Analysis of human urine metabolites using SPE and NMR spectroscopy

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Nuclear magnetic resonance (NMR) spectroscopic analysis of metabonome/metabolome has widespread applications in biomedical science researches. However, most of NMR resonances for urinary metabolites remain to be fully assigned. In the present study, human urine samples from two healthy volunteers were pre-treated with C18 solid-phase extraction and the resultant 5 sub-fractions were subjected to one- and two-dimensional NMR studies, including ¹H J-Resolved, ¹H-¹H COSY, ¹H-¹H TOCSY, ¹H-¹³C HSQC, and HMBC 2D NMR. More than 70 low molecular weight metabolites were identified, and complete assignments of ¹H and ¹³C resonances including many complex coupled spin systems were obtained.

urine metabolites, NMR spectroscopy, solid phase extraction, metabonomics/metabolomics

1 Introduction

Metabolite analysis has long been an important aspect of biological sciences and the nuclear magnetic resonance (NMR)-based metabonomics have been proven as a powerful technology for measuring the metabolic responses of living systems to internal or external stimuli^[1–5]. This is because (1) the changes in gene and protein levels are often manifested into much greater changes in the metabonomes offering a better opportunity to detect such alterations, and (2) the number of metabolites is also conceived smaller than that of genes and proteins. Nevertheless, metabonomics is still under rapid development in both theory and technology aspects^[1–13]. Conventional metabolite analysis requires complicated sample preparations but often results in information on some limited number of metabolites. The rapid development of analytical technologies makes it possible for metabonomic analysis of a large number of samples containing many metabolites. Amongst many metabonomic detecting techniques, NMR^[9,14–21] and

mass spectrometry^[22–26] remain the most important.

As one of the most widely used analytical techniques in both chemistry and biosciences, NMR provides information on molecular structure, dynamics and interactions with unbiased detection. For example, ¹H NMR spectra show proton signals of all constituents in a complex mixture with the same response coefficients. The position of signals in NMR spectra, described by chemical shift, is directly related to the electron environment of the given atoms and 2D NMR techniques provide direct atomic connectivity^[27] in the metabolite molecules, thus structure, whereas the signal intensity is linearly proportional to the quantity of the analytes. Therefore, the NMR spectra can be considered as quantitative “finger-prints” for a metabonome. Due to its non-destructive nature, NMR can be used to analyze the

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composition of metabolites under or close to the physiological conditions. With some spectral editing techniques^[27], one can also separate signals of metabolites according to their physical properties without separating samples^[15,27,28]. With high resolution magic-angle spinning NMR and *in vivo* magnetic resonance techniques, the metabolites in biological tissues can also be analyzed which are exemplified in a number of studies of biological tissues such as the liver^[9,19,28–30] and intestine^[31,32].

Urine samples are one of the most frequently used biofluids for metabonomics studies, which consist of metabolites including amino acids, hydroxycarboxylic acids, carbohydrates and inorganic salts. The changes of these metabolites can be directly employed to reflect the biochemical changes in a living system. The NMR spectra of urine samples are normally assigned using a catalogue of 2D NMR methods and *in situ* “spiking” experiments. However, the complexity of urinary composition makes the complete assignments of the urinary spectra difficult with spiking alone or even with the combination of 2D NMR and spiking methods. For example, *in situ* spiking experiments require the so-called metabolite standards which are not necessarily available all the time. In particular, the standards for unknown metabolites, by default, are not available at all. Consequently, for the time being, only about one-third of detectable urinary metabolites have been assigned unambiguously. In order to improve the spectral assignment, this paper is aimed to provide a different strategy by employing pre-treatment of urine with solid phase extraction techniques to simplify urine samples according to their polarity and enhance the concentration of some minor metabolites together with a catalogue of 2D NMR techniques.

2 Experimental section

2.1 Materials

C18 solid phase extraction columns (or cartridges) were purchased from IST (International Sorbent Technology, UK) in the format of 0.5 g (6 mL) and 2 g (12 mL). Eluting solvents were aqueous formate buffer (formic acid and ammonium formate mixture, pH=2.8) containing acetonitrile (CH₃CN). Formic acid and ammonium formate were purchased from Fisons Scientific U.K. Limited with purity of 98% and 99.3%, respectively. CH₃CN was purchased from BDH Laboratory Supplies

with purity of 99.9%. D₂O (99.9% D) was purchased from Goss Scientific Instruments Ltd whilst sodium 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate (TSP, 98%) was from Cambridge Isotope Laboratory Inc. Urine samples from two healthy male adult volunteers (northern Chinese, 36 and 38 years old) were collected once in the morning and afternoon on two separate days respectively without any diet or lifestyle control, and stored at –40°C prior to analysis.

2.2 Sample pre-treatment and SPE fractionation

C18 SPE cartridges (2 g, 6 mL) were activated with 24 mL CH₃CN with elution rate of 6 mL/min followed by conditioning with 25 mL formate buffer (0.1%, pH=3) containing 2% CH₃CN with the eluting rate of 6 mL/min. Urine samples (6 mL) were mixed with 0.12 mL CH₃CN and their pH was adjusted to pH=3 with formic acid prior to being loaded onto the SPE cartridge. The column was eluted with 15 mL formate buffer containing 2% CH₃CN to obtain the first fraction (Fraction 1). The column was then eluted with 15 mL formate buffer containing 15% CH₃CN to yield the second fraction (Fraction 2) and with 15 mL the same buffer containing 35% CH₃CN to collect the third fraction (Fraction 3). The cartridge was further eluted with 15 mL formate buffer containing 50% CH₃CN to obtain the fourth fraction (Fraction 4) followed with 20 mL formate (containing 75% CH₃CN) elution to obtain the final fraction (Fraction 5). After evaporating CH₃CN in a fume hood, the fractions were lyophilized and the resultant powers were added with D₂O followed with centrifugation to retrieve the supernatant for NMR experiments.

2.3 NMR spectroscopy

All NMR spectra were recorded on a Bruker AV600 NMR spectrometer equipped with a 5 mm conventional inverse triple resonance probe at 298 K and a 3 mm inverse CryoprobeTM at 292 K. For the 5 mm probe, the sub-fractions were reconstituted to 550 µL in a standard 5 mm NMR tube, whereas they were made up to 150 µL sample in an 1.7 mm capillary NMR tube for the cryoprobe and handled with a Bruker MATCHTM device. Both probes were equipped with a shielded gradient coil in Z-axis capable of delivering about 50 G/cm (1G = 10^{–4} T) pulse gradient. The length of 90° pulses was adjusted to about 10 µs unless specifically stated other-

wise.

One dimensional ^1H NMR spectra were acquired using a standard 1D-NOESY pulse sequence with pre-saturation (NOESYPR1D, RD-90°- t_1 -90°- t_m -90°-Aq), where the recycle delay, RD, was 2 s, t_1 was 4 μs , and mixing time, t_m , was 0.1 s. In the case of the first fraction of urine, the 90° pulse was 13 μs due to high salt concentration. 64 transients were collected into 32768 data points for each spectrum with a spectral width of 16 ppm unless specified otherwise. Water suppression was achieved with a weak continuous wave irradiation, equivalent to 50 Hz, on the water resonance during both the recycle delay and mixing time. The free induction decays (FIDs) were multiplied by an exponential function with a 0.3 Hz line-broadening factor prior to Fourier transformation.

Two dimensional (2D) NMR experiments were performed on a 600 MHz spectrometer equipped with a 3 mm cryoprobe. ^1H - ^1H COSY 2D NMR spectra were acquired using a gradient selected pulse sequence (RD-90°- t_1 -gp1-90°-gp2- t_2 -Aq) in the magnitude mode. Typical parameters include recycle delay of 1.1 s, gradient pulse length of 1.5 ms. The gradient pulses were sine-shaped with the strength of 3 G/cm and the spectra widths were 10.5 ppm on both dimensions. 256 increments with the 2048 data points in the acquisition dimensions were collected with 40 transients per increment. ^1H - ^1H TOCSY 2D NMR spectra were recorded with 2048 data points and 40 scans per increment with 128 increments; spectral width for both dimension was 10.5 ppm. MLEV-17 sequence^[33] was employed as spin-locking scheme with mixing time of 90 ms in the TPPI mode, and the recycle delay was 1.1 s.

^1H - ^{13}C HSQC 2D NMR spectra were recorded at 600.22 MHz for ^1H and 150.92 MHz for ^{13}C with gradient selection in the echo-antiecho mode. 304 scans were collected into 2048 data points each increment with 11 ppm for ^1H and 200 ppm for ^{13}C for 128 increments. The recycle delay was 1.1 s with GARP as the decoupling scheme. ^1H - ^{13}C HMBC 2D NMR spectra were recorded using gradient selection, magnitude mode and optimized for the long-range coupling of 6 Hz. 128 increments were recorded with 480 scans and 2048 data points per increment; the spectral width was 11 ppm for ^1H and 220 ppm for ^{13}C , and the recycle delay was 1.1 s.

All 2D data sets were applied appropriate sine-bell or phase shifted sine-bell functions depending on the phase

mode of evolution dimension prior to Fourier transformation into 2048×2048. Forward linear prediction was employed for the increment dimension to improve resolution for the second dimension.

3 Results and discussion

3.1 ^1H NMR spectroscopy of SPE fractions of human urine

Figure 1 shows the ^1H NMR spectra of the five SPE fractions from human urine. It is obvious that the spectra are significantly different for all fractions and signals became weaker as the elution progressed. The first fraction shows the strongest proton signals from the polar metabolites since the highest concentration metabolites are mostly from the polar ones in urine samples. Spectrum of the Fraction 1 is dominated by signals in the $\delta(2.5-4.5)$ region corresponding to polar metabolites, such as amino acids and hydroxycarboxylic acids whereas aromatic signals at $\delta(6.5-8)$ ppm are barely visible. Fraction 3 appears to be mainly containing fatty acids with resonances at $\delta(0.7-2)$ ppm, whilst Fraction 4 and Fraction 5 only have some weak signals, showing a few metabolites in these fractions. These observations are consistent with the fractionation feature of the reverse-phase chromatographic separation of the urine metabolites and with the fact that urinary metabolites are dominated by relatively polar compounds.

3.2 2D NMR spectroscopy of the urinary SPE fractions

2D NMR techniques provide information on atomic connectivity with improved spectral resolution, assisting metabolite identification unambiguously. Figure 2 shows the examples for the ^1H - ^1H COSY and TOCSY 2D NMR spectra for Fraction 1 of one of the urine samples providing the proton connectivity, whereas Figure 3 shows ^1H - ^{13}C HSQC and HMBC 2D NMR spectra of the same fraction providing information on the H-C connectivity and long range atomic connectivity. Figure 4 shows the J-resolved spectra of the fraction providing information about the coupling patterns for each proton signal.

From detailed analysis of these 2D NMR spectra, more than 70 metabolites have been identified with detailed NMR information such as ^1H and ^{13}C chemical shifts, ^1H coupling patterns, and coupling constants (Figures 5, 6). Compared with “spiking” method, the

Figure 1 ^1H NMR spectra of urinary fractions.

Figure 2 ^1H - ^1H COSY and TOCSY (right hand) 2D NMR spectra of Fraction 1.

SPE-NMR methods used here do not require standards, and are relatively simple and potentially useful for detection of some metabolites otherwise not observable.

Amongst these 73 metabolites, 49 of them were detected in Fraction 1, whereas 18 metabolites were assigned from Fraction 2. Only 7 metabolites were assigned from Fraction 3, 4 and 5, which is largely consistent with the fact that most of the urinary metabolites are polar ones. From Fraction 1, most polar amino acids

were observed, such as histidine and its methyl derivative, glycine and its dimethyl derivative, alanine and its 3-methylamino derivative. Hydrophobic amino acids such as phenylalanine, tyrosine and valine were not detected. Generally, many amino acids were not widely reported from the urine samples, especially when NMR was used, due to their low concentration. However, the SPE methods used in this study provided a way of concentrating some of the weak metabolites aiding their

Figure 3 ^1H - ^{13}C HSQC and HMBC (right hand) 2D NMR spectra of Fraction 1.

Figure 4 ^1H J-Resolved 2D NMR spectra of Fraction 1 (regional expansions).

NMR detection. β -amino-isobutyrate was detected in this urine sample, which is consistent with the fact that this sample was donated from a Northern Chinese. Creatine and creatinine were also observed together with carboxylic acids including citrate, pyruvate, malonate and a derivative of butyrates, which is consistent with the results normally reported from the NMR studies. In addition, basic metabolites, such as trimethylamine and its derivatives trimethylamine oxide and dimethylamine, were also detected. Small amounts of glucose and mannitol were also detected together with methylnicotinamide and methylnicotinate. In contrast, Fraction 2 contained a number of phenylacetate derivatives, including hippurate, phenylacetate, para-hydroxyhippurate, and 4-hydroxyphenylacetate, which originated from the gut microflora metabolites or co-metabolism between host and gut microbiota. Other metabolites from gut microflora were also detected, such as para-methylphenol^[34,35]. Tryptophan was also detected in this fraction. Fraction 3 contained 3-(3-methoxy-4-hydroxyphenyl) propionate

and indole-3-acetate^[29]. It is noteworthy that lactate, which has high polarity and good water solubility, appeared in Fraction 4 but not in the earlier fraction probably owing to its intermolecular hydrogen bonding effects or some protein-binding effects. It is also worth pointing out that all fractions studied here were in acidic condition, and therefore chemical shifts may differ to various extents from that under neutral pH^[36]. The most noticeable such examples were histidine and its derivatives, such as 1-methylhistidines, anserine, and 3-methylhistidines (Table 1). This reminds one that when metabolomics studies were carried out, attention had to be paid to the pH effects on the chemical shifts.

3.3 Conclusions

Spectral assignment is fundamentally important for metabolomics studies. SPE fractionation combined with 2D NMR spectroscopic methods is a simple but useful way to identify the metabolites which otherwise may not be able to be detected or unambiguously assigned. Such

Figure 5 The 1D- ^1H NMR of fraction 1 of SPE. $\delta(0.8-9.5)$ ppm (1); $\delta(6.5-9.4)$ ppm (2); $\delta(4.4-6.5)$ ppm (3); $\delta(3.0-4.4)$ ppm (4) (A: anserine, B: 1-methyl-*L*-histidine, C: 3-methyl-*L*-histidine, D: histidine); $\delta(0.9-3.0)$ ppm (5).

method provides a strategy which is not dependent on the standard “spiking” method. SPE fractionation not only simplified spectral assignment, but also enhanced

the detecting sensitivity for some minor metabolites by enhancing their concentration together with the intrinsic sensitivity enhancement with the cryogenic NMR tech-

Figure 6 The $^1\text{D-H}$ NMR of fraction 2 of SPE, $\delta(9-0)$ ppm.

Table 1 The pH effects on the chemical shifts of histidine and its derivatives

Metabolites	Acidic	Neutral	Acidic	Neutral	Acidic	Neutral
	H-2 (ring)		H-4 (ring)		methyl group	
Anserine	8.66	7.95	7.32	6.97	3.86	3.72
Histidine	8.72	7.83	7.44	7.08	-	-
3-methylhistidine	8.67	7.63	7.39	7.00	3.91	3.69
1-methylhistidine	8.74	7.82	7.47	7.01	3.89	3.69

nology. Finally, NMR data from more than 70 metabolites obtained from this study will provide useful information for the future metabolite assignment.

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