; 3-IAA: 3-Indolacetic Acid; RSD: Relative Standard Deviation; SD: Standard Deviation; 5-HIAA: 5-Hydroxyindolacetic Acid.

Introduction

The Ultra-High-Performance Liquid Chromatography (UHPLC) is widely used in biomedical chemistry. It is used in the diagnostics of diseases and treatment progress monitoring, in human biomonitoring or in therapeutic drug monitoring [1,2]. UHPLC is used also for the fractionation of biological materials, e.g., urine, serum and plasma [3-5]. The most important advantage of UHPLC is the sensitive, accurate and quick separation of complex mixtures of biologically active substances. The effort of modern medicine is the application of biological fluids that do not require invasive sampling. The analysis of urine samples takes advantage with respect to other biological matrices both because the analytes are more stable in urine and because urine sampling is less invasive. Urine requires minimal sample pretreatment and contains substances reflecting the physiological/pathological condition of the organism. The endogenous metabolites are most frequently determined with High Performance Liquid Chromatography interfaced with electrochemical detection [6] or fluorescence detection [7]. Recently, a number of analytical methods using HPLC coupled to tandem mass detection (HPLC-MS/MS) have been described and applied for analysis of compounds in the neurotransmitter family with targeted metabolomics [8], catecholamines and their metabolites [9,10]. The goal of the present study was to develop and validate a suitable UHPLC method for the simultaneous quantification of selected metabolites of phenylalanine, tyrosine and tryptophan in human urine. Selection of metabolites was based on studies of their presence at various pathological conditions of organism.

Currently, metabolomics of urine represents rapid development in prediction, detection and monitoring of diseases. This noninvasive approach revealed homeostatic imbalance of biological systems and enables it to provide comprehensive information of potential biomarkers for noninvasive monitoring of diseases. An imbalance in tyrosine and tryptophan metabolites is associated with cancer [11], neurological [12] and inflammatory disorders [13]. The accurate and precise measurement of these compounds in biological specimens is a powerful tool to understand the biochemical state in several diseases.

The aromatic amino acids phenylalanine and tyrosine are precursors for catecholamines norepinephrine, epinephrine and dopamine (Figure 1). In acute stress, the synthesis of catecholamines is rapidly increasing, and therefore these amines are referred to as the stress hormones [14]. Determination of urinary and plasma catecholamines plays an important role in clinical diagnostics [15,16]. The main endproduct of degradation of NE and E is vanillylmandelic acid, the degradation product of dopamine is homovanillic acid, the degradation product of dopamine is homovanillic acid.

Intermediate of the metabolic breakdown of Tyr and Phe is homogentisic acid [17-19]. High levels of these urinary metabolites predict suspicion of pheochromocytoma [20-23], neuroblastoma [24-26], ganglioneuroma [27,28], parangangioma [29], phenylketonuria [30,31] and alkaptonuria [17-19]. The precursor of a large number of biologically active metabolites from the serotonin, kynurenine and indolic pathways is tryptophan (Trp; Figure 2). Elevated levels of urinary Trp metabolites have been demonstrated in patients with carcinoid [32,33], breast cancer [34], bladder cancer [35] or autistic symptom [36]. Trp deficiency is associated with depressive conditions, bipolar affective disorder, and also with HIV infection [37,38]. Immune-mediated activation of Trp catabolism via the kynurenine pathway is a consistent finding in all inflammatory disorders and this activation leads to the production of several immune-modulating metabolites [13,39].

*Corresponding author: Marcela Valko-Rokytovská, Department of Chemistry, Biochemistry and Biophysics, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovak Republic, Tel: +421905561511; E-mail: marcela.valko.rokytovska@gmail.com

Received May 02, 2018; Accepted May 10, 2018; Published May 15, 2018

Citation: Valko-Rokytovská M, Očenáš P, Salayová A, Kostecká Z (2018) New Developed UHPLC Method for Selected Urine Metabolites. J Chromatogr Sep Tech 9: 404. doi: 10.4172/2157-7064.1000404

Copyright: © 2018 Valko-Rokytovská M, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Imbalance of kynurenic acid level in the kynurenine pathway have been related with several pathological conditions like schizophrenia, major depression, autism and epilepsy or Alzheimer disease [5].

Indole urine metabolites, 3-indoxyl sulphate and 3-indoleacetic acid, are converted to indole from Trp. IS increases the rate of progression of renal failure - chronic kidney disease and 3-IAA is associated with cardiovascular disease in patients with chronic kidney disease [40]. Pathologically elevated urinary IS indicates the rotting processes in the body that may occur in intestinal obstructions, diarrhea, Hartnup disease [41], gastric carcinoma or Escherichia coli proliferation [42]. Pathologically elevated urinary concentration of 5-hydroxyindoleacetic acid (5-HIAA), serotonin metabolite, is used as a biochemical test for the diagnosis of a carcinoid tumor [43-47] and appendicitis [48,49]. Decreased urine levels of 5-HIAA were studied in children with Down syndrome [50], in irritable bowel syndrome and in pathogenesis of functional bowel diseases [51].

In summary, the accurate measurement of these compounds in urine is a powerful tool to understand the biochemical state in several diseases and various cancer diseases. Therefore, the aim of this study was to develop simultaneous, qualitative and quantitative determination of selected metabolites in urine, such as tyrosine, tryptophan and their metabolites.

### Experimental

#### Materials

Creatinine, tyrosine, phenylalanine, vanillylmandelic acid, homovanillic acid, homogentisic acid, tryptophan, 5-hydroxyindoleacetic acid, indoxyl sulphate, kynurenic acid, 3-indolacetic acid and formic acid were purchased from Sigma-Aldrich (USA). Methanol of HPLC grade was purchased from Fisher (Fisher Scientific UK Ltd, Loughborough, UK) and water of HPLC grade from central water production of UVLF (RegPur s.r.o.) was used. All reagents were HPLC grade.

Stock solutions of creatinine, Tyr, Phe, VMA, HVA, HGA, Tryp, 5-HIAA, IS, KYNA and 3-IAA were prepared by diluting of given compounds to concentration of 1 mg/mL in deionized water. Stock solutions of these standards were diluted in an ultrasonic bath. Mobile phases were degassed in an ultrasonic bath.

#### Instrumentation and chromatography

The UHPLC separations were performed using UHPLC Dionex UltiMate 3000 RS (Thermo Fisher Scientific Waltham, MA, USA)
equipped with autosampler, intelligent pump, diode array detector and fluorescence detector. The chromatographic column Thermo Scientific Acclaim™ 120 C18 (Thermo Fisher Scientific, Germany; column length 150 mm, inner diameter 3 mm, particle size 3 µm, pore size 120 Å) was used. The column was thermostatted and the precision of the temperature adjustment was ± 0.5°C. The collection and evaluation of data was performed using Chromeleon 7.2 Chromatography Data System software. The DAD detection was carried out within spectral range of 190-800 nm. Fluorescence detection was performed at excitation wavelength of $\lambda_{\text{ex}}$=280 nm and emission wavelength of $\lambda_{\text{em}}$=350 nm. Samples were injected in a volume of 10 µL. The mobile phase of water (containing 0.1% formic acid, A) and methanol (B) in gradient conditions was applied: 0-50% B (0-20 min), 100% A (20-25 min). Flow rate was set at 0.6 mL/min and column temperature was 37°C. The overall analysis time was 25 min.

Urine sample preparation

Urine sample was obtained from healthy volunteer. Urine sample was taken under standard conditions as first morning urine. Urine sample was subjected to commercial biochemical semiquantitative analysis. Sample was stored at -50°C. After thawing and centrifugation at 10,000 rpm (10,621 rcf) for 5 min at laboratory temperature (Eppendorf Centrifuge 5430, Germany), sample was filtered by PVDF syringe filters with pore size of 0.25 µm and diluted with mobile phase A to 15% (v/v) for UHPLC analysis.

Calibration curves

Calibration curves for all standards in urine matrix were prepared. The calibration curve was obtained for a series of solutions with concentration ranging from 0.01 to 20 µg/mL. The calibration points based on the physiological values of the individual metabolites were selected (Table 1). The number of curve points was n=8 and every analyte was injected two times. The volume of the solution in every single injection was 10 µL. The limit of detection and limit of quantitation were determined. The internal standard was not present in the urine matrix, so that the added standards of metabolites were the only source of the standard. In clinical analysis, creatinine is frequently considered to be the best natural internal standard for normalizing the excretion of other metabolites in urine.

Results and Discussion

According to our goal of study, new method for simultaneous separation and determination of phenylalanine, tyrosine, tryptophan and their metabolites present in urine at physiological/pathological conditions of the organism has been developed. This work was focused to develop of the new UHPLC method in urine matrix. The determined parameters, i.e., linearity, recovery, precision, limit of detection, and limit of quantification were established.

Diode array and fluorescence detectors serially connected were used. The five analytes (creatinine, KYNA, HVA, Phe and HGA) were determined by DAD. Signals from six analytes (Tyr, VMA, Trp, 5-HIAA, IS and 3-IAA) were registered using the FLD. The measurements were registered using of DAD at different wavelength (220, 230, 240, 280 nm), but the selected method at wavelength 220 nm.
The chromatographic method allows one to separate and assay creatinine, Tyr, Phe, VMA, HVA, HGA, Trp, 5-HIAA, IS, KYNA and 3-IAA (10 µg/mL, Figure 3). Examined metabolites were well separated in the proposed chromatographic method and HPLC chromatogram showing the resolution of selected five metabolite standards (10 µg/mL) in urine matrix recorded using DAD is shown in Figure 4. A chromatogram of mixture of six metabolites (10 µg/mL) recorded with a FLD is shown in Figure 5. No interfering peaks were observed in the chromatograms of the blank urine samples from healthy people. Matrix interference, caused by endogenous substances in the urine samples, was evaluated by comparing the peak areas of spiked standards with those of the standards of pooled blank urine concentrations.

The signal-to-noise ratio for the lower limit of detection was 3 and for the lower limit of quantitation was 10. The range of LOD values for examined metabolites was 10-15 ng/mL and for LOQ values was 29-45 ng/mL. The results of calibration curves, retention times, LOD and LOQ values for each metabolite are summarized in Tables 2 and 3.

Calibration curves for all metabolites were obtained by plotting the peak areas against the concentrations used. Eight different concentrations of the standard solutions were analyzed in duplicate. Linearity was studied in the range from 0.01 to 20 µg/mL for all used standards. All standards showed good linearity in the tested range and the calibration graphs were analyzed by regression analysis. Correlation coefficient and slope were calculated and the square correlation coefficient ($R^2$) was always greater than 0.9985 (Tables 2 and 3).
The precision of the method was evaluated by intra- and interday tests. Precision and recovery were determined by duplicate analysis of the urine samples (n=5) in which the values of examined metabolites were calculated on 3 consecutive days. Repetitive injections of urine sample containing 0.01 µg/mL of the mixed solution of all standards from the same vial performed on the same day (Table 4). Precision was expressed as relative standard deviation (RSD%) for each standard. The mean values of RSD were within the ranges of 2.02-10.22% with recovery from 98.7 to 107.2% (Tables 2 and 3). A value of RSD within 10% is generally acceptable.

The purpose of this paper was to describe a method that was developed for the analysis of 11 metabolites for the simultaneous monitoring of their concentration. Determination of this metabolites combination in the mixture is poorly described in literary sources. Mostly, individual or mixture of some metabolites was determined by similar methodology. In our studies, FLD and DAD detection were used for the identification and quantification of urinary catecholamines and tryptophan metabolites. Baranowska and Plonka reported the detection of HVA and 5-HIAA using by fluorescence detector [1,52,53]. The sensitivity of this method was LOD (8 ng/mL) and LOQ (24 ng/mL), it was comparable to our results LOD (12 ng/mL) and LOQ (37 ng/mL) for 5-HIAA and LOD (10 ng/mL) and LOQ (29 ng/mL) for HVA. The study of Yan et al. described the simultaneous quantification of multi-class neurotransmitters associated with dopamine, tryptophan and glutamate-γ-aminobutyric acid pathways [54]. The sensitivity of LC-MS/MS was comparable to our results. Only the values of LOD (25 ng/mL) and LOQ (75 ng/mL) for KYNA were higher.

UHPLC method was obtained for the identification and quantification of urine sample, which will reduce analysis times and workload for the laboratories charged with the diagnosis of different diseases and which can, if necessary, be coupled to a MS-detector for a more thorough characterization.

**Conclusion**

In this study, a rapid, accurate and sensitive method based on ultra-high-performance chromatography for the analysis of the metabolism...
of tyrosine and tryptophan has been developed and validated. The study method has been validated in biological matrix—health human urine. The method allows the quantification of selected metabolites of phenylalanine; tyrosine and its metabolites: vanillylmandelic acid, homovanillic acid, homogentisic acid; tryptophan and its metabolites 5-hydroxyindolacetic acid, indoxyl sulphate, kynurenic acid, 3-indolacetic acid. Diode array and fluorescence detectors serially connected were used. The five analytes were determined by DAD. Signals from six analytes were registered using the FLD. The method was evaluated in terms of recovery, precision, linearity, limit of detection, limit of quantification. The wide coverage of Trp and Tyr metabolism, together with optimal analysis time, low sample volume, simple sample preparation and satisfactory quantitative results make this developed procedure useful for rapid and reliable clinical studies.

### Conflict of Interest

The authors declare that they have no conflict of interest.

---

### Table 2: Retention times and calibration curve parameters for five urine metabolites (diode array detector, DAD).

| Analyte | Retention time (t<sub>R</sub>, min) | Slope | Correlation coefficient (R<sup>2</sup>) | RSD (%) | LOD (ng/mL) | LOQ (ng/mL) |
|---------|----------------------------------|-------|----------------------------------------|--------|-------------|-------------|
| Creatinine | 0.717 | 0.3889 | 0.9997 | 2.516 | 10 | 30 |
| Phe | 4.563 | 0.3387 | 0.9991 | 4.596 | 14 | 41 |
| HGA | 4.750 | 0.2595 | 0.9962 | 10.224 | 10 | 30 |
| KYNA | 9.427 | 1.0333 | 0.9998 | 2.020 | 12 | 36 |
| HVA | 10.767 | 0.5156 | 0.9996 | 3.093 | 10 | 29 |

### Table 3: Retention times and calibration curve parameters for six urine metabolites (fluorescence detector, FLD).

| Analyte | Retention time (t<sub>R</sub>, min) | Slope | Correlation coefficient (R<sup>2</sup>) | RSD (%) | LOD (ng/mL) | LOQ (ng/mL) |
|---------|----------------------------------|-------|----------------------------------------|--------|-------------|-------------|
| Tyr | 2.425 | 4590.4682 | 0.9988 | 5.459 | 11 | 32 |
| VMA | 3.762 | 2118.4295 | 0.9985 | 5.782 | 12 | 37 |
| IS | 6.752 | 183019.6362 | 0.9993 | 3.922 | 15 | 45 |
| Trp | 7.485 | 241663.4940 | 0.9990 | 4.980 | 12 | 35 |
| 5-HIAA | 8.665 | 17127.7056 | 0.9993 | 4.613 | 12 | 37 |
| 3-IAA | 15.049 | 550159.4074 | 0.9989 | 5.316 | 12 | 37 |

### Table 4: Intraday and interday precision for metabolites in urine.

| Analyte | Intraday | Interday |
|---------|----------|----------|
|         | Measured (mean ± SD, ng/mL) | Recovery (%) | Measured (mean ± SD, ng/mL) | Recovery (%) |
| Creatinine | 10.3 ± 0.3 | 103.1 | 10.7 ± 0.3 | 107.2 |
| Phe | 9.9 ± 0.5 | 99.2 | 10.5 ± 0.2 | 105.1 |
| HGA | 10.2 ± 0.4 | 102.6 | 10.1 ± 0.2 | 101.3 |
| KYNA | 10.5 ± 0.4 | 104.9 | 10.3 ± 0.1 | 103.3 |
| HVA | 10.1 ± 0.2 | 100.9 | 9.8 ± 0.4 | 98.7 |
| Tyr | 10.2 ± 0.1 | 102.3 | 10.4 ± 0.3 | 104.2 |
| VMA | 10.0 ± 0.1 | 100.7 | 10.2 ± 0.3 | 102.3 |
| IS | 10.6 ± 0.4 | 106.3 | 10.1 ± 0.2 | 101.8 |
| Trp | 9.9 ± 0.2 | 99.0 | 10.1 ± 0.3 | 101.0 |
| 5-HIAA | 10.3 ± 0.2 | 103.1 | 10.2 ± 0.3 | 102.1 |
| 3-IAA | 10.4 ± 0.2 | 104.4 | 10.6 ± 0.4 | 106.4 |
Acknowledgements
The realization of the experiment was supported by Internal Grant Agency - IGA UVLF 05/2018, Urinary biochemical profile of cancer diseases with potential use in diagnostic practice, Kosice, Slovakia.

References
1. Baranowska I, Plonka J (2016) Monitoring of biogenic amines and drugs of various therapeutic groups in urine samples with use of HPLC. Biomed Chromatogr 30: 652-657.
2. Mathias PI, Connor TH, B'Tymer C (2018) A review of high performance liquid chromatographic-mass spectrometric urinary methods for anticancer drug exposure of health care workers. J Chromatogr B Anal Technol Biomed Life Sci 1060: 316-324.
3. Katsuki A, Yoshimura R, Kishi T, Hori H, Umene-Nakano W et al. (2012) Serum levels of brain-derived neurotrophic factor (BDNF), BDNF gene Val66Met polymorphism, or plasma catecholamine metabolites, and response to mirtazapine in Japanese patients with major depressive disorder (MDD). CNS Spectr 17: 155-163.
4. Abdel-Hamid NM, Shehata DE, Abdel-Ghany AA, Ragaa A, Wahid A (2016) Serum serotonin as unexpected potential marker for staging of experimental hepatocellular carcinoma. Biomed and Pharmacother 83: 407-411.
5. Marcos J, Renau N, Valverde O, Aznar-Lain G, Gracia-Rubio I, et al. (2016) Targeting tryptophan and tyrosine metabolism by liquid chromatography tandem mass spectrometry. J Chromatogr A 1434: 91-101.
6. Parrot S, Neuzeret PC, Denory L (2011) A rapid and sensitive method for the analysis of brain monoamine neurotransmitters using ultra-fast liquid chromatography coupled to electrochemical detection. J Chromatogr B Analyt Technol Biomed Life Sci 879: 3871-3878.
7. Zhao XE, Suo YR (2008) Simultaneous determination of monoamine and amino acid neurotransmitters in rat endbrain tissues by pre-column derivatization with high-performance liquid chromatographic fluorescence detection and mass spectrometric identification. Talanta 76: 690-697.
8. Moriarty M, Lehane M, O’Connell B, Keely H, Furey A (2012) Development of a nano-electrospray MSn method for the analysis of serotonin and related compounds in urine using a LTQ-orbitrap mass spectrometer. Talanta 90: 1-11.
9. Grouzmann E, Lamine F (2013) Determination of catecholamines in plasma and urine. Best Pract Res Clin Endocrinol Metab 27: 713-723.
10. Clark ZD, Cutler JM, Pavlov IV, Strathamn FG, Frank EL (2018) Simple dilute-and-shoot method for urinary vanillylmandelic acid and homovanillic acid by liquid chromatography tandem mass spectrometry. Clin Chim Acta 468: 201-208.
11. Wiggins T, Kumar S, Markar SR, Antonowicz S, Hanna GB (2015) Tyrosine, phenylalanine, and tryptophan in gastroesophageal malignancy: a systematic review. Cancer Epidemiol Biomarkers Prev 24: 32-38.
12. Oto J, Suzue A, Inui D, Fukuta Y, Hosotsubo K, et al. (2008) Plasma proinflammatory and anti-inflammatory cytokine and catecholamine concentrations as predictors of neurological outcome in acute stroke patients. J Anesth 22: 207-212.
13. Heng B, Lim CK, Lovejoy DB, Bessede A, Gluch L, et al. (2016) Understanding the role of the kynurenine pathway in human breast cancer immunobiology. Oncotarget 7: 6506-6520.
34. Nam H, Chung BC, Kim Y, Lee K, Lee D (2009) Combining tissue transcriptomics and urine metabolomics for breast cancer biomarker identification. Bioinformatics 25: 3151-3157.

35. Chung KT, Gadupudi GS (2011) Possible roles of excess tryptophan metabolites in cancer. Environ Mol Mutagen 52: 81-104.

36. Mulder EJ, Anderson GM, Kempannen RF, Oosterloo-Duinkerken A, Minderaa RB et al. (2010) Urinary excretion of 5-hydroxyindoleacetic acid, serotonin and 6-sulphatoxyxylomin in normosrotomatic and hyperserotonemic autidividuals. Neuropsychobiology 61: 27-32.

37. Davies NWS, Guillemin G, Brew BJ (2010) Tryptophan, Neurodegeneration and HIV-Associated Neurocognitive Disorder. Int J Tryptophan Res 3: 121-140.

38. Douet V, Tanizaki N, Franke A, Li X, Chang L (2016) Polymorphism of Kynurenine Pathway-Related Genes, Kynurenic Acid, and Psychopathological Symptoms in HIV. J Neuroimmune Pharmacol 11: 549-561.

39. Brady N, Grant R (2018) Kynurenine pathway metabolism and neuroinflammatory disease. Neuronal Regen Res 12: 39-42.

40. Hung SC, Kuo KL, Wu CC, Tamg DC (2018) Indoxyl Sulfate: A Novel Cardiovascular Risk Factor in Chronic Kidney Disease. J Am Heart Assoc 6: e005022.

41. Orbak Z (2010) H atrup disease masked by kwashiorkor. J Health Nutr Publ 28: 413-415.

42. Kim KB, Yang JY, Kwack SJ, Park KL, Kim HS, et al. (2010) Toxicmetabolisms of urinary biomarkers for human gastric cancer in a mouse model. J Toxicol Environ Health A 73: 1420-1430.

43. Bhattacharyya S, Troupanakis C, Chilkuna D, Caplin ME, Davar J (2011) Risk factors for the development and progression of carcinoid heart disease. Am J Cardiol 107: 1221-1226.

44. van Dijk SC, de Herder WW, Kweeboom DJ, Zillikens MC, Feelders RA, et al. (2012) 5-HIAA excretion is not associated with bone metabolism in carcinoid syndrome patients. Bone 50: 1260-1265.

45. Kinova S, Kovacova M, Caprnda M, Koren M (2015) Management of treatment in patients with neuroendocrine neoplasmas of digestive tract. Vnitr Lek 61: 12-20.

46. Adaway JE, Dobson R, Walsh J, Cuthbertson DJ, Monaghan PJ, et al. (2016) Serum and plasma 5-hydroxyindoleacetic acid as an alternative to 24-h urine 5-hydroxyindoleacetic acid measurement. Ann Clin Biochem 53: 554-560.

47. Shi DD, Yuppda DP, Dutton T, Biais LK, Minden SL, et al. (2018) Retrospective review of serotoninergic medication tolerability in patients with neuroendocrine tumors with biochemically proven carcinoid syndrome. Cancer 123: 2735-2742.

48. Apak S, Kazez A, Ozel SK, Ustundag B, Akpoltal N, et al. (2005) Spot urine 5-hydroxyindoleacetic acid levels in the early diagnosis of acute appendicitis. J Pediatr Surg 40: 1436-1439.

49. Xu H, Zhang W, Wang D, Zhu W, Jin L (2007) Simultaneous determination of 5-hydroxyindoleacetic acid and 5-hydroxytryptamine in urine samples from patients with acute appendicitis by liquid chromatography using poly (bromophenol blue) film modified electrode. J Chromatogr B Analytical Biomed Life Sci 846: 14-19.

50. Tasdemir HA, Cetinkaya MC, Polat C, Belet U, Kalyaci AG, et al. (2004) Gallbladder motility in children with Down syndrome. J Pediatr Gastroenterol Nutr 39: 187-191.

51. Moskwa A, Chojnacki J, Wielis-Jarosinski M, Stec-Michalska K, Szadkowski K, et al. (2007) Serum serotonin concentration and urine 5-hydroxyindoleacetic acid excretion in patients with irritable bowel syndrome. Pol Merkur Lekar 22: 366-368.

52. Baranowska I, Plonka J (2008) Determination of biogenic amines and vitamins in urine samples with HPLC. J Liq Chromatogr Relat Technol 31: 2974-2987.

53. Baranowska I, Plonka J (2008) Determination of Levodopa and Biogenic Amines in Urine Samples Using High-Performance Liquid Chromatography. J Chromatogr Sci 46: 30-34.

54. Yan J, Kuzhiamparambil U, Bhandokar S, Solowj N, Fu S (2018) Development and validation of a simple, rapid and sensitive LC-MS/MS method for the measurement of urinary neurotransmitters and their metabolites. Anal Bioanal Chem 409: 7191-7199.