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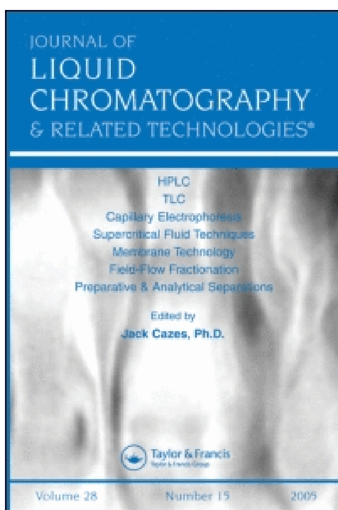

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LC-MS in Metabonomics: Optimization of Experimental Conditions for the Analysis of Metabolites in Human Urine

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LC-MS in Metabonomics: Optimization of Experimental Conditions for the Analysis of Metabolites in Human Urine

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Abstract: The analysis of metabolic pathways for dysfunction has been used for many years in the scientific and medical community to determine overall health. Metabonomics (metabolomics), the global profiling of metabolites, has experienced a rekindling of interest due, in part, to advances in analytical instrumentation for conducting measurements and informatics available for interpretation of the data acquired in this area of biomedical research. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) based approaches are two primary analytical methods of choice for conducting metabonomic measurements. To overcome the complexity and wide dynamic range of concentrations of metabolites present in biological samples, a common practice is to couple online an analytical separation, typically high performance liquid chromatography (HPLC), with the mass spectrometer. Hence, of critical importance are not only the MS acquisition parameters, but also optimization of those variables that impact the analytical HPLC separation as well. A systematic investigation of a number of variables related to HPLC, such as mobile phase composition and flow rate, gradient time, column dimensions, and packing material properties has been conducted. The results of this study show that 10 cm long × 1 mm inner diameter (i.d.), C_{18} reversed-phase columns provide higher resolution than C_8 or C_4 columns for the analysis of urine samples. The results also show that longer columns and extended mobile phase gradients allowed detection of a greater number of metabolites. As expected, MS analysis of the same urine sample using positive and negative ionization modes resulted in detection of a different ensemble of metabolites. Though prior dilution of rat and mouse urine is a common

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practice in conducting HPLC-MS metabonomic analyses, our results suggest that a greater number of species may be observed using undiluted urine. The matrix (composition) of urine collected from different individuals affected the reproducibility of retention times. The variability in metabolite retention times using internal standards, although improved, was not completely corrected.

Keywords: Metabonomics, Metabolomics, Urine, Mass spectrometry, HPLC-MS

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INTRODUCTION

Metabonomics has been defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification."[1] Simply stated, it is the metabolic response of living systems to drug toxicity or disease. Technological advances in nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), chromatography, and electrophoresis are enabling the measurement of alterations in the concentration of metabolites found in biological samples to unsurpassed levels.

Target or metabolite specific studies depend upon *a priori* knowledge of the biological system or pathway utilized for use as a successful diagnostics tool.[2–5] Global metabonomics relies on non-biased, quantitative analysis of metabolites in biological samples obtained from healthy (control) and diseased individuals, where the aim is to identify disease related differences in one or more of the numerous endogenous metabolites found in biological fluids.[6–8]

In global metabonomics, the entire metabolite component of a biological sample is separated, detected, quantified, and identified by various methods, each of which has its' own strengths and weaknesses. Currently, two of the most commonly used platforms for metabolite analysis are NMR spectroscopy and high performance liquid chromatography (HPLC) coupled online with MS.[9–16] Other methods of analysis have included gas chromatography (GC) coupled with MS,[17] electrochemical cells designed for disposable electrodes,[18] and capillary electrophoresis (CE)-MS[19] for the analysis and identification of metabolites from different biological specimen such as plants, mice, and humans.[20–22] Though powerful in their own right, each of these methods is only capable of measuring a fraction of the metabolites in a complex mixture. For example, GC-MS is only capable of measuring volatile metabolites, while electrochemical detection is only useful for charged metabolites.

Metabonomics analysis of biological systems, as with other "-omics" techniques, typically results in a plethora of data that can be overwhelming in its abundance. For any meaningful interpretation of the data, the appropriate statistical tools must be employed to manipulate the large raw data sets to provide a useful, understandable, and workable format. Different multidimensional and multivariate statistical analyses and pattern recognition programs

have been developed to distill the large amounts of data, in an effort to interpret the complex metabolic pathway information from the measurements.[1,5,23–30]

A critical parameter to the analysis of any complex biological sample is how it is prepared prior to analysis. For example, urine samples have been analyzed neat,[5] centrifuged, and diluted,[4,31] passed through solid phase extraction (SPE) columns to separate the hydrophilic fraction from the hydrophobic fraction,[32] or treated and incubated with various reagents prior to analysis.[33] Since biological fluids contain hundreds to thousands of hydrophilic and hydrophobic compounds, sample pretreatment to separate these two groups might be required in order to simplify the complexity of the mixture and to more readily detect the expected, but potentially low abundant metabolic changes. Also, of further concern is that these fluids (serum, plasma, urine, cerospinal, etc) contain varying amounts of salts, proteins, lipids, and other compounds that may interfere with metabolite detection by NMR spectroscopy or MS and negatively impact chromatographic reproducibility.

Another critical parameter when dealing with complex biological samples is how they are fractionated prior to analysis, particularly when using MS. As with any chromatographic analysis, the experimental parameters (column dimensions, packing material properties, solvent composition, gradient times, temperature, etc.) may vary greatly between laboratories, or even from one experiment to another. Optimal conditions for the best resolution and peak separation must take these factors into consideration, as well as others, such as history of the sample, matrix effects, etc., when choosing which analytical technique and instrumentation to use. For example, Wilson et al.[16] demonstrated an increased peak resolution and shorter analysis time by performing the separation of urine metabolites using ultra performance liquid chromatography (UPLC) ca. 12000 psi, compared to traditional HPLC, which is performed at a much lower back pressure.

The focus of this study was the optimization of the experimental parameters for maximum metabolite detection, and the evaluation of the effect of sample preparation and matrix on the overall reproducibility of the results. For example, Williams et al.[3] reported diluting rat urine samples 1 : 4 prior to HPLC/MS analysis. Results from our investigation, however, suggest that dilution of human urine samples may result in decreased peak intensities and a total loss of signal of lower abundance metabolites. One important aspect of the HPLC-MS and NMR analysis of urine or serum samples from different individuals for a metabonomics study is the variability (i.e., reproducibility) of metabolite retention times and NMR signals due to subtle chemical differences, an issue that requires addressing. Smith et al.[31] and Katajamaa and Orešič,[27] in recent studies, developed bioinformatic systems to deal with this problem. Experimental HPLC-MS parameters were also evaluated in terms of the effect of the urine matrix on internal standard (IS) and metabolite retention times, sample preparation (neat vs.

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SPE-treated), varying the mobile phase gradient (linear vs. modified step), time of day for urine collection, fasting vs. not fasting, chromatographic parameters, the MS ionization modes used (positive vs. negative), and sample concentration (dilute vs. neat).

EXPERIMENTAL

Materials

Acetonitrile (HPLC grade) was purchased from Fisher Scientific (NJ, USA), ammonium acetate, formic acid, 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid, and phenylaceturic acid were purchased from Sigma-Aldrich (MO, USA). Distilled water was obtained from an in-house Barnstead International NANOpure water filtration system (IA, USA). Dansyl-17-β-estradiol was prepared in-house as an internal standard. Urine samples were collected from healthy volunteers following NCI guidelines.

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Instrumentation

An Agilent 1100 Series HPLC system was coupled online with a hybrid triple-quad time-of-flight (QqTOF) mass spectrometer (QSTAR XL, Applied Biosystems, Inc., Framingham, MA) for the HPLC-MS analysis of the urine samples. All samples were scanned over a mass range of 100–1500 m/z with an ion spray voltage (IS) = 5000, declustering potential (DP) = 70, focusing potential (FP) = 150, ion source gas (GS1) = 20, curtain gas (CUR) = 25, and collision gas (CAD) = 3. Several HPLC columns with a dimension of 100 × 1 mm (Thermo Hypersil-Keystone Betabasic Pioneer, Thermo Electron Corp., Waltham, MA) packed with C_{18} (3 μm and 5 μm), C_8 (3 μm), and C_4 (5 μm) stationary phases, and a 50 × 2 mm column packed with 2 μm C_{18} stationary phase (Phenomenex Synergi Hydro-100, Phenomenex, Torrance, CA) were placed in line from the LC system and attached directly to the MS electrospray ionization source. A flow rate of 50 μL/min was maintained for all sample analyses. All NMR spectra were recorded using a 500 MHz NMR spectrometer (Varian Unity INOVA, Varian Inc., Palo Alto, CA, etc.) equipped with a gradient, triple resonance HCN cryogenic probe.

Sample Preparation and Analysis

Three urine specimens were collected from a healthy male volunteer over a 12 hour period at 7:00 am (fasting), 1:00 pm (not fasting), and 7:00 pm (not fasting). No preservatives were added to the urine samples since they were frozen at −40°C immediately after collection. At the time of analysis

samples were thawed on ice and centrifuged at 3000 rpm for 20 minutes at 5°C. Six SPE cartridges (Water Oasis HLB Extraction Plate, 30 mg, part #WAT058951) were conditioned with 1 mL of CH_3OH, then 1 mL of 10 mM ammonium acetate, pH 4.01. Each urine specimen (500 μL) was placed in two cartridge wells and allowed to gravity feed onto the cartridge. Each well was washed with 1 mL of the ammonium acetate buffer that was saved and stored at −80°C for further analysis of polar metabolites. The hydrophobic fraction was eluted with 1 mL of CH_3OH, lyophilized at ambient temperature, resolubilized in 500 μL acetonitrile/0.1% formic acid in H_2O (1 : 1v/v), and analyzed by HPLC-MS (5 μL total injected volume).

The capacity of the SPE cartridge was determined by passing through increasing amounts of urine (100, 250, and 500 μL) and washed with 1 mL buffer. The flow through and buffer were collected, divided into two equal aliquots, and lyophilized at ambient temperature. One aliquot of the polar eluent was resuspended in acetonitrile/0.1% formic acid in H_2O (1 : 1v/v) and analyzed by HPLC-MS (5 μL total injected volume). The second aliquot was analyzed by NMR spectroscopy. The sample (not lyophilized) was prepared by adding 46.8 μL deuterated water (D_2O) and 13.2 μL of 10 mM 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP) stock (as an internal standard, prepared in D_2O) to 600 μL of the 500 μL aqueous buffer wash (giving a final concentration of 10% D_2O and 0.2 mM TSP). Data was acquired at 25°C using a non-selective, gradient 1D NOESY experiment[34] with an equilibrium delay of 5 s during which water was saturated for 4 s. A spectral width of 6000 Hz was used, with an acquisition time of 5 s and 64 transients. A 20 ms Carr-Purcell-Meiboom-Gill (CPMG) pulse train of fast spin-echoes[35] was sandwiched between the first and second 90 degree pulses to improve water suppression. The resulting NMR data was zero-filled once and processed with 0.5 Hz line broadening.

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Chromatographic Conditions

Prior to urine analysis, the reversed-phase (RP)-HPLC columns were equilibrated with 98 : 2 mobile phase A/mobile phase B (A = 0.1% formic acid in H_2O, B = 0.1% formic acid in acetonitrile). Experiments were performed to determine the optimum solvent gradient that would result in the maximum number of detectable peaks in the chromatogram. Three different linear gradients were tested first to evaluate the effect of gradient slope on metabolite resolution, (2% B → 98% B in 40 minutes, 2% B → 98% B in 60 minutes, and 2% B → 98% B in 80 minutes) using untreated urine samples (not passed through the SPE cartridge or diluted). A step gradient (0–10 min = 95% A : 5% B, 65 min = 60% A : 40% B, 70 min = 2% A : 98% B, 80 min = 95% A : 5% B, where mobile phase A and B composition are the same as above) was also evaluated. Untreated and SPE-treated urine (5 μL) from each time collection point were injected and the results

compared. An aliquot of each was diluted 1 : 4 with ddH_2O, injected under the same conditions, and the results were compared with the undiluted samples.

Two aliquots of the acetonitrile-solubilized, SPE-treated urine (7:00 am collection) were injected and analyzed, first using MS-positive, then MS-negative mode, to determine if any compounds are being missed if only one detection method is used.

RESULTS AND DISCUSSION

Optimization of Chromatographic Parameters

Column Selection

High performance liquid chromatography columns that varied in length, particle size, diameter, and hydrophobicity of the stationary phase were evaluated for attributes such as resolution and back pressure at a flow rate of 50 μL/min using the following step gradient: 0–10 min = 95% A : 5% B, 65 min = 60% A : 40% B, 70 min = 2% A : 98% B, 80 min = 95% A : 5% B. Visual comparison of the results obtained using two different hydrophobicity columns

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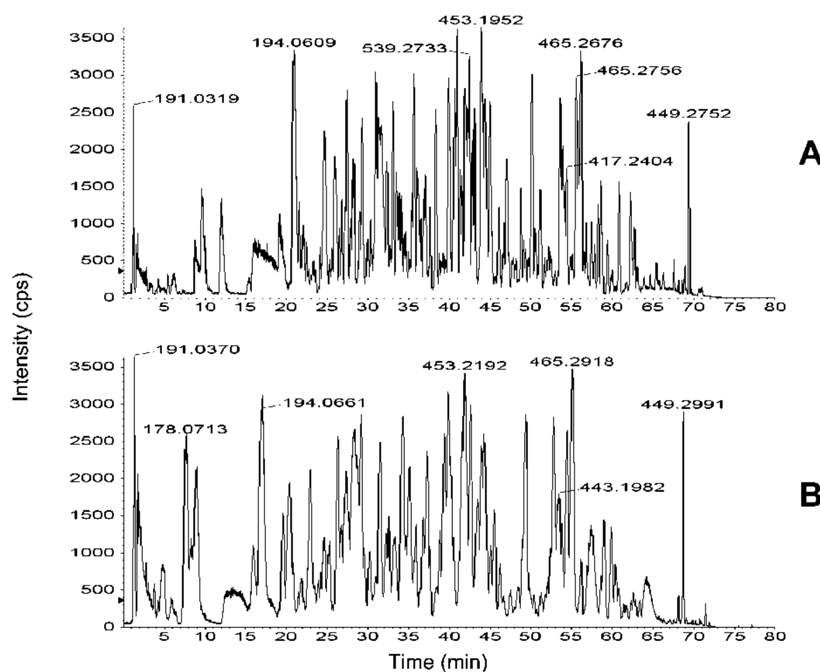

Figure 1. Chromatographic comparison of urine sample using a C_{18} column (A) and C_4 column (B) packed with 5 μm stationary phase.

(C_{18}–100 × 1 mm, 5 μm particle size vs. C_4–100 × 1 mm, 5 μm particle size) from the same manufacturer indicated that the C_{18} column resulted in a higher resolution separation (Fig. 1). The other evaluation was to explore the impact of stationary phase particle diameter, specifically C_{18} 100 × 1 mm, 3 μm particle size vs. C_{18} 100 × 1 mm, 5 μm particle size. The results indicated comparable separations; however as expected the smaller diameter stationary phase had higher back pressure, ranging from 160–190 psi for the 3 μm column, and 105–130 psi for the 5 μm column. Also, two other columns; C_8 100 × 1 mm, 3 μm particle size and a Phenomenex Synergi Hydro-100 C_{18} column, 50 × 2 mm, 2 μm particle size were tested. Taken together, these results (Fig. 2) indicate that in terms of resolution and back pressure the C_{18}–100 × 1 mm column packed with 5 μm stationary phase possesses the most optimal performance. Chromatograms obtained using the Synergi Hydro-100 column (50 × 2 mm, 2 μm stationary phase) showed that this column did not provide the optimal peak separation, with very broad, unresolved peaks at 25 and 35 min and longer retention times of the hydrophobic compounds.

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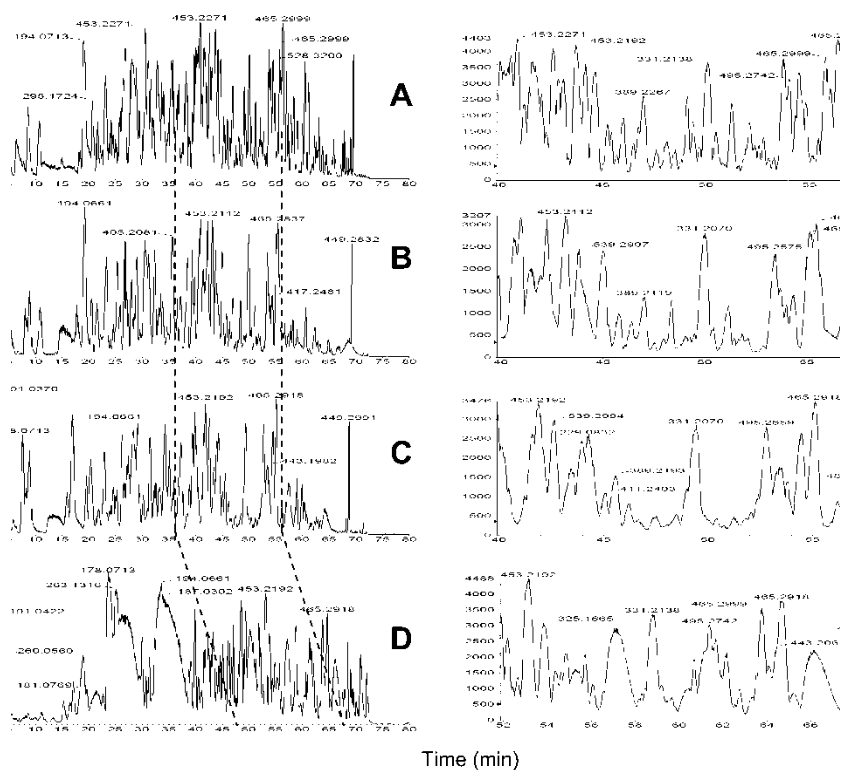

Figure 2. Chromatographic comparison of urine sample using C_{18} (A), C_8 (B), C_4 (C) and Hydro-100 C_{18} columns (D) (left) and expanded regions (right).

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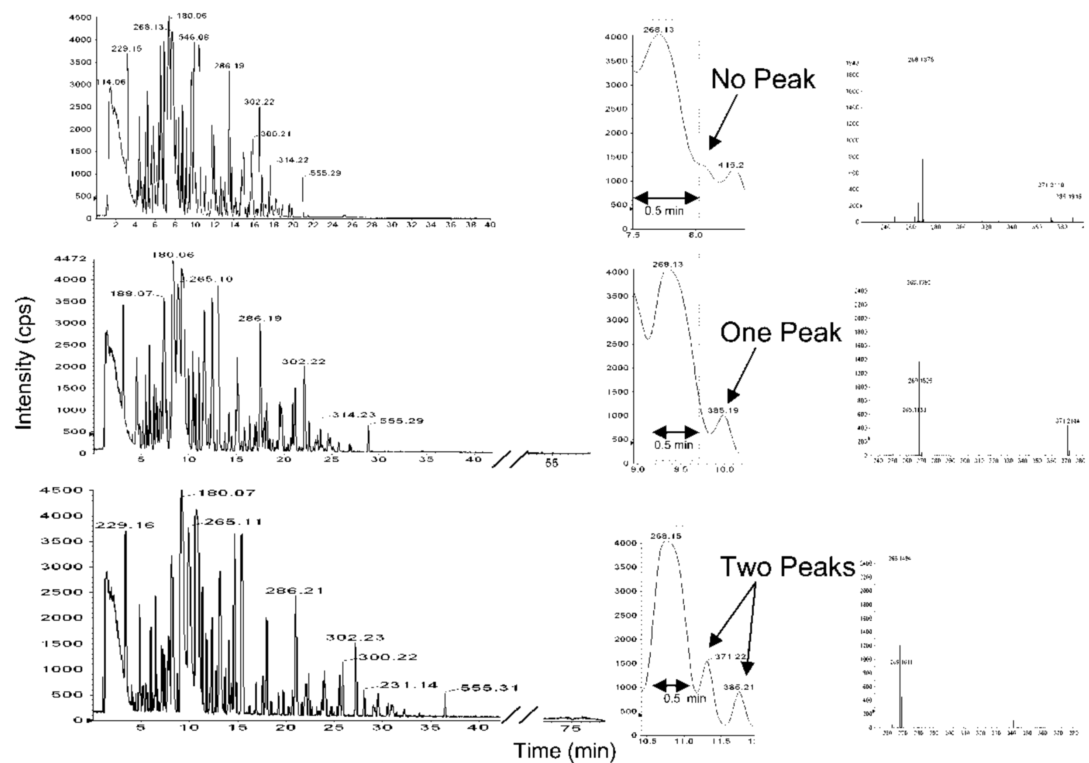

Figure 3. Effect time of the linear mobile phase gradient on peak resolution: A) 40 minutes; B) 60 minutes; and C) 80 minutes. Other conditions as in text.

Mobile Phase Selection

Experiments were performed to determine the optimal mobile phase composition and gradient that would provide the highest resolution separations. Due to the configuration of the LC system used in this study, an acetonitrile/H_2O mobile phase was preferred over a CH_3OH/H_2O and THF/H_2O since it resulted in lower back-pressure. Initially, three linear gradients from 2%–98%B in 40, 60, and 80 minutes were used. Longer times were not evaluated because they are not conducive for high throughput analysis of large sample sets. The results showed that decreasing the slope of the gradient, i.e., increasing the elution time, provided better peak resolution and resulted in a larger peak capacity. For example, a single chromatographic peak appeared as a shoulder in a 40 minute gradient, this peak was fully resolved into two peaks and three peaks using the 60 and 80 minute gradients, respectively, (Fig. 3).

Experimentation with different flow rates and linear gradient compositions indicated that a linear gradient was not the most efficient method for achieving optimal peak resolution. Therefore, it was decided to explore a step gradient elution profile to see if peak resolution could be improved. After further experimentation, and based on the three linear gradient results,

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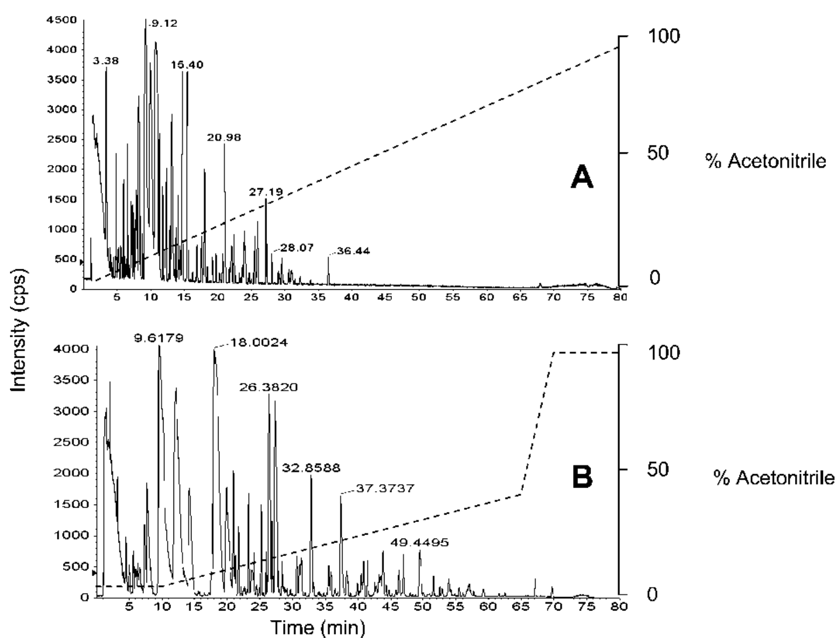

Figure 4. Chromatographic comparison mobile phase gradients (dotted line) using a linear (A) vs. a step (B) gradient.

the following step gradient was selected: 0–10 min = 95% A : 5% B, 65 min = 60% A : 40% B, 70 min = 2% A : 98% B, 80 min = 95% A : 5% B. This step gradient resulted in base line separation of a larger number of peaks than any of the three linear gradients (Fig. 4). We find that normal day-to-day operations in our laboratory have shown it to be a robust method of analysis that provides highly reproducible data using urine samples collected from a previous study (Fig. 5).

Analysis of Urine Samples Using Different MS Ionization Modes

Wilson et al.[16] and Plumb et al.[33] reported using negative and positive MS ionization modes to analyze mouse urine. As expected, these two studies indicate that each ionization mode gave different results. Hong and

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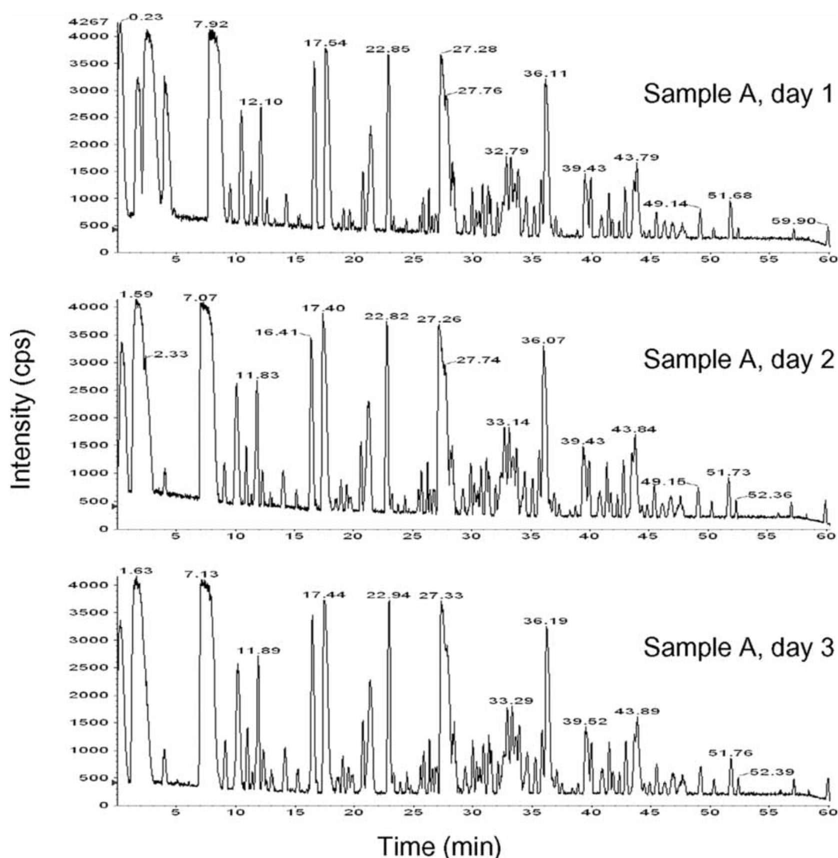

Figure 5. Day-to-day reproducibility of chromatographic profile of an undiluted urine sample using a C_{18} column and step gradient elution. Details as in text.

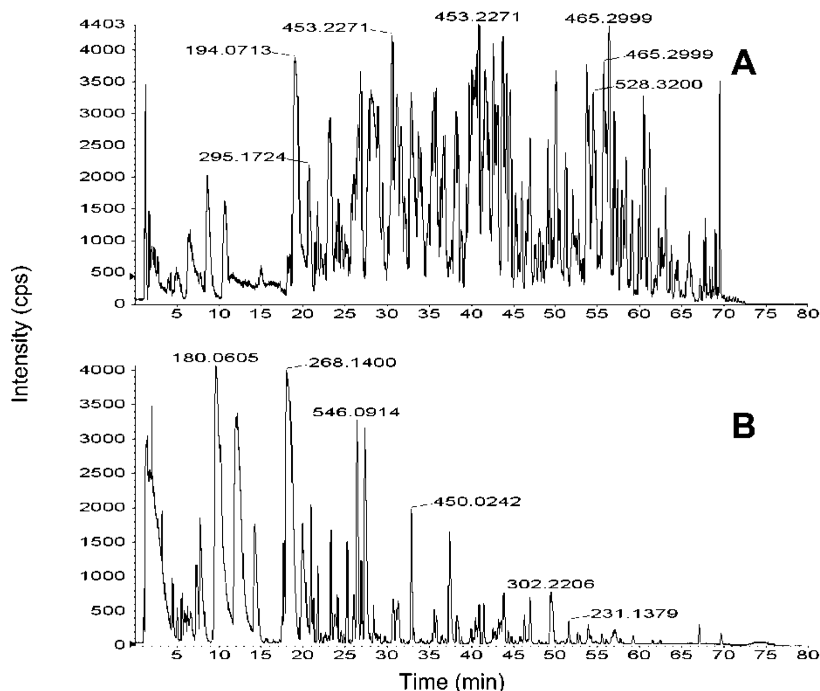

Figure 6. Untreated, undiluted urine chromatograms using a C_{18} column, step gradient, and negative (A) and positive (B) MS ionization modes.

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Mitchell[2] used positive ion mode for human urine, while Plumb et al.[17] used negative ion mode for rat urine analysis. In the present evaluation, we sought to explore the impact of ionization mode for the profiling of human urine. Demonstrated in Fig. 6 are the chromatographic differences in positive vs. negative ion modes of ESI-MS, expectedly showing that while some peaks could be detected in both modes, other peaks were unique to each MS ionization mode. Our results indicate that generally a greater number of peaks are identified in the early portion ($t_r < 25$ minutes) of the chromatogram using the positive ionization mode, while the negative ionization mode gave much richer peak abundances in the middle and later portions of the chromatogram ($t_r > 25$ minutes).

Effect of Urine Matrix on Retention of Metabolites

Urine samples obtained from different individuals are potentially quite variable in their chemical composition, including such non-metabolite molecules as proteins and salts. Since these differences may affect the chemistry of the HPLC column, it was decided to study what influence, if

any, the matrix had on the reproducibility of the retention times of the metabolites between samples. The first part of the study dealt with the reproducibility of the HPLC-MS procedure. A single urine sample was analyzed in the negative MS mode by repeatedly injecting 5 μL aliquots twenty consecutive times under the same experimental conditions. The results (Fig. 7) indicated excellent run-to-run reproducibility, demonstrating the robustness of our method. Also, this reproducibility was confirmed by periodically monitoring variances in retention times using a Glu-fibrinopeptide B standard (data not shown).

Matrix effects on the reproducible analysis of urine samples obtained from different individuals were evaluated by spiking each sample with two internal standards, (phenylaceturic acid and dansylated-17-β-estradiol). These standards were selected based on their elution during the early and later parts of the chromatogram. The two internal standards were first

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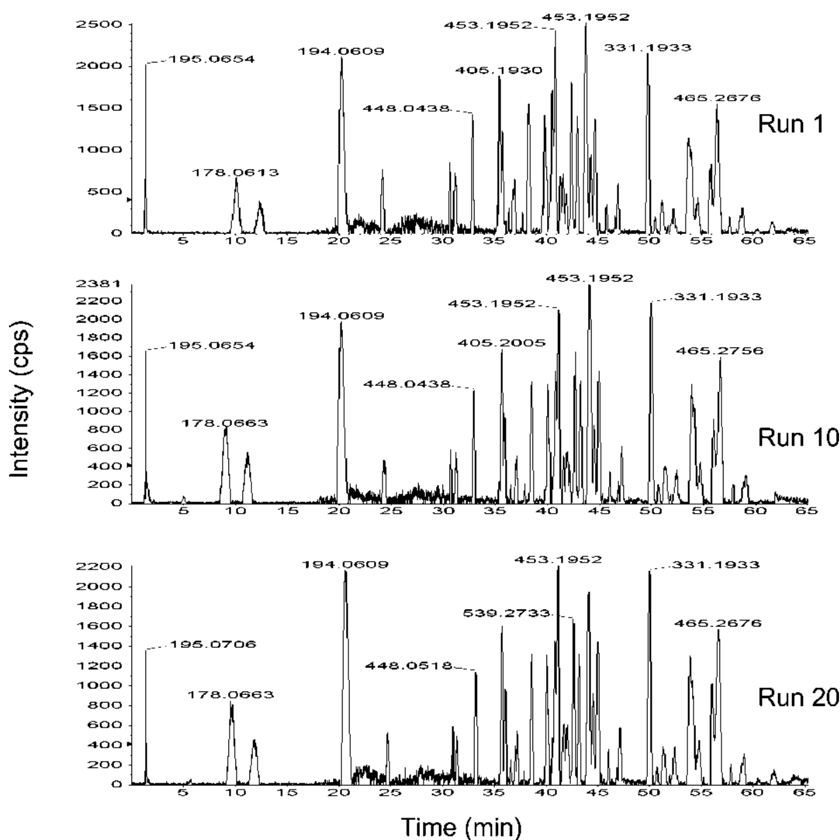

Figure 7. Reproducibility of chromatographic profile of a neat urine sample during same day runs.

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Table 1. Retention time variance for two internal standards and five endogenous matabolites from HPLC/MS of human urine uncorrected and normalized (Bold Italics) to internal standards

Sample	180	194*	265	*m/z* 271	464	387	504*
		14.33					71.81
6	9.31/***9.43***	14.14	11.35/***11.49***	31.26/***31.24***	41.48/***41.45***	61.87/***61.83***	71.85
15	8.95/***9.26***	13.84	11.05/***11.43***	31.34/***31.34***	41.53/***41.52***	61.90/***61.89***	71.82
30	9.05/***9.36***	13.84	11.15/***11.53***	31.30/***31.29***	41.49/***41.47***	61.87/***61.83***	71.85
45	9.19/***9.43***	13.94	11.30/***11.61***	31.23/***31.21***	41.49/***41.48***	61.90/***61.87***	71.84
60	9.42/***9.49***	14.20	11.49/***11.57***	31.19/***31.19***	41.48/***41.48***	61.90/***61.90***	71.80
74	9.22/***9.44***	13.97	11.30/***11.58***	31.22/***31.23***	41.51/***41.51***	61.93/***61.94***	71.80
AVG	9.19/***9.40***	13.99	11.27/***11.54***	31.26/***31.25***	41.50/***41.48***	61.89/***61.88***	71.83
MIN	8.95/***9.26***	13.84	11.05/***11.43***	31.19/***31.19***	41.43/***41.39***	61.83/***61.78***	71.80
MAX	9.42/***9.59***	14.20	11.49/***11.61***	31.34/***31.34***	41.53/***41.52***	61.93/***61.94***	71.87
STDV	0.17/***0.08***	0.15	0.15/***0.06***	0.06/***0.05***	0.02/***0.03***	0.02/***0.04***	0.02
%CV	1.87/***0.87***	1.08	1.37/***0.56***	0.18/***0.17***	0.05/***0.06***	0.03/***0.07***	0.03

*Internal standard.

dissolved in acetonitrile/H_2O (1 : 1v/v) and analyzed in the positive mode by HPLC/MS using the step gradient described above. The retention times of the internal standards were determined to be 14.33 min for phenylaceturic acid and 71.81 min for the dansylated 17-β-estradiol. Urine samples obtained from six different individuals were spiked with the two internal standards and analyzed under the same experimental conditions. The results (Table 1) indicated a variation in the retention times that ranged from 13.84 to 14.20 min, with an average retention time and standard deviation of 13.99 and 0.17, respectively, for the phenylaceturic acid standard ($m/z = 194$) and 71.80 to 71.85 min, with an average retention time and standard deviation of 71.83 and 0.02, respectively, for dansylated 17-β-estradiol ($m/z = 504$). Monitoring the retention times of five selected metabolites ($m/z = 180, 265, 271, 387$, and 464) from the urine of six different individuals resulted in different retention times for each metabolite (Table 1). These results clearly indicate that the urine matrix has an effect on retention time reproducibility between samples from different individuals. A set of 74 urine samples from different individuals that were analyzed under the above experimental conditions gave variation in the retention times of both standards and metabolites that ranged from approximately 5–70 seconds (Table 2).

This variation in retention times may be attributed to the different chemical properties of each of the urine samples. Such retention time variation for each of the metabolites would introduce an error in metabolite assignment unless corrected using internal standards, MS identification, or statistical methods. Smith et al.[31] suggested that the use of internal standards is not necessarily the best choice for peak retention time correction, so they developed a statistical approach for correcting the retention times of metabolites from plasma samples using previously collected data sets.

We decided to explore the possibility of using two internal standards to normalize the retention times of the six selected metabolites shown in Table 1. The normalization procedure was carried out as follows: each of the metabolite retention times are multiplied by a factor that is determined

Table 2. Computed statistical data from entire urine sample set (n = 74) for five endogenous metabolite peaks and two internal standards

	180	194[a]	265	m/z 271	464	387	504[a]
AVG	9.24	14.04	11.35	31.27	41.48	61.89	71.84
MIN	8.94	13.75	10.97	31.19	41.12	61.39	71.80
MAX	10.09	14.90	12.50	31.34	41.58	61.98	71.87
STDV	0.21	0.19	0.27	0.04	0.08	0.07	0.02
%CV	2.25	1.39	2.34	0.13	0.19	0.11	0.03

[a]Denotes Internal Standards – Phenvlaceturic Acid (194) and 17 β-Estradiol-Dansvl (504).

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for each sample by the following equation:

$$ISt_{rw}/ISt_{ru} \times t_{rm1\rightarrow n}$$

where ISt_{rw} and ISt_{ru} are the retention times of the internal standard in water/acetonitrile and in urine, respectively, and $t_{rm1\rightarrow n}$ are the retention times of the metabolites.

The results indicated that after normalization the retention times were not completely corrected, but there was an improvement in the deviation and coefficient of variation of the peaks at *m/z* 180 and 265, while those at *m/z* 271 and 464 remained relatively unchanged. Examination of the results indicated that the early eluting peaks had the largest spread (difference between fastest and slowest elution times of the same *m/z* peak) in retention times before normalization (0.47 minutes for *m/z* 180, and 0.44 minutes for *m/z* 265), while for the peaks at *m/z* 271, 464, and 387 the spread was 0.15, 0.05, and 0.03 minutes, respectively. These results indicate that the less hydrophobic compounds (early eluting peaks) are more influenced by the urine matrix than the late eluting peaks, due in part to the varying quantities of salts and other interfering compounds present in the samples. Also, the normalization of the early eluting peaks was more effective than the normalization of the late eluting peaks, as evident from Table 1. This result may be due to the fact that the internal standard (*m/z* 194) eluted at about 14 minutes, while the second internal standard eluted at about 71 min; later than the elution time of the majority of the peaks observed in the urine samples. Since the late eluting peaks (i.e., after 32 minutes) had a spread of less than 10 sec, we felt that normalization of these retention times was not absolutely necessary.

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Effect of Sample Preparation

Since urine is a complex mixture of hydrophobic and hydrophilic molecules, salts, proteins, and other compounds, it was decided to study the effect of introducing a sample clean-up or dilution step prior to HPLC/MS analysis. Williams et al.[3] used a 1:4 dilution of rat urine prior to HPLC/MS analysis, while Idborg-Björkman et al.[34] used SPE to selectively extract the hydrophobic metabolites. Such a procedure would simplify the complexity of the urine sample and remove any interfering compounds that might poison the chromatographic column and affect the sensitivity of MS detection. In this study, we were interested in analyzing the hydrophobic fraction of urine and not the hydrophilic fraction, which will be the subject of another study. The urine samples were processed according to a published procedure.[34] Briefly, a urine specimen was aspirated through a C_{18} SPE cartridge, followed by a buffer wash, and elution of the fraction of interest with CH_3OH. To prevent loss of metabolites it was important to determine the capacity of the C_{18} SPE cartridge for extracting the hydrophobic metabolites.

This capacity was measured by passing 100 μL, 250 μL, and 500 μL of urine through the SPE column. Analysis of the combined aqueous-buffer wash fraction of the 500 μL sample by HPLC/MS indicated that a minimum number of hydrophobic compounds broke through the column, as is clear from Fig. 8. The early eluting peaks evident in the chromatogram are from the polar fraction of urine. Therefore, it is clear that this C_{18} SPE cartridge can extract the hydrophobic fraction of 500 μL of urine without any appreciable losses.

Further analysis was performed to determine the differences between neat and SPE-treated samples. The chromatograms of an undiluted, untreated urine sample and an undiluted, treated urine sample (Fig. 9) show that the untreated urine sample base peak chromatogram (BPC) displays more intense peaks than the SPE treated sample, although some of the treated sample peaks are sharper and, therefore, presumably cleaner. The extracted ion chromatograms (XIC) of some of the peaks that were less intense or not visible in the BPC of

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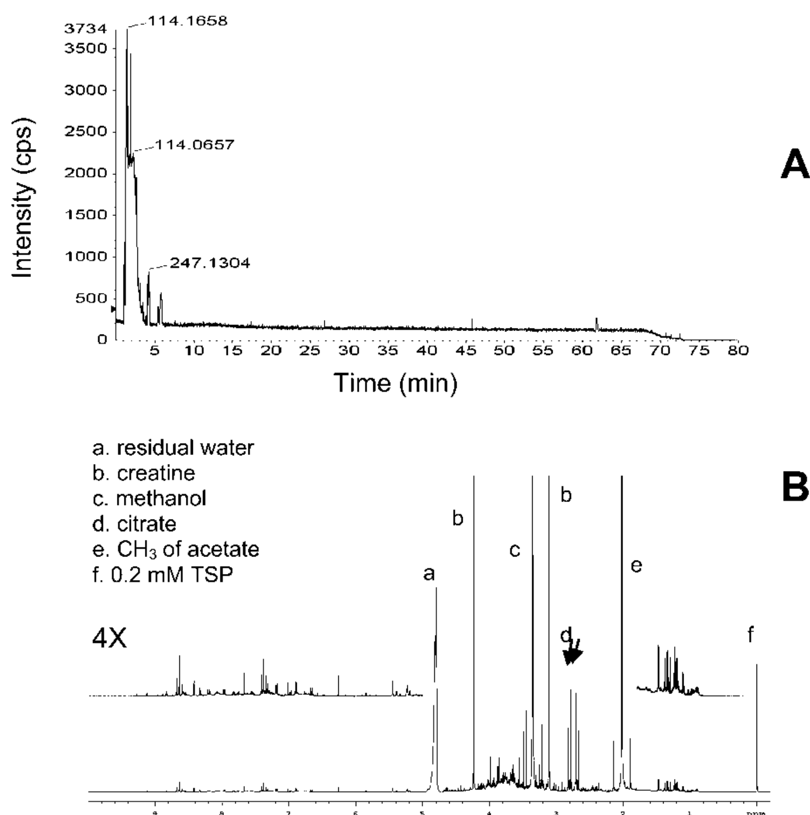

Figure 8. HPLC Chromatogram (A) and ^{1}H NMR spectra (B) of aqueous wash from 500 μL of urine passed through an SPE column.

the treated urine are detectable, with an expected decrease in intensity (data not shown). Although, the chromatogram of neat untreated urine is more intense, injection of neat urine onto an HPLC column, without column washing between injections, is not recommended because of the poisoning of the HPLC column that will affect reproducibility and the MS detection sensitivity.

Effect of Dilution on Urine Samples

Plumb et al.[29] and Williams et al.[3] diluted rat urine samples at least 1 : 4 before analysis. It was of interest to see what effect dilution had on the overall chromatogram of human urine. Analysis of neat and 4-fold diluted

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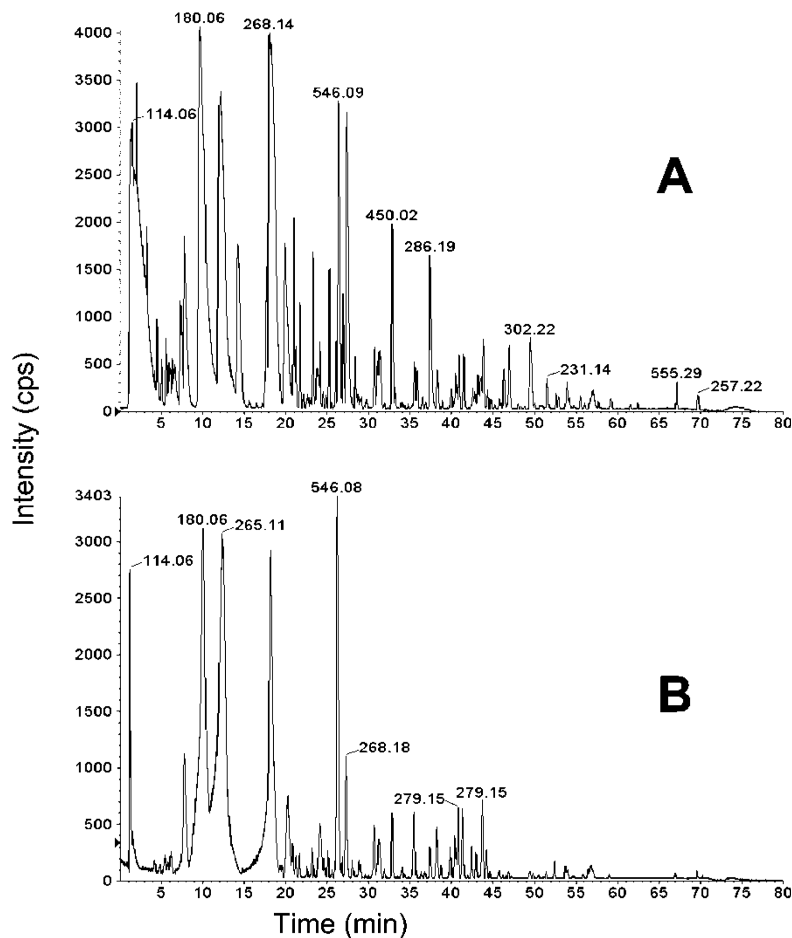

Figure 9. Chromatograms of neat (untreated) urine (A) vs. SPE-treated urine (B).

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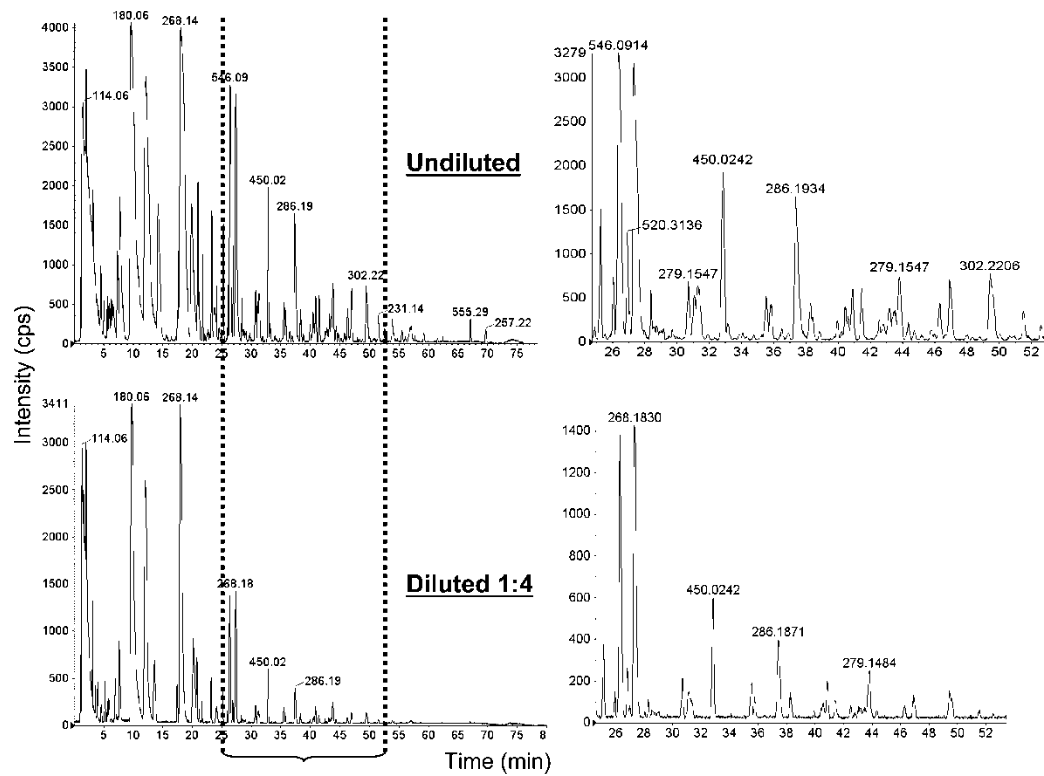

Figure 10. Chromatograms of undiluted urine sample vs. diluted (1 : 4) urine sample (left) and expanded chromatogram regions (right).

human urine shows that there was a difference in the peak profile between the two samples (Fig. 10). Upon close examination of the BPC, the neat sample displayed more peaks of greater intensity than the diluted sample. Some peaks were not detected in the diluted sample, however, they were observed in the undiluted one. While Plumb et al.[29] reported obtaining good results from HPLC/MS analysis of rat urine after 1 : 4 dilution, human urine is less concentrated than mouse or rat urine. Therefore, based on the experiments presented here, a 1 : 4 dilution of human urine may not be the optimum for the detection of lower abundance metabolites.

Effect of Time of Day of Urine Collection and of Diet

Another factor pertaining to a metabonomic study of biological samples analysis (urine, plasma, or serum) is what effect does the time of day for specimen collection have on the results (profile) of the sample. It has been

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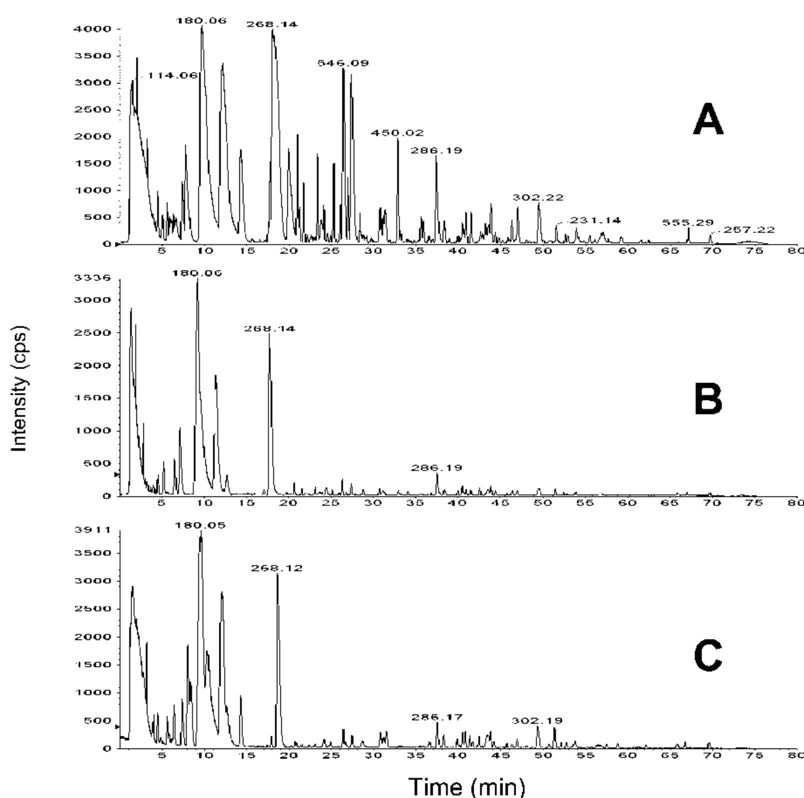

Figure 11. Chromatograms of untreated, undiluted urine samples collected at different times: 7:00 am (A), 1:00 pm (B), and 7:00 pm (C).

demonstrated by Plumb et al.,[33] that through metabolic analysis it is possible to differentiate morning from afternoon urine specimens. In the present study, urine specimens were collected from a healthy male volunteer at 7 : 00am (fasting), 1 : 00 pm (immediately after lunch), and 7 : 00 pm (immediately after dinner). Results of the analysis (Fig. 11) demonstrate distinct differences in the chromatographic profiles of urine collected at different times. The 7 : 00 am collection gave a much higher number of detectable peaks than the 1 : 00 pm collection. This is possibly due to the fact that the test subject consumed a moderate amount of water prior to lunch, and collected the (diluted) specimen immediately thereafter, before much of the digestive and metabolic processes had time to occur. The 7 : 00 pm collected sample showed the detection of some of the earlier detected peaks, although they are still not as numerous or intense as the 7 : 00 am collection.

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CONCLUSIONS

The present study indicates that for a comprehensive metabonomic study using urine samples it becomes important to first optimize the experimental conditions for maximum peak detection. Our results indicate that a step gradient (0–10 min = 95% A : 5% B, 65 min = 60% A : 40% B, 70 min = 2% A : 98% B, 80 min = 95% A : 5% B) where solvent A is 0.1% formic acid in H_2O and solvent B is 0.1% formic acid in acetonitrile, resulted in a larger number of detected peaks than a linear gradient of 2%A : 98% B in 60 minutes. Also, a 100 × 1 mm column packed with C_{18} derivatized silica particles gave better results than columns of the same dimensions packed with C_8 or C_4 derivatized silica particles. Separation of the urine samples into two main fractions, hydrophobic (non-polar) and hydrophilic (polar), simplified the complex mixture of metabolites and resulted in sharper (cleaner) peaks. The use of two MS ionization modes for metabolite detection is recommended, since the positive mode resulted in a different profile than the negative mode albeit some m/z values that was detected by both ionization modes.

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