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Short communication

Development of rat urinary HPLC-UV profiling for metabonomic study on Liuwei Dihuang Pills

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ABSTRACT

Since urine contains thousands of metabolites, there are no universal analytical techniques to analyze these compounds simultaneously. Therefore, a readily obtained and simple sample pretreatment high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection urinary profiling with good reproducibility was developed for metabonomic study in this paper. The results of current study clearly showed that there were different phenotypes of metabolites based on HPLC-UV urinary profiling after administration of Liuwei Dihuang Pills (LWPs) or Carrageenan-stimulated inflammation model, and those could be conveniently discriminated by principal component analysis (PCA). In addition, the results also showed that LWPs could restore the metabolite network that disturbed by inflammation, which would be a proof of therapeutic efficacy of LWPs to inflammation by metabonomics study. Our study indicated that HPLC-UV method could be used as a potentially powerful tool for urinary profiling on metabonomic studies.

1. Introduction

Metabonomics is a broad new System Biology technology after "genomics" and "proteomics". It is a non-targeted global analysis of tissues or biofluids for organic metabolites of low molecular mass, and to reveal in the patterns of metabolic markers associated with toxic responses to drug therapies, pathological changes, disease diagnosis and ecology [1,2]. In China, Traditional Chinese Medicine (TCM) has been practised for thousands of years and shows the advantages over western medicine in the treatment of some multi-gene and multi-factor diseases [3]. Since TCM is based on "holism" philosophy instead of "reductionism", it is philosophically conceivable that metabonomic techniques may play some roles in providing important information to the TCM in an integrated manner [4]. However, there were few reports on TCM study based on this technology.

In order to optimize and utilize metabonomics, good reproducibility of metabolite profilings must be readily available. High-resolution ^{1}H NMR spectroscopy was extensively used as it permits rapid, multi-component analysis of samples with minimal sample pretreatment [5,6]. More recently, high-performance liquid chromatography (HPLC)–MS and GC–MS have been employed as useful tools with great separation capacity and higher sensitivity when compared with NMR [7–9]. It was generally accepted that there was not a single analytical technique could provide all information of metabolites in urine for its complex nature. Ultraviolet (UV) detection is generally available to a great population of scientists and perhaps the most rugged technique, which makes it an attractive alternative for urine profiling. There have been some reports about the influences of metabolism by rats on herbs [10,11] and urinary metabolites target analysis [12] using HPLC-UV method. However, to the best of our knowledge, with no prior knowledge of the analyzed compounds, it has not been applied for discrimination among urinary profilings for the purpose of metabonomic study.

In the present study, rat HPLC-UV urinary profiling with simple sample preparation courses and good profile reproducibility was developed. In order to investigate the possible potential for metabonomic study on TCM, urinary profilings before and after administration of Liuwei Dihuang Pills (LWPs) and/or injection of Carrageenan as inflammation model were readily obtained. After peak alignment, the entire chromatographic data were normalized by four algorithms and performed to discriminate among samples from different treatments by principal component analysis (PCA).

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2. Experimental

2.1. Materials and LWPs preparation

LWPs were purchased from Tongrentang Group Co., Ltd. (Beijing, China). Carrageenan was purchased from Sigma Chemical Co. (USA). Methanol (HPLC grade) was from Ludu Chemical Factory (Shanghai, China). 105 g of grinded powder of LWPs were accurately weighted and extracted ultrasonically by 300.0 mL 50% (v/v) alcohol–water solution for 30 min. This extraction was repeated twice and the extracted solution was mixed. The solution was filtered and evaporated under vacuum at 55 °C, then 150.0 mL water was added to the dried residues and the suspension of LWPs (0.70 g/mL) was obtained.

2.2. In vivo experiments and urine sample preparation

20 male Sprague–Dawley rats weighing 180–210 g, were kept in metabolism cages individually, under controlled humidity (30–70%) and temperature (22 ± 2 °C) with a 12 h light–dark cycle. After being accommodated to environment for 3 days, rats were randomly divided into four groups as follows: (1) Control group received intragastric administration of water (1.0 mL) once a day at 9.00 a.m. for 3 successive days. (2) Normal LWPs-treated group received administration of LWPs (1.0 mL, 3.5 g/kg) once a day for 3 successive days. (3) LWPs-treated inflammation group received administration of LWP (1.0 mL, 3.5 g/kg) once a day for 3 successive days and subcutaneous injection of 0.1 mL 1.0% Carrageenan (a pro-inflammatory agent, dissolved in 0.9% NaCl) in the sub-plantar region of hind paw at 9.00 a.m. on the third day. (4) Untreated inflammation group received administration of water (1.0 mL) once a day for 3 successive days and injection of Carrageenan. 12-h urine samples were collected at 9.00 p.m. without any treatments as normal variation. Samples of other groups were collected on the third night 0–12 h after administration. Rats had free access to food (Standard rat feeds, Sichuan University, China) and water during the experimental period.

The collected urine samples were centrifuged immediately at 5,000 rpm for 10 min at 10 °C, and 1.0 mL of the supernatant was frozen at −40 °C before being lyophilized by the Savant ModulyoD (Thermo Savant, USA) for 24 h. Then the dried samples were extracted ultrasonically by 1.0 mL methanol for 2 min. The formed suspension was centrifuged at 14,000 rpm for 10 min at 10 °C. Finally, the sample for HPLC injection was obtained by diluting 0.2 mL supernatant with 1.0 mL water and kept at −20 °C until analysis.

2.3. Rat urinary HPLC-UV profiling acquisition

HPLC-UV profiling of rat urine was acquired on a Varian series ProStar HPLC system (Varian, USA) which consists of a ProStar 210 solvent delivery module and a photodiode array detector (DAD). The chromatographic separation of samples was achieved by a reversed-phase HPLC column (Hypersil ODS C18, 250 mm × 4.6 mm, 5 μm particle size, Elite, Dalian, China) protected by a pre-column (Chromguard C18 column, Varian, USA). The sample injection volume was 20 μl and the column was maintained at 40 °C. Solvent A consisted of 5.0 mM ammonium acetate (adjusted to pH 4.0 with formic acid), while Solvent B was methanol. The mobile phase flow rate was 0.8 mL/min and the gradient elution conditions was 0–5 min, isocratic 5% B; 5–30 min, linear gradient 5–80% B; 30–31 min, linear gradient 80–90% B; 31–38 min, isocratic 90% B.

2.4. Peak alignment and data preprocessing for PCA

The obtained data is a matrix for samples. The row of dataset represents UV-intensity (variable) at one point in retention time, and one column represents a sample. A typical chromatogram in dataset (Fig. 1A) was selected and 18 common peaks of all samples were aligned according to it as reference by the *Computer Aided Similarity Evaluation System*, and a new chromatographic profiling dataset (6000 × 30) for samples would be reconstructed by cubic spline data interpolation technique [13–15]. Four algorithms for data normalization in each column were listed as follows [16]: (1) $x_{j,\mathrm{new}} = x_{i,\mathrm{old}}/\mathrm{sum}(x_{j,\mathrm{old}})$, constant sum method (CS). (2) $x_{j,\mathrm{new}} = x_{j,\mathrm{old}}/x_{j,\mathrm{max}}$, maximum method (MX). (3) $x_{j,\mathrm{new}} = (x_{j,\mathrm{old}} - m_j)/V_j$, auto-scaled method (AS). (4) $x_{j,\mathrm{new}} = (x_{j,\mathrm{old}} - x_{j,\mathrm{min}})/(x_{j,\mathrm{max}} - x_{j,\mathrm{min}})$, range method (RG). Where x_j represents one variable in one column, m_j and V_j are the mean and variance of the one column data. All routines for data preprocessing and PCA in this study were performed under the MATLAB 6.5 environment (The MathWorks Inc., USA).

3. Results and discussion

3.1. Optimization of urine pretreatment method

A problem associated with analysis of urine is that it contains varieties of materials including diverse cells, macromolecules such as protein and polypeptide, and thousands of compounds of different polarities. Therefore, in order to remove macromolecules and obtain low molecular mass organic metabolites in urine that can be retained on reversed-phase stationary phases such as C_{18} column for separation, solid-phase extraction (SPE) was commonly used prior to HPLC-based method analysis [17]. However, it is a high-cost and tedious process for routine analysis.

In our work, to achieve the above-mentioned aims, urine samples were freeze-dried and the dry residues were extracted by organic solution. Different solvent systems, including methanol, acetoacetate and acetone were employed to optimize the extraction condition and enhance the extraction efficiency. Generally, a good extraction solvent was able to obtain more peaks in the chromatogram and the results suggested that methanol was a better choice (Fig. 1A).

For a urinary profiling-based metabonomics study, the metabolic profile becomes more descriptive with the increased number of reproducible analytes. Although freeze-drying might produce the loss of some volatile compounds, in our study, more peaks could be obtained in a single run using freeze-dried coupled with methanol extraction method, also, with easy operation and non-destruction to urine advantages when comparing with SPE (Fig. 1D). The pretreatment method is equally applicable to other HPLC-based profilings such as HPLC (UPLC)-DAD (MS/MS) for metabonomic study.

3.2. Optimization of chromatographic conditions

In this work, optimization of parameters in HPLC was achieved through investigating the influences of the mobile phase and detection wavelength. Methanol or acetonitrile was chosen as the mobile phase. Considering the complex of the urine sample, buffer solution was employed in the mobile phase to reduce the ionization and polarity of some compounds. Three concentration levels (20 mM, 10 mM and 5 mM) and three pH values (2.5, 4.0, 6.0) of ammonium acetate with methanol or acetonitrile were investigated. The results showed that more peaks and higher resolution could be attained using methanol with 5 mM ammonium acetate (pH 4.0) as mobile

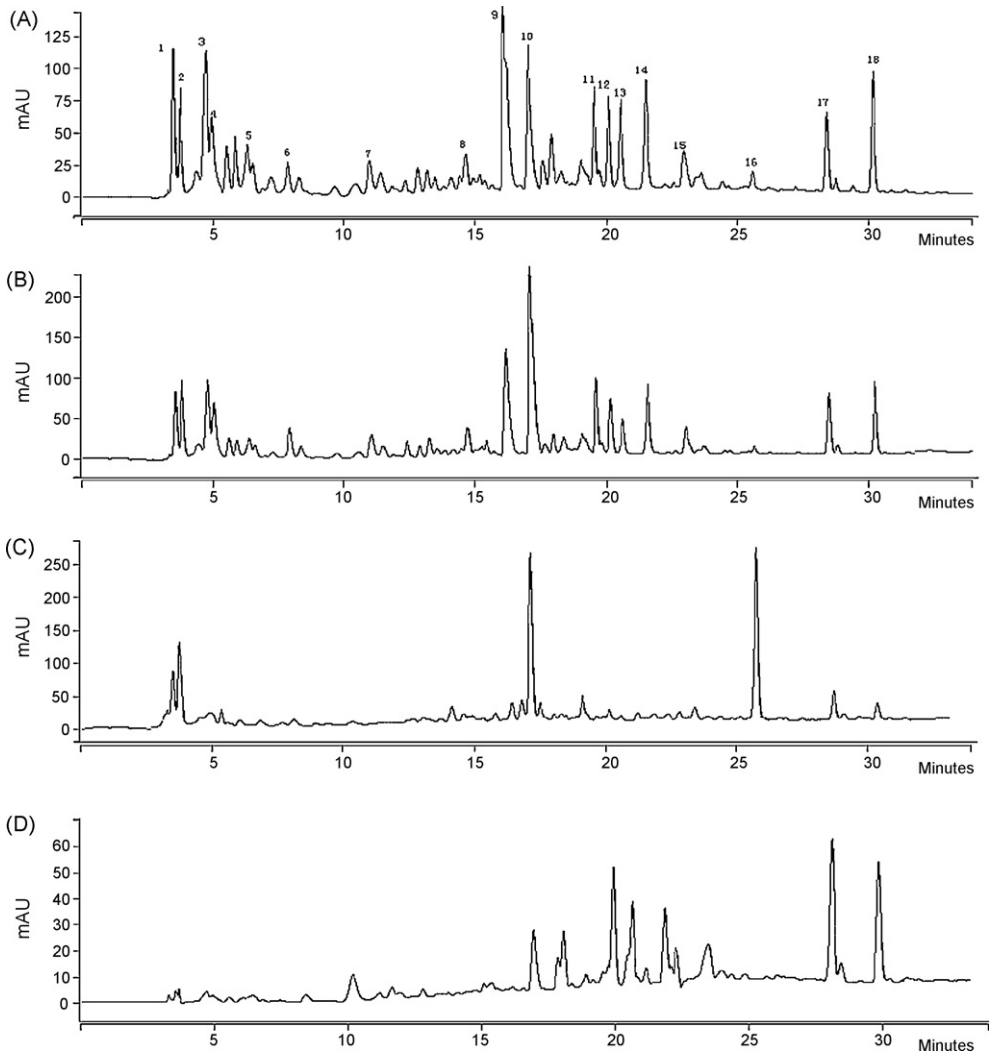

Fig. 1. Typical HPLC-UV chromatogram of rat urine. Urine sample collected before any treatment was freeze-dried and extracted by methanol before analysis. (A) Detected wavelength setting at 265 nm. (B) Detected wavelength setting at 254 nm. (C) Detected wavelength setting at 238 nm. (D) Urine sample was extracted by SPE. Chromatographic condition as described in Section 2.3.

phase (data not shown). In order to obtain a sufficient number of detectable and stronger peaks on the HPLC chromatogram, the spectra of all peaks in the chromatogram were acquired with DAD and 265 nm was selected as detection wavelength at which there were the most amount of effective peaks (number of theoretical plates >1000, Fig. 1). The optimal condition was presented in detail in Section 2.3.

3.3. Methodology validation

Method precision was based on analysis of the sample solution for five times. The relative standard deviation (R.S.D.) values of 18 common peaks (Fig. 1A) height and retention time were better than 2.6% and 1.0%, respectively. The repeatability was assessed by analyzing five independently prepared samples from one urine sample. R.S.D. values of 18 common peaks height and retention time were lower than 4.0% and 1.2%, respectively. Sample stability for metabonomic study was critically important and storage stability of samples under different storage conditions (3 days at 4 °C, 1 month at −20 °C) was investigated. The corresponding R.S.D. of peaks height and retention time of 18 common peaks were 6.0% and 4.0%, respectively.

In addition, to understand reproducibility of the urinary profiling from different rats, 12-h morning and night spot urine samples were collected from diurnal variation groups without any treatments. No new peak appeared or disappeared in chromatograms of the urine samples from different rat. The relationship among a set of chromatographic fingerprints could be analyzed through comparisons in terms of similarity of the objects with a certain reference, and correlation coefficient is the most commonly used standards for evaluation of similarity of the multivariate systems [15]. Average similarities of 10 different samples randomly selected in normal variation group were above 0.80. The above results demonstrated that the sample pretreatment and HPLC separation conditions could be successfully applied to rat urine profiling for metabonomic study with ease of sample preparation and good profiling reproducibility.

3.4. Application of metabonomic study to LWPs

LWPs is a classical TCM prescription and was widely used for hundreds years in preventing and curing many diseases, involving diabetes, dizziness, inflammation, etc. [18], but the effective constituents and pharmacologic mechanism are still undefined. As

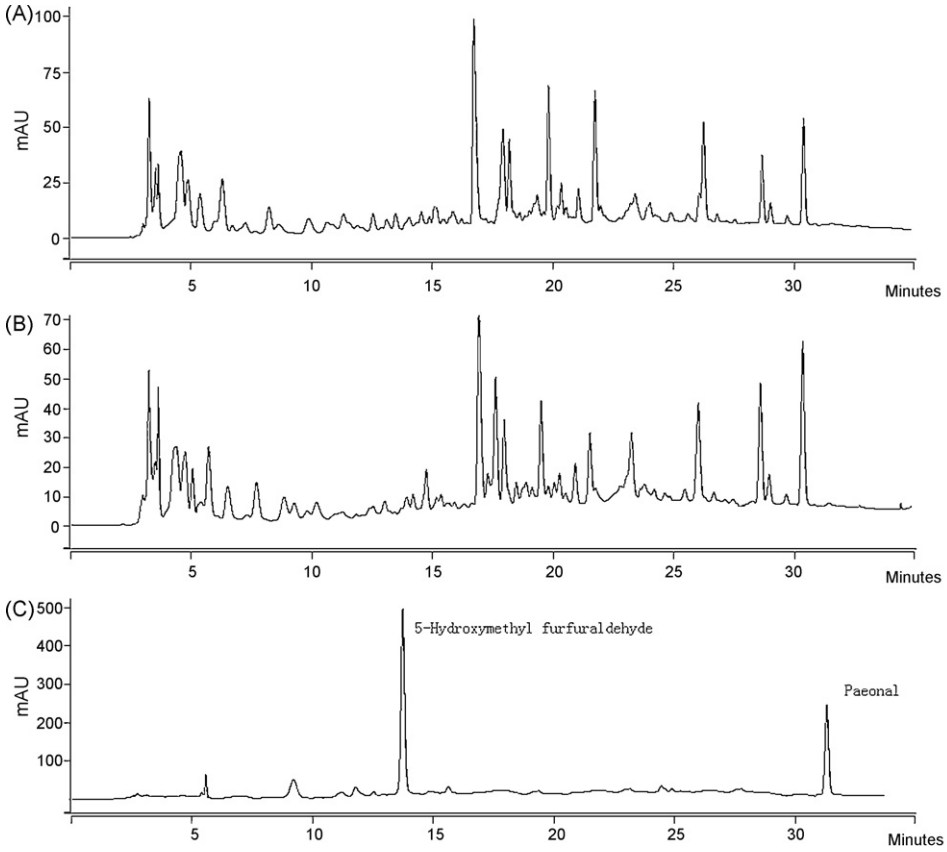

Fig. 2. HPLC-UV chromatogram of rat urine collected before and after oral-administered LWPs. Chromatographic condition as Section 2.3. (A) Before LWPs administered, (B) after LWPs administered and (C) the fingerprint of LWPs administered to rat.

a "proof of principle" study for TCM, the method developed was applied to the profiling of rat urine after oral administration of LWPs. Since most of compounds administered in LWPs such as Paeonol were less than 0.1 mg under the dosage which was suggested by manufacturer and their low oral bioavailability [19], there were few clearly detectable peaks of original compounds and/or their metabolites (Fig. 2B and C) after LWPs treatment. However, as shown in Fig. 2, we could see that the perturbations to rat urinary metabolites after LWPs administered were mainly from the differences of relative intensities of some common peaks. In addition, to reveal the possible influences of LWPs in inflammation state based on HPLC-UV urinary profiling, Carrageenan-induced inflammation model was established [20] and the interferences of LWPs were investigated in this study. Obviously, there were some differences between urinary profiling of inflammation and that of normal rat (Fig. 3). The variations of analyte concentration

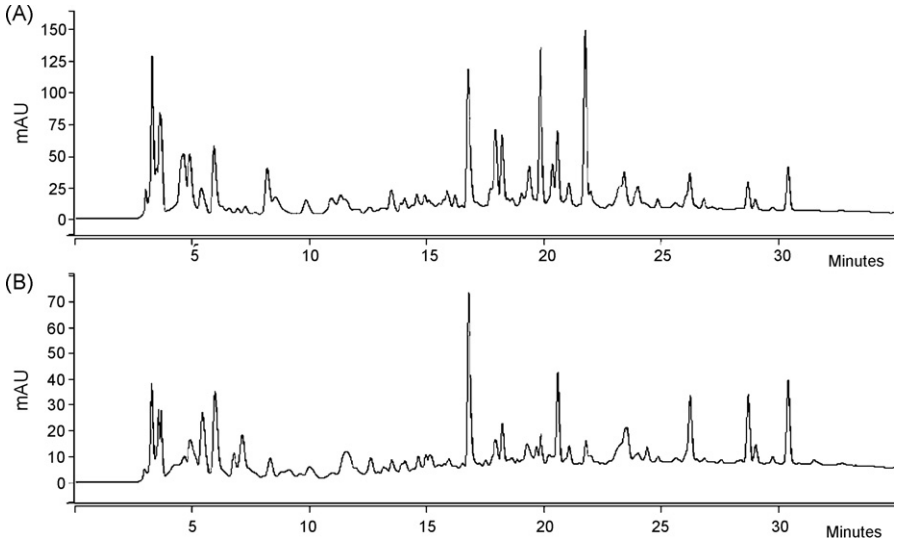

Fig. 3. HPLC-UV chromatogram of urine collected before and after injection of 1.0% Carrageenan as an inflammation model. Chromatographic condition as Section 2.3. (A) Before injection and (B) after injection.

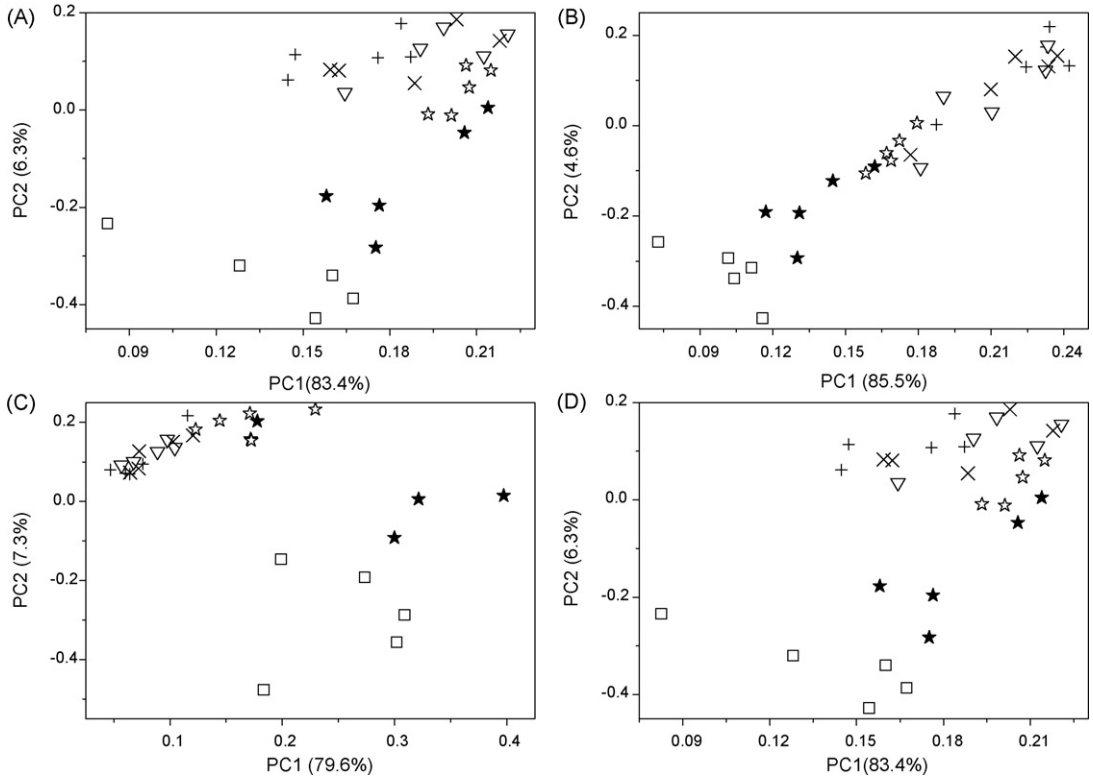

Fig. 4. Scores plot of PCA based on four algorithms for data normalization. (A): MX-normalized data. (B) CS-normalized data. (C) AS-normalized data. (D) RG-normalized data (+) Urine samples collected from rat without any treatment in daytime period. (×) Urine samples collected from rat without any treatment in nighttime period. (▽) Control group received administration of water (1.0 mL). (☆) Normal LWPs-treated group received administration of LWPs (1.0 mL, 3.5 g/kg). (★) LWPs-treated inflammation group received administration of LWP (1.0 mL, 3.5 g/kg) and subcutaneous injection of Carrageenan. (□) Untreated inflammation group received administration of water (1.0 mL) and injection of Carrageenan.

in urine by LWPs/Carrageenan stimuli, indicated that there were probably different phenotypes of metabolites. To probe the relationship between these urinary profilings from different groups, pattern recognition method such as PCA should be employed [21].

3.4.1. Peak alignment and data preprocessing

Recently, to achieve PCA, more attention has been paid to data-analysis methods in entire chromatogram. In this study, retention time shifts which could not be neglected in HPLC [22,23] were aligned as described in Section 2.4 after these common peaks checked by DAD data. Data normalization of each sample was commonly used to modify data derived from all samples and make them directly comparable with each other. There were many algorithms for data normalization and CS method by normalizing each point to the total sum of the variables were extensively used for removing or minimizing infinite dilution effect on the analytes such as urine [24]. In our study, in order to obtain an optimum separation of various sample classes, four data normalization algorithms of each observation were employed for classification of different samples by PCA.

3.4.2. Classification of samples

The consequences calculated by four normalization algorithms were used for PCA, the results were shown in Fig. 4. In these scores plots, samples were mapped in the space spanned by the first two principal components PC1 versus PC2 as they could describe over 80% of the variances from the original data. From these scores plots, we could see that diurnal variation and control group samples were almost clustered together in the same region and were away from other groups, which indicated the metabolite phenotypes based on HPLC-UV in normal states were similar to each other.

As shown in Fig. 4, urine samples collected from rats after LWPs treatment were clustered together as a group and exhibited a separation from samples collected before administration as well as control group. The result suggested that the variations of analyte concentration in urine caused by LWPs stimulus were greater than those without treatment. Furthermore, inflammation group was far away from other groups but the group of inflammation treated by LWPs was closer to normal groups in scores plot. These results obviously indicated that diversities of urinary profiling by inflammation stimulus were different and greater than those by LWPs. It was more important that LWPs could restore the metabolite network which was disturbed by inflammation based on HPLC-UV urinary profilings, which would be a proof for therapeutic efficacy of LWPs to inflammation convinced by metabonomics study. When comparing different data preprocessing methods, nearly the same classification results were obtained (Fig. 4). Relatively, MX and RG method were better to classify different groups than others. Because the CS method changed the correlation to original data, it got the poorest results in classification. This effect in "closed" data sets has been discussed previously [24]. The results of our study also indicated that normalization of urinary metabolic data should be best considered as a data transformation which minimizes inter-sample variations due to differences in gross urinary concentration among samples.

The aim of this paper was development of a general and readily obtained HPLC-UV method to enable discrimination of metabolic urine profiles before and after LWPs stimuli with no prior knowledge of compounds being analyzed. The results clearly showed that based on HPLC-UV urinary profiling and PCA, different phenotypes of metabolites after treatment of LWPs/Carrageenan stimuli could be recognized and discriminated. It is perfectly possible to detect these differences of phenotypes of metabolite by chemo-

metrics approaches. Thus, with the help of semi-preparative HPLC and HPLC-DAD–MS/MS as well as NMR, some critical peaks responsible for classification by different phenotypes of metabolites based on HPLC-UV profiling were successfully isolated and identified by our group. And we used those biomarkers as cues to investigate the biochemical response of rat to LWPs, explore the possible pharmacological mechanism of LWPs and which will be described in another report.

4. Conclusion

Metabonomics has been demonstrated enormous potential in furthering the understanding of disease processes, toxicological mechanisms, biomarkers discovery and so on. Obviously, development of rapid, advanced analytical tools to comprehensively profile biofluid metabolites such as urine is highly appreciated. Since urine contains thousands of metabolites, there are no universal analytical techniques to analyze these compounds simultaneously. Therefore, in this study, a HPLC-UV urinary profiling method with simple sample pretreatment and good reproducibility was developed for metabonomic study. The results showed that combined with PCA, HPLC-UV method could be used as a potentially analytical tool in metabonomic studies as others for recognization and discrimination of different phenotypes of metabolites produced by stimuli of LWPs/Carrageenan.

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References

[1] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Nat. Rev. Drug Discov. 1 (2002) 153–161.
[2] D.G. Robertson, M.D. Reily, J.D. Baker, J. Proteome Res. 6 (2007) 526–539.
[3] C.T. Keith, A.A. Borisy, B.R. Stockwell, Nat. Drug Rev. Discov. 4 (2005) 71–78.
[4] J. Qiu, Nature 448 (2007) 126–128.
[5] E. Holmes, T.M. Tsang, S.J. Tabrizi, NeuroRX 3 (2006) 358–372.
[6] K.E. Price, S.S. Vandaveer, C.E. Lunte, C.K. Larive, J. Pharm. Biomed. Anal. 38 (2005) 904–909.
[7] I.D. Wilson, R. Plumb, J. Granger, H. Major, R. Williams, E.M. Lenz, J. Chromatogr. B 817 (2005) 67–76.
[8] J. Yang, G.W. Xu, Y.F. Zheng, H.W. Kong, C. Wang, Z.J. Zhao, T. Pang, J. Chromatogr. A 1084 (2005) 214–221.
[9] X.Y. Wang, M.M. Su, Y.P. Qiu, Y. Ni, T. Zhao, M.M. Zhou, A.H. Zhao, S.L. Yang, L.P. Zhao, W. Jia, J. Proteome Res. 6 (2007) 3449–3455.
[10] J.L. Zhang, M. Cui, Y. He, H.L. Yu, D.A. Guo, J. Pharm. Biomed. Anal. 36 (2005) 1029–1035.
[11] X.N. Li, C.H. Huo, Q. Wang, X.W. Zhang, X.N. Sheng, L.T. Zhang, J. Pharm. Biomed. Anal. 45 (2007) 268–274.
[12] J. Yang, G.W. Xu, Y.F. Zhang, H.W. Kong, T. Pang, S. Lv, Q. Yang, J. Chromatogr. B 813 (2004) 59–65.
[13] F. Gong, Y.Z. Liang, Y.S. Fung, F.T. Chau, J. Chromatogr. A 1029 (2004) 173–183.
[14] F. Gong, Y.Z. Liang, P.S. Xie, F.T. Chau, J. Chromatogr. A 1002 (2003) 25–40.
[15] Y.Z. Liang, P.S. Xie, K.J. Chan, J. Chromatogr. B 812 (2004) 53–70.
[16] Y.L. Wang, E. Holmes, J.K. Nicholson, O. Cloarec, J. Chollet, M. Tanner, B.H. Singer, J. Utzinger, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 12676–12681.
[17] H. Idborg, L. Zamani, P.O. Edlund, I.S. Koistinen, S.P. Jacobsson, J. Chromatogr. B 828 (2005) 9–13.
[18] W. Nie, Y. Zhang, J. Herb. Pharmacol. Chin. J. Herb. Parmacol. Ther. 14 (1998) 41–44.
[19] C.M. Riley, T.C. Ren, J. Chromatogr. 489 (1989) 432–437.
[20] P. Arumugam, N.G. Priya, M. Subathra, A. Ramesh, Environ. Toxicol. Phar. 26 (2008) 92–95.
[21] Z. Ramadan, D. Jacobs, M. Grigorov, S. Kochhar, Talanta 68 (2006) 1683–1691.
[22] D.K.W. Mok, F.T. Chau, Chemom. Intell. Lab. Syst. 82 (2006) 210–217.
[23] C. Malmquist, R. Danielsson, J. Chromatogr. A 687 (1994) 71–88.
[24] A. Craig, O. Cloarec, E. Holmes, J.K. Nicholson, J.C. Lindon, Anal. Chem. 78 (2006) 2262–2267.