Simultaneous determination of 4-hydroxyphenyl lactic acid, 4-hydroxyphenyl acetic acid, and 3-4-hydroxyphenyl propionic acid in human urine by Ultra high performance liquid chromatography with fluorescence detection

Yang Yongli[1], Liu Fan[2*], Wan Yiqun[1, 2*]

(1. College of Chemistry, Nanchang University, Nanchang Jiangxi P. R. China 330047,

2. Center of Analysis and Testing, Nanchang University, Nanchang, Jiangxi, P. R. China 330047)

[*]Corresponding author:

Yiqun Wan

College of Chemistry, Nanchang University, Nanchang Jiangxi P. R. China 330047

Tel.: +86 791 88321370

Fax: +86 791 88321370

E-mail addresses: yqwanoy@sina.com

Fan Liu

Center of Analysis and Testing, Nanchang University, Nanchang, Jiangxi, P. R. China 330047

E-mail addresses: fan_liu@ncu.edu.cn

Abstract:

A simple and reliable method was established for simultaneous determination of 4-hydroxyphenyl acetic acid, 4-hydroxyphenyl lactic acid, and 3-4-hydroxyphenyl propionic acid in human urine by high-performance liquid chromatography with fluorescence detection. Solid-phase extraction was used to eliminate the interferences in urine. The separation of three analytes was achieved using a C_{18} column and a mobile phase formed by a 95:5 v/v mixture of 50 mmol/L ammonium acetate buffer at pH 6.8 that contained 5 mmol/L tetrabutyl ammonium bromide and acetonitrile. Under the optimized conditions, the detection limits of 4-hydroxyphenyl acetic acid, 4-hydroxyphenyl lactic acid, and 3-4-hydroxyphenyl propionic acid were 4.8×10^{-3}, 8.80×10^{-3}, and 9.00×10^{-3} mg/L, respectively, and the recoveries were in the range of 85.0–120.0% with relative standard deviations of 1.5–3.1%. This method was used to analyze urine samples from breast cancer patients, healthy people and postsurgery breast cancer patients. Significant differences of urinary levels of 4-hydroxyphenyl acetic acid and 4-hydroxyphenyl lactic acid could be found between the breast cancer patients group and other two groups. No effect of age and sex was observed on the urinary levels of 4-hydroxyphenyl acetic acid and 4-hydroxyphenyl lactic acid. This method might be helpful for cancer biomarkers discovery in urine.

Keywords: biomarkers; breast cancer; High-performance liquid chromatography; urine;

Abbreviation List

Abbreviation	Full name
PHPAA	4-hydroxyphenyl acetic acid
PHPLA	4-hydroxyphenyl lactic acid
PHPA	3-4-hydroxyphenyl propionic acid
FLD	fluorescence detection

1. Introduction

Amino acids play an important role in the process of human metabolism, so they are important substances which related to human health [1–4]. Studies have shown that amino acid metabolism diseases and malignant tumors had certain relevance to some tyrosine metabolite. [5–9] At present, the studies of tyrosine metabolites mainly focused on the serum samples [10–14], but the analysis of tyrosine metabolites in urine samples were less reported. Urine is viewed as the ultrafiltrate of plasma, and the changes of urinary materials may relate to some diseases [15]. Moreover, large amount of urine can be obtained noninvasively. So, urine has been an important body fluid for disease biomarker discovery [16–18]. 4-Hydroxyphenyl acetic acid (PHPAA), 4-hydroxyphenyl lactic acid (PHPLA) and 3,4-hydroxyphenyl propionic

acid (PHPA) are very important metabolites of tyrosine [19]. So exploring a simple and reliable method for the determination of PHPAA, PHPLA and PHPA in urine has important clinical significance.

The determination of PHPLA, PHPAA and PHPA were mainly carried out by HPLC methods, such as HPLC–UV [20–21], HPLC with fluorescence detection (FLD) [22–24] and HPLC–MS [25–27]. Among them, the sensitivity of UV method is low, so the UV method is not suitable for the determination of trace amount of tyrosine metabolites in urine; MS method is sensitive, but the MS equipment is expensive [28–30]. Because tyrosine metabolites have autofluorescence, some HPLC–FLD methods [31–32] have been reported for the determination of tyrosine metabolites in other body fluids but not in urine.

In this paper, a HPLC–FLD method was developed for the determination of PHPLA, PHPAA and PHPA in urine. After optimization, this method was applied to analysis of human urine. And the levels of PHPAA, PHPLA and PHPA in urine from breast cancer patients, healthy people and postsurgery breast cancer patients were investigated. To the best of our knowledge, it is the first time that PHPLA, PHPAA and PHPA in urine were determined by HPLC–FLD.

2. Materials and Methods

2.1 Instruments and Reagents

Agilent 1290 Infinity system (Agilent Technologies, Amstelveen, The Netherlands); pHS-3C Precision pH meter (Shanghai Lei magnetic Instrument Factory); Milli-Q ultrapure water system (Millipore, Bedford, Massachusetts, United states of America); TDL-40B low-speed large-capacity desktop centrifuge (Shanghai Anting Instrument); ENVI-18 cartridges (bed wt. 500 mg, 3 mL) were supplied by Supelco (Bellefonte, Pennsylvania, United states of America); Generik C8/BCX (bed wt. 500 mg, 6 mL) were supplied by Sepax (Sepax Technologies), Domestic 717 type anion exchange resin were supplied by Shanghai win glory resin factory.

4-Hydroxyphenyl acetic acid, 4-hydroxyphenyl lactic acid and 3,4-hydroxyphenyl propionic acid standards were supplied by Acros Organics (Morris plains, United States of America); methanol and acetonitrile (HPLC grade) was purchased from Tedia company (Fairfield, USA); All the other chemicals or solvents were of analytical grade.

2.2 Preparation of standard solutions

The 100 mg/L stock solutions of PHPAA, PHPLA and PHPA were prepared by dissolving 2.5 mg of each standard in 25 mL 1% formic acid, and stored at 4°C. The standard working

solutions were prepared by dilution of the stock solutions with 50 mmol/L acetic acid/ammonium acetate (pH = 6.0) to the required concentrations.

2.3 Chromatographic conditions

LC of PHPAA, PHPLA and PHPA was accomplished using a ZORBAX SB–C_{18} quick separation column (100 × 2.1 mm, 1.8 μm; Agilent companies). The column temperature was maintained at 25°C. An isocratic mobile phase consisting of a 95:5 v/v mixture of 50 mmol/L ammonium acetate buffer at pH 6.8 containing 5 mmol/L tetrabutyl ammonium bromide and acetonitrile was used at the flow rate of 0.2 mL/min.

The mobile phase was prepared daily, purified by a 0.22 mm membrane filter, and further degassed by sonication for 10 min. The fluorescence detector was operated at excitation and emission wavelengths of 277 and 316nm, respectively, and the injection volume was 5 μL. The concentrations of the analytes were calculated from peak areas. All data were collected and processed using Agilent ChemStation B.04.02.

2.4 Urine sample collection and pretreatment

The urine samples of normal persons were collected from student volunteers in Nanchang University (P. R. China). Breast cancer patients' urine samples were provided by Jiangxi province tumor hospital (P. R. China) and the breast cancer patients did not receive any treatment. Urine samples were kept at –80°C in the dark until analysis.

Urine samples were adjusted pH to 3.0 with acetic acid solution before preparation. Then the urine samples were centrifuged at 4000 rpm for 10 min. After that, 1.0 mL urine sample was passed through Generik C_8/BCX SPE column which was preconditioned with 6 mL methanol followed by 6mL ultrapure water. The cartridge was washed with 2 mL ultrapure water and eluted with 8 mL acetic acid/ammonium acetate solution (50 mmol/L, pH = 6.0) subsequently. The elution was filtered through a 0.22 μm filter before injection into the HPLC system.

3. Results and discussion

3.1 Optimization of chromatographic conditions

To optimize the separation of PHPLA, PHPAA and PHPA, the pH, mobile phase composition (buffer solution and organic phase ratio) were investigated. Firstly, trials were performed with ammonium formate (50 mM) and ammonium acetate (50 mM), the best results were observed by using ammonium formate (50 mM) solution. To avoid the tailing and irregular shape of the signals, various solvents (acetonitrile, methanol) and ratios between them were tested, and the best conditions were obtained by using 95:5 v/v mixture of 50 mmol/L ammonium acetate buffer at pH 6.8. As the urine containing huge number of hydrophilic compounds, to separate target compounds from impurities effectively, the ion pair reagent, tetrabutylammonium bromide, was added into ammonium acetate buffer to enhance the

analytes retention on column. And the concentration of tetrabutylammonium bromide was optimized, but the results showed no marked differences when 5, 10 or 20 mmol/L tetrabutylammonium bromide was added. Taking into the damage of the column, 5 mmol/L of tetrabutylammonium bromide was chosen. Furthermore the buffer pH value was optimized, and the best results were obtained with pH=6.8. When pH <6.7, the retention times were too long, when pH> 7.0, analytes were interfered by the impurities in urine. So, pH = 6.8 ammonium acetate buffer (50 mmol/L, with 5 mmol/L tetrabutylammonium bromide)/acetonitrile 95:5 v/v was chosen as the mobile phase. And under those conditions, tyrosine did not interfere with the target compounds. Figure 1 was the chromatogram of three analytes under the optimized separation conditions.

3.2 Selection of Sample Pretreatment Methods

Human urine matrix was very complex. Therefore, it is necessary to choose an effective clean technique to remove interferences for the successful analysis of tyrosine metabolites. Since the separation column could be easily contaminated by the proteins in urine, the sample was firstly centrifuged at 4000 rpm for 10 min to eliminate the proteins. To further eliminate other interferences, three sample preparation methods as following were explored in this paper:

(1) The urine sample was diluted eightfold by acetic acid/ammonium acetate buffer (pH = 6.0), then purified by a 0.22 mm membrane filter.

(2) The urine sample was cleaned by an ENVI –18 SPE column, and then purified by a 0.22 mm membrane filter.

(3) The urine sample was cleaned by a Generik C_8/BCX SPE column, and then purified by a 0.22 mm membrane filter.

The procedures of methods (2) and (3) were the same as the one described in Section 2.4.

The results showed that some impurities could interfere with the analysis of the three target compounds when the samples were cleaned by method (1) or (2). Because in method (1), the membrane filter can only remove the insoluble impurities in the urine. And the ENVI –18 SPE column only contains C_{18} packing material, since the interaction between the analytes and packing materials was weak, the urine could not been cleaned effectively by method (2). But the impurities could be removed effectively by method (3). Because the Generik C_8/BCX SPE column contains both C_8 and benzenesulfonic acid packing materials, the target compounds could be adsorbed on the packing materials by both electrostatic and hydrophobic interaction, and be separated with the impurities effectively. So the Generik C_8/BCX SPE column was chosen to purify the urine samples.

Furthermore, to obtain high recovery with less impurity, the procedures of the cleaning method were optimized. Since the target compounds were polar analytes, and could be

eluted from the column easily, the urine sample was adjusted to pH=3.0 by acetic acid to enhance the interaction between analytes and packing materials, previously. Then, the effects of four types of elution solutions including acetic acid –ammonium acetate (50mmol/L, pH = 6.0), acetic acid/ammonium acetate (50 mmol/L, pH = 6.0)/methanol 95:5 v/v, acetic acid/ammonium acetate (50 mmol/L, pH = 6.8, with 5 mmol/L tetrabutylammonium bromide) and acetic acid/ammonium acetate (50 mmol/L, pH = 6.8, with 5 mmol/L tetrabutylammonium bromide)/methanol 95:5 v/v on the recovery efficiency of analytes were investigated. The results showed that the highest recovery efficiency was obtained when acetic acid/ammonium acetate (50 mmol/L, pH = 6.0) was used. Elution was performed using different volumes of eluent (2, 4, 6, 8 and 10 mL). The results showed that the target compounds could be completely eluted by using 8 mL eluent. The recoveries of PHPLA, PHPAA and PHPA were in the range of 85–120%. So, 8 mL acetic acid/ammonium acetate (50 mmol/L, pH = 6.0) was used to elute the analytes in the following experiments.

3.3 Linearity and detection limits

The linearity of each analyte was achieved by analyzing a series of concentration of mixed standard solutions under the optimized conditions. And the calibration curves were plotted by peak area (y) against the concentration of the analyte (x). The results in Table 1 showed that good linear relationships were achieved in the range of 0.05–4.00 mg/L for PHPAA and

PHPLA, and 0.10–4.00 mg/L for PHPA. And the LODs (S/N=3) of PHPAA, PHPLA and PHPA were in the range of 0.0048–0.0090 mg/L.

3.4 Recovery and Precision

To test the method recovery and precision, experiments were carried out by adding three levels of concentrations of analytes for PHPAA, PHPLA and PHPA in urine, and the results were shown in Table 2 and Figure S1. The recoveries of the three analytes were in the range of 85–120%, and the RSDs (n=3) were less than 3.1%.

3.5 Sample determination

Human urines from 26 healthy people, 7 breast cancer patients and 13 postsurgery breast cancer patients were analyzed. Each sample was analyzed three times, and the p values were calculated based on a t-test (p values are listed in Table S2). The average concentrations of PHPAA, PHPLA and PHPA in urine from healthy people, breast cancer patients and postsurgery breast cancer patients are listed in Table S1. The mean urinary levels of PHPAA were 8.34, 23.30 and 9.73 mg/L, and PHPLA were 0.48, 1.63 and 0.57 mg/L from healthy people, breast cancer patients and postsurgery breast cancer patients, respectively. But PHPA was not detected in all the urine samples.

The box plots of the urinary levels of PHPAA and PHPLA are shown in Figures 2 and 3. Obviously, compared to healthy people and postsurgery breast cancer patients, breast cancer

patients showed much higher urinary levels of PHPAA and PHPLA. In other words, the breast cancer patients could be discriminated by the determination of PHPAA and PHPLA in human urine. Urinary levels of PHPAA and PHPLA from breast cancer patients showed very significant difference from that from healthy people and postsurgery breast cancer patients ($p < 0.01$). The urinary levels of PHPLA from breast cancer patients showed significant difference from that from postsurgery breast cancer patients ($p < 0.05$). The urinary levels of PHPAA and PHPLA from healthy people showed no significances difference from those from postsurgery breast cancer patients.

Furthermore, to eliminate the individual differences, we investigated the affection of sex and age on the urinary levels of PHPLA and PHPAA. Firstly, the healthy people were separated to male group and female group, the differences of the urinary levels of PHPAA and PHPLA between the two groups were used to evaluate the sexual affection. Then, the healthy people were separated to three groups based on age, and the affection of age was evaluated by the differences of the urinary levels of PHPAA and PHPLA among the groups. The results in Table S3 showed that no differences could be observed between the sexual groups or among the age groups. So, there were no effect of age and sex on the urinary levels of PHPAA and PHPLA.

4. Conclusion

Studies have shown that tyrosine metabolites in human body fluids have certain relation with cancer [15]. And urine has been regarded as one of the important body fluids for disease biomarker discovery, because of acquiring advantage. But the relationships of urinary levels of PHPAA, PHPLA and PHPA to breast cancer have not been studied. A simple and reliable method was established in this paper for the determination of three important tyrosine metabolites, including PHPAA, PHPLA and PHPA in urine by HPLC–FLD system. And the urinary levels of PHPAA, PHPLA and PHPA from breast cancer patients, healthy people and postsurgery breast cancer patients were investigated by this method. The results showed that there were significant differences of urinary levels of PHPAA and PHPLA between breast cancer patients and healthy people. And there were no affection of sex and age on the urinary levels of PHPAA and PHPLA. PHPAA and PHPLA in urine might be the potential biomarkers of breast cancer.

jssc201700014-sup-0001-SuppInfo.docx

Supplementary material

Acknowledgements

The financial support for this study from the Natural Science Foundation of P.R. China (No: 21465017), Research Program of State Key Laboratory of Food Science and Technology in Nanchang University (No: SKLF-ZZA-201612) are gratefully acknowledged.

Reference

[1] Jin, R., Banton, S., ViLinh, T. T., Konomi, J. V., Li, S. Z., Jones, D. P., Vos, M. B., Amino Acid Metabolism is Altered in Adolescents with Nonalcoholic Fatty Liver Disease—An Untargeted, High Resolution Metabolomics Study. *J. Pediatr.* 2016, 172, 14-9.

[2] Martin-Lorenzo, M., Gonzalez-Calero, L., Maroto, A. S., Martinez, P. J., Zubiri, I., Cuesta, F. D. L., Mourino-Alvarez, L., Barderas, M. G., Heredero, A., Aldamiz-Echevarría, G., Vivanco, F., Alvarez-Llamas, G., Cytoskeleton deregulation and impairment in amino acids and energy metabolism in early atherosclerosis at aortic tissue with reflection in plasma. *Bba-Biomembranes*. 2016, 1862, 25-732.

[3] Jegatheesan, P., Beutheu, S., Ventura, G., Sarfati, G., Nubret, E., Kapel, N., Waligora-Dupriet, A. J., Bergheim, I., Cynober, L., De-Bandt, J. P., Effect of specific amino acids on hepatic lipid metabolism in fructose-induced non-alcoholic fatty liver disease. *Clin. Nutr*. 2016, 35, 75-182.

[4] Domingues, D. S., Crevelin, E. J., Moraes, L. A., Hallak, J. E., Crippa, J. A., Queiroz, M. E., Simultaneous determination of amino acids and neurotransmitters in plasma samples from schizophrenic patients by hydrophilic interaction liquid chromatography with tandem mass spectrometry. *J. Sep. Sci.* 2015, 38, 780–787.

[5] Geck, R. C., Toker, A., Nonessential amino acid metabolism in breast cancer. *Advances in Biological Regulation*. 2016, 62, 11-17.

[6] Das, A. M., Pharmacotherapy of inborn errors of metabolism illustrating challenges in orphan diseases. *J. Pharmzcal. Toxicol*. 2016, 81, 9-14.

[7] Harden, J. L., Lewis, S. M., Lish, S. R., Suarez-Farinas, M., Gareau, D., Lentini, T., Johnson-Huang, L. M., Krueger, J. G., Lowes, M. A., The tryptophan metabolism enzyme L-kynureninase is a novel inflammatory factor in psoriasis and other inflammatory diseases. *J. Allergy. Clin. Immunol*. 2016, 137, 1830-1840.

[8] Zolodz, M. D., Jia, M., Liu, H. P., Henderson, G. N., Stacpoole, P. W., A GC–MS/MS method for the quantitative analysis of low levels of the tyrosine metabolites maleylacetone, succinylacetone, and the tyrosine metabolism inhibitor dichloroacetate in biological fluids and tissues. *J. Chromatogr. B*. 2006, 837, 125-132.

[9] Li, X. C., Chen, Z.G., Yang, F., Pan, J. B., Li, Y.B., Development of a microchip-pulsed electrochemical method for rapid determination of L-DOPA and tyrosine in Mucuna pruriens. *J. Sep. Sci*. 2013, 36, 1590–1596.

[10] Meesters, R. J. W., Wolfe, R. R., Deutz, N. E. P., Application of liquid chromatography-tandem mass spectrometry (LC–MS/MS) for the analysis of stable isotope enrichments of phenylalanine and tyrosine. *J. Chromatogr. B*. 2009, 877, 43-49.

[11] Van Erp, N. P., Wit, D. D., Guchelaar, H. J., Gelderblom, H., Hessing, T. J., Hartigh, J. D., A validated assay for the simultaneous quantification of six tyrosine kinase inhibitors and two active metabolites in human serum using liquid chromatography coupled with tandem mass spectrometry. *J. Chromatogr. B*. 2013, 937, 33-43.

[12] Qin, J. C., Su, H., Zhang, Y. M., Gao, J. M., Zhu, L., Wu, X., Pan, X. Y., Li, X., Highly brominated metabolites from marine red algaLaurencia similis inhibit protein tyrosine phosphatase 1B. *Bioorg. Med. Chem. Let*. 2010, 20, 7152–7154.

[13] Mu, S., Li, Y., Tang, A. G., Xiao, L. D., Ren, Y. P., Simultaneous determination of tyrosine, tryptophan and 5-hydroxytryptamine in serum of MDD patients by high performance liquid chromatography with fluorescence detection. *Clin. Chim. Acta*. 4 (2012) 973–977.

[14] Shroads, A. L., Henderson, G. N., Cheung, J., James, M. O., Stacpoole, P.W., Unified gas chromatographic–mass spectrometric method for quantitating tyrosine metabolites in urine and plasma. *J. Chromatogr. B*. 2004, 808, 153-161.

[15] Rodríguez-Suárez, E., Siwy, J., Zürbig, P., Mischak, H., Urine as a source for clinical proteome analysis: From discovery to clinical application. *Bba-Proteins Proteom*. 2014, 1844, 884-898.

[16] J Bauc¸ J. M., Martı´nez-Morillo, E., Diamandis, E. P., Peptidomics of Urine and Other Biofluids for Cancer Diagnostics. *Clin Chem*.2014, 60, 1052-1061.

[17] Xue, S. W., Luo, L., Xu L., Magnetic solid-phase extraction of angiotensin II receptor antagonists in human urine and plasma with a reversed-phase/cation-exchange mixed-mode sorbent. *J. Sep. Sci*. 2016, 39, 4812–4818

[18] Abbasian, M., Balali-Mood, M., Mozaffari, S. A., Amoli, H.S., Solid-phase microextraction of ultra-trace amounts of tramadol from human urine by using a carbon nanotube/flower-shaped zinc oxide hollow fiber. *J. Sep. Sci*. 2016, 39, 4449–4457

[19] Feng, L., He, Y. Q., Xu, G. H., Hu, H., Guo, L., Wan, Y. Q., Determination of Tyrosine and Its Metabolites in Human Serum with Application to Cancer Diagnosis. *Anal Lett*. 2014, 47, 1275-1289.

[20] Contin, M., Mohamed, S., Albani, F., Riva, R., Baruzzi, A., Simple and validated HPLC–UV analysis of levetiracetam in deproteinized plasma of patients with epilepsy. *J. Chromatogr. B*. 2008, 873, 129-132.

[21] Contin, M., Mohamed, S., Candela,C., Albani, F., Riva, R., Baruzzi, A., Simultaneous HPLC–UV analysis of rufinamide, zonisamide, lamotrigine, oxcarbazepine monohydroxy derivative and felbamate in deproteinized plasma of patients with epilepsy. *J. Chromatogr. B*. 2010, 878, 461-465.

[22] Yoshida, H., Nohta, H., Harada, Y., Yoshitake, M., Todoroki,K., Yamagata, K., Yamaguchi, M., A simple liquid chromatographic method based on intramolecular excimer-forming derivatization and fluorescence detection for the determination of tyrosine and tyramine in urine. *J. Chromatogr. B*. 2005, 821, 88-93.

[23] Kanamori, Y., Isokawa, M., Funatsu, T., Tsunoda, M., Development of analytical method for catechol compounds in mouse urine using hydrophilic interaction liquid chromatography with fluorescence detection. *J. Chromatogr. B*. 2015, 985, 142-148.

[24] Kassem, M. G., Ezzeldin, E., Korashy, H. M. Mostafa, G. A. E., High-performance liquid chromatographic method for the determination of dasatinib in rabbit plasma using fluorescence detection and its application to a pharmacokinetic study. *J. Chromatogr. B*. 2013, 939, 73-79.

[25] Taylor, P. J., Franklin, M. E., Graham, K. S., Pillans, P. I., A HPLC–mass spectrometric method suitable for the therapeutic drug monitoring of everolimus. *J.Chromatogr.B*. 2007, 848, 208-214.

[26] Segall, S.D., Artz, W.E., Raslan, D. S., Ferraz, V. P., Takahashi, J. A., Analysis of triacylglycerol isomers in Malaysian cocoa butter using HPLC–mass spectrometry. *Food. Res. Int*. 2005, 38, 167-174.

[27] Kokarnig, S., Tsirigotaki, A., Wiesenhofer, T., Lackner, V., Francesconi, K.A., Pergantis, S.A., Kuehnelt, D., Concurrent quantitative HPLC–mass spectrometry profiling of small selenium species in human serum and urine after ingestion of selenium supplements. *J Trace Elem Med Bio*. 2015, 29, 83-90.

[28] Lionetto, L., Lostia, A. M., Stigliano, A., Cardelli, P., Simmaco, M., HPLC–mass spectrometry method for quantitative detection of neuroendocrine tumor markers: Vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid. *Clin. Chim. Acta*. 2008, 398, 53-56.

[29] Emmerechts, G., Herdewijn, P., Rozenski, J., Pseudouridine detection improvement by derivatization with methyl vinyl sulfone and capillary HPLC–mass spectrometry. *J. Chromatogr. B*. 2005, 825, 233-238.

[30] Galba, J., Michalicova, A., Parrak, V., Novak, M., Quantitative analysis of phenylalanine, tyrosine, tryptophan and kynurenine in rat model for tauopathies by ultra-high performance liquid chromatography with fluorescence and mass spectrometry detection. *J. Pharmaceut. Biome*. 2016, 117, 85-90.

[31] Wang, H., McNeil, Y. R., Yeo, T. W., Anstey, N. M., Simultaneous determination of multiple amino acids in plasma in critical illness by high performance liquid chromatography with ultraviolet and fluorescence detection. *J. Chromatogr. B*. 2013, 940, 53-58.

[32] Guo, X. F., Wang, J. Y., Wang, H., Zhang, H. S., Simultaneous determination of primary and secondary phenethylamines in biological samples by high-performance liquid chromatographic method with fluorescence detection. *J. Chromatogr. B*. 2014, 967, 69-74.

Figure captions

Figure 1. Chromatogram of standard mixture of tyrosine metabolites : (1) PHPAA (1.5mg/L) ; (2) PHPLA (2mg/L) ; (3) PHPA (2mg/L).

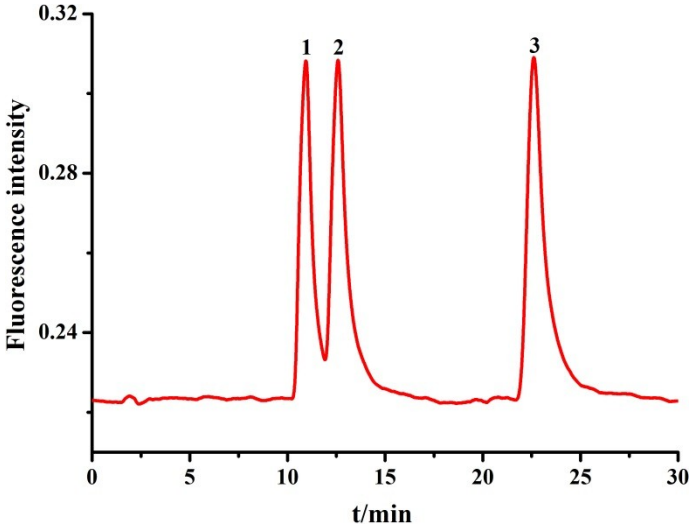

Figure 2. Box plot of urinary levels of PHPAA from healthy people, breast cancer patients and postsurgery breast cancer patients

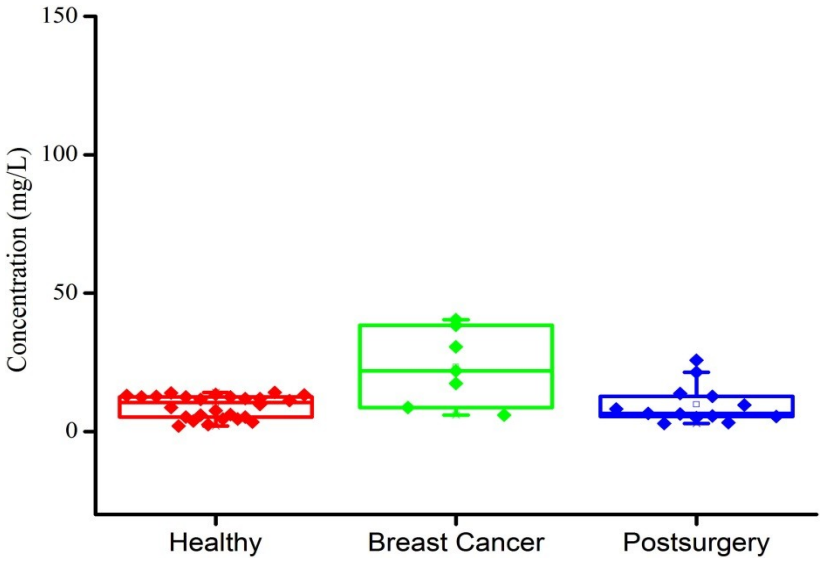

Figure 3. Box plot of urinary levels of PHPLA from healthy people, breast cancer patients and postsurgery breast cancer patients

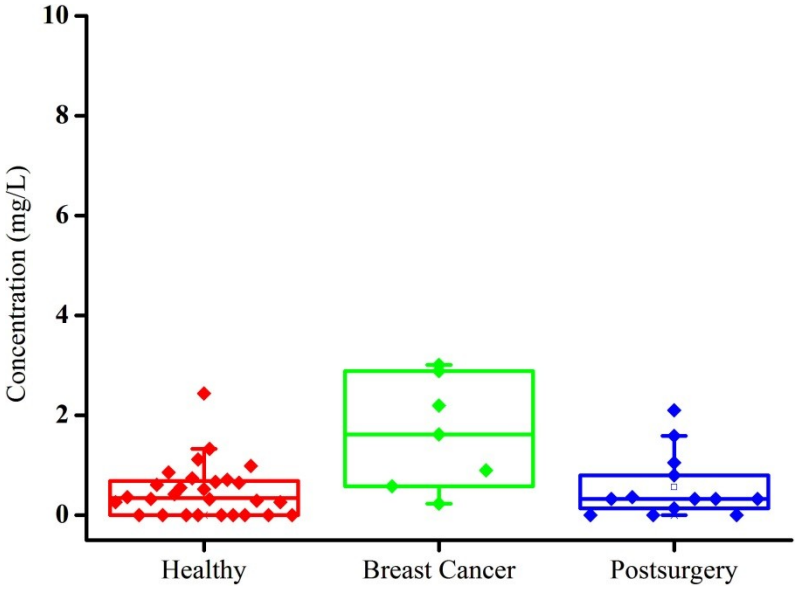

Table1 Linear equation, correlation coefficients, limits of detection (LODs, S/N=3) and limits of quantification (LOQs, S/N=10) for PHPAA、PHPLA and PHPA

Analytes	Linearity range (mg/L)	Linear equations	Correlation coefficient	LOD （mg/L）	LOQ （mg/L）
PHPAA	0.05-4.00	y=2.530x+0.0468	0.9997	4.80×10-3	0.02
PHPLA	0.05-4.00	y=3.503x+0.1161	0.9997	8.80×10-3	0.03
PHPA	0.10-4.00	y=3.928x+0.1074	0.9993	9.00×10-3	0.03

Table 2 Recovery results of the three analytes for PHPAA, PHPLA and PHPA in human urine (n=3)

Analytes	Background（mg/L）	Added（mg/L）	Found（mg/L）	Recovery（%）	RSD（%）
PHPAA	9.30	5.00	14.37	101.4	3.1
		10.00	19.61	103.1	1.3
		12.00	22.58	110. 7	2.8
PHPLA	0.28	0.80	0.96	85.0	2.3
		2.00	2.46	109.0	1.3
		8.00	8.16	98.5	2.4
PHPA	ND*	0.80	0.96	120.0	1.2
		2.00	1.76	88.0	2.7
		8.00	7.73	96.6	1.5