

Metabolic profile associated with glucose and cholesterol lowering effects of berberine in Sprague–Dawley rats

Zhangjian Jiang · Feng Liu · Eng Shi Ong ·
Sam Fong Yau Li

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Abstract In this study, we present an integrated strategy to deconvolute the metabolic signatures associated with the cholesterol lowering effect of berberine in the livers of Sprague–Dawley rats. The rats were dosed with berberine at 50 mg/kg. Urine samples and liver tissues were collected for the analysis of metabolite contents, while livers and kidneys were collected for histopathology. Metabolites such as fatty acids, cholesterol, glucose and others in liver were analyzed by gas chromatography/mass spectrometry. The urinary metabolites were analyzed using targeted profiling with liquid chromatography/tandem mass spectrometry and non-targeted profiling with proton nuclear magnetic resonance (^1H NMR). Our results demonstrated that analysis of metabolites in rat urine samples using liquid chromatography/mass spectrometry (LC/MS) and ^1H NMR produced complementary, consistent and reliable results. The administration of berberine resulted in a reduction of glucose, maltose, fatty acids (saturated and unsaturated) and cholesterol in the rat liver samples. The analysis of urinary metabolic profiles on different days showed that before the cholesterol reduction in the rat livers, a high rate of carbohydrate usage was found to be an early event (day 2). The results suggested that the animals

utilized alternative energy sources by altering the synthesis and consumption of amino acids and fatty acids. In addition, changes in the level of glutamine for the treated animals on day 2 suggested that glutamine and glutamate metabolism could be affected. Since glutamine is a precursor for nucleotides synthesis and nucleotides are required for cell growth and replication, the results are consistent with the observed cholesterol lowering effect and weight reduction. Finally, our results demonstrated that the combination of LC/MS and ^1H NMR provided a unique metabolic profile associated with the cholesterol lowering effect of berberine in rat livers.

Keywords Berberine · LC/MS · GC/MS · ^1H NMR · Metabolic profile · Glucose/cholesterol lowering

1 Introduction

Berberine, an isoquinoline alkaloid of the protoberberine type, is derived from the root, rhizome and stem bark of many plant species such as *Coptis chinensis* Franch., *Coptis japonica* Makino., *Berberis thunbergii* DC., *Hydrastis canadensis* L., and *Thalictrum lucidum* Ait. Berberine is commonly used in traditional Chinese medicine due to its wide distribution all over the world and various therapeutic effects (National Commission of Chinese Pharmacopoeia 2005). Multiple pharmacological properties were attributed to berberine, such as anti-diabetic (Sach and Froehlich 1982; Yin et al. 2008; Zhou et al. 2008), anti-microbial (Yan et al. 2008), anti-inflammatory (Kuo et al. 2004), anti-cancer (Tan et al. 2006). At the same time, it was reported to be a cholesterol lowering drug working through a unique mechanism distinct from statins (Kong et al. 2004). Berberine lowered total cholesterol by

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Z. Jiang · F. Liu · S. F. Y. Li (✉)
Department of Chemistry, National University of Singapore,
3 Science Drive 3, Singapore 117543, Republic of Singapore
e-mail: chmlifys@nus.edu.sg

E. S. Ong
Department of Epidemiology and Public Health, Yong Loo Lin
School of Medicine, National University of Singapore, MD3,
16 Medical Drive, Singapore, Republic of Singapore

29% and low-density lipoprotein (LDL)-cholesterol by 25% in 91 hypercholesterolemic patients after 3 months treatment with 500 mg berberine given orally twice a day. The mechanism of lowering circulating cholesterol proposed by Kong et al. (2004) was an increase in hepatic LDL receptors, increasing LDL-cholesterol hepatic uptake and catabolism.

Previous studies showed that berberine could be a cholesterol lowering drug acting through the stabilization of the low-density lipoprotein receptor messenger RNA (Zhou et al. 2008; Kong et al. 2004, 2008). It was found to increase mRNA and proteins as well as function of hepatic low-density lipoprotein receptor in human hepatoma derived cell lines. Treatment of hyperlipidemic animals such as hamster with berberine by oral administration for 10 days resulted in dose dependent decreases in serum total cholesterol and low-density lipoprotein cholesterol (Kong et al. 2004). Berberine was also found to restore the increased blood glucose, total cholesterol, triglyceride, low density lipoprotein—cholesterol in diabetic rats to near control values.

Berberine has also been found to result in a reduction of cholesterol in the rat liver samples collected (Kong et al. 2008). The liver plays an important role in the whole-body lipid metabolism by affecting the synthesis, oxidation, transport and excretion of lipids. Cholesterol is one of the important regulators of lipid organization and mammals have developed sophisticated and complex mechanism to maintain cellular cholesterol levels in membranes within a narrow range. However, the structure of cholesterol is very different from that of other membrane lipids as it may help to stabilize boundaries between coexisting lipid domains. The lipid components of biological membranes are important for normal cell function and their improper distribution or metabolism can have serious effects for cells and organisms (Goldstein and Brown 2001; Strott and Higashi 2003; Maxfield and Tabas 2005). Together with cholesterol, fatty acids can exist in its free form and are found as fatty acyl esters in complex molecules such as triacylglycerol. Free fatty acids can be oxidized by the liver to provide energy. Polyunsaturated free fatty acids participate in normal functioning of the cell which include the control of intracellular cell signaling (Goldstein and Brown 2001; Szachowicz-Petelska et al. 2007). A decrease in glucose and polyunsaturated fatty acids, an increase in lipid triglycerides and mono-unsaturated fatty acids were observed in mouse livers as a result of drug induced hepatotoxicity (Coen et al. 2003; Waters et al. 2005, 2006). Similarly, an increase in fatty acid accumulation and decrease in glucose storage, gluconeogenesis and cholesterol synthesis was observed in livers samples obtained from PPAR- α null mouse (Atherton et al. 2009).

A combination of simvastatin with berberine improved the lipid-lowering efficacy in rats and hypercholesterolemic patients as compared with either monotherapy (Kong et al. 2008). The pharmacokinetics of berberine and its main metabolites have been identified in rats and human (Zuo et al. 2006; Tsai and Tsai 2004; Qiu et al. 2008).

On the other hand, studies on the toxicity of berberine have also been reported. Berberine could cause jaundice, kernicterus and brain damage in infants by displacing bilirubin from serum binding proteins (Bateman et al. 1998; Chan 1993, 1994). The developmental toxicity of berberine in rats and mice has been evaluated by Jahnke et al. (2006). They reported that in rats, maternal, but not fetal adverse effects were noted. There were no maternal deaths. The rat maternal lowest observed adverse effect level (LOAEL), based on reduced maternal weight gain, was 7,250 ppm (531 mg/kg/day).

High resolution nuclear magnetic resonance (NMR) approaches have been used extensively for metabolic profiling of mouse or rat urine for the monitoring of diet effects and evaluation of toxicity of chemical substances (Nicholson et al. 1999; Williams et al. 2003; Bollard et al. 2005; Waters et al. 2005, 2006; Wei et al. 2009). It has been used for the investigation of the effects of gender, diurnal variation and age in human urine (Slupsky et al. 2007) and the susceptibility of human metabolic phenotypes to dietary modulation (Stella et al. 2006). The combination of liquid chromatography/tandem mass spectrometry (LC/MSMS) with multivariate statistical tools has been used to compare metabolic signatures in biological samples (Bennett. 2005; Plumb et al. 2002; Lafaye et al. 2003; Jonsson et al. 2005; Lin et al. 2009; Lenz et al. 2004a, b, 2005, 2007). Analytical techniques such as ^1H NMR and LC/MS were preferred for the analysis of metabolites in urine samples as minimal sample preparation is needed. At the same time, a combination of ^1H NMR and LC/MS had been used for the investigation of metabolic profiles associated with the effects of drugs and chemical substances in rat urine samples (Lenz et al. 2004a, b, 2005, 2007). We have observed that additional information can be obtained using a combination of ^1H NMR and LC/MS for the profiling of metabolites in human urine samples. However, the manual processing and exporting of LC/MS data can be rather tedious (Lenz et al. 2004a, b). It was demonstrated that a targeted profiling with ^1H NMR produced robust models, generated accurate metabolite concentration data and provided information that can be used to help understand metabolic differences in a healthy population (Slupsky et al. 2007; Um et al. 2009; Slupsky et al. 2009). However, the use of targeted profiling with LC/MS has not attracted much attention until recently (Mal et al. 2011; Lv et al. 2011). In addition, the link between metabolites involved in nucleotide metabolism with cholesterol lowering drugs has not been extensively studied. At the same time, metabolic

profiles associated with the effect of cholesterol lowering drugs in rats have rarely been reported.

As the manual processing of LC/MS can be rather tedious, we used an approach based on targeted profiling with LC/MS. Selected metabolites obtained from the proposed approach using targeted profiling with LC/MS was compared with non-targeted profiling with ^1H NMR. A comparison of urinary metabolites profiles from normal rats and rats administered with berberine (50 mg/kg) using LC/MS and ^1H NMR will be obtained. In addition, the current approach is used to study the urinary metabolic profiles associated with the glucose reduction, lipid and cholesterol lowering effects of berberine in the livers of Sprague–Dawley rats.

2 Experimental

2.1 Chemicals

Berberine, xylene, paraffin wax, D_2O , chloroform, ethyl acetate (EA) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were the products of Sigma-Aldrich (Science Park II, Singapore). Pure water was obtained from Millipore Alpha-Q water system (Bedford, MA, USA). Methanol, ethanol and acetonitrile of HPLC grade were purchased from APS (NSW, Australia). Formic acid, phosphate and pyridine were purchased from Merck (Darmstadt, Germany). Valeric acid, L-leucine, D-serine, homocysteine, lysine, creatinine, phenylalanine and hippuric acid were obtained from Sigma (St. Louis, MO, USA).

2.2 Animal studies

Rats (male, Sprague–Dawley) were obtained from Laboratory Animals Centre, National University of Singapore. Animals were acclimatized in standard rodent cages with individual ventilation. The animal room was maintained at $25 \pm 2^\circ\text{C}$ with natural day/night cycle. Following a 7 day acclimatization period, animals were randomly allocated into 2 groups comprising 8 animals each. Standard commercial pelleted feed and water (Laboratory Animal Centre, National University of Singapore) were provided ad libitum. The treated group and control group were administered berberine at a dose of 50 mg/kg and water by intraperitoneal injection on 0 and 48 h, respectively. All animals were housed individually in metabolism cages for the ease of urine collection from 9 am to 12 noon. After that, the rats were transferred to the standard rodent cages and urine samples accumulated in the metabolism cages were immediately transferred into the sterile tubes. The urine samples collected were stored in dry ice initially and then frozen at -20°C prior to analysis.

Body weights of individual rats in each group were measured at the beginning of the experiment, weekly and at the time of sacrifice. All animals were sacrificed at the end of the experiment. Post-mortem examination was done on all animals. The livers and kidneys of each rat from the control and treated groups were preserved in 10% buffered formalin solution for histopathological examination. The rat livers were collected for GC/MS analysis.

2.3 Histopathological examination

The livers and kidneys of control and treated animals were dehydrated in a series of ethanol solutions (50%, 60%, 70%, and 75%). The tissue samples were cleared in xylene and embedded in paraffin wax. Serial sections (5 μm thick) were cut, stained with hematoxylin and eosin. Sections were examined under the light microscope.

2.4 Analysis of urine samples by reversed-phased LC/MS

A 20 μL aliquot of rat urine was diluted to 100 μL with distilled water. The mixture was centrifuged at $161,000\times g$ for 10 min and the supernatant was used for LC/MS analysis. An Agilent 1200 RRLC system (Waldbronn, Germany) equipped with a binary gradient pump, auto-sampler, column oven and diode array detector was coupled with an Agilent 6410 triple quadrupole mass spectrometer. The gradient elution involved a mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The initial condition was set at 5% of (B), gradient up to 100% in 10 min and returning to initial condition for 5 min. Oven temperature was set at 50°C and flow rate was set at 200 $\mu\text{L}/\text{min}$. For all experiments, 5 μL of samples was injected. The column used for the separation was a reversed-phase Zorbax SB18, 50×2.0 mm, 1.8 μm (Agilent Technologies, USA). The ESI/MS was acquired in the positive and negative ion mode. The product ions of m/z range from 100 to 600 were collected. The heated capillary temperature was maintained at 350°C , the drying gas and nebulizer nitrogen gas flow rates were 10 L/min and 50 psi, respectively.

2.5 Analysis of urine samples by ^1H NMR

300 μL of rat urine samples were buffered with 300 μL of 0.2 M phosphate buffer/ D_2O (pH 7.4) prior to analysis by ^1H NMR. The mixtures were kept for 10 min and centrifuged at $13,000\times g$ for 10 min to remove any precipitates. The supernatant was pipetted into NMR tubes (5 mm OD, 7 in. length, Sigma-Aldrich) and one dimensional ^1H NMR spectra were obtained on a Bruker DRX500 operating at 500.15 MHz observation frequency. Solvent suppression

was achieved by applying the standard Noesyprsat pulse sequence (Bruker Biospin Ltd) with secondary irradiation of the dominant water signal during the mixing time of 150 ms and the relaxation delay of 2 s. Spectra were referenced to the internal reference standard TSP dissolve in D₂O to provide a field-frequency lock.

2.6 Analysis of liver samples by GC/MS

Individual liver (1 g) was dried in freeze dryer and extracted with 0.5 mL of CHCl₃/CH₃OH (3:1). After centrifugation at 161,000×*g* for 5 min, the supernatant was lyophilized. The dried sample (lipid fraction) was kept at −30°C prior to analysis using GC/MS. For the liver tissue extract, the dried sample (1 mg) was reconstituted in 50 μL EA. 50 μL of BSTFA, pyridine and EA (3:1:1, v/v/v) mixture was added to the tissue extract together with the standard solutions. The resulting solution was vortexed for 1 min at room temperature and transferred to an amber glass vial for analysis using GC/MS.

1.0 μL aliquot of the derivatized sample with standard was injected using the splitless mode with an Agilent 7683 Series autosampler (Agilent Technologies) into an Agilent 6890 GC system equipped with a Agilent HP-1MS capillary column (15 m × 0.25 mm ID × 0.25 μm). The inlet temperature was set at 300°C. Helium was used as the carrier gas with a constant flow rate 1.40 mL/min through the column. The initial temperature was set at 100°C, 1 min after injection the GC temperature was increased at a rate of 10°C/min to 300°C and held for 3 min at 300°C. The transfer line temperature was set at 300°C. Detection was achieved using MS in electron impact mode and full scan monitoring (*m/z* 50–800). The temperature of the ion source was set at 200°C, and the quadrupole was set at 150°C.

2.7 Chemometric analysis

Fourier transformed ¹H NMR spectra were manually phased and baseline corrected using XWINNMR 3.5 (Bruker Biospin, Rheinstetten, Germany). Each spectrum was integrated between 0.5–4.5 and 5.1–10 ppm. The spectral region containing the water resonance was removed from all data sets prior to normalization and multivariate data analysis in order to eliminate variation due to water suppression efficiency. The resulting two-dimensional data, ¹H chemical shift, and peak heights were generated.

For GC/MS, each sample was represented by a GC/MS total ion chromatogram (TIC). The data was exported to Genespring 1.1.1 (Agilent Technologies, USA) for the determination of perturbed metabolites. Among the detected peaks, a multi-dimensional vector was constructed to characterize the biochemical pattern. Each vector was normalized to the total sum of vector intensity, thereby partially

accounting for concentration due to the different sample size used. Peaks due to column bleed and derivatization reagent were removed. The identification of peaks was based on the use of reference standards and NIST98 library. The mass spectra obtained were inspected manually and only those molecules with probability matching higher than 90% were considered. The current approach was consistent with earlier works reported (Ong et al. 2009, 2010).

The resulting LC/MS data served as raw data for PCA analysis. The LC/MS data were peak-detected and noise-reduced such that only true analytical peaks were further processed by the PCA software. To reduce the number of data points required for the processing of LC/MS data, targeted profiling was used. For targeted profiling, a list of the peak areas of the peaks detected from Table S1 and S6 (Supporting information) and *m/z* 100–110, 200–210, 300–310 and 400–410 were then generated manually. The data was tabulated into Microsoft Excel for each sample run, using the retention time (RT) and *m/z* data pairs as the identifier for each peak. A total of 130 data points were obtained. The peak areas for each peak detected were then normalized within each sample, to the sum of the peak area in that sample. Normalization was required to remove concentration differences between dilute and concentrated urine samples. To account for any difference in concentration between samples, all ¹H NMR and LC/MS data were normalized to a total value of 100. The resulting three-dimensional data for ¹H NMR and LC/MS were analyzed by PCA. The resulting data were then exported to Simca-P+ Software package (Umetrics, Umea, Sweden) for subsequent processing by unsupervised and supervised method. For PCA, the data are reduced to 2 latent variables (or principal components, PCs) that will describe maximum variation within the data. The PCs which are obtained from the scores will highlight clustering, trends and outliers in the observation direction in the data set.

2.8 Statistical analysis

From the normalized data obtained from LC/MS and ¹H NMR, indication of significance was based on a two-tailed Student *t* test performed with SPSS 14.0 for Windows (SPSS, Chicago, IL). For the identification of potential biomarkers, two-tailed Student *t* test (*p* < 0.05 and *p* < 0.01) were used.

3 Results

3.1 Histopathology

The dose of berberine at 50 mg/kg used in the current work is based on other reports (Yin et al. 2008; Zhou et al. 2008;

Kong et al. 2004, 2008; Zuo et al. 2006; Jia et al. 2008). In a previous study, treatment with berberine at higher concentrations than 50 mg/kg was investigated, and 150 and 300 mg/kg were found to have no obvious toxic effects but improved damaged liver tissues in diabetic rats (Zhou et al. 2008). Since our study was focused on metabolic profiling of berberine rather than toxicity effects, we did not repeat the investigations on different concentration as already performed previously (Zhou et al. 2008). After administering 50 mg/kg berberine, a decrease in whole body weight of the rats was observed initially (Fig. 1a). Since the same food and water was provided ad libitum to all rats, weight loss was not correlated with food intake. Histopathological findings after the administration of berberine are summarized in Fig. S1 (supplementary information). It is noted that no obvious differences compared with the normal livers and kidneys could be observed from the treated rats. The current observations are consistent with other reports where significant changes in liver and kidney pathology and potential toxic effects were not observed after administration of berberine (Zhou et al. 2008; Kong et al. 2004; Jia et al. 2008). In addition, it had been noted that the use of other plants with alkaloids to reduce cholesterol and glucose depends on the specific situations. Jia et al. (2008) reported that berberine and plant stanols, when combined, markedly decreased plasma cholesterol apparently through a synergistic action and produced an additive effect on plasma TAG reduction. Kong et al. (2008) reported that combining berberine with simva increased the LDLR mRNA expression to a higher level as compared with the monotherapies. This combination significantly improved LDL-c-lowering efficacy in an additive manner.

3.2 Analysis of metabolites in rat liver samples by GC/MS

In the present study, livers samples of individual rats on day 5 were collected and subjected to GC/MS analysis. For the analysis of tissues samples, GC/MS was selected for the analysis of cholesterol, various fatty acids and sugars. Quality controls were performed using known standards, reagent blank control and method blank control and the current approach was similar to our earlier work (Ong et al. 2009; Lu et al. 2010). Representative GC/MS chromatograms are shown in Fig. S2 (supplementary information) for liver tissue extract from the two different groups. After data normalization, PCA was carried out using multivariate analysis. The PCA score plot (Fig. 1b) shows two distinct clusters for data obtained from normal rat livers and livers treated with berberine. With the assistance of Genespring, metabolites from the liver samples that are observed to be significantly different (based on a two-tailed Student's *t* test) from the two groups are summarized in Fig. 1c.

These include glucose, maltose, fatty acids and cholesterol in the liver samples collected.

3.3 Analysis of metabolites in rat urine samples by ¹H NMR (non-targeted)

Typical ¹H NMR spectra of the control and berberine treated groups' urine samples acquired using the standard 1D pulse sequence for water suppression are shown in Fig. 2a. The PCA score plot shows two distinct clusters for data obtained from the two groups on pre-dose, days 2 and 4 (Fig. 2b; Fig. S3, supplementary information).

The observed changes in endogenous metabolites in the rat urine samples collected on different days are summarized in Tables S1–S5 (supplementary information). Data points for days 1 and 3 were omitted from Fig. 2c–e as it was noted that higher concentrations of berberine and its related metabolites were present in the urine samples collected after the injection of berberine on days 1 and 3. These may interfere with the determination of other metabolites (Tables 1, 2) in the rat urine samples collected. Representative metabolites detected by ¹H NMR that which were also detected by the LC/MS method in this study were listed in Tables 1 and 2, respectively. Finally, a two-tailed Student *t* test ($p < 0.05$ and $p < 0.01$) was used to identify metabolites that were expected to be perturbed between the two groups (Tables S1–S5, supplementary information). The variations of TCA cycles intermediates such as glucose, lactate and pyruvate over time were shown in Fig. 2c–e, respectively. A higher level of glucose and lactate are found in the urine samples from the treated animals.

3.4 Analysis of metabolites in rat urine samples by LC/MS (targeted)

Urine samples collected on pre-dose, day 1, day 2, day 3 and day 4 after the start of berberine treatment were subjected to LC/MS analysis. The discrimination of mouse urine samples resulting from differences in gender, strain and diurnal variation has been reported (Plumb et al. 2003). Hence, all urine samples were collected at the same time interval. Berberine and its related metabolites can be found in TIC for the positive ESI/MS of urine samples for day 1 and day 3. The metabolites observed in the rat urine samples for the current work are consistent with other reports (Tsai and Tsai 2004; Zuo et al. 2006; Qiu et al. 2008). These include peaks from berberrubine (m/z 322), thalifendine (m/z 322), demethyleneberberine (m/z 324) and jatrorrhizine (m/z 338). The concentration of berberine and its main metabolites in the plasma were found to decrease from 24 to 48 h post-dose (Tsai and Tsai 2004; Qiu et al. 2008). As applied in our earlier work (Law et al. 2008), to minimize interfering peaks from berberine and its metabolites from the rat urine

Fig. 1 a The change characteristics of body weight of rats. The results shown are average with standard deviation ($n = 8$), **b** PCA scores plot based on GC/MS analysis of rat liver samples from all the control ($n = 8$) and treated group ($n = 8$) (PCA component 1: 34.7% variance, PCA component 2: 17.1% variance), and **c** Simplified pathway illustrating perturbed metabolites in the rat liver samples between the control and treated group. The treated group was found to have a lower amount of cholesterol, glucose, maltose and fatty acids. The statistics are as follows: significance difference between the control group ($n = 8$) and treated group ($n = 8$) is based on two-tailed Student t test ($*p < 0.05$; $**p < 0.01$)

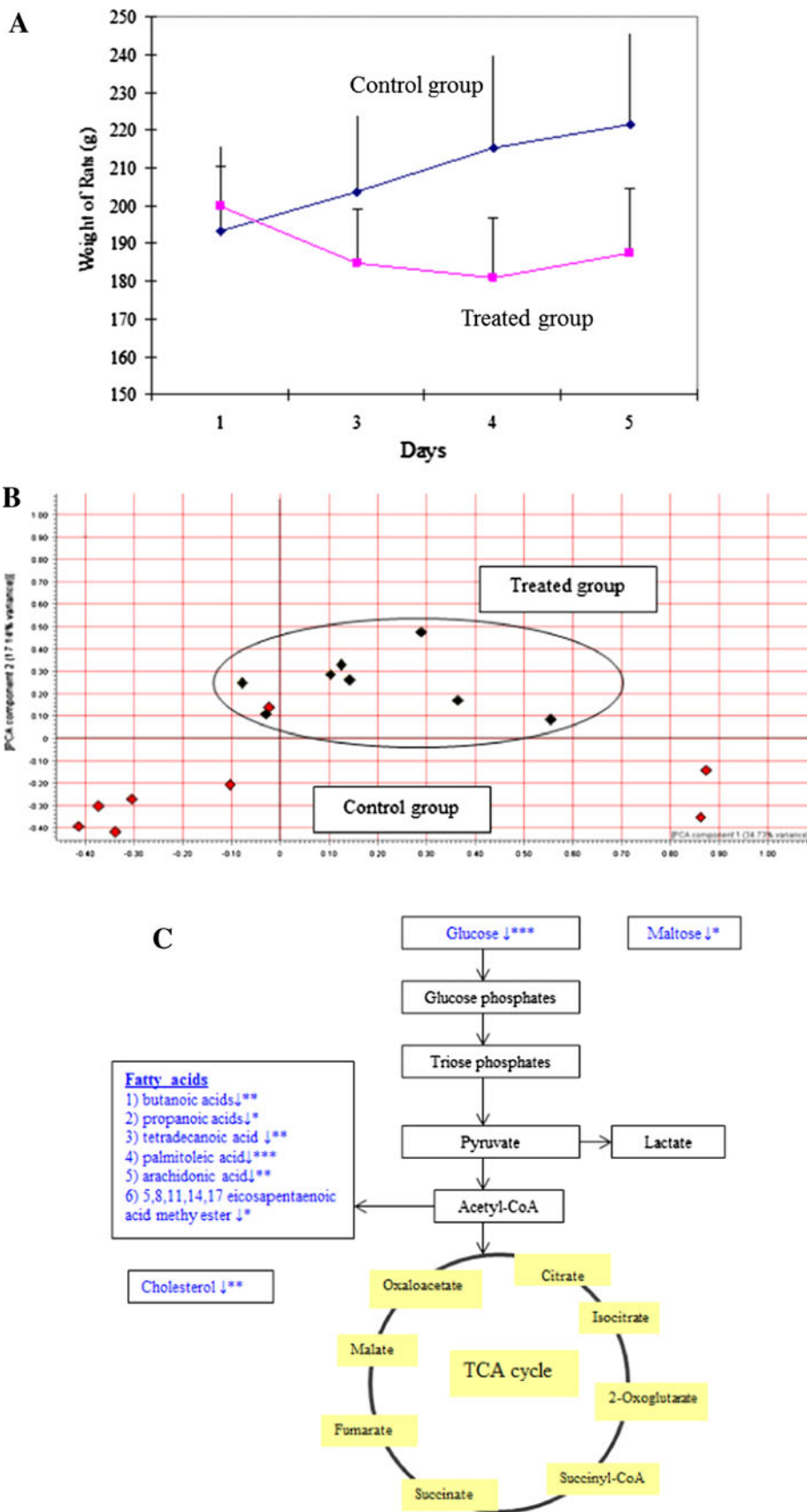
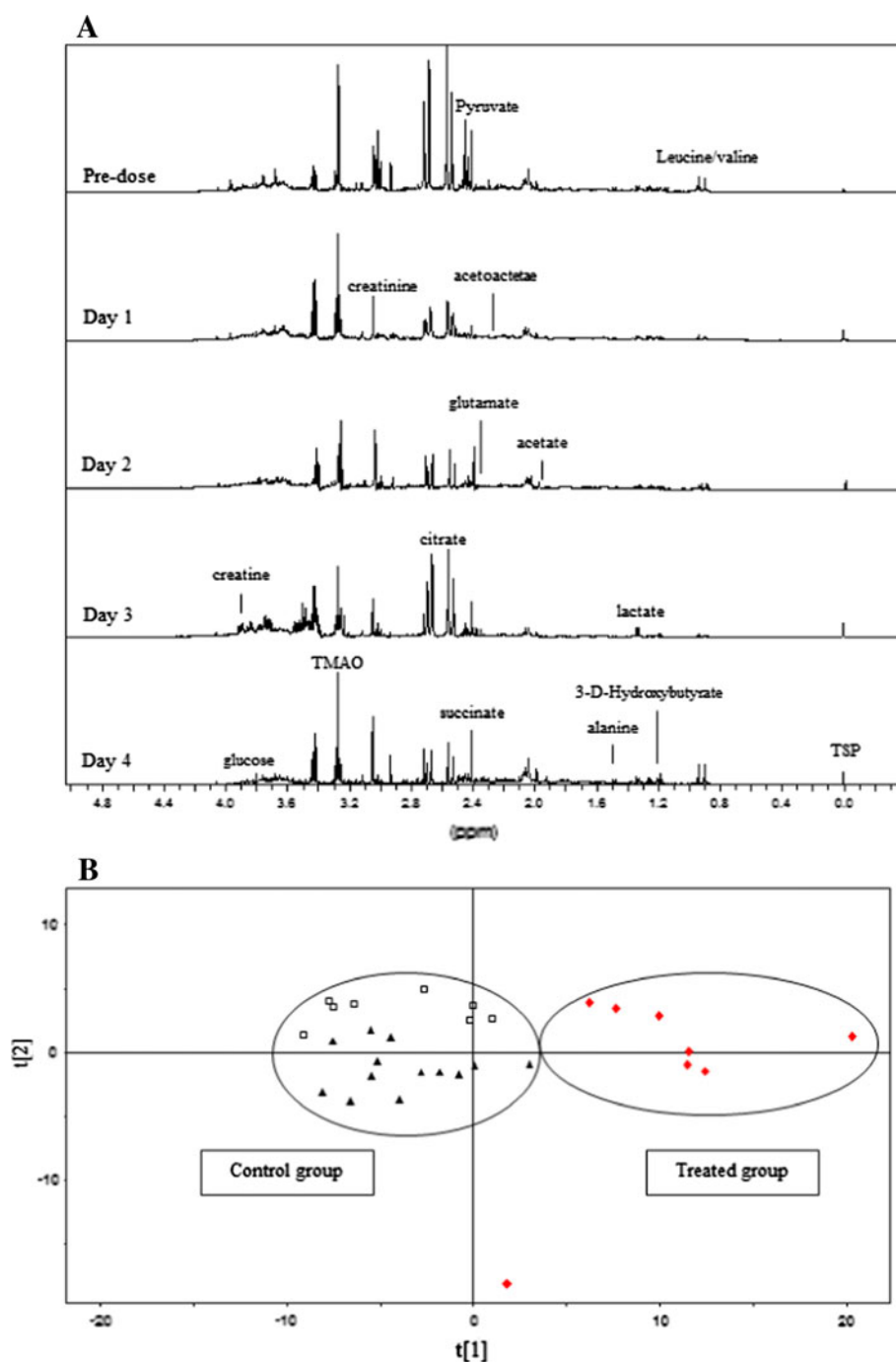


Fig. 2 a Typical ^1H NMR spectrum of rat urine samples obtained from pre-dose and treated group (day 1, 2, 3 and 4), **b** PCA scores plot for data from all rat urine samples collected (*triangle*: control group from day 0, *open square*: control group at day 4 and *diamond*: treated group at day 4) ($R^2X[1] = 0.346$, $R^2X[2] = 0.114$). Changes of selected metabolites as measured by ^1H NMR on different days, **c** glucose (**), **d** pyruvate and **e** lactate (**). The statistics are as follows: significance difference between the control group ($n = 8$) and treated group ($n = 8$) is based on two-tailed Student t test ($*p < 0.05$; $**p < 0.01$)



samples collected, peaks that were not found in the previous control group were not included into the treated group for data normalization purposes.

A comparison of the TIC for the positive and negative ESI/MS of urine samples demonstrated qualitative differences between the control and treated groups (Fig. 3a). Visual inspection of the data suggested that the control and berberine treated group exhibited unique metabolic profiles. For the current work, profiling of metabolites using a targeted LC/MS method based on selected m/z was used. A

list of compounds that have been characterized in rat urine samples using this approach is shown in Tables S6–S15 (supplementary information). They belong to various chemical families, namely, amino acids (lysine, phenylalanine, serine), vitamins such as riboflavin, organic acids (adipic acid, kynurenic acid, citric acid and hippuric acids), sulfoconjugates of phenolic compounds (phenol sulfates), fatty acids, bile salts and metabolites involved in purine metabolism. The identities of these species were confirmed by comparison with authentic standards and interpretation

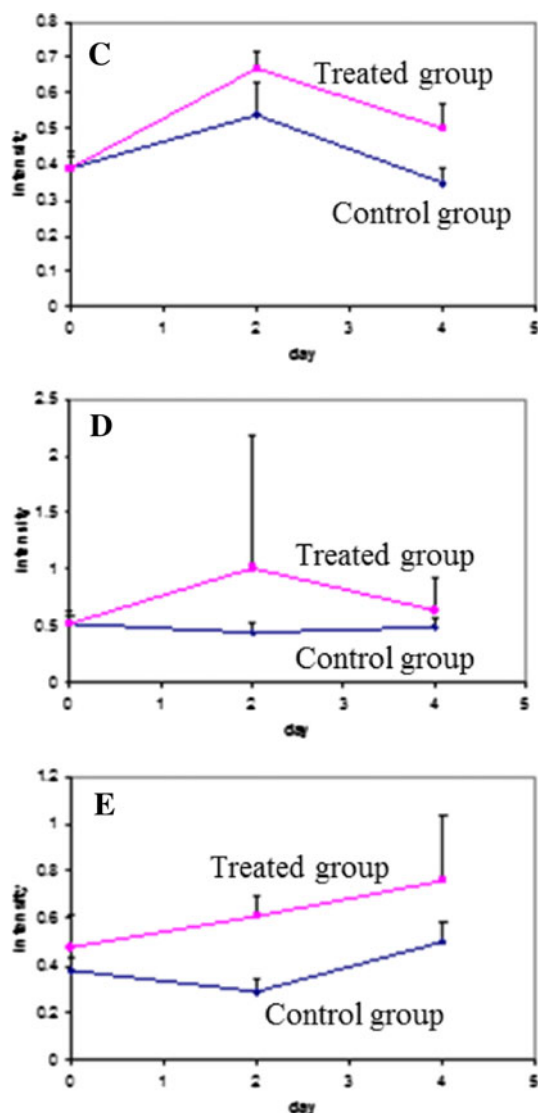


Fig. 2 continued

of the MS/MS spectra. After data normalization, multivariate statistical method such as PCA was carried out. The plot of the first two PCA scores for the positive mode ESI/MS data are shown in Fig. 3b and c.

For all rat urine samples collected, it was noted that the control and treated groups were scattered into 2 distinct clusters (Fig. 3b, c). These data suggested that metabolic profiling using LC/MS in the positive mode with a targeted approach provided clear clustering showing significant differences between the two groups. A two-tailed Student *t* test ($p < 0.05$ and $p < 0.01$) was used to identify metabolites that were perturbed between the two groups (Tables S6–S15, supplementary information). Excretion of selected metabolites such as adipic acid, lauric acid, palmitoleic acid, linoleic acid, fumaric acid, citrate, hypoxanthine, xanthine, uric acid, guanine and N1-methyladenosine over time is shown in Fig. 4. For the rat urine samples collected, it was noted that the administration of berberine (50 mg/kg)

increased the excretion of fatty acids and metabolites involved in nucleotides metabolism.

The current study demonstrates that analysis of metabolites in rat urine samples from two different groups using with LC/MS (targeted) produces consistent and reliable results. Based on the targeted ions selected with LC/MS, the PCA score plots from different days were consistent with that obtained from using the non-targeted approach with ^1H NMR (Fig. 3; Fig. S3, supplementary information) where distinctive clusters are observed. For the current dataset, metabolites such as lysine, creatinine, fumarate and citrate were identified by ^1H NMR and LC/MS, respectively. The close agreement of values obtained for lysine, creatinine, fumarate and citrate using different techniques shows that ^1H NMR has good specificity for the selected metabolites, and also the reliability of the current method of normalization. The current observation is consistent with our early work using human urine samples and zebrafish livers where non targeted approaches were used (Law et al. 2008, 2009; Ong et al. 2010). These observations further confirm that using a multiple analytical approach does not just provide additional information, but enhance the confidence of the data obtained. Based on Tables 1 and 2, the quality of the data and the reliability of the current approach using normalization to a constant sum with a bigger dataset are demonstrated.

Other than normalization to a constant sum, the other approach includes normalization of the selected peaks from the ^1H NMR spectra obtained to creatinine methylene singlet (Bollard et al. 2010; Clayton et al. 2009). For the current work, the selected peaks from the ^1H NMR spectra were normalized to creatinine methylene singlet and a trend similar to normalization to a constant sum was observed (data not shown). Hence, for the comparison with other reports (Chan 1994; Jahnke et al. 2006; Nicholson et al. 1999; Williams et al. 2003; Coen et al. 2003), normalization to a constant sum was finally selected for the current study. The combination of ^1H NMR and LC/MS allows us to study a number of metabolites present in carbohydrate metabolism, amino acid metabolism, TCA cycle, fatty acid metabolism, purine metabolism, as well as metabolism and synthesis of major bile salts (Fig. 5).

4 Discussion

From the rat liver samples obtained on day 5 for the treated group, a decrease in the level of cholesterol was accompanied by changes in a number of fatty acids (Fig. 1c). The depletion of liver glucose and maltose in the current work suggested extensive glycogenolysis and glycolysis. Without inducing significant histopathological changes in the livers, variations in several fatty acids such as butanoic acids, propanoic acids

Table 1 Selected metabolites identified in rat urine samples for both the control group and treated group as measured by LC/MS (positive and negative mode)

No.	Metabolite	Retention time (min)	<i>m/z</i>	Normalized peak intensity ^a (%)			
				Control average	±SD	Treated average	±SD
Pre-dose							
1	Fumaric acid	1.00	115	0.0227	0.0118	0.0263	0.0205
2	Creatinine	0.80	114	6.09	1.08	5.55	1.10
3	Lysine	0.62	147	0.038	0.018	0.050	0.017
4	Citric acid	0.94	191	6.10	1.51	5.97	1.71
Day 1							
1	Fumaric acid	1.00	115	0.024	0.0071	0.024	0.0170
2	Creatinine*	0.80	114	3.88	0.56	6.49	2.51
3	Lysine	0.62	147	0.052	0.018	0.056	0.018
4	Citric acid**	0.94	191	6.24	1.66	10.33	2.96
Day 2							
1	Fumaric acid*	1.00	115	0.0504	0.0316	0.0167	0.0165
2	Creatinine**	0.80	114	4.24	0.38	10.42	1.89
3	Lysine**	0.62	147	0.051	0.027	0.182	0.087
4	Citric acid	0.94	191	6.93	1.21	5.39	3.09
Day 3							
1	Fumaric acid**	1.00	115	0.0345	0.0303	0.1690	0.0912
2	Creatinine**	0.80	114	4.02	1.16	10.10	3.02
3	Lysine	0.62	147	0.112	0.104	0.113	0.092
4	Citric acid**	0.94	191	4.61	1.92	26.42	12.61
Day 4							
1	Fumaric acid	1.00	115	0.0392	0.0245	0.0413	0.0247
2	Creatinine**	0.80	114	4.68	1.24	9.55	2.00
3	Lysine*	0.62	147	0.074	0.056	0.158	0.075
4	Citric acid	0.94	191	6.74	1.81	8.08	10.17

^a The relative intensity of metabolites is expressed with their normalized peak area. Values are represented as mean ± SD (standard deviation). The statistics are as follows: significance difference between the control group ($n = 8$) and treated group ($n = 8$) is based on two-tailed Student *t* test (* $p < 0.05$; ** $p < 0.01$)

and others stated in Fig. 1c showed alteration in the fatty acids metabolism, glucose catabolism and storage in the treated animals. Hence, it was proposed that the administration of berberine changes the β -oxidation of fatty acids (saturated and unsaturated) in the rat livers.

Metabolic profiles of biofluids such as urine samples reflect the impact of chemical substances on single or multiple organ systems. To obtain a snapshot of the glucose reduction, lipid and cholesterol lowering effects of berberine in the livers of Sprague–Dawley rats, urinary metabolic profiles on different days were obtained. Lactate which is the final product of anaerobic glycolysis in many organisms has been proposed as an active metabolite and a modulator of energy production (Sola-Penna 2008). It is also noted that lactate can be used for energy production through the Cori cycle. A change in energy metabolism with an increase in the levels of glucose and lactate in the rat urine samples have been observed as a result of drug induced hepatotoxicity

(Williams et al. 2003; Bollard et al. 2005, 2010; Waters et al. 2005, 2006) and nephrotoxicity (Lenz et al. 2004a, b, 2005). In addition, a combination of changes in urinary levels of taurine, hypotaurine, dimethylglycine, citrate and succinate indicated a result of drug induced hepatotoxicity (Williams et al. 2003; Bollard et al. 2005; Waters et al. 2005, 2006). For the current work, it was observed that berberine changed the urinary metabolic profiles of the treated group on day 2 with an increase in the level of glucose, fatty acids and other metabolites (Figs. 2, 5). At the same time, changes in the urinary metabolites such as glucose and fatty acids could be the result of glucose reduction, lipid and cholesterol lowering effects of berberine in the rat livers. It is well known that higher amounts of glucose in the urine could probably be an indicator of kidney injury. However, according to Ma et al. (2010), potential biomarkers in urine metabolites related with kidney injury included creatinine, hippuric acid, phenylacetyl glycine, kynurenic acid, homocysteine,

Table 2 Selected metabolites identified in rat urine samples for both control and treated group as measured by ^1H NMR

No.	Metabolite	Chemical shift (ppm)	Multiplicity	Normalized peak intensity ^a			
				Control average	\pm SD	Treated average	\pm SD
Pre-dose							
1	Fumarate	6.53	s	0.18	0.04	0.17	0.04
2	Creatinine	3.08	s	0.15	0.03	0.15	0.07
3	Lysine	3.74	t	0.41	0.21	0.38	0.26
4	Citrate	2.60	d	0.32	0.05	0.34	0.06
	Citrate	2.50	d	0.37	0.07	0.32	0.08
Day 1							
1	Fumarate	6.53	s	0.26	0.03	0.23	0.09
2	Creatinine	3.08	s	0.21	0.03	0.17	0.06
3	Lysine	3.74	t	0.65	0.27	1.79	1.48
4	Citrate	2.60	d	0.34	0.04	0.28	0.08
	Citrate	2.50	d	0.41	0.09	0.49	0.38
Day 2							
1	Fumarate**	6.53	s	0.27	0.02	0.13	0.06
2	Creatinine**	3.08	s	0.19	0.03	0.33	0.04
3	Lysine**	3.74	t	0.71	0.10	1.08	0.19
4	Citrate	2.60	d	0.28	0.12	0.32	0.08
	Citrate	2.50	d	0.36	0.09	0.41	0.03
Day 3							
1	Fumarate	6.53	s	0.20	0.03	0.31	0.33
2	Creatinine*	3.08	s	0.25	0.03	0.13	0.12
3	Lysine*	3.74	t	0.91	0.11	2.58	1.94
4	Citrate	2.60	d	0.32	0.13	0.15	0.13
	Citrate	2.50	d	0.30	0.05	0.17	0.16
Day 4							
1	Fumarate	6.53	s	0.15	0.04	0.14	0.07
2	Creatinine**	3.08	s	0.18	0.03	0.27	0.06
3	Lysine**	3.74	t	0.48	0.06	0.77	0.17
4	Citrate	2.60	d	0.30	0.03	0.44	0.21
	Citrate	2.50	d	0.36	0.14	0.39	0.07

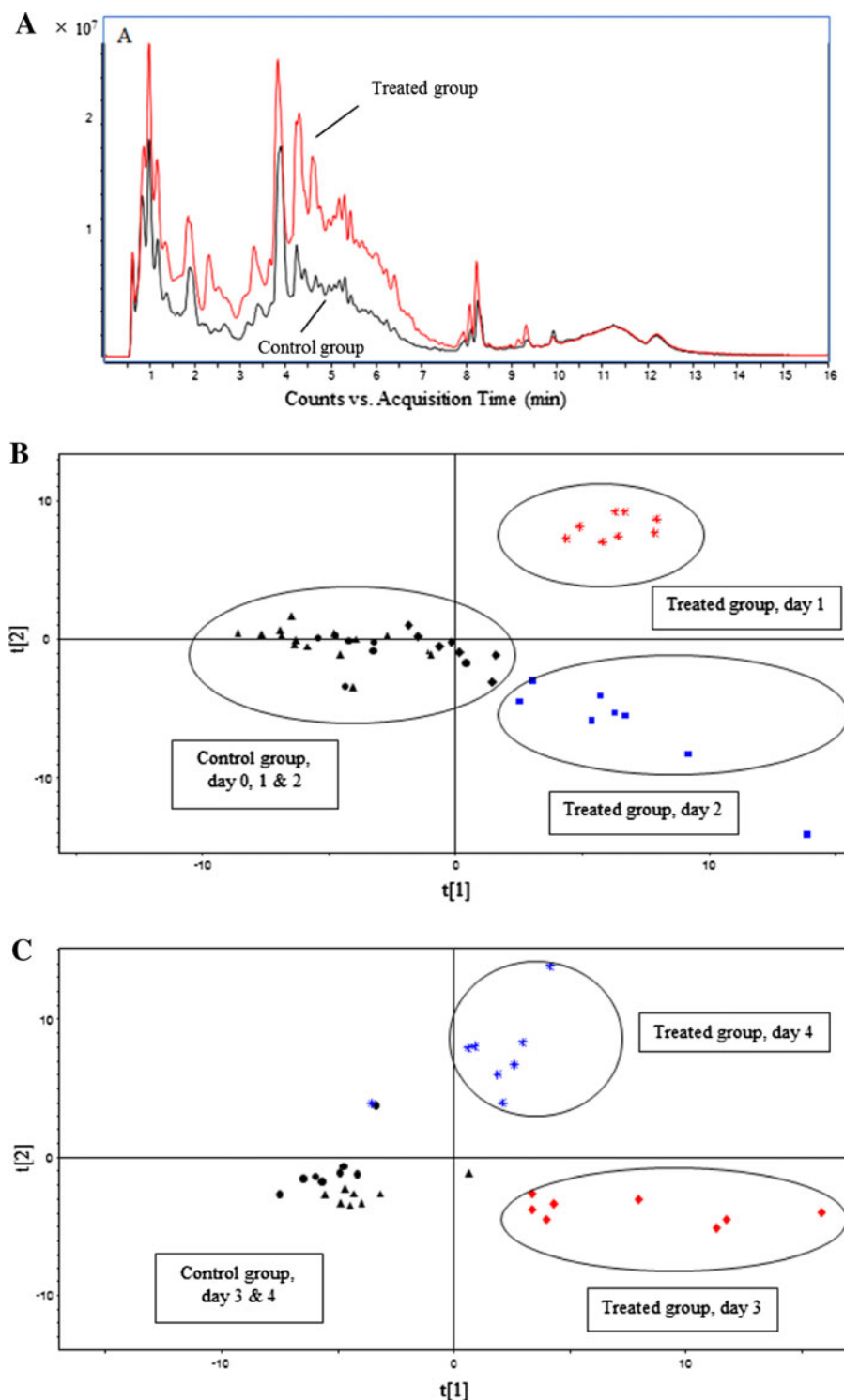
^a The relative intensity of metabolites is expressed with their normalized peak area. Values are represented as mean \pm SD (standard deviation). The statistics are as follows: significance difference between the control group ($n = 8$) and treated group ($n = 8$) is based on two-tailed Student t test ($*p < 0.05$; $**p < 0.01$)

deoxycholic acid, cholic acid, phenylalanine, adrenalin, adenosine and hypoxanthine. Lenz et al. (2005) reported that observed perturbations in the urinary metabolite profiles related with kidney damage included glucose, lactate, citrate, N-acgp, TMAO and betaine. Sieber et al. (2009) reported that identified candidate kidney biomarkers included kidney injury molecule (Kim-1), clusterin, lipocalin-2, and tissue inhibitor of metalloproteinase 1 (Timp-1). Urinary lipocalin-2 and Kim-1 appeared to be the most sensitive and specific indicators of gentamicin-induced kidney injury. In contrast, clusterin and Timp-1 were less sensitive than glucose and BUN to detect gentamicin-mediated renal toxicity. Serum creatinine and γ -glutamyl transferase were the least responsive. Based on previous studies (Lenz et al.

2005; Ma et al. 2010; Sieber et al. 2009), elevated glucose level in urine alone cannot conclusively indicate kidney injury since there would have to be other accompanying changes in metabolites indicating such injury. In view of the lack of specific biomarkers detected (e.g. urinary lipocalin-2 and Kim-1), and the histopathology result in this study, we believed that no kidney injury was observed at the dosage of 50 mg/kg.

Hence, we propose that the administration of berberine resulted in a shift in the energy metabolism with changes in glucose, lactate and metabolism of fatty acids (Fig. 5) on day 2. In addition, the urinary metabolic profiles are consistent with the histopathological data of the livers where hepatotoxicity is not observed.

Fig. 3 **a** Typical total ion chromatograms (TIC) of rat urine samples obtained from control group and treated group and **b** PCA scores plot for data from all rat urine samples collected (*triangle*: control at day 0, *circle*: control at day 1, *diamond*: control at day 2, *star*: treated at day 1, *square*: treated at day 2 ($R2X[1] = 0.177$, $R2X[2] = 0.128$), and **c** PCA scores plot for data from all rat urine samples collected (*triangle*: control at day 3, *circle*: control at day 4, *diamond*: treated at day 3, *star*: treated at day 4) ($R2X[1] = 0.209$, $R2X[2] = 0.140$)



One of the prominent features in the current work is that the administration of berberine changes glucogenic amino acids such as alanine, arginine, valine, methionine, glycine, serine, glutamine and glutamate in the rat urines samples collected (Fig. 5a). Amino acids are not only cell signaling molecules but also regulators of gene expression and the

protein phosphorylation cascades. Many amino acids can also be used as precursors for the TCA cycle. In addition, amino acids are key precursors for synthesis of hormones and low molecular weight nitrogenous substances that have enormous biological importance. Other than their roles as building blocks for proteins and peptides, some amino

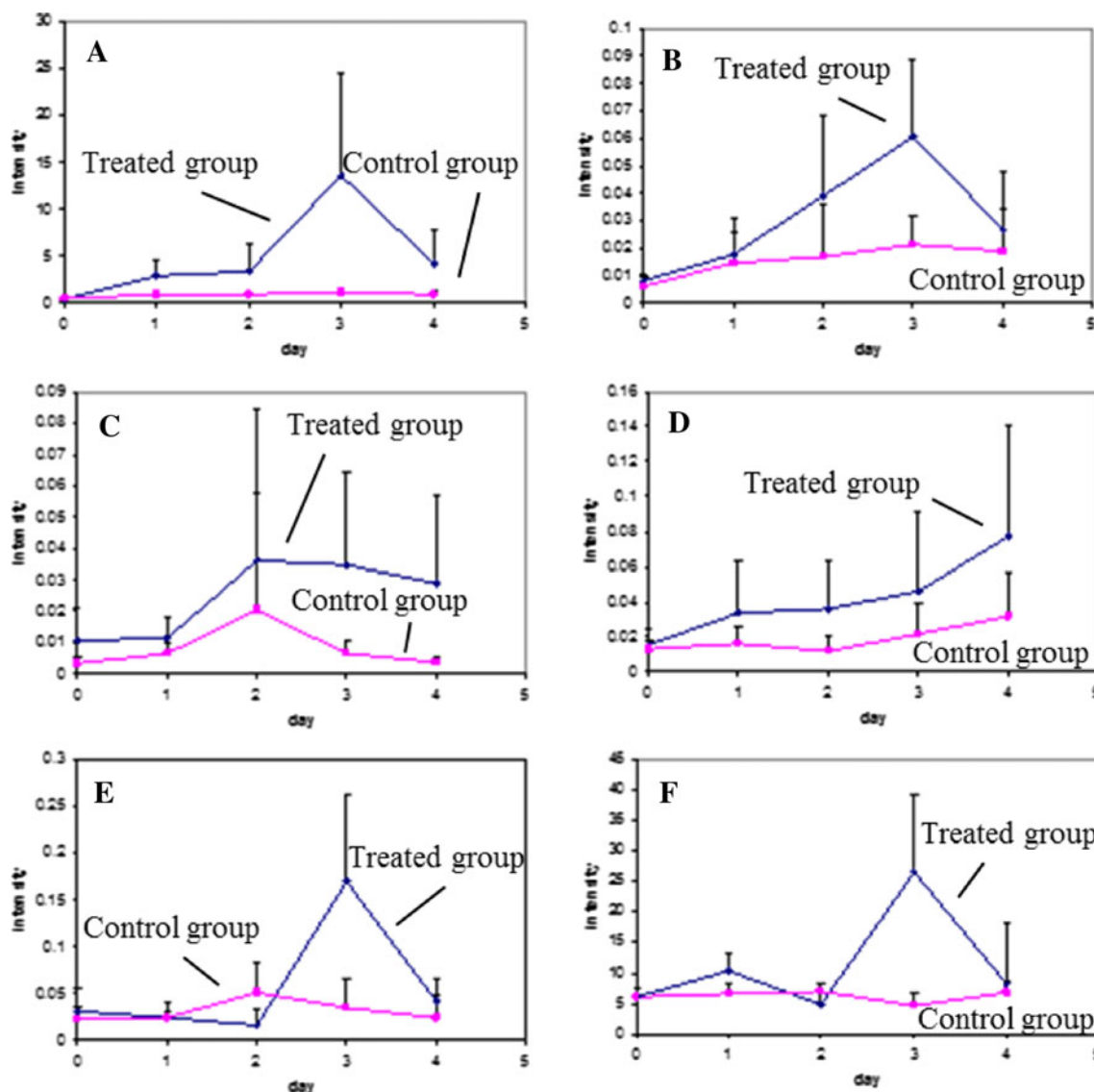


Fig. 4 Changes of selected metabolites as measured by LC/MS on different days. **a** adipic acid (*), **b** lauric acid, **c** palmitoleic acid (*), **d** linoleic acid (*), **e** fumaric acid (*), **f** citrate, **g** hypoxanthine (*), **h** xanthine (*), **i** uric acid (**), **j** guanine and **k** N1-methyladenosine

(**). The statistics are as follows: significance difference between the control group ($n = 8$) and treated group ($n = 8$) is based on two-tailed Student t test (* $p < 0.05$; ** $p < 0.01$)

acids regulate key metabolic pathways that are necessary for maintenance, growth, reproduction and immunity (Wu 2009). The 5 carbon amino acids such as glutamine and glutamate are considered to be equally important for maintenance and promotion of cell growth (Yuan et al. 2009). The functions of glutamine include substrate for protein synthesis, anabolic precursor for muscle growth, acid base balance in the kidney, substrate for hepatic and renal gluconeogenesis, precursors for nucleotide synthesis and others (Newsholme et al. 2003; Bronsnan 2003). Glutamate is produced from glutamine through glutaminase activity and it can be converted into γ -amino butyric acid, ornithine, α -ketoglutarate, glucose and others

(Newsholme et al. 2003; Bronsnan 2003). Changes in metabolites such as glutamine, glutamate, α -ketoglutarate and arginine in the rat urine samples collected suggested that the glutamine and glutamate metabolism is affected in the treated animals. At the same time, ketogenic amino acids such as lysine and leucine were affected in the treated group (Fig. 5a). Hence, it is clear that the administration of berberine resulted in a shift in transamination and amino acid metabolism.

Nucleotides are required for cell growth and replication and glutamine is a precursor for nucleotides synthesis. In addition, nucleotides play an important role as energy currency in the cell and are important regulatory

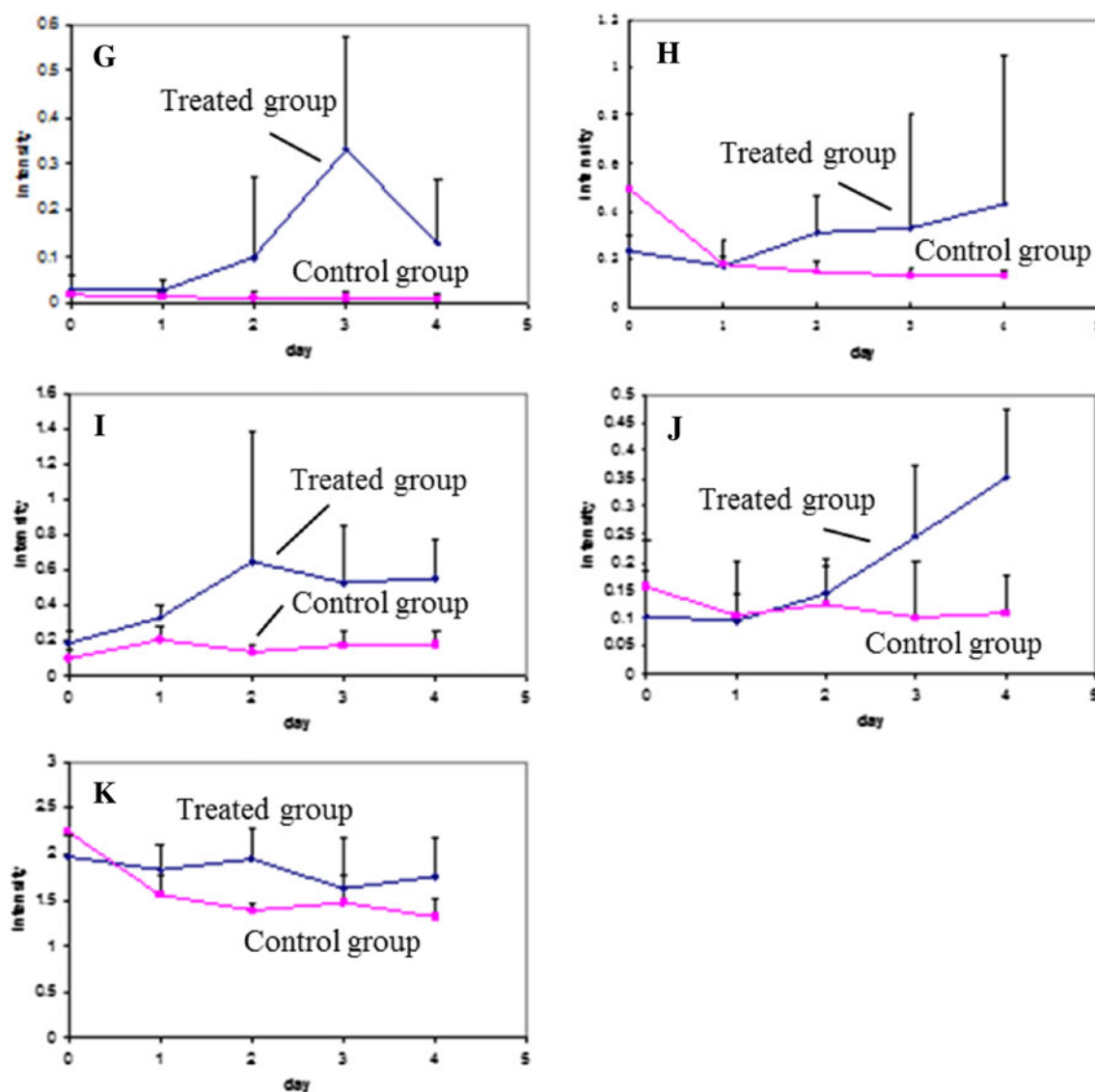


Fig. 4 continued

compounds for many of the pathways of intermediary metabolism (Huang and Graves 2003). Purine bases are converted into xanthine and to uric acid in the urine for excretion. From Figs. 4h–k and 5b, it is noted that the cholesterol lowering effect of berberine causes a change in glutamine, hypoxanthine, xanthine, guanine, N1-methyladenosine and finally uric acid. This resulted in a change in the nucleotide metabolism for the treated animals on day 2 where a shift in the transmission of ATP and synthesis of nucleic acids could have taken place. The current work provides direct evidence that the administration of berberine generates a change in the nucleotide metabolism in treated rats.

It was noted that it is not possible to detect the different classes of metabolites stated in Fig. 5 with any single

analytical technique. The changes of a number of metabolites in the current study are very similar to those observed in urine or liver samples of drug induced hepatotoxicity and liver samples from PPAR- α null mouse. However, it is clear that the metabolic profiles associated with the glucose reduction, lipid and cholesterol lowering effects of berberine in the livers of Sprague–Dawley rats is rather unique. From the analysis of rat urine samples obtained (Fig. 5), a high rate of carbohydrate usage was proposed to be an early event. The synthesis of amino acids and fatty acids was altered at the same time. A change in the nucleotide metabolism for the treated animals provided the evidences for a shift in the transmission of ATP and synthesis of nucleic acids. Finally, it is suggested that the increase of a number of metabolites detected from ^1H

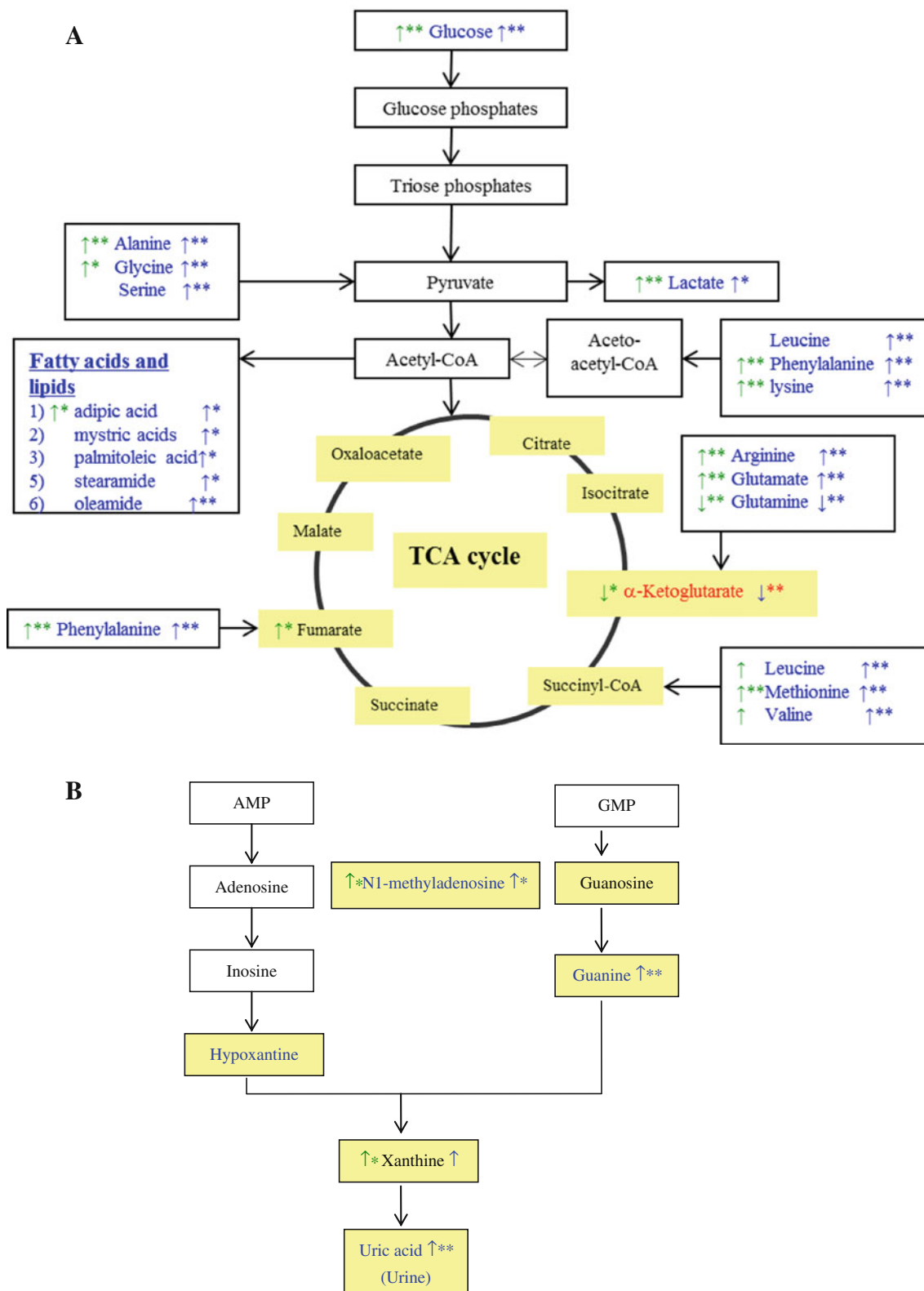


Fig. 5 a Simplified pathway illustrating perturbed metabolites in the rat urine samples on day 2 (↑↓ on the left side of metabolites) and day 4 (↑↓ on the right side of metabolites) between the control and treated group and **b** Simplified pathway illustrating metabolites involved in

purine metabolism in the rat urine samples on day 4 between the control and treated group. The statistics are as follows: significance difference between the control group ($n = 8$) and treated group ($n = 8$) is based on two-tailed Student t test ($*p < 0.05$; $**p < 0.01$)

NMR and LC/MS in the rat urine samples collected (Fig. 5) reflect the impact of berberine on single or multiple organ systems.

5 Conclusions

Our results showed that metabonomics could be an attractive approach to understand the many biologic sequences of a cholesterol lowering drug administration in vivo while minimizing both time and compound requirements necessary to get answers. We were able to identify signature metabolic shifts associated with glucose reduction as well as lipid and cholesterol lowering effects of berberine in the livers of Sprague–Dawley rats. Urinary metabolic profiles on different days also provided a snapshot of the glucose reduction, lipid and cholesterol lowering effects of berberine in the livers of treated animals. Finally, the use of both ^1H NMR and LC/MS with pattern recognition tools such PCA provided a comprehensive picture of metabolic changes in the urine samples between the two groups.

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References

- Atherton, H. J., Gulston, M. K., Bailey, N. J., Cheng, K. K., Zhang, W., Clarke, K., et al. (2009). Metabolomics of the interaction between PPAR- α and age in the PPAR- α -null mouse. *Molecular System Biology*, 5, 259–268.
- Bateman, J., Chapman, R. D., & Simpson, D. (1998). Possible toxicity of herbal remedies. *Scottish Medical Journal*, 43(1), 7–15.
- Bennett, D. (2005). Growing pains for metabolomics. *The Scientist*, 19, 25–28.
- Bollard, M. E., Contel, N. R., Ebbels, T. M. D., Smith, L., Beckonert, O., Cantor, G. H., et al. (2010). NMR-based metabolic profiling identifies biomarkers of liver regeneration following partial hepatectomy in the rat. *Journal of Proteome Research*, 9(1), 59–69.
- Bollard, M. E., Keun, H. C., Beckonert, O., Ebbels, T. M. D., Antti, H., Nicholls, A. W., et al. (2005). Comparative metabonomics of differential hydrazine toxicity in the rat and mouse. *Toxicology and Applied Pharmacology*, 204(2), 135–151.
- Bronsnan, J. T. (2003). Interorgan amino acid transport and its regulation. *Journal of Nutrition*, 133(6), 2068S–2072S.
- Chan, E. (1993). Displacement of bilirubin from albumin by berberine. *Biology of the Neonate*, 63(4), 201–208.
- Chan, T. K. Y. (1994). The prevalence use and harmful potential of some Chinese herbal medicines in babies and children. *Veterinary and Human Toxicology*, 36(3), 238–240.
- Clayton, T. A., Barker, D., Lindon, J. C., Everett, J. R., & Nicholson, J. K. (2009). Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proceedings of the National Academy of Sciences of the United States of America*, 106(34), 14728–14733.
- Coen, M., Lenz, E. M., Nicholson, J. K., Wilson, I. D., Pognan, F., & Lindon, J. C. (2003). An integrated metabonomic investigation of acetaminophen toxicity in the mouse using NMR spectroscopy. *Chemical Research in Toxicology*, 16(3), 295–303.
- Goldstein, J. L., & Brown, M. S. (2001). The cholesterol quartet. *Science*, 292(5520), 1310–1312.
- Huang, M., & Graves, L. M. (2003). De novo synthesis of pyrimidine nucleotides; emerging interfaces with signal transduction pathways. *Cellular and Molecular Life Science*, 60(2), 321–336.
- Jahnke, G. D., Price, C. J., Marr, M. C., Myers, C. B., & George, J. D. (2006). Developmental toxicity evaluation of berberine in rats and mice. *Birth Defects Research*, 77(3), 195–206.
- Jia, X. M., Chen, Y. F., Zidichouski, J., Zhang, J. Z., Sun, C. H., & Wang, Y. W. (2008). Co-administration of berberine and plant stanols synergistically reduces plasma cholesterol in rats. *Atherosclerosis*, 201(1), 101–107.
- Jonsson, P., Bruce, S. J., Moritz, T., Trygg, J., Sjöström, M., Plumb, R., et al. (2005). Extraction, interpretation and validation of information for comparing samples in metabolic LC/MS data sets. *Analyst*, 130(5), 701–707.
- Kong, W. J., Wei, J., Abidi, P., Lin, M. H., Inaba, S., Li, C., et al. (2004). Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nature Medicine*, 10(12), 1344–1351.
- Kong, W. J., Wei, J., Zuo, Z. Y., Wang, Y. M., Song, D. Q., You, X. F., et al. (2008). Combination of simvastatin with berberine improves the lipid-lowering efficacy. *Metabolism Clinical and Experimental*, 57(8), 1029–1037.
- Kuo, C. L., Chi, C. W., & Liu, T. Y. (2004). The anti-inflammatory potential of berberine in vitro and in vivo. *Cancer Letters*, 203(2), 127–137.
- Lafaye, A., Junot, C., Gall, B. R. L., Fritsch, P., Tabet, J. C., & Ezan, E. (2003). Metabolite profiling in rat urine by liquid chromatography/electrospray ion trap mass spectrometry. Application to the study of heavy metal toxicity. *Rapid Communications in Mass Spectrometry*, 17(22), 2541–2549.
- Law, W. S., Huang, P. Y., Ong, E. S., Ong, C. N., Li, S. F. Y., Pasikanti, K. K., et al. (2008). Metabonomics investigation of human urine after ingestion of green tea with gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry and ^1H NMR spectroscopy. *Rapid Communications in Mass Spectrometry*, 22(16), 2436–2446.
- Law, W. S., Huang, P. Y., Ong, E. S., Sethi, S. K., Saw, S., Ong, C. N., et al. (2009). Combination of ^1H nuclear magnetic resonance spectroscopy and liquid chromatography/mass spectrometry with pattern recognition techniques for evaluation of metabolic profile associated with albuminuria. *Journal of Proteome Research*, 8(4), 1828–1837.
- Lenz, E. M., Bright, J., Knight, R., Westwood, F. R., Davies, D., Major, H., et al. (2005). Metabonomics with ^1H -NMR spectroscopy and liquid chromatography–mass spectrometry applied to the investigation of metabolic changes caused by gentamicin-induced nephrotoxicity in the rat. *Biomarkers*, 10(2/3), 173–187.
- Lenz, E. M., Bright, J., Knight, R., Wilson, I. D., & Major, H. (2004a). Cyclosporin A-induced changes in endogenous metabolites in rat urine: A metabonomic investigation using high field ^1H NMR spectroscopy, HPLC-TOF/MS and chemometrics. *Journal of Pharmaceutical and Biomedical Analysis*, 35(3), 599–608.
- Lenz, E. M., Bright, J., Knight, R., Wilson, I. D., & Major, H. (2004b). A metabonomic investigation of the biochemical effects of mercuric chloride in the rat using ^1H NMR and HPLC-TOF/MS: Time dependant changes in the urinary profile of

- endogenous metabolites as a result of nephrotoxicity. *Analyst*, 129(6), 535–541.
- Lenz, E. M., Williams, R. E., Sidaway, J., Smith, B. W., Plumb, R. S., Johnson, K. A., et al. (2007). The application of microbore UPLC/oa-TOF-MS and ^1H NMR spectroscopy to the metabolomic analysis of rat urine following the intravenous administration of pravastatin. *Journal of Pharmaceutical and Biomedical Analysis*, 44(4), 845–852.
- Lin, Y. P., Si, D. Y., Zhang, Z. P., & Liu, C. X. (2009). An integrated metabolomic method for profiling of metabolic changes in carbon tetrachloride induced rat urine. *Toxicology*, 256(3), 191–200.
- Lu, C. F., Wang, Y. M., Sheng, Z. G., Liu, G., Fu, Z., Zhao, J., et al. (2010). NMR-based metabolomic analysis of the hepatotoxicity induced by combined exposure to PCBs and TCDD in rats. *Toxicology and Applied Pharmacology*, 248(3), 178–184.
- Lv, H., Palacios, G., Hartil, K., & Kurlans, I. J. (2011). Advantages of tandem LC–MS for the rapid assessment of tissue-specific metabolic complexity using a pentafluorophenylpropyl stationary phase. *Journal of Proteome Research*, 10(4), 2104–2112.
- Ma, C., Bi, K. S., Zhang, M., Su, D., Fan, X. X., Ji, W., et al. (2010). Toxicology effects of Morning Glory Seed in rat: A metabolomic method for profiling of urine metabolic changes. *Journal of Ethnopharmacology*, 130(1), 134–142.
- Mal, M., Koh, P. K., Cheah, P. Y., & Chan, E. C. (2011). Ultra-pressure liquid chromatography/tandem mass spectrometry targeted profiling of arachidonic acid and eicosanoids in human colorectal cancer. *Rapid Communications in Mass Spectrometry*, 25(6), 755–764.
- Maxfield, F. R., & Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature*, 438(1), 612–621.
- National Commission of Chinese Pharmacopoeia. (2005). *Pharmacopoeia of People Republic of China*. Beijing: CI. Press.
- Newsholme, P., Procopio, J., Lima, M. M. R., Pithom-Curi, T. C., & Curi, R. (2003). Glutamine and glutamate—Their central role in cell metabolism and function. *Cell Biochemistry and Function*, 21(1), 1–9.
- Nicholson, J. K., Lindon, J. C., & Holmes, E. (1999). ‘Metabonomics’: Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica*, 29(11), 1181–1189.
- Ong, E. S., Chor, C. F., Zou, L., & Ong, C. N. (2009). A multi-analytical approach for metabolomic profiling of zebrafish (*Danio rerio*) livers. *Molecular Biosystems*, 5(3), 288–298.
- Ong, E. S., Zou, L., Li, S., Cheah, P. Y., Eu, K. W., & Ong, C. N. (2010). *Molecular & Cellular Proteomics*, in press.
- Plumb, R., Granger, J., Stumpf, C., Wilson, I. D., Evans, J. A., & Lenz, E. M. (2003). Metabonomic analysis of mouse urine by liquid-chromatography–time of flight mass spectrometry (LC–TOFMS): Detection of strain, diurnal and gender differences. *Analyst*, 128(7), 819–823.
- Plumb, R. S., Stumpf, C. L., Gorenstein, M. V., Castro-Perez, J. M., Dear, G. J., Anthony, M., et al. (2002). Metabonomics: The use of electrospray mass spectrometry coupled to reversed-phase liquid chromatography shows potential for the screening of rat urine in drug development. *Rapid Communications in Mass Spectrometry*, 16(20), 1991–1996.
- Qiu, F., Zhu, Z. Y., Kang, N., Piao, S. J., Qin, G. Y., & Yao, X. S. (2008). Isolation and identification of urinary metabolites of berberine in rats and humans. *Drug Metabolism and Disposition*, 36(11), 2159–2165.
- Sach, R. B., & Froehlich, J. L. (1982). Berberine inhibits intestinal secretory response of *Vibrio cholerae* and *Escherichia coli* enterotoxins. *Infection and Immunity*, 35(2), 471–475.
- Sieber, M., Hoffmann, D., Adler, M., Vaidya, V. S., Clement, M., Bonventre, J. V., et al. (2009). Comparative analysis of novel noninvasive renal biomarkers and metabolomic changes in a rat model of gentamicin nephrotoxicity. *Toxicological Sciences*, 109(2), 336–349.
- Slupsky, C. M., Cheyesh, A., Chao, D. V., Fu, H., Rankin, K. N., Marrie, T. J., et al. (2009). *Streptococcus pneumoniae* and *Staphylococcus aureus* pneumonia induce distinct metabolic responses. *Journal of Proteome Research*, 8(6), 3029–3036.
- Slupsky, C. M., Rankin, K. N., Wagner, J., Fu, H., Chang, D., Weljie, A. M., et al. (2007). Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. *Analytical Chemistry*, 79(18), 6995–7004.
- Sola-Penna, M. (2008). Metabolic regulation by lactate. *IUBMB Life*, 60(9), 605–608.
- Stella, C., Beckwith-Hall, B., Cloarec, O., Holmes, E., Lindon, J. C., Powell, J., et al. (2006). Susceptibility of human metabolic phenotypes to dietary modulation. *Journal of Proteome Research*, 5(10), 2780–2788.
- Strott, C. A., & Higashi, Y. (2003). Cholesterol sulfate in human physiology: What’s it all about? *Journal of Lipid Research*, 44(7), 1268–1278.
- Szachowicz-Petelska, B., Sulkowski, S., & Figaszewski, Z. A. (2007). Altered membrane free unsaturated fatty acid composition in human colorectal cancer tissue. *Molecular and Cellular Biochemistry*, 294(1–2), 237–242.
- Tan, Y. L., Goh, D., & Ong, E. S. (2006). Investigation of differentially expressed proteins due to the inhibitory effects of berberine in human liver cancer cell line HepG2. *Molecular Biosystems*, 2(5), 250–258.
- Tsai, P. L., & Tsai, T. H. (2004). Hepatobiliary excretion of berberine. *Drug Metabolism and Disposition*, 32(4), 405–412.
- Um, S. Y., Chung, M. W., Kim, K. B., Kim, S. H., Oh, J. S., Oh, H. Y., et al. (2009). Pattern recognition analysis for the prediction of adverse effects by nonsteroidal anti-inflammatory drugs using ^1H NMR-based metabolomics in rats. *Analytical Chemistry*, 81(12), 4734–4741.
- Waters, N. J., Waterfield, C. J., Farrant, R. D., Holmes, E., & Nicholson, J. K. (2005). Metabonomic deconvolution of embedded toxicity: Application to thioacetamide hepato- and nephrotoxicity. *Chemical Research in Toxicology*, 18(4), 639–654.
- Waters, N. J., Waterfield, C. J., Farrant, R. D., Holmes, E., & Nicholson, J. K. (2006). Integrated metabolomic analysis of bromobenzene-induced hepatotoxicity: Novel induction of 5-Oxoprolinosis. *Journal of Proteome Research*, 5(6), 1448–1459.
- Wei, L., Liao, P. Q., Wu, H. F., Li, X. J., Pei, F. K., Li, W. S., et al. (2009). Metabolic profiling studies on the toxicological effects of realgar in rats by ^1H NMR spectroscopy. *Toxicology and Applied Pharmacology*, 234(3), 314–325.
- Williams, R. E., Jacobsen, M., & Lock, E. A. (2003). ^1H NMR pattern recognition and ^{31}P NMR studies with D-serine in rat urine and kidney, time- and dose-related metabolic effects. *Chemical Research in Toxicology*, 16(10), 1207–1216.
- Wu, G. Y. (2009). Amino acids: Metabolism, functions, and nutrition. *Amino Acids*, 37(1), 1–17.
- Yan, D., Jin, C., Xiao, X. H., & Dong, X. P. (2008). Antimicrobial properties of berberines alkaloids in *Coptis chinensis* Franch by microcalorimetry. *Journal of Biochemical and Biophysical Methods*, 70(6), 845–849.
- Yin, J., Xing, H. L., & Ye, J. P. (2008). Efficacy of berberine in patients with type 2 diabetes mellitus. *Metabolism Clinical and Experimental*, 57(5), 712–717.
- Yuan, J., Doucette, C. D., Fowler, W. U., Feng, X. J., Piazza, M., Rabitz, H. A., et al. (2009). Metabolomics-driven quantitative analysis of ammonia assimilation in *E. coli*. *Molecular System Biology*, 5, 302–317.

- Zhou, J. Y., Zhou, S. W., Zhang, K. B., Tang, J. L., Guang, L. X., Ying, Y., et al. (2008). Chronic effects of berberine on blood, liver glucolipid metabolism and liver PPARs expression in diabetic hyperlipidemic rats (pharmacology). *Biological and Pharmaceutical Bulletin*, 31(6), 1169–1176.
- Zuo, F., Nakamura, N., Akao, T., & Hattori, M. (2006). Pharmacokinetics of berberine and its main metabolites in conventional and pseudo germ-free rats determined by liquid chromatography/ion trap mass spectrometry. *Drug Metabolism and Disposition*, 34(12), 2064–2072.