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Metabolic fingerprinting of rat urine by LC/MS Part 1. Analysis by hydrophilic interaction liquid chromatography–electrospray ionization mass spectrometry

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Abstract

Complex biological samples, such as urine, contain a very large number of endogenous metabolites reflecting the metabolic state of an organism. Metabolite patterns can provide a comprehensive signature of the physiological state of an organism as well as insights into specific biochemical processes. Although the metabolites excreted in urine are commonly highly polar, the samples are generally analyzed using reversed-phase liquid chromatography mass spectrometry (RP-LC/MS). In Part 1 of this work, a method for detecting highly polar metabolites by hydrophilic interaction liquid chromatography–electrospray ionization mass spectrometry (HILIC/ESI-MS) is described as a complement to RP-LC/ESI-MS. In addition, in an accompanying paper (Part 2), different multivariate approaches to extracting information from the resulting complex data are described to enable metabolic fingerprints to be obtained. The coverage of the method for the screening of as many metabolites as possible is highly improved by analyzing the urine samples using both a C_{18} column and a ZIC®-HILIC column. The latter was found to be a good alternative when analyzing highly polar compounds, e.g., hydroxyproline and creatinine, to columns typically used for reversed-phase liquid chromatography.

Keywords: High-throughput profiling; Metabonomics; Metabolomics; Metabolite profiling; Metabolic profiling; Metabolic fingerprints; Biomarker; Biological fluids; Hydrophilic interaction; HILIC; LC/MS; ZIC®-HILIC column

1. Introduction

In drug research and development, there is an intense focus on the identification and validation of biomarkers for drug effect and toxicity. Metabolic profiling of body fluids using NMR or MS as analytical platforms allows the simultaneous detection of hundreds of low molecular weight species, resulting in the generation of endogenous metabolic profiles reflecting physiological status, toxic insult, or disease processes.

It is neither possible nor necessary to determine the individual level of every single endogenous metabolite in a biofluid; instead, a "fingerprint" of each biofluid can be a more feasible route [1]. In this methodology a generic analytical method for rapid profiling of a biological sample (Part 1) is needed, together with an appropriate chemometric method (Part 2) for data evaluation [2]. Today, metabolic fingerprinting using NMR has gained wide acceptance and is well documented in the literature [3,4]. However, liquid chromatography mass spectrometry (LC/MS) as a complementary technique in metabolic fingerprinting is advancing and there are a few publications in the field of small molecules [5–8]. To obtain a metabolic fingerprint, preferably as many metabolites as possible should be detected. The aim is, of

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course, to do this using only one fast generic method. However, due to the large differences in the chemical and physical properties of metabolites, several methods need to be combined.

Reversed-phase (RP) chromatography is the most commonly used separation technique for analyzing metabolites by LC/MS [9] and was used in previous work by us [6,8]. Although metabolites found in urine are expected to be polar compounds, it was found that many metabolites could be detected using a C_{18} column for separation. In that previous work the majority of the metabolites seemed to be retained on the column [10]; however, solid-phase extraction (SPE) preceded the LC/MS analysis and the highly polar metabolites were discarded during sample preparation. The most polar components would not have been retained on the analytical column either if the urine were injected directly without pretreatment.

To enable the analysis of highly polar compounds while still using mass spectrometry as a comprehensive and sensitive detector, hydrophilic interaction liquid chromatography (HILIC) is one alternative [11–13]. HILIC is similar to normal phase chromatography as a polar stationary phase is used and the separation is based on the distribution between stationary and mobile phases, although it differs in that aqueous mobile phases can be used. In this work a ZIC®-HILIC column was used, which is supposed to give reversed retention compared to RP-LC [14]. It separates compounds according to their weak electrostatic interactions between charged analytes and the zwitterionic stationary phase combined with hydrophilic interaction liquid chromatography. The influence of structural parameters upon retention and resolution when using this column is being evaluated in an ongoing project and will not be discussed here. However, retention times for a few known compounds have been reported below to illustrate the selectivity of the ZIC®-HILIC column.

This work focuses on the analysis of highly polar metabolites that are not retained on a C_{18} column. In previous studies these highly polar compounds were ignored during screening for biomarkers and drug metabolites. In this work (Part 1) two complementary chromatographic methods were used with the aim of increasing the number of metabolites to be detected. With this method, it is possible to analyze the less polar compounds using reversed phase, and the more polar fraction, i.e., the wash fraction from SPE, can be analyzed using the ZIC®-HILIC column.

2. Experimental

The workflow of the analysis is summarized in Fig. 1.

2.1. Urine samples

Urine from 11 male Wistar rats dosed with vehicle control (rats #1–5) or 130 mg of experimental drug X/kg/day (rats #6–11) for 14 consecutive days was collected. It was collected on one occasion prior to dosing and on day 14 of the study.

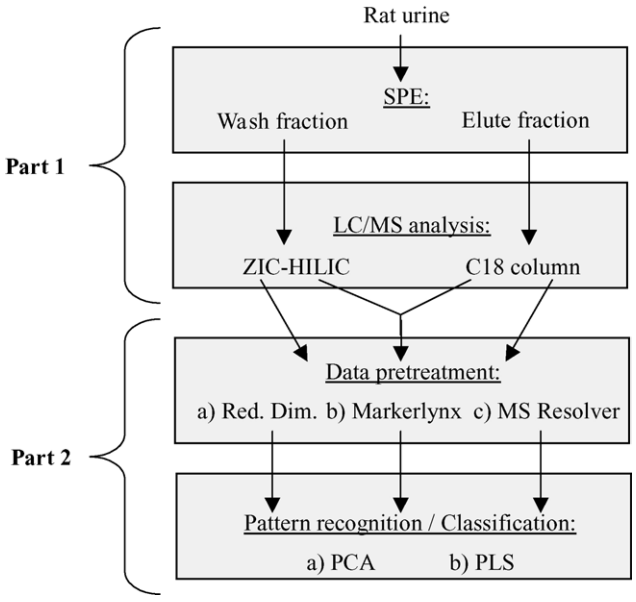

Fig. 1. Summary of the workflow. In this work Part 1 is described in detail. Part 2 is presented in an accompanied paper [10].

2.2. Standard solutions

Nicotine (CAS no.: 54-11-5), creatinine (CAS no.: 60-27-5), tryptophane (CAS no.: 54-12-6), lidocaine (CAS no.: 137-58-6), β-hydroxyethyl theophylline (CAS no.: 519-37-9), caffeine (CAS no.: 58-08-2), salicylic acid (CAS no.: 69-72-7), *p*-nitrophenol (CAS no.: 100-02-7) and cholic acid (CAS no.: 81-25-4) were purchased from Sigma and hydroxyproline (CAS no.: 51-35-4) and uric acid (CAS no.: 69-93-2) were purchased from Fluka. Standard stock solutions of approximately 10 mM were prepared in DMSO and diluted with Milli-Q water to approximately 0.1 mM solutions.

2.3. Sample preparation

Solid-phase extraction was used for sample preparation. The adsorbent was activated and conditioned first with 1 mL methanol and then with 1 mL ammonium acetate buffer (10 mM, pH 4). An aliquot of 0.5 mL of rat urine was loaded onto the SPE column (1 cc, 30 mg, Waters Oasis HLB). In the washing step a 0.5 mL ammonium acetate buffer (10 mM, pH 4) was used and 0.5 mL methanol was used for elution. Both the wash fraction and the eluate were collected separately and filtered through syringe filters (Gelman GHP Acrodiscs, 13 mm i.d., 45 μm, Merck) before injection. Samples awaiting preparation and newly prepared samples were stored at approx. 8 °C prior to analysis. For longer periods of storage, e.g., more than two days, the fractions were kept at −80 °C.

2.4. LC/ESI-MS analysis

For the LC/MS analysis, a Waters 2790 LC system was coupled to a Quattro Micro mass spectrometer (Waters, Milford, MA, USA).

2.4.1. Hydrophilic interaction liquid chromatography (HILIC)

For the HILIC analysis, a ZIC®-HILIC column (3.5 μm, 2.1 mm × 100 mm) from Sequant AB (Umeå, Sweden) was used together with a C_4 pre-column (5 μm, 2.1 mm × 10 mm) from Thermo (www.thermo.com). The injection volume was 5 μl. Mobile phase A consisted of 5 mM ammonium acetate (adjusted to pH 4 with formic acid), while mobile phase B consisted of acetonitrile and 0.025% formic acid. The gradient started with 10% A and increased linearly to 90% A within 15 min. The mobile phase was kept at isocratic conditions (90% A) for 4 min before the gradient was allowed to reach 10% A in 30 s and then equilibrated for 10.5 min. The total analysis time was 30 min and the flow rate was 100 μl/min.

2.4.2. Reversed-phase liquid chromatography (RP-LC)

For the RPLC analysis, an Xterra C18 column (3.5 μm, 2.1 mm × 150 mm) from Waters (Milford, MA, USA) was used together with a pre-column (3.5 μm, 2.1 mm × 10 mm). The injection volume was 5 μl. Mobile phase A consisted of 10 mM ammonium acetate (adjusted to pH 4 with formic acid), while mobile phase B consisted of acetonitrile. The gradient started with 10% B for 2 min and then linearly increased to 90% B within 15 min. The mobile phase was kept at isocratic conditions (90% B) for 8 min before the gradient was allowed to reach 10% B in 30 s and then equilibrated for 6.5 min. The total analysis time was 30 min and the flow rate was 200 μl/min.

2.4.3. Electrospray mass spectrometry

An electrospray ionization (ESI) interface was used. The capillary voltage was set to 2.5 kV and the cone voltage to 25 V. The source temperature was 110 °C. Profile data for negative ions from *m/z* (Thomson values) 50 to 600 were recorded at a speed of 1 s/scan. In the same run positive ions were collected, i.e., switching between negative and positive ion modes.

3. Results and discussion

Solid-phase extraction was used for sample preparation of the urine samples. The wash fraction was injected onto

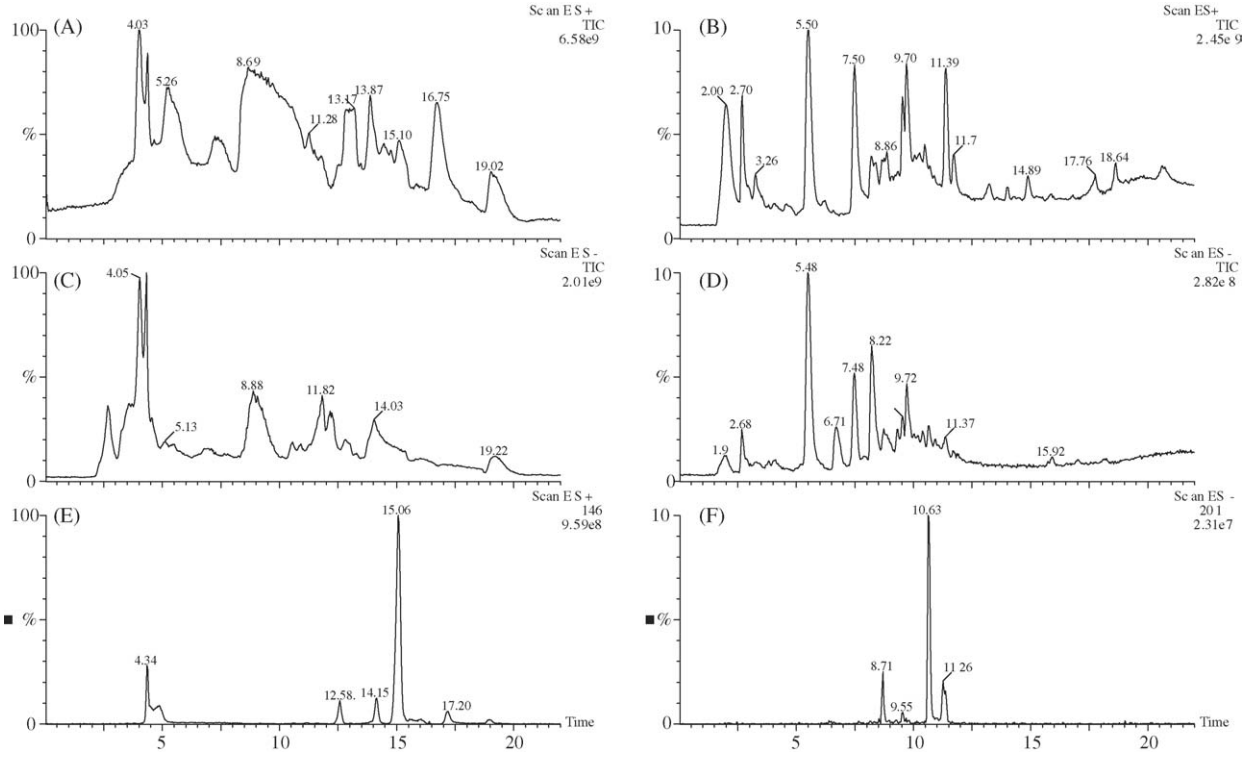

Fig. 2. Urine sample from rat dosed for 14 days was analysed after SPE. The wash fraction was analyzed on a ZIC®-HILIC column (A, C and E). Chromatograms to the right were obtained for the eluate fraction by RP-LC (B, D and F). The upper TIC (A and B) was obtained in positive ionization mode and parts C and D in negative. To illustrate the chromatography of a single, randomly chosen, mass number the RIC for *m/z* 146 in positive mode using ZIC®-HILIC are shown to the bottom left (E) and RIC of *m/z* 201 in negative mode using RP-LC are shown to the bottom right (F).

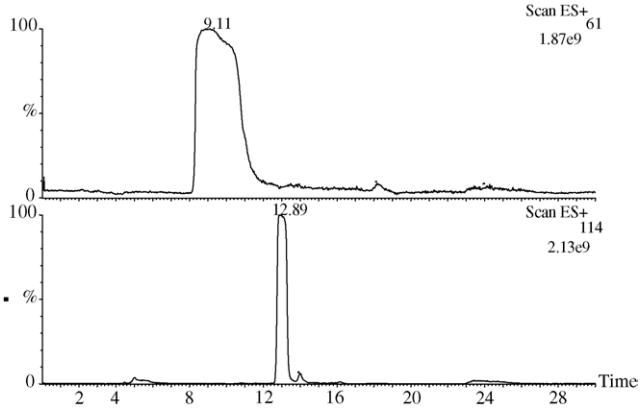

Fig. 3. Reconstructed ion chromatograms of *m/z* 61 (upper) and 114 (lower) are shown. A mass number of 61 is expected for urea and *m/z* 114 is expected for creatinine.

Table 1
Retention times for 11 compounds using HILIC and RP-HPLC, respectively, are shown

Compound	Retention time[a]		log *D* (pH 4)[b]
	HILIC	RP-HPLC	
Hydroxyproline ($C_5H_9NO_3$)	12.7	1.9	−4.34
Uric acid ($C_5H_4N_4O_3$)	10.15	2.06	−3.33
Nicotine ($C_{10}H_{14}N_2$)	14.6	2.6	−2.4
Creatinine ($C_4H_7N_3O$)	11.5	2.2	−2.38
Tryptophane ($C_{11}H_{12}N_2O_2$)	13.4	4.3	−1.47
Lidocaine ($C_{14}H_{22}N_2O$)	11.2	9.3	−0.73
β-Hydroxyethyl theophylline ($C_9H_{12}N_4O_3$)	3.4	5.4	−0.5
Caffein ($C_8H_{10}N_4O_2$)	3.3	7.5	−0.08
Salicylic acid ($C_7H_6O_3$)	2.7	7.5	1.03
p-Nitrophenol ($C_6H_5NO_3$)	3.2	11.7	1.57
Cholic acid ($C_{24}H_{40}O_5$)	3.3	13.5	2.54

[a] The void volume corresponding time (t_0) was calculated to 2.3 min for ZIC®-HILIC and 2.1 min for RP-LC which might differ slightly from experimental results.

[b] The distribution coefficient D is given as $D = [\text{R–COOH}_{org}]/([\text{R–COOH}_{aq}] + [\text{R–COO}^-_{aq}])$ for an acid or $\log D = c\log P - \log\{1 + \text{antilog}(\text{pH} - \text{p}K_a)\}$ where P is the partition coefficient to octanol. pK_a and $c\log P$ values were obtained from SciFinder and calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67.

the ZIC®-HILIC column and the eluate was injected onto a C_{18} column. Even though the sample solutions injected were strong eluents for each column, no chromatographic problems were observed as only 5 μl was injected. Two total ion chromatograms (TICs) obtained using ZIC®-HILIC in negative and positive ionization modes, respectively, are shown in Fig. 2A and C. The large peak at around 9 min in positive mode might correspond to urea, and creatinine elutes as an overloaded peak at 13 min. Reconstructed ion chromatograms (RICs) of corresponding mass number are shown in Fig. 3. In Fig. 2E and F additional RICs of randomly chosen mass numbers are shown to give an example of a chromatographic peak elution profile.

Although it is likely that a majority of metabolites can be analyzed using RP-LC, some metabolites are not retained and are more suitable for analysis using HILIC. In Table 1 retention times for a number of compounds retained on both columns are listed to give an overview of the retention on a ZIC®-HILIC column compared to a C18 column. Compounds in the upper part of Table 1 is not retained on the C18 column and compounds in the lower part of the table is not retained on the ZIC®-HILIC column under these conditions. The retention of different compounds is evaluated in detail in an ongoing work; however, simply by studying the ZIC®-HILIC chromatograms from a rat urine sample in Fig. 2, it is obvious that a lot of analytes are neglected if only RP-LC is used. The peaks in Fig. 2A and C would have been discarded as waste as they were detected in the wash fraction. Even though no sample preparation would have been used, i.e., direct injection of urine, these compounds would still had been rejected as, if the compounds are not retained on the SPE sorbent, which is a reversed-phase sorbent, they would show no retention on the analytical C18 column. The TIC obtained using RP-LC (Fig. 2C and D) shows that even in the eluate there are compounds that are not retained on the C18 column and elute in the void volume. This is due to that the SPE sorbent is not identical with the stationary phase of the analytical column. The SPE sorbent is a hydrophilic–lipophilic based (HLB) copolymer that is more suitable for polar compounds. It would of course have been advantageous to use the same stationary phase both for the sample preparation and for the analytical column. (When the wash fraction was injected and RP-LC was used, the amount of non-retained compounds was higher compared to analyzing the eluate.) By using these two complementary setups, with a ZIC®-HILIC column for analysis of the wash fraction and a C_{18} column for the analysis of the eluate, the number of metabolites to be detected could be increased as described in Part 2.

The retention time shifts were larger on the ZIC®-HILIC column compared to the C18 column. The standard deviation of the retention time of a peak at 6.3 min on the ZIC®-HILIC column obtained from six different rats was 0.2 min, while the C18 column gave a standard deviation of 0.02 min for a peak at 5.5 min. This might complicate the data analysis when comparing different samples, indicating the need for proper alignment of peaks or the use of appropriate data analysis tools (discussed in Part 2).

To illustrate the performance of the ZIC®-HILIC column when the analysis is made as general as possible and the conditions are not optimized for a certain compound, the peak shapes are reported in Table 2. The peak shapes are sufficient for this application as, when screening for a wide range of unknown compounds, it is not possible to obtain perfect chromatographic conditions for all compounds. However, compared to, for example, a porous graphitic carbon column [15], the ZIC®-HILIC column will give a better peak shape in general.

Table 2
Data obtained using ZIC®-HILIC illustrating the performance for 11 compounds

Compound	Physical properties[a]	RT[b]	Assymetry[c]	N[d]	LOD[e]	±Mode
Hydroxyproline ($C_5H_9NO_3$)	pK_a = 2.14, $c\log P$ = −1.84	12.7	1.5	4223	4.7	$[M+H]^+$
Uric acid ($C_5H_4N_4O_3$)	pK_a = 5.6, $c\log P$ = −2.37	10.2	2.3	1838	0.6	$[M-H]^-$
Nicotine ($C_{10}H_{14}N_2$)	pK_a = 8.0, $c\log P$ = 0.72	14.6	1.9	4724	0.4	$[M+H]^+$
Creatinine ($C_4H_7N_3O$)	pK_a = 4.4, $c\log P$ = −1.8	11.5	1.5	4817	0.9	$[M+H]^+$
Tryptophane ($C_{11}H_{12}N_2O_2$)	pK_a = 2.31, $c\log P$ = 1.04	10.4	1.5	1658	1.5	$[M+H]^+$
Lidocaine ($C_{14}H_{22}N_2O$)	pK_a = 8.53, $c\log P$ = 2.36	11.2	1.4	2216	0.1	$[M+H]^+$
β-Hydroxyethyl theophylline ($C_9H_{12}N_4O_3$)	pK_a = 0.61, $c\log P$ = −0.5	3.4	10	1211	0.2	$[M+H]^+$
Caffein ($C_8H_{10}N_4O_2$)	pK_a = 1.39, $c\log P$ = −0.08	3.3	1.1	770	1.2	$[M+H]^+$
Salicylic acid ($C_7H_6O_3$)	pK_a = 3.01, $c\log P$ = 2.06	2.7	1.3	1010	0.3	$[M-H]^-$
p-Nitrophenol ($C_6H_5NO_3$)	pK_a = 7.23, $c\log P$ = 1.57	3.2	1.6	778	0.1	$[M-H]^-$
Cholic acid ($C_{24}H_{40}O_5$)	pK_a = 4.76, $c\log P$ = 2.62	3.3	1.2	466	0.4	$[M-H]^-$

[a] pK_a and $c\log P$ values were obtained from SciFinder and calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67.
[b] Retention time obtained on a ZIC®-HILIC column. The void volume (t_0) was 2.3 min.
[c] Asymmetry calculated as, assymetry = 1/2(1 + B/A), where A and B were evaluated at 5% of the peak height. A and B were the width from RT to the beginning and the end of the peak, respectively.
[d] Number of theoretical plates are calculated using following equation: $N = 5.54(RT/W_{1/2})^2$, where RT was the retention time and $W_{1/2}$ was the peak width at its half-peak height.
[e] Limit of detection (LOD) in nanograms injected analyte was calculated as three times the noise level.

4. Conclusions

To cover the complexity of endogenous metabolite signatures in body fluids such as urine, a variety of different analytical approaches are required. Employing LC/MS as an analytical platform, different chromatographic and data-analysis techniques can be used. In this work ZIC®-HILIC was found to be a good stationary phase for the separation of highly polar compounds in a generic way and, in combination with a C18 column, a wider range of analytes could be detected. More automated dual-column approaches are an extension of this work and the additional number of metabolites will, of course, improve the quality of the metabolic fingerprints and facilitate classification. To further improve the extraction of information, different data-analysis methods can be used to obtain complementary information or to verify results, which will be discussed in Part 2 of this work.

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