Development of a non-targeted metabolomics method to investigate urine in a rat model of polycystic kidney disease

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Abstract

Aim: The purpose of this research was to use metabolomics to investigate the cystic phenotype in the Lewis polycystic kidney rat.

Methods: Spot urine samples were collected from four male Lewis control and five male Lewis polycystic kidney rats aged 5 weeks, before kidney function was significantly impaired. Metabolites were extracted from urine and analysed using gas chromatography–mass spectrometry. Principal component analysis was used to determine key metabolites contributing to the variance observed between sample groups.

Results: With the development of a metabolomics method to analyse Lewis and Lewis polycystic kidney rat urine, 2-ketoglutaric acid, allantoin, uric acid and hippuric acid were identified as potential biomarkers of cystic disease in the rat model.

Conclusion: The findings of this study demonstrate the potential of metabolomics to further investigate kidney disease.

Keywords: biomarker; gas chromatography; metabolite; polycystic kidney disease; urine

The advent of metabolomics, also termed metabolic profiling, has been in light of not only recent technological advancements in the separation and identification of low-molecular-weight molecules, but also in the software and hardware for processing the resultant large quantities of data.[1]As the physicochemical properties of metabolites, such as polarity, volatility and solubility, vary greatly, metabolomic analysis can involve the use of several complimentary analytical techniques.[2] Gas chromatography–mass spectrometry (GC-MS) is a commonly employed method for the non-targeted screening of metabolites due to its ability to resolve hundreds of metabolites with a single injection of sample.[3–5] Coupled with the sensitivity and ability of MS to provide structural information, the detection, separation and identification of metabolites are now possible.[2]Metabolomics allows for the analysis of small sample volumes, enabling the development of less invasive studies of animal and human subjects. There is, however, no agreed-upon method for the selection, collection and preparation of metabolomics samples. Method optimization is therefore a key issue that needs to be established.

Polycystic kidney diseases (PKD) are inherited diseases characterized by renal tubular defects[6] for which there is no cure. In humans, advances in molecular genetics and studies arising from animal models have allowed the characterization of the three specific PKD genes: *PKD1*, *PKD2* and *PKHD1*.[7] Autosomal dominant polycystic kidney disease (ADPKD) has an incidence of 1 in 400–1000[8] and is caused by mutations in the *PKD1* or *PKD2* genes,[9] presenting

typically in adulthood. Autosomal recessive polycystic kidney disease (ARPKD) is due to mutations in the *pkhd1* gene and has an incidence of 1 in 20 000,[7] accounting for 5–8% of patients requiring kidney dialysis and/or transplantation.[7] Unlike ADPKD, ARPKD is most commonly evident *in utero* or at birth, but has also been characterized in later childhood and in patients over 20 years of age.[7] Other cystic diseases include the nephronophthisis (NPHP) cystic group, for which 13 genes have been identified,[10] and syndromes such as Bardet–Biedl syndrome or Meckel–Gruber syndrome which also have cystic kidneys as one of the pathologic features.[8,10]

Much current research has centred on determining the molecular/cellular basis underlying cyst development[7,11] and therapeutic interventions for patients suffering from PKD.[7,11] The use of non-targeted metabolomics as a tool to diagnose, monitor or investigate cystic renal disease mechanisms is not established. The purpose of this study was to use urinary metabolomics as a method to investigate the cystic phenotype in the recently characterized Lewis polycystic kidney (LPK) rat.[12] A key component in the process of this work was to optimize the methodology, increasing its utility as an application for renal disease metabolite profiling.

Methods

Chemicals

Chemicals were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). *n*-hexane (>95%) and methanol (>99%) were purchased from LabScan (Seacliff, SA, Australia).

Experimental

A total of 13 urine samples were used. The experimental group consisted of six biological replicates and the control group consisted of seven biological replicates. A subsample of each urine sample was taken and pooled for method development experiments. For each experiment, three technical replicates were used.

Samples

Spot urine samples were obtained from male 5 week Lewis (n = 7) and LPK (n = 6) rats, held at the Animal Resources Centre (ARC), Perth, WA, Australia. Samples were collected with approval from the relevant ethics committee and experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition. Samples were stored at −80°C until processing.

Metabolite extraction

Unless otherwise stated, 50 μL of urine was treated with 50 μL of a urease solution (1 U/μL). Samples were incubated for 30 min at 37°C and centrifuged for 10 min at 4000 rpm. Chilled methanol containing internal standard (ribitol, 29.25 μg/mL) was added to the sample. Samples were agitated for 10 min at 1200 rpm and subsequently centrifuged for 10 min at 4°C and 12 000 rpm. Aliquots (50 μL) of the sample were dried in an Eppendorf Concentrator Plus rotary vacuum concentrator (Eppendorf South Pacific Pty Ltd, North Ryde, NSW, Australia).

Derivatization

Metabolites were treated with 20 μL of methoxylamine-HCl (20 mg/mL in pyridine) and agitated for 90 min at 1200 rpm at 30°C in an Eppendorf Thermomixer Comfort. Following this, 40 μL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was added and the metabolites were incubated at 37°C for 30 min in the thermomixer. The metabolites were agitated for the first 1–2 min of this incubation period to mix the MSTFA. The 60 μL sample was then transferred to a vial containing 5 μL of alkane mix in hexane (C_{10}–C_{36}) to allow the calculation of a Kovat's index for each metabolite.

Instrumentation

An Agilent 6890 series Gas Chromatograph coupled to an Agilent 5973N mass selective detector (Palo Alto, CA, USA) was used. A Varian Factor Four-fused silica capillary column VF 5MS (30 m × ID = 0.25 mm × DF = 0.25 μm + 10 m EZ-Guard; Agilent Technologies, Palo Alto, CA,

USA6) was used. The derivatized sample (1 μL) was injected splitless into the inlet at a temperature of 230°C. The initial column temperature was set to 70°C. A temperature ramp of 1°C/min for 5 min followed by a temperature ramp of 5.63°C/min to a final temperature of 330°C was then set. The carrier gas used was helium at a flow rate of 1 mL/min (constant flow). The transfer line was set to 330°C and the ion source was set to 230°C. Ionization was achieved with a 70 eV electron beam and the mass spectrometer was set to scan ion masses in the range of *m/z* 45–600 at a scan rate of 1.56 scans per second.

Data analysis

Gas chromatography chromatograms and mass spectra were viewed using AnalyzerPro v2.2.0.7 (SpectralWorks, Runcorn, UK). All unique components of the chromatogram were identified and, where possible, matched to an in-house library of metabolite standards (Metabolomics Australia Node; Murdoch University, Perth, WA, Australia). In addition, spectra were compared with the National Institute of Standards and Technology (NIST) 2005 Mass Spectral library to aid in the identification of unknown metabolites. Data were normalized to an internal standard, ribitol, and to creatinine. Univariate statistical analyses were conducted using spss v17.0 using a paired-samples *t*-test (significance level $P = 0.05$) or a one-way anova with Bonferroni *post hoc* multiple comparisons (significance level $P < 0.05$) where appropriate. Pearson correlation was employed (significance level $P < 0.01$) where appropriate. Multivariate principal component analysis (PCA) was conducted using the Unscrambler v9.8 (CAMO, Oslo, Norway). Data were log-transformed using the equation $x = \log(x + 1)$.

Results

Extraction optimization

Urease was added to a 200 μL pooled urine sample at a concentration of 0.3 U/μL of urine and incubated at 37°C for 10 min. The sample was then dried and derivatized with MSTFA as described

in *Experimental*. The resulting chromatogram (not shown) showed an overloaded peak in the region of RT (retention time) 16.7–19.8 min. The experiment was repeated with three modifications: (i) the amount of urease added per microlitre of urine was increased to 1 U; (ii) the incubation period was increased to 30 min; and (iii) the volume of urine treated with urease was decreased to 50 μL. The resulting chromatogram (Fig. 1) showed that the urea peak, located at RT 16.7 min, did not exhibit excessive overloading.

Initial experiments involving catalysed urea hydrolysis highlighted the need to investigate the extraction volume of urine to reduce overloading. There was a significant difference ($P < 0.05$) in the number of components between each of the urine volumes (10, 25, 50, 200 μL) tested, and there was a significant correlation ($P < 0.01$) between the number of components found and the urine volume, demonstrating good linearity ($r = 0.99$) of the assay method. There was no significant difference between the number of library-matched components identified for the volumes 25 μL, 50 μL and 200 μL. At an earlier stage, it was concluded that extracting 200 μL of urine resulted in an overload of material on the GC column; however, it also yielded the greatest total number of components. A 20:1 split ratio of a 200 μL urine extract ($n = 3$) yielded 179 ± 7 components and 18 ± 0 library-matched components, significantly less ($P < 0.05$) than the splitless injection mode which yielded 473 ± 7 components. Extracted urine volumes of 10, 25 and 50 μL showed significantly less components than 200 μL ($P < 0.01$); however, there was much less evidence of overloading with these smaller extraction volumes. A 50 μL extract is shown in Figure 1 with a maximum ion intensity of 3×10^7.

Some debate has centred on the effect of centrifuging urine prior to metabolomic analysis, specifically the potential loss of metabolites in the sediment of the urine during centrifugation.[13] Urine samples (200 μL, in triplicate) with and without an initial centrifugation step (as described in *Metabolite extraction*) were prepared for extraction. Centrifugation resulted in 473 ± 7 components and 44 ± 1 library-matched components detected, and the non-centrifuged samples resulted in 436 ± 33 components and 43 ± 4 library-matched components detected. There was no significant difference between the number of components ($P = 0.14$) and library-matched components ($P = 0.68$) found

between the centrifuged and non-centrifuged groups. It is noted, however, that there is greater variance in the number of detected components in the non-centrifuged group.

Data analysis

Internal standards are used as a measure of method reproducibility. Trimethylsilylated ribitol was used as an internal standard in these experiments. The relative standard deviation of the ribitol peak area in previously prepared mannitol standards was 2.68% which indicated that the assay was highly reproducible. There was a significant correlation ($r = 0.62$; $P < 0.01$) between the sum of the peak area of all components in the urine samples to the respective ribitol peak area. Based on this result, it was assumed that if ribitol was affected by sample preparation and instrument variability, all peaks were subsequently affected. The peak area of ribitol in each of the urine samples showed a relative standard deviation of 14.02%. To further normalize the data and correct for varying hydration states, peaks were normalized to creatinine. The sum of all peak areas in the sample was correlated to the respective creatinine peak area. This was found to be significant ($r = 0.65$; $P < 0.01$) after ribitol normalization.

Principal component analysis

Figure 2 shows the PCA scores plot of 5 week-old male Lewis and LPK rat urine metabolite profiles. This output was generated using peak area data normalized to the internal standard and to creatinine which was subsequently log-transformed. The scores plot shows grouping of the urinary metabolite profile of the LPK rat samples. The top 12 metabolites contributing to variance in PC-1 are shown (Fig. 3). The X-loadings revealed that the metabolites attributing to the variance between sample groups included 2-ketoglutaric acid, uric acid and allantoin.

Discussion

In this study, we have developed a method for non-targeted metabolite profiling of rat urine, using the approach to investigate the cystic phenotype of the LPK rat. Importantly, our results are comparable

with those of Taylor *et al*.[14] and Toyohara *et al*.,[15] who used metabolite profiling to investigate the cystic phenotype in *jck* mice and Cy/+ rats respectively, validating this approach as a means for identification of biomarkers of diagnostic and therapeutic potential.

Our determination of the extensive optimization required for urine sample preparation prior to analysis is a key outcome of this study. Alvarez-Sanchez *et al*.[16,17] and Walsh *et al*.[18] have reviewed the challenges in metabolomics, particularly in the selection, collection and preparation of samples. As we have shown, initial centrifugation did not significantly affect the number of metabolites detectable in Lewis and LPK rat urine by GC-MS. A *et al*.[13] found that metabolites such as myoinositol, urate, hippurate, glycerate, p-hydroxyphenylacetate and a number of unidentified compounds were found in greater concentrations in non-centrifuged rat urine. However, A *et al*.[13] also found that centrifugation improved the response of metabolites such as creatinine, phosphorate and allantoin and proposed that this was due to improved trimethylsilylation for analytes at lower concentrations in the centrifuged supernatants. As a key aim of this study was to develop a method for non-targeted urinary metabolic profiling, it was decided that the centrifugation method should be adopted for effective trimethylsilylation, particularly because creatinine was to be used for normalization and allantoin has been previously identified as a potentially important metabolite in the cystic phenotype.[14,15]

Biofluids such as plasma, serum and cerebrospinal fluid are physiologically controlled; however, urine can be much more varied. This is mainly due to water consumption, but also as a result of other factors such as breathing pattern[19] and blood pressure.[20,21] It was expected that hydration state would affect the concentration of metabolites in the urine and potentially cause significant variability in the metabolic profiles. To correct for this, creatinine was identified as a normalization strategy. As the focus of this study uses a model of kidney failure, there was concern that creatinine levels would be significantly lower in the LPK animals than Lewis rats. The result of a one-way anova found that there was no significant difference in urinary creatinine at 5 weeks of age between strains. This finding was supported by Phillips *et al*.,[12] who found that serum creatinine was not significantly increased in the LPK rat until 12 weeks of age.

Our data indicate that PCA is a powerful tool for visualizing metabolomics data. The model (Fig. 2) is representative of approximately 100 metabolites for 13 individual animals. PCA is commonly used to identify specific metabolites which are attributed to variations between disease states;[4,20,22,23] however, it can also be used as a method for data mining to identify interferences due to assay method.[5] The X-loadings for PC-1 revealed the top 12 metabolites attributing to the variance between sample groups (Fig. 3). The presence of mannitol is an indication that there was overloading in the samples resulting in the misidentification of this peak as higher eukaryotes do not synthesize or catabolize mannitol.[24,25]The peak identified as mannitol was most likely a closely eluting carbohydrate with a similar fragmentation pattern. For this reason, mannitol was not excluded from the data analysis. Mannitol may be present in urine if it has been taken up from the rats' diet or other external source; however, as it is not involved in metabolism, it would not provide information about the PKD phenotype with respect to disease pathogenicity or progression.

The presence of 2-ketoglutaric acid, uric acid, allantoin, glucose and hippuric acid are indicative that the groups are likely to be separated due to disease state. 2-ketoglutaric acid (α-ketoglutaric acid) and hippurate (hippuric acid) have been linked to hypertension by Akira *et al*.,[20] and hypertension has been identified as a clinical symptom of cystic kidney disease in the LPK rat by Phillips *et al*.[12] even at this young age. 2-ketoglutaric acid is involved in the tricarboxylic acid (TCA) cycle and the urea cycle, two pathways which are exceedingly relevant to the study of kidney disease.[26] Toyohara *et al*.[15] identified 2-ketoglutaric acid (2-oxoglutarate) as significantly different between cystic and control rats as well as citrate, hippurate and trimethylamine *N*-oxide which were also identified by Akira *et al*.[20] as significantly different between hyper- and normotensive rats. It was postulated that differences in hippurate and trimethylamine *N*-oxide between these rats were due to strain differences in intestinal microfloral populations. The results of this study, comparable with those of Taylor *et al*.[14] and Toyohara *et al*.,[15] however, are evidence that these metabolites are more likely to be attributed to either hypertension or kidney disease. Interestingly, we know hippuric acid to be a marker of chronic kidney disease and it is also involved in inhibiting glucose usage.[27] These metabolites demonstrate the link between kidney disease and hypertension and also, including glucose

as a metabolite which contributes to variance between LPK and control animals, suggest a link between kidney disease and diabetes. Furthermore, allantoin, a marker of oxidative stress in humans,[28] and uric acid have been shown to be linked to kidney and/or liver dysgenesis.[14,15,27] In particular, Forbes *et al.*[29] have argued that oxidative stress is key in the development of diabetic kidney disease. Certainly, a change in purine metabolism in the LPK male rat is evident here, as also found by Taylor *et al.*[14] The involvement of purine metabolism and glycolysis in cystic kidney disease has been demonstrated previously;[26,30,31] however, it is now possible to directly measure the metabolites involved in these pathways.

While these findings provide a solid foundation for investigating cystic kidney disease using metabolomics, food and water intake, urine collection technique (i.e. spot urine) and sample preparation cannot be ruled out as factors influencing variance. Walsh *et al.*[18] and Holmes *et al.*[21] have identified urinary metabolomics as an effective method of determining and assessing dietary intake which suggests that food and water should be either withheld or strictly controlled to eliminate this as a factor contributing to variance. Alvarez-Sanchez *et al.*[16,17] have reported on the selection and preparation of metabolomics samples and Walsh *et al.*[18] and Bando *et al.*[32] have identified the need to investigate the difference between various urine collection techniques. It has been determined that, with respect to selection, urine is often chosen as it is non-invasive and therefore preferred in clinical applications. With respect to collection, common techniques in urinary metabolomics include spot collection[18] and, with the use of metabolic cages, 4 h,[32] 12 h[13] and 24 h pooled collection.[20,32] The 24 h samples have been shown to exhibit little variation in a control group[32] as this method potentially overcomes diurnal effects; however, a shorter collection time has shown greater relative concentrations of metabolites.[32] Samples used in this study were obtained by spot collection and from an external source. Therefore, factors such as collection technique and diurnal variation may contribute to metabolite profiles and influence the discrimination of sample groups shown in Figure 2. Similarly, sample handling, in particular the use of urease[5,13] to break down urea and eliminate overloading, peak distortion, matrix effects and ion suppression,[33] can alter metabolite profiles.[34] The metabolites diminished by urease treatment outlined by Kind *et al.*,[34] however, have not been

identified as potentially important in this study or a previous study which used a metabolomics approach to investigate urine in PKD without the use of urease treatment.[14]

Clearly, there are advantages in the collection and analysis of urine samples; however, it is suggested that in global metabolite profiling, a number of biofluids should be analysed for increased coverage of the metabolome.[16] By collecting blood samples, for example, intermediates of metabolism can be measured rather than the end products which are typical of urine. With the inclusion of intermediate metabolites, the biochemistry of disease progression can be interpreted with more confidence.

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Figure 1. Gas chromatography–mass spectrometry (GC-MS) chromatogram of a 50 µL urine metabolite extract showing a total of 230 compounds. Of these, 108 were library matched.

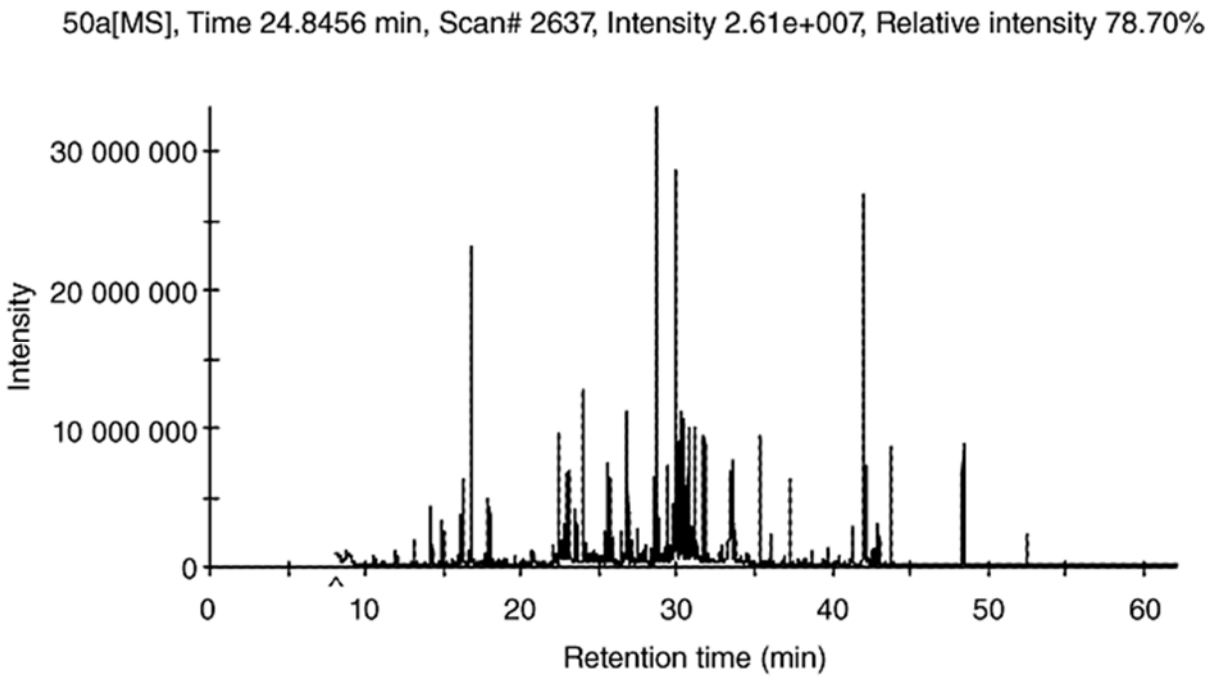

Figure 2. Principal component analysis (PCA) scores plot and corresponding loadings plot of 5 week-old male Lewis and Lewis polycystic kidney (LPK) rat urine metabolite profiles. This output was generated using log-transformed peak area data normalized to the internal standard, ribitol, and to creatinine.

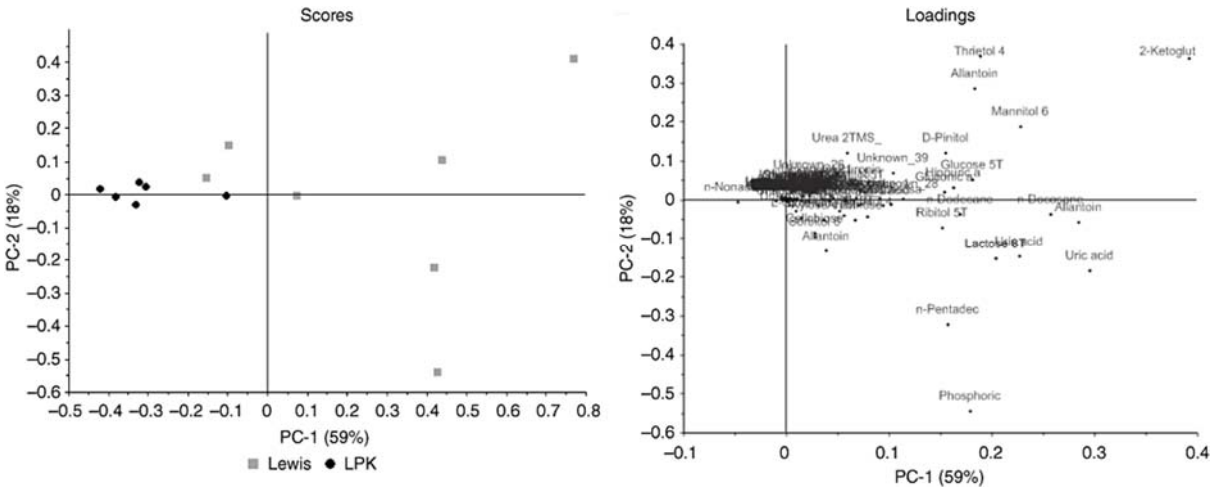

Figure 3. The top 12 metabolites (2-ketoglutaric acid, uric acid, allantoin, mannitol, lactose, thrietol, glucose, phosphoric acid, hippuric acid and pinitol) contributing to variance in PC-1 are shown.