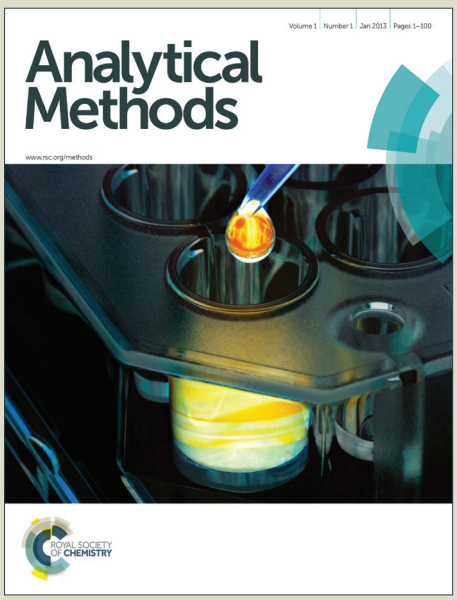
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Communications

Rapid assay of neopterin and biopterin in urine by wooden-tip electrospray ionization mass spectrometry

Bi-cheng Yang [+], Fa-ying Liu [+], Jiu-bai Guo, Lei Wan, Juan Wu, Feng Wang, Huai Liu*, Ou-ping Huang**

Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi, 330006, P. R. China.

* Corresponding author. Tel.: +86 791 86310570; fax: +86 791 86310570. E-mail: lh8830@126.com

** Corresponding author. Tel.: +86 791 86296519; fax: +86 791 86296519. E-mail:jxxs6519@126.com

+ Bi-cheng Yang and Fa-ying Liu contributed equally to the work, and should be considered as first authors.

Abstract A rapid and sensitive method based on wooden-tip electrospray ionization mass spectrometry (WT-ESI-MS) has been established for the quantitative detection of neopterin and biopterin in urine with only little sample preparation. The limit of detections (LOD) for the analysis of neopterin and biopterin were determined to be 30 ng mL^{-1} and 50 ng mL^{-1} (S/N≥3), respectively. Acceptable relative standard deviation (RSD) values (6.3~9.5%) and the recovery values (90~133.6%) were obtained for direct measurement of neopterin and biopterin in raw urine. Moreover, neopterin and biopterin were directly detected from 9 clinical urine samples by WT-ESI-MS. A single sample analysis was completed within a few minutes, indicating that the present WT-ESI-MS method is a promising strategy for the rapid analysis of clinical sample.

Keywords: neopterin, biopterin, WT-ESI-MS, urine

Neopterin (Neo) and biopterin(Bio) belong to a group of unconjugated pterins derived from guanosine triphosphate by guanosine triphosphate cyclohydrolase I. Neopterin and biopterin, occur normally in body fluids including urine. Neopterin is synthesized mainly by activated monocytes/macrophages after stimulation by the cytokine interferon-gamma (IFN-γ), which is released by natural killer cells and T-lymphocytes.[1] Neopterin is a useful biomarker for the intensity of the immune response mediated by Th-1 type cells. Biopterin is produced by nonenzymatic oxidation of tetrahydrobiopterin. Synthesis also takes place in cells such as T-cells, B-cells, endothelium, smooth muscle cells, fibroblasts, and potentially in liver and kidney.[2, 3] Recent research studies have focused on the detection and monitoring of concentration of neopterin and biopterin in human fluids as diagnostic markers for prognosis of a host of diseases and assessing treatment efficacy.[4-7] Increasing levels of biopterin and neopterin in human serum and urine were reported in patients with some cancers.[8] The ratio of urinary biopterin-to-neopterin in urine is an important marker for diagnosis of hyperphenylalaninemia, since hyperphenylalaninemia is caused not only by defective by phenylalanine 4 monooxygenase as an classical phenylketonuria (PKU), but also about 2% by tetrahydrobiopterin deficiency (BH4D) as atypical PKU,[9] these patients require different treatments.[10] Differential diagnosis is most commonly performed by analysis of urinary neopterin and biopterin.[11] Quantification of urinary neopterin and biopterin has been successful performed by high-performance liquid chromatography (HPLC) coupled with electrochemical, fluorescence or MS.[12-15] Due to the complicated matrix, extensive sample pre-treatment, including extraction, pre-concentration, and chromatographic separation, etc., which can take tens of minutes or even hours is usually required. The cost of consumables, such as consumable kits for extraction of samples and solvents for sample extraction and chromatographic separation, is also relatively high. Therefore, development of novel assay methods that are simple, rapid, accurate and sensitive is highly beneficial to quantification of urinary neopterin and biopterin.

Electrospray ionization mass spectrometry (ESI-MS) is a useful analytical tool for the analysis of complex mixtures, providing information on the molecular weights and chemical structures of the analytes. In conventional ESI, a sample solution is introduced into a capillary and usually with the assistance of gas. In the late 1990s, use of a copper wire as solid-substrate ESI emitter was firstly introduced by Shiea et al.[16] Recent non-capillary ESI techniques with solid substrates, such as metal needle,[17] wooden tip,[18] paper,[19]

aluminium foil,[20] and other solid materials[20-23] have been successfully developed as emitters for ESI and applied for analysis of various complex samples.

In this study, a simple wooden-tip ESI-MS (WT-ESI-MS) was adopted for the quantitative determination of neopterin and biopterin in urine. The disposable wooden tips (purchased from Nanchang supermarket) used are cheap, are readily available, and can be directly mounted on commercial nano-ESI ion source device; the angle between wooden tip and the MS inlet was ninety degrees like previous methods.[18, 25, 26] Briefly, sample solutions were loaded to the sharp tip-end by pipetting. Upon application of a high voltage (+3.5 kV) to the wooden tip, spray ionization was generated and mass spectrum was observed by a triple-quadrupole mass spectrometer (Waters xevo TQD). Furthermore, internal standard method by WT-ESI-MS for quantitation of trace amount of analytes in urine has been successfully confirmed in previous study.[26] Herein, the raw urine samples spiked with neopterin, biopterin, and tyrosine (as internal standard[27]) were diluted with methanol. An aliquot of 2 μL of the prepared sample solution was applied onto a wooden-tip for analysis. As shown in the MS spectrum (Fig. 1a) of raw urine with spiked neopterin (0.1 ng/mL), biopterin (0.1 ng/mL), and tyrosine (0.1 ng/ml), the predominant peaks are ions of urea and/or creatinine. Because concentrations of urea and creatinine are very high in urine,[28] resulting in intensities of protonated neopterin (m/z 254) and biopterin (m/z 238) are relatively weak, thus their peaks are hardly seen in the same scale. However, ions of m/z 254, 238 and 182 were selected for MS/MS studies. The main fragments recorded at m/z 254 (Fig.1b) and m/z 238 (Fig.1c) were similarly generated in MS/MS experiments by loss of H_2O or/and NH_2CN from protonated neopterin and protonated biopterin due to their similar structures, the fragmentation pathways were shown in inset of Fig. 1. These MS/MS data were in agreement with previous results.[27] The peaks at m/z 165 and 136 in MS/MS could be produced by loss of H_2O and HCOOH from the protonated tyrosine, respectively (Fig.1d). The MS/MS experiments of neopterin, biopterin, and tyrosine were also confirmed with authentic compounds (Sigma, St. Louis, MO, USA). Therefore, the experimental data show that trace amounts of neopterin and biopterin in the urine samples can be rapidly detected using WT-ESI-MS.

In selected-reaction monitoring (SRM) mode with selected reactions, m/z 254→206, m/z 238→220 and m/z 182→136 were used for quantification of neopterin, biopterin, and tyrosine, respectively.[27] The

calibration curves for quantitation of urinary neopterin and biopterin were constructed by averaging five sets of experimental data, while each set of data was obtained by applying samples containing different concentrations of the analytes (concentration of neopterin, biopterin in urine: 50, 100, 200, 500, 1000, 2000, 5000 ng mL^{-1}) and a fixed amount of the internal standard (concentration of tyrosine in urine: 200 ng mL^{-1}) onto disposable wooden tip. The internal standard was used to compensate variations in instrumental responses, which were mainly caused by different sample loadings and the use of different wooden tips. With tyrosine as the internal standard, the calibration curves of neopterin (Fig. 2a) and biopterin (Fig. 2b) were obtained with excellent linearities (R^2 >0.99). The linear regression equations for the concentration ranges of neopterin (50–5000 ng mL^{-1}) and biopterin (50–5000 ng mL^{-1}) were y = 0.0028x + 0.6689 and y = 0.0015x + 0.3235, respectively (n = 5). This linear range achieved is comparable to the HPLC-MS methods in previous studies.[13]

To investigate the accuracy and precision of the WT-ESI-MS method in rapid quantitation of urinary neopterin and biopterin, raw urine samples with spiked analytes in low, medium, and high concentration ranges were assayed. Each sample was determined five times using disposable wooden tip, and the data obtained were averaged for comparison. As shown in table. 1, the accuracy and precision determined were in the range of 90~133.6% and 6.3~9.5%, respectively, which were comparable to the HPLC-MS studies.[13] The LOD of the assay was estimated as three times the signal-to-noise ratio (S/N). The LOD determined for neopterin and biopterin were experimentally found to be 30 ng mL^{-1} and 50 ng mL^{-1}, respectively, which were suitable for assay of real samples.

Clinic urine samples, which were collected from patients (from Jiangxi Provincial Neonatal Screening Center, all patients signed an informed consent for research purposes), were investigated in this study under the approval by the Ethics Committee of Jiangxi Provincial Maternal and Child Health Hospital. As shown in the clinical data (Table 2), neopterin and biopterin were successfully detected in 9 hyperphenylalaninemic patients (6 with classical PKU; 3 with 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, the most common form of atypical PKU). Assay results of detection of Phe dried blood concentration by Neonatal Phenylalanine kits (PerkinElmer Wallac Inc, Turku, Finland) were shown in Table 2. All patients have increased Phe concentrations (i.e., > cut-off level of 120 μmol/L). Compared

with classical PKU patients, PTPS deficiency patients showed significant lower biopterin-to-neopterin ratios; this findings are in good agreement with reported values for similar patients.[29] In addition, previous study showed that the biopterin-to-neopterin ratio decreased with age and severity in Alzheimer's Disease patients.[30]

A rapid, sensitive method based on wooden-tip electrospray ionization mass spectrometry (WT-ESI-MS) has been established for the quantitative detection of neopterin and biopterin in urine with only little sample preparation. The wooden tips used in WT-ESI-MS are cost-effective, are readily available, and can be directly coupled to various commercial mass spectrometers. The sampling of this method is very simple and convenient. Wooden-tip ESI allows direct sample loading, ionization with little sample preparation prior to MS analysis, providing a rapid way to obtain quantitative information on the neopterin and biopterin content in the clinical urine. The analytical performances, including the linear range, accuracy, precision, LOD of the method were well acceptable for analysis of clinical urine samples. The single sample analysis was completed within a few minutes, indicating that the present WT-ESI-MS method is a promising strategy for the rapid analysis of clinical sample. Further investigation will be performed to further optimize this technique for analysis of more small molecules in clinical samples.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No.: 81460232).

References

1. G. Hoffmann, B. Wirleitner and D. Fuchs, Inflamm. Res., 2003, 52, 313-321.
2. P. Marsalek, M. Svoboda, M. Smutna, J. Blahova and V. Vecerek, J. Anim. Sci., 2011, 89, 1758-1762.
3. H. Fujioka, H. Shintaku, H. Nakanishi, T. J. Kim, S. Kusuda and T. Yamano, Brain. Dev., 2008, 30, 1-6.
4. L. Bonafe, B. Thony, W. Leimbacher, L. Kierat and N. Blau, Clin. Chem., 2001, 47, 477-485.
5. N. Longo, J. Inherit. Metab. Dis., 2009, 32, 333-342.
6. K. Azumagawa, S. Suzuki, T. Tanabe, E. Wakamiya, N. Kawamura and H. Tamai, Brain. Dev., 2003, 25, 200-202.
7. S. Ogiwara, T. Nagatsu, R. Teradaira, K. Fujita and T. Sugimoto, Biol. Chem., 1992, 373, 1061-1065.
8. H. Rokos, K. Rokos, H. Frisius and H. J. Kirstaedter, Clin. Chim. Acta, 1980, 105, 275-286.
9. D. M. Danks and R. G. H. Cotton, Enzyme, 1987, 38, 296-301.

10. Y. Matsubara, J. A. Heininger and Y. Y. Lin, Clin. Chem., 1984, 30, 278-280.

11. T. Opladen, B. Abu Seda, A. Rassi, B. Thony, G. F. Hoffmann and N. Blau, J. Inherit. Metab. Dis., 2011, 34, 819-826.

12. M. Zhang, S. Ding and G. Yi, Chin. J. Chromatogr., 2004, 22, 416-419.

13. J. Tomandl, J. Tallova, M. Tomandlova and V. Palyza, J. Sep. Sci., 2003, 26, 674-678.

14. M. Hibiya, R. Teradaira, T. Sugimoto, K. Fujita and T. Nagatsu, J. Chromatogr. B, 1995, 672, 143-148.

15. W. E. Slazyk and F. W. Spierto, Clin. Chem., 1990, 36, 1364-1368.

16. C. M. Hong, C. T. Lee, Y. M. Lee, C. P. Kuo, C. H. Yuan, J. Shiea. Rapid Commun. Mass Spectrom., 1999, 13, 21–25.

17. K. Hiraoka, K. Nishidate, K. Mori, D. Asakawa and S. Suzuki, Rapid Commun. Mass Spectrom., 2007, 21, 3139-3144.

18. B. Hu, P.-K. So, H. Chen and Z.-P. Yao, Anal. Chem., 2011, 83, 8201-8207.

19. J. Liu, H. Wang, N. E. Manicke, J.-M. Lin, R. G. Cooks and Z. Ouyang, Anal. Chem., 2010, 82, 2463-2471.

20. B. Hu, P. K. So and Z. P. Yao, Anal. Chim. Acta, 2014, 817, 1-8.

21. J. J. Liu, H. Wang, R. G. Cooks and Z. Ouyang, Anal. Chem., 2011, 83, 7608-7613.

22. B. Hu, Y. H. Lai, P. K. So, H. Chen and Z.-P. Yao, Analyst, 2012, 137, 3613-3619.

23. H. K. Chen, C. H. Lin, J. T. Liu and C. H. Lin, Int. J. Mass Spectrom., 2013, 356, 37-40.

24. B. Hu, L. Wang, W. C. Ye and Z. P. Yao, Sci. Rep., 2013, 3. 2104.

25. B. Hu, P. K. So and Z. P. Yao, J. Am. Soc. Mass Spectrom., 2013, 24, 57-65.

26. P.-K. So, T. T. Ng, H. Wang, B. Hu and Z. P. Yao, Analyst, 2013, 138, 2239-2243.

27. S. Bourcier, J. F. Benoist, F. Clerc, O. Rigal, M. Taghi and Y. Hoppilliard, Rapid Commun. Mass Spectrom., 2006, 20, 1405-1421.

28. J. L. Pezzaniti, T. W. Jeng , L. McDowell, G. M. Oosta, Clin Biochem., 2001, 34, 239-46.

29. N. Blau, J. B. Hennermann, U. Langenbeck and U. Lichter-Konecki, *Mol. Genet. Metab.*, 2011, **104** Suppl, S2-9.

30. R. A. Armstrong, R. J. Cattell, S. J. Winsper and J. A. Blair, *Pteridines*, 1995, **6**, 185-189.

Figure Captions

Fig.1 a) Mass spectrum of raw urine with spiked neopterin (0.1 μg mL^{-1}), biopterin (0.1 μg mL^{-1}), and tyrosine (0.1 μg mL^{-1}); MS/MS mass spectra obtained for 0.1 μg mL^{-1} of (b) neopterin (c) biopterin and (d) tyrosine in raw urine, inserted structures shows the fragmentation pathways of these ions.

Fig. 2 Calibration curves obtained for (a) neopterin and (b) biopterin in urine

Figures

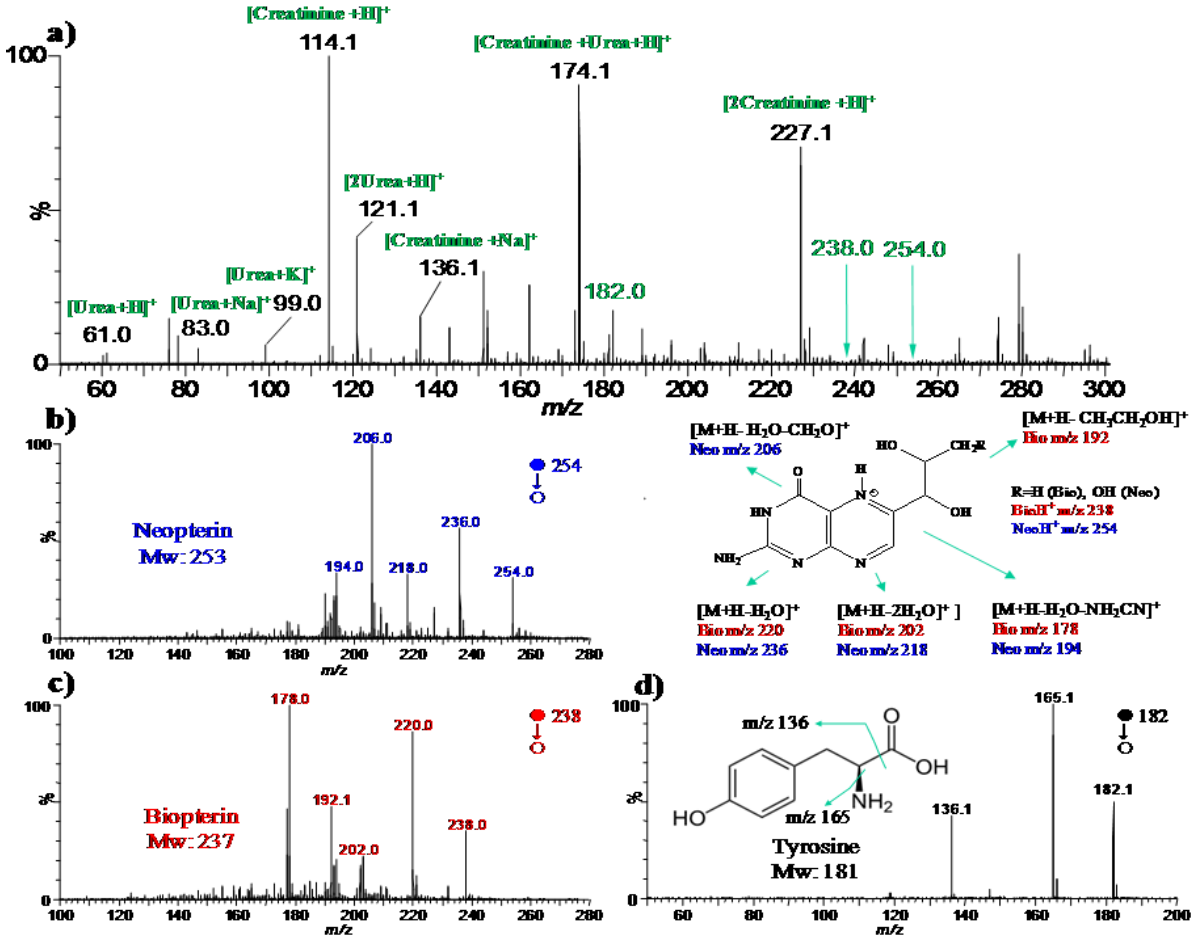


Fig. 1

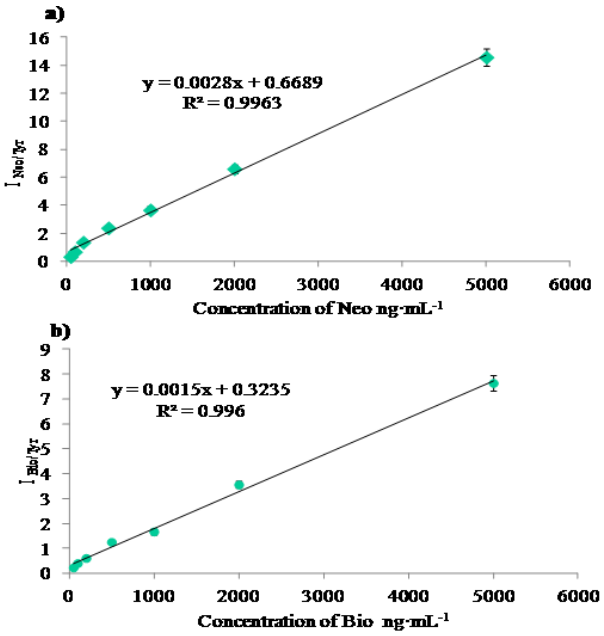


Fig. 2

Tables

Table 1. Experimental data for determination of accuracy and precision of the WT-ESI-MS method in quantitation of Neo and Bio in urine

Spiked ng mL^{-1}	Detection ng mL^{-1}		Accuracy %		RSD (n=5)	
	Neo	Bio	Neo	Bio	Neo	Bio
100	133.6±8.2	90.3±9.5	133.6	90	8. 2	9.5
600	645.8±48	682.6±52	107.5	113.7	7.5	7.6
3000	2780±220	3320.6±209	92.7	110.6	7.9	6.3

Table 2. assay results for urine specimens from nine patients with Hyperphenylalaninemia

Diagnosis	Age (years)	Bio ng mL^{-1} (n=3)[a]	Neo ng mL^{-1} (n=3)[a]	Ratio of Bio to Neo	Phe μmol L^{-1}[b]
Classical PKU	4	95.2±5.7	112.1±25.6	0.85	311
Classical PKU	3	303.7±32.3	197.6±66.4	1.54	864
Classical PKU	4	101.7±11.1	135.4±30.4	0.75	326
Classical PKU	1	140.1±19.2	190.3±51.2	0.74	407
Classical PKU	5	83.2±10.9	154.9±28.8	0.54	302
Classical PKU	7	59.7±5.4	86.7±17.7	0.69	187
PTPS	9	55.4±9.1	120.0±24.1	0.46	216
PTPS	11	52.1±10.7	193.3±31.9	0.27	337
PTPS	14	52.0±7.6	182.3±5.9	0.29	198

[a] obtained by WT-ESI-MS.
[b] Phe dried blood concentration was obtained by Neonatal Phenylalanine kits.