High-Throughput UHPLC/MS/MS-Based Metabolic Profiling Using a Vacuum Jacketed Column

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ABSTRACT: In UHPLC, frictional heating from the eluent flowing through the column at pressures of ca. 10−15 Kpsi causes radial diffusion via temperature differences between the center of the column and its walls. Longitudinal dispersion also occurs due to temperature gradients between the inlet and outlet. These effects cause band broadening but can be mitigated via a combination of vacuum jacketed stainless steel tubing, reduced column end nut mass, and a constant temperature in the column from heating the inlet fitting. Here, vacuum jacketed column (VJC) technology, employing a novel column housing located on the source of the mass spectrometer and minimized tubing from the column outlet to the electrospray probe, was applied to profiling metabolites in urine. For a 75 s reversed-phase gradient separation, the average peak widths for endogenous compounds in urine were 1.2 and 0.6 s for conventional LC/MS and VJC systems, respectively. The peak tailing factor was reduced from 1.25 to 1.13 when using the VJC system compared to conventional UHPLC, and the peak capacity increased from 65 to 120, with a 25% increase in features detected in urine. The increased resolving power of the VJC system reduced co-elution, simplifying MS and MS/MS spectra, providing a more confident metabolite identification. The increased LC performance also gave more intense MS peaks, with a 10−120% increase in response, improving the quality of the MS data and detection limits. Reducing the LC gradient duration to 37 s gave peak widths of ca. 0.4 s and a peak capacity of 84.
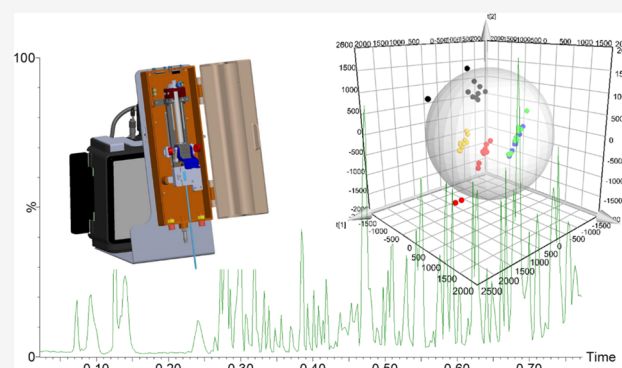
1. INTRODUCTION

Very high-throughput LC/MS(MS) methods are becoming increasingly important in areas where large numbers of biological samples require analysis. However, rapid analysis can be achieved without sacrificing the high data content of the lower-throughput assays currently employed[1]. There are several driving factors for this desire for speed: whether due to the need for results as soon as possible after sampling, e.g., analysis in forensic toxicology, hospital point of care for overdosing, poisonings, etc., or due to the sheer numbers of samples requiring analysis, such as the profiling of large cohort epidemiological studies or biobanks[2]. Several approaches have been developed in an attempt to address the issue of throughput, such as direct infusion MS (DIMS),[3] thermal desorption MS,[4] acoustic ejection MS,[5] desorption electrospray ionization MS (DESI),[6] and rapid evaporative ionization MS (REIMS).[7] These approaches each have their merits and advantages; however, in the absence of a preceding separation, all of these types of analysis can suffer from ion suppression, resulting in reduced sensitivity compared with LC/MS. In addition, these direct methodologies are often unable to address the issues of isomers/isobaric mass interferences and

poor quantitative performance. It is for these reasons that liquid chromatography coupled to mass spectrometry has become the premier technology for the analysis of, e.g., pharmaceuticals, their metabolites, and endogenous compounds in biofluids and tissue extracts. Complex mixture analysis in particular has benefited from LC advances such as sub 2 μm LC/MS[8,9] facilitating high-resolution separations in the 5−10 min time scale. The coupling of ion mobility (IM) separations with UHPLC/MS[10] represents a further refinement that has allowed for either an increase in peak capacities or shorter analysis times. Further developments such as rapid microbore metabolic profiling combined with MS (RAMMP/MS)[11] and RAMMP/IM/MS[12] have also been described. Performed using 1 mm-scale UHPLC at increased mobile phase linear velocities, RAMMP methods provide increased

sensitivity and lower solvent consumption as a result of the reduction in column geometries. Using them, metabolomic/drug metabolite analysis times as low as 3 min/sample have been obtained, e.g., ref 11.

The ability to access the full potential of these rapid LC/MS methodologies is, however, impeded by peak dispersion, both within the column (caused by frictional heating) and post column. Such peak dispersion results in increased peak widths and peak tailing and can significantly reduce the overall peak capacity of the system. Post column dispersion can be attributed to tubing connections between the column outlet and MS probe as well as features such as liquid junctions/connections with the electrospray probe itself. The use of LC columns situated within the source of the mass spectrometer, such as those found in chip-based LC, has been shown to substantially reduce post column peak broadening.[13] However, as frictional heating occurring within the LC column is caused by the mobile phase being forced through the stationary phase at extremely high pressures (10−15 Kpsi), it is not attenuated by this type of solution. This frictional heating results in both radial diffusion (due to differences in the temperature at the center of the column and the walls of the column) and longitudinal dispersion as a result of the difference in temperature between the inlet and outlet of the column.[14] A comprehensive theoretical explanation of both radial and longitudinal diffusion is given elsewhere.[15] In an attempt to address these frictional heating issues, Gritti *et al.* used a combination of vacuum jacketed stainless steel tubing and reduced mass of the column end nuts to address the heating losses.[15] Longitudinal heating effects were addressed by applying thermal energy to the inlet fitting to provide a constant temperature throughout the column. This combination resulted in a very significant improvement in LC performance, with up to a 30% increase in column efficiency.[15,16]

Here, we have combined the vacuum jacketed column (VJC) technology with a novel column housing located on the source of the mass spectrometer and continuous connecting tubing from the outlet of the column to the electrospray tip on the MS probe. This was done with the aim of better controlling peak dispersion in order to facilitate high-throughput information-rich analysis that could be employed for complex mixture analysis. Here, we have performed a preliminary evaluation of the vacuum jacketed LC/MS system applied to the rapid LC/MS metabolic profiling of human urine following oral administration of acetaminophen (paracetamol, *N*-(4-hydroxyphenyl) acetamide, APAP). In addition, various selected endogenous metabolites were profiled. Particular attention was paid to chromatographic performance, MS peak response, and MS spectral quality.

2. EXPERIMENTAL SECTION

2.1. Materials. LC/MS grade water, methanol (MeOH), acetonitrile (ACN), ammonium acetate, formic acid (FA), and leucine enkephalin (LeuEnk) were sourced from Thermo Fisher Scientific (Franklin, USA). Sodium formate was used to calibrate a time-of-flight (TOF) mass spectrometer (Waters Corp., Milford, USA). Distilled water was prepared in-house using a Millipore System (Millipore, Burlington, MA).

2.2. Sample Preparation. Timed mid-stream human urine samples, obtained following the oral administration of 400 mg to a healthy male, were collected predose and at 1, 3, and 24 h post-dose into a 50 mL sterile plastic container and

processed immediately following collection. Samples (100 μL) were diluted 1:10 (v/v) with distilled water containing 0.1% formic acid, vortex mixed, and centrifuged (25,000*g*, 5 min). Each urine time point was analyzed in duplicate in a randomized order and with five replicate analyses for each sample. A batch QC was constructed by mixing 100 μL of urine from each time point. The resulting QC mixture was then processed as described above, with a QC analysis being performed prior to the beginning of the analysis and then following every fifth sample.[17]

2.3. Chromatography. Sample analysis was performed on a Waters ACQUITY I-Class binary chromatography system equipped with a flow through needle (Waters Corp, USA). The separations were performed on either a "conventional" 2.1 × 30 mm ACQUITY HSS T3 1.7 μm C18 column or a vacuum jacketed stainless-steel 2.1 × 30 mm ACQUITY HSS T3 1.7 μm C18 column (Waters Corp, USA). Both columns were identical in construction with the conventional column formed by the removal of the outer vacuum sleeve of a vacuum jacketed column. Both columns were packed using the same batch of stationary phase, using the same column packing operation, and on the same day.

The conventional column was housed in a Waters ACQUITY column manager thermostatically controlled to 40 °C and connected to the mass spectrometer using standard fittings (50 cm of 100 μm ID tubing from the column to the probe and 35 cm of 120 μm tubing within the probe). The VJC was housed in a prototype column holder located on the source of the mass spectrometer with the column effluent transferred to the MS probe via one continuous piece of capillary tubing (6 cm of 50 μm ID tubing from the column to the probe and 35 cm of 75 μm ID within the probe). The inlet temperature of the vacuum jacketed column was maintained at 55 °C and the outlet temperature at 40 °C. The columns were eluted with a multilinear gradient using 0.1% (v/v) aqueous FA (mobile phase A) and acetonitrile, also containing 0.1% formic acid (v/v) (mobile phase B) at a flow rate of 1.0 mL/min for the 75 s separation (overall analysis time, with column washing and re-equilibration 96 s, MS data were collected for 96 s) and 1.1 mL/min for the 37 s gradient separation (overall analysis time, with column washing and re-equilibration 48 s, data was collected for 48 s), which was only performed on the vacuum jacketed column. This methodology was adapted from that previously described by Want *et al.*[18] The gradient conditions employed for urine analysis are described in Tables 1 and 2. An 8 μL injection volume was employed for all separations.

2.4. Mass Spectrometry. MS data were acquired on a Xevo G2-XS QTof mass spectrometer (Waters Corporation, Wilmslow, UK) using positive electrospray ionization (+ve

Table 1. Chromatographic Conditions for the 75 s Gradient Analysis

time (s)	flow (mL/min)	%A	%B
0	1.0	99	1
8.4	1.0	99	1
25	1.0	85	15
50	1.0	50	50
75	1.0	5	95
90	1.0	5	95
96	1.0	99	1

Table 2. Chromatographic Conditions for the 37 s Gradient Analysis

time (s)	flow (mL/min)	%A	%B
0	1.1	99	1
4.2	1.1	99	1
13	1.1	85	15
25	1.1	50	50
37	1.1	5	95
45	1.1	5	95
48	1.1	99	1

ESI) at a capillary voltage of 3.0 kV and source temperature of 100 °C with a cone gas (nitrogen) flow of 50 L/h. The desolvation gas flow was 600 L/h at a temperature of 300 °C, selected to minimize the decomposition of drug metabolites, and the desolvation and nebulizer gas flows were set at 6 bar. MS experiments were performed over the m/z range 50−1200 Da. Sodium formate was used for the calibration of the TOF region. These data were collected in continuum mode using a low collision energy of 4 eV (function 1) with a collision energy ramp (19−45 eV) used to obtain elevated energy data (function 2). Each of these functions employed a scan time of 0.1 s, and this acquisition rate was selected as it provided the best compromise between the number of points across the chromatographic peak and ion statistics required for mass accuracy. LeuEnk (m/z 556.2771) provided the external lock mass, and a scan was collected every 30 s using a fixed cone voltage of 40 V.

2.5. Metabolite Identification. APAP and its metabolites were identified using MS and MS/MS with comparison to spectra obtained for authentic standards.[19] Endogenous metabolites were similarly identified based on MS and MS/MS data and comparison with those of authentic standards.

2.6. Data Analysis. The data were collected using MassLynx vs 4.1 (Waters Corp., Wilmslow, UK), while data processing and visualization were conducted using Progenisis QI vs 3.0. The multivariate statistical analyses were performed on EZInfo vs 2.0 (Sartorius, Gottingen, Germany). Principle component analysis (PCA) was performed using Pareto scaling over data ranges of 0−80 s for the 96 s UHPLC/MS analysis and 0−40 s for the 4 s UHPLC/MS analysis.

3. RESULTS AND DISCUSSION

3.1. Initial Method Development. As is well known, as column length is reduced and the gradient steepness increased (column volumes/minute), the chromatographic peak volume of the eluting analytes is significantly reduced, resulting in sharper peaks. In conventional systems, however, the sharpness of these smaller peak volumes is adversely impacted by the intrinsic dispersion of the chromatographic system and the attendant post column tubing as well as on-column thermal dispersion due to frictional heating. Here, the short 2.1 × 30 mm VJC column constructed using a vacuum jacketed stainless-steel tubing and packed with a sub 2 μm porous stationary phase was designed to reduce these effects. Thus, the column inlet and outlet temperatures were maintained at different temperatures to minimize thermal dispersion and the column itself was located on the MS source with a single length of fused silica capillary used to connect it to the MS capillary sprayer, minimizing extra column dispersion. In order to evaluate the effect of this new configuration on the analysis of complex mixtures of drug and endogenous metabolites, the

previously published 12 min UPLC analysis[18] was scaled to give a 75 s gradient time with an overall 96 s cycle time. For this, the column length was reduced by a factor of 3.3 and the gradient time reduced by a factor of 10. The flow rate was then increased from 0.6 to 1.0 mL/min, resulting in the number of column volumes defining the gradient in this "high-throughput" method becoming 24 compared to the 41 employed by Want *et al.*[18] The separation obtained from this new approach was then compared to that obtained from a conventional column, with the same dimensions and operated under the same conditions.

To determine if the use of the new VJC conferred benefits over those provided by conventional UPLC/MS methodology, human urine samples collected following the administration of a single 400 mg dose of acetaminophen were analyzed using both systems. This preliminary evaluation was undertaken using the 75 s gradient with an overall cycle time of 96 s. By using a complex biological sample such as urine, the utility of the VJC approach was examined for the analysis of the drug and both drug-derived and endogenous metabolites excreted in the urine. Analysis of these data showed that the number of features detected (drug-related and endogenous) increased from the 9373 for the conventional UHPLC configuration to 11,673 with the VJC system (Table 3), representing a ca. 25%

Table 3. Comparison of Chromatographic Performance between Conventional and VJC (37 and 75 s Separations) Analysis

component	conventional UPLC system (75 s analysis)	VJC UPLC system (75 s analysis)	VJC UPLC system (37 s analysis)
peak width (s)	1.3	0.6	0.4
peak capacity	55	120	87
number of detected features	9373	11,673	8723
peak intensity (APAP glucuronide)	1.12×10^6	1.25×10^6	1.1×10^6
peak tailing	1.250	1.125	1.130

increase in features detected by the latter. Representative chromatograms for the conventional and VJC system separations of the 1 h post-dose urine sample are shown in Figure 1. Pooled QC samples were analyzed every five injections to determine the variability of the assay,[17] giving a total of 12 QC sample analyses throughout the course of the analytical batch. Analysis of the VJC data showed that, for each sampling occasion, more of the features detected in the PCA had lower CVs than the equivalent conventional data. Therefore, for, e.g., the 3 h time point, 82% of the data had CVs ≤30% with the corresponding figure for the 4 h time point being 88%, while in the case of the QC samples, 86% of the features had a CV <30%. These results suggest that the analytical variability of the individual time points was essentially identical to that of the QC samples. For the conventional system, the equivalent figures from the PCA for a CV of ≤30% were 76% and 73% for the 3 and 4 h time points, respectively, while for the QC samples, 78% of the features had a CV <30%.

While the number of data features obtained from the VJC system at ca. 12,000 was lower than the ca. 18,000 features obtained from a conventional 15 min UPLC/MS cycle time,[18] this reduction should be weighed against the ca. 10-fold
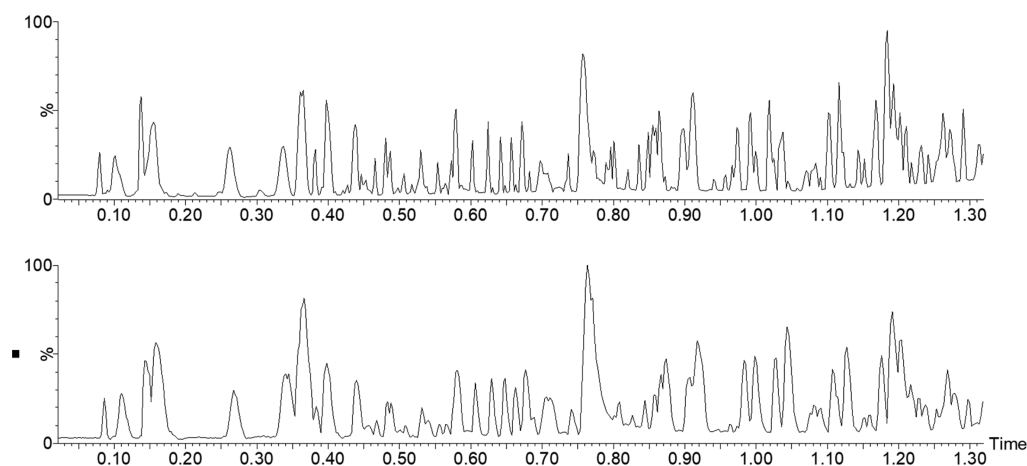
Figure 1. Comparison of mass chromatograms obtained from the UPLC/MS (ESI+) analysis of the 1 h post-dose urine sample (400 mg of APAP) to a male human (time scale in minutes). Upper trace: Data from the VJC method (HSS T3 2.1 mm × 30 mm 1.8 μm column). Lower trace: Data from the conventional method (HSS T3 2.1 mm × 30 mm 1.8 μm column).
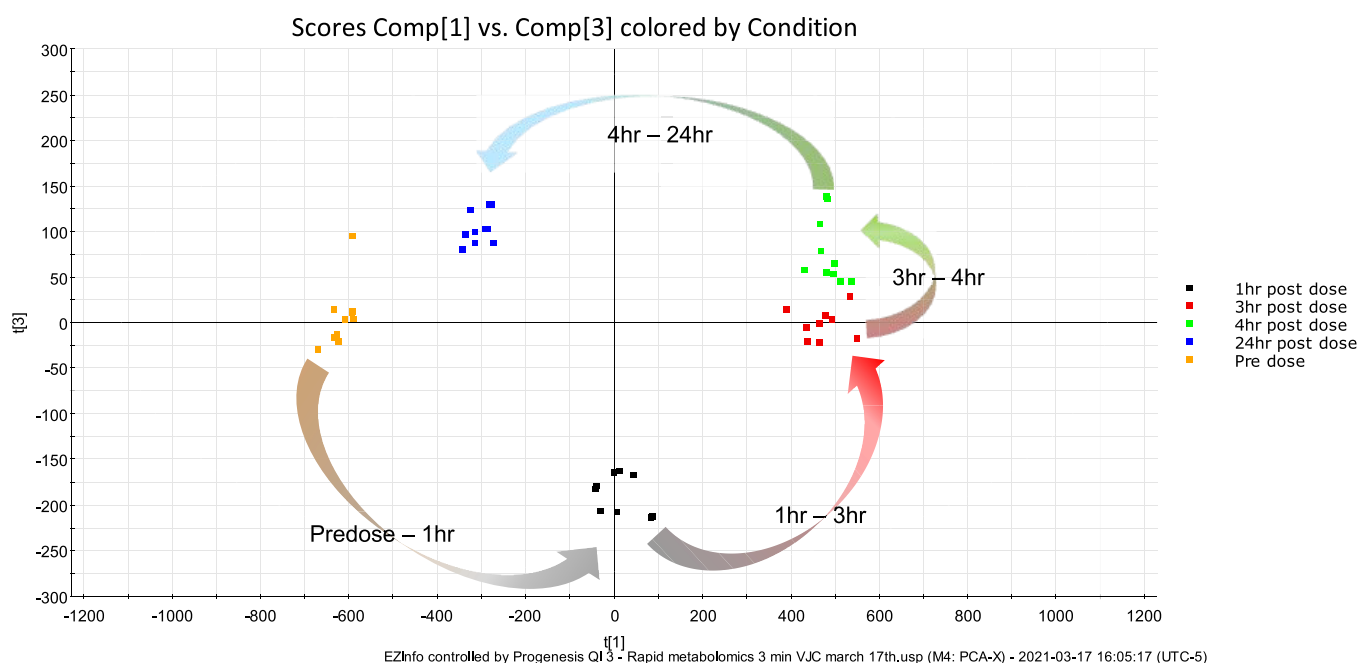

Figure 2. Principal component analysis (PCA) plots of the first (PC1) versus the third (PC3) principal components of the same subset of human urine samples taken from an acetaminophen study analyzed via the VJC method using ESI+. PC1 accounted for 27.1% of the variance in the data with PC3 accounting for a further 20.0%.

increase in throughput and excellent discrimination of the time points provided by this ultrahigh-throughput LC assay on the VJC.

As Figure 1 shows, the combination of the improved control of thermal dispersion (radial and longitudinal) and reduced peak broadening due to the transfer tubing yielded significant gains in LC performance. For example, a visual inspection of the chromatogram for the region between 0.6 and 1.1 min demonstrates that the VJC system yielded greatly improved performance compared to the conventional UPLC system. Thus, e.g., two peaks eluting at ca. 0.92 min were clearly resolved by the VJC system whereas they co-eluted on the conventional column. This increased resolving power of the VJC system can be attributed to the reduced thermal and connection tubing dispersion of the system directly leading to narrower peak widths. The average chromatographic peak width obtained from the VJC column was 0.6 s at the base compared to 1.2 s for the conventional column, giving peak capacities of 120 and 55, respectively (based on a 1.3 min separation window); the resulting data are summarized in Table 3.

When the data for the analysis of urine samples covering the period from predose to 24 h post-dose of 400 mg of acetaminophen (APAP) were analyzed statistically, the principal component analysis (PCA) plots for the data obtained by both separation platforms showed generally good discrimination between the time points, as illustrated in Figure S1. However, the PCA performed on the VJC data (Figure 2 and Figure S1) showed significantly tighter clustering than that obtained from the conventional UPLC/MS system and greater resolution of the 3 and 4 h time points (see Figure S1). While the individual time points and QC samples were
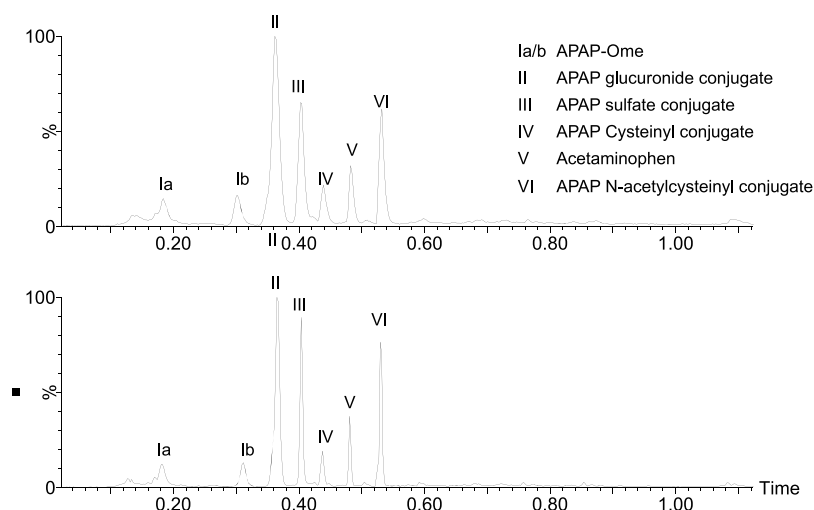
Figure 3. Comparison of the extracted ion chromatographic acetaminophen metabolite traces obtained from the UPLC/MS (ESI$^+$) analysis of 1 h post-dose urine following 400 mg of acetaminophen to a male human using VJC and conventional methods (time scale in minutes). Data from the conventional method, performed on a HSS T3 2.1 mm × 30 mm 1.8 μm column, is shown in the upper trace, and that from the VJC method, performed on a HSS T3 2.1 mm × 30 mm 1.8 μm column, is shown in the lower trace.

clearly separated using PC1/PC2 (data not shown), PC1/PC3 provided the best visualization of the variance due to the metabolic effects of drug administration. As can be seen from the PCA scores plot for the VJC separation, the samples showed a typical, and clearly time-related, response to acetaminophen administration with the trajectory moving rapidly away from the predose urine, with the 3 and 4 h samples clustering closely together and the 24 h samples returning to a position near the predose samples. Statistical analysis showed that between them, PC1 and PC2 accounted for 27.1 and 25.5% of the variation, respectively, with PC1 and PC3 accounting for 27.1 and 20.0% of the variation, respectively. Further statistical analysis of these data via orthogonal projection to latent structures discriminant analysis (OPLS-DA), predose vs 1 h and predose vs 3 h samples, showed that although the acetaminophen sulfate and acetaminophen glucuronides were significantly increased in abundance for the post-dose samples, they did not significantly contribute to the observed statistical separation at these time points. It is evident from these figures that the data for the individual sample groups clustered more closely together when analyzed using the VJC for profiling than the conventional UPLC/MS system. Thus, for the conventional system, the more widely dispersed data caused the 3 and 4 h post-dose samples to be intermingled while they were well separated by the VJC (Figure S1).

In this type of analysis it is common practice for "column conditioning", whereby multiple injections of matrix (most often the QC samples) are made to stabilize retention times and signal intensities, to be undertaken before commencing analysis in metabolic phenotyping.[20] One of the major benefits of this methodology is that a large number of conditioning injections can be made onto the LC system very rapidly if effecting this "conditioning" is required, thus improving assay performance without requiring a long "run in" period.

3.2. APAP and Metabolites. A more detailed study of the samples for signals resulting from APAP and its metabolites was then undertaken. A common OTC analgesic and antipyretic drug APAP has a long history of hepatotoxicity when used above the maximum recommended dose of 4 g/

day. The cause of this drug-induced liver injury (DILI) is that the major detoxication pathways for APAP (glucuronidation and sulfation) are overwhelmed, leading to the production of large quantities of the highly reactive N-acetyl-p-benzoquinone imine (NAPQI) metabolite through oxidation by Cytochrome P450 2E1. NAPQI is usually neutralized by reaction with glutathione (GSH), and the glutathionylated adduct is then further metabolized to its cysteinyl and mercapturate conjugates. Any NAPQI that escapes this detoxication however reacts with cellular macromolecules with, if amounts are high enough, cell death and organ failure as consequences. The glutathione-derived metabolites of APAP are increasingly being seen as a potential "biomarker" for APAP-derived DILI and, as a result, assays for the drug and its major metabolites (the glucuronide (APAP-G), sulfate (APAP-S), GSH (APAP-GS), cysteinyl (APAP-C), N-acetylcysteinyl (APAP-NAC), me-thoxy-(APAP-OMe) and, for some species such as the pig,[19] the phenolic glucuronide of the N-deacetylated metabolite p-aminophenol (PAP-G) abound e.g.[21] The results obtained here using the VJC system are illustrated in Figure 3. Compared with our recent 7 min bioanalytical method for the determination of APAP and its metabolites,[22] the data acquired here showed that the VJC system produced an obvious improvement in peak shape. The drug and six metabolites detected in these post-dose urine samples were resolved and eluted in under 40 s with an average peak width of 0.016 min. A similar result was seen for the conventional method, but the peak width was 0.022 min (Table S3), giving a 27% reduction in peak width for the former. The narrower peak width produced by the VJC system also resulted in an average 10% increase in peak intensity for the metabolite peaks. This is illustrated by the increase in signal intensity obtained from the acetaminophen sulfate conjugate (Figure S2) where peak intensity increased from 1.12 × 10^6 for conventional UHPLC/MS analysis to 1.25 × 10^6 for the VJC system. The increase in peak intensity is again most likely due to a combination of narrower LC peaks and reduced ion suppression. The narrower peaks produced by the VJC system also produced more intense MS spectra for this metabolite, with fewer interferences (Figure S3). The urinary concen-
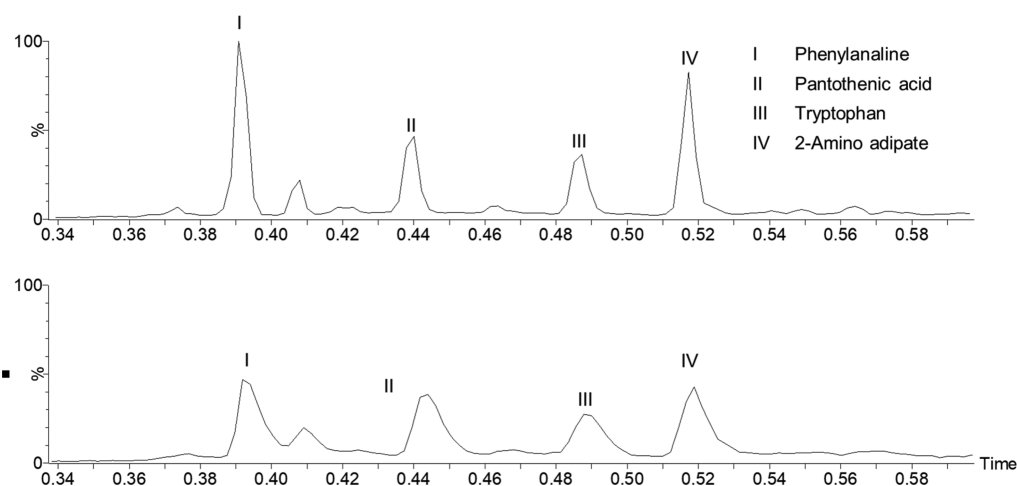
Figure 4. Comparison of extracted ion chromatographic traces for (I) phenylanaline, (II) pantothenic acid, (III) tryptophan, and (IV) 2-aminoadipate obtained from the UPLC/MS (ESI+) analysis of urine 1 h post-dose following the administration of 400 mg of acetaminophen to a male human using VJC and conventional UPLC/MS (time scale in minutes). Data from the VJC method, performed on a HSS T3 2.1 mm × 30 mm 1.8 μm column, is shown in the upper trace, and that from the conventional method, performed on a HSS T3 2.1 mm × 30 mm 1.8 μm column, is shown in the lower trace.
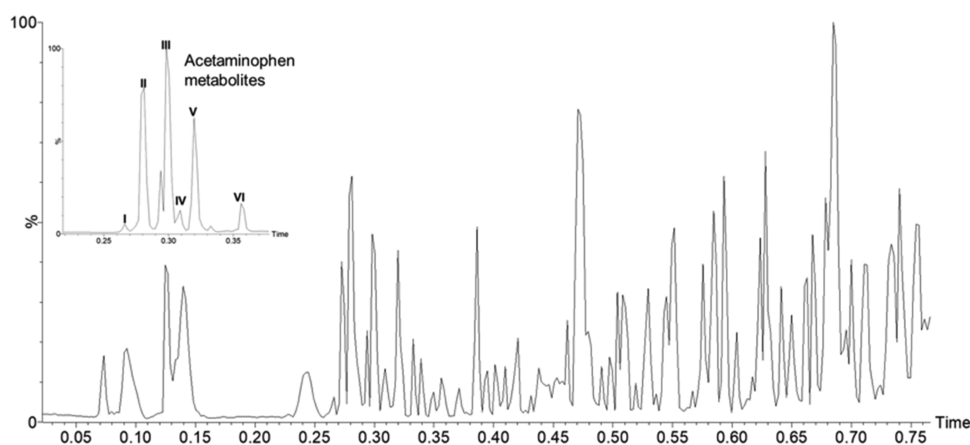

Figure 5. Chromatographic traces obtained from the UPLC/MS (ESI⁺) analysis of a urine sample obtained 1 h post-dose 400 mg/kg of APAP to a male human using the "sub 1 min" VJC method performed on a HSS T3 2.1 mm × 30 mm 1.8 μm column (time scale in minutes). The inset trace shows the extracted ion chromatogram of the acetaminophen metabolites with (I) APAP-OMe, (II) APAP glucuronide conjugate, (III) APAP sulfate conjugate, (IV) APAP cysteinyl conjugate, (V) acetaminophen, and (VI) APAP N-acetylcysteinyl conjugate, data collected for 45 s.

trations of acetaminophen in urine peaked 1 h after dosing, declining thereafter, and were undetectable in the 24 h sample. In the case of the sulfate and glucuronide conjugates, the highest concentrations were observed in the sample collected 3 h after dosing and were also undetectable in the 24 h sample. The cysteinyl and N-acetyl cysteinyl conjugates were present at low concentrations in the 1 h samples, peaking in concentration at 4 h post-dose, but were still detectable in the 24 h urine. The VJC chromatographic system was also able to resolve the three and four isomers of the minor 3-methoxy-APAP conjugate, which eluted with retention times of 0.17 and 0.30 min (Figure 3 and Figure S4).

3.3. Endogenous Metabolites. As mentioned above, in addition to APAP and its metabolites, the MS data for the peaks of a number of endogenous compounds were also obtained from these urine samples. As examples, the chromatographic peak tailing factor was determined for both VJC and conventional systems for four endogenous metabolites, namely, 2-aminoadipic acid, phenylaniline, tryptophan, and pantothenic acid. As illustrated in Figure 4 and Figures

S5−S8, the analysis of these data showed that, as for APAP and its metabolites, the average peak tailing for the endogenous compounds was reduced from 1.25 with the conventional UPLC to 1.13 with the VJC system (Table 3). The narrower chromatographic peaks produced by the VJC system also resulted in an increase in peak response in the MS detector ranging from 20% for pantothenic acid to 120% for phenylaniline (Table S2). As discussed for the APAP and drug-related peaks, this increase in intensity is probably due to a combination of the narrower peak width producing a taller LC peak and the reduction in analyte co-elution also reducing the influence of any in-source ion suppression. The endogenous metabolite identities were confirmed by comparison of the low and high collision energy MS spectra to in-house library data (Figure S9). The improved LC performance obtained from the 75 s gradient VJC UPLC separation compared to the conventional LC system is in agreement with that predicted by a chromatographic theory.[14] This increase in performance is most significant for steep LC gradients where the change in column volumes/min is high, compared to

conventional, longer, separations where the number of column volumes/min is low. This is illustrated in Figures S10 and S11 where the improvement in LC performance has been modeled (using an in-house program) for a 3 cm column using a 1 min gradient and a 10 cm column using a conventional 15 min gradient. The derived data predicted that while using the VJC system should result in a 60% improvement in peak capacity for the 3 cm column/1 min methodology, it would only result in a 3% improvement in performance for the 10 cm column/15 min methodology.

In addition, we looked at the potential for APAP administered at this dose to interfere with the metabolism of gut microbially derived metabolites such as *p*-cresol or phenol.

As discussed earlier, APAP detoxification occurs primarily though conjugation to sulfate and glucuronate. These conjugations also represent major biotransformations for the detoxication of both *p*-cresol and phenol, and the competition for sulfation with such gut microbiota-derived metabolites and drugs such as APAP was identified as a major interaction, leading to the concept of pharmacometabonomics.[23] However, in this instance, statistical analysis by OPLS-DA showed that the amounts of neither *p*-cresol nor phenol sulfates or glucuronides were significantly increased or decreased in relative abundance nor did the ratio of sulfate/glucuronide change during the study. This suggests that the dose 400 mg of APAP used here had no effect on the sulfation capacity of the subject in this study.

3.4. Sub 1 min Analysis Using the VJC. To further examine the potential of the VJC platform for rapid analysis, the chromatographic gradient duration was reduced such that the organic solvent (%B) reached 50% after 25 s and 95% after 37 s, and additionally the flow rate increased to 1.1 mL/min. Thus, the number of column volumes defining the gradient was reduced from 24 with the 96 s analysis to 13 with this "sub 1 min" analysis. The full scan separation LC/MS data produced for the analysis of the 1 h urine sample is given in Figure 5, with all of the urinary peaks eluting within 37 s, and then an organic wash was employed prior to the next analysis. The average peak produced in this shortened analysis was determined to be 0.4 s at the base, giving a peak capacity of 84, which is 70% of that obtained with the 96 s analysis. The combined extracted ion chromatogram for the acetaminophen metabolites showed that this rapid system still had sufficient chromatographic resolving power to separate the acetaminophen metabolites (see inset to Figure 5). The extremely narrow peak widths produced by the 37 s VJC gradient separation resulted in just four to five data points across the MS peak, although this could have been increased if the MS instrument had been operated in TOF-MS mode only rather than MSe mode (where the instrument alternates between collecting low and high collision energy data[24]). Data collection in LC-TOF-MS mode would effectively halve the duty cycle of the MS detector, thereby doubling the number of points obtained across the peak. These extremely narrow LC peaks do, however, highlight the need for fast acquisition MS detection to eliminate the potential of missing the apex of the LC peak and thus possibly underestimating the peak intensity. In quantitative assays employing ^{13}C or ^{15}N labeled internal standards, this problem might be attenuated to some extent, but this would need further investigation.

Clearly, in the drive for ever-increasing sample throughput in MS-based analysis, there is a tension between the speed of analysis and the amount of data obtained. For example,

although both thermal desorption MS and ultrasonic sampling can deliver analysis times in a range of 0.5−3 s/sample,[4,5] both require either single analytes for library analysis[25] or (extensive) sample preparation/isolation when faced with biological matrices. Likewise, "infusion" MS that takes advantage of the sensitivity of nanospray MS[26] also requires sample preparation/isolation prior to data acquisition when presented with complex matrices for analysis. Successful examples of the application of such approaches include high-throughput analysis via nanospray infusion coupled with tandem quadrupole MS/MS[26] and the use of this technology following HPTLC for the analysis of meningioma ganglio-sides.[27] Thus, in the case of biological samples, the bottleneck is simply transferred from the analytical to the sample preparation step. Direct approaches such as DIMS, DESI, and REIMS remove the need for sample preparation to some extent, depending upon the matrix to be analyzed, allowing samples to be analyzed *in situ* or from a medium such as paper, by spraying a stream of charged solvent droplets onto the surface of the sample (see the example of the use of REIMS for direct cell line analysis).[28] However, our comparison of, e.g., DIMS vs RAMMP/LC/MS for urine analysis clearly illustrated the advantage of even a short a chromatographic step in improving the quality of the result.[16] Thus, LC/MS helps to address the issues of ion suppression and isomer resolution, thereby improving assay specificity and selectivity. For proteinaceous matrices, both DIMS and LC must be subject to sample preparation for protein removal (typically solvent precipitation), which can be automated in fast liquid chromatographic approaches such as RapidFire MS.[29] Thus, RapidFire employs on-line chromatographic extraction for the targeted analysis of analytes such as immunosuppressants in whole blood, achieving analysis times as low as 15 s/sample. To date however, this methodology has not addressed more complex multi-analyte methods. As previously demonstrated, the use of short chromatographic columns and rapid analysis times[10,11] results in the inevitable loss of some metabolome coverage compared to the more conventional 12 or 25 min analysis times routinely employed in metabolomic studies.[11] As peak capacity is lost, analyte co-elution results in increased matrix effects. However, our earlier studies on the use of UPLC in metabolic phenotyping[8] had already indicated the potential of rapid UPLC/MS on a 50 mm 2.1 mm id column with a 1.5 min analysis, providing average peak widths of 1.5 s and a peak capacity of ca. 60 (similar to the result obtained in the present study for the non-vacuum jacketed UPLC/MS system). This result was comparable with that obtained by conventional HPLC/MS with a peak capacity of ca. 75 and a 10 min analysis time. In the intervening period, we have used miniaturization and rapid gradients to shorten analysis times with greater efficiency than achievable with our earlier studies, e.g., refs 11 and 12 However, the data obtained in the present study with the VJC system shows that even greater gains in throughput are achievable, with limited modification to the existing LC/ MS system (see Figure S12). Applications such as metabonomics/metabolomics, lipidomics, proteomic profiling of biofluids, biobanking, drug metabolism, toxicology screening, and basic disease understanding in animal models and large cohort clinical studies may all benefit from this type of system.

4. CONCLUSIONS

Application of a vacuum jacketed column (VJC), located at the source of the mass spectrometer, to the analysis of human urine has demonstrated the potential for high-throughput methods for drugs, their metabolites, and endogenous compounds. A 75 s gradient VJC reversed-phase LC/MS analysis yielded an average peak width of 0.6 s and a peak capacity of 120 compared to just 55 for a conventional non-vacuum jacketed system. This translated to a 25% increase in features detected in a urinary metabolic profiling study and facilitated the baseline resolution of acetaminophen and its major urinary metabolites in less than 0.6 min. Compared to an equivalent conventional UPLC/MS system, the increased resolving power of the VJC system reduced co-elution and increased MS peak intensity (10−120%), resulting in simplified MS and MS/MS spectra, allowing for more confident metabolite identification. The low dispersion characteristics of the VJC system offer the potential to drive the analytical throughput to even greater levels by reducing the LC/MS run time to less than 1 min while maintaining analyte resolution.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c01982.

> (Figures S1−S12) PCA plots of human urine analyzed via conventional or VJC methods plus comparisons of extracted ion chromatograms for APAP sulfate via VJC and conventional UHPLC; spectra of APAP sulfate, the 3/4 isomers of methoxy-acetaminophen, 2-aminoadipic acid, pantothenic acid, tryptophan, and phenylaniline; computer models for 1 and 15 min separations of conventional and VJC UHPLC separations; and image of the VJC assembly on a mass spectrometer and (Tables S1 and S2) comparison of MS response for APAP, APAP metabolites, and endogenous metabolites using VJC and conventional UPLC systems (PDF)

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Author Contributions

The manuscript was written through contributions from all authors. LCMS experiments were performed by R.S.P. and T.M.; experimental design was by R.S.P., L.A.G., K.A.J. and I.D.W.; and VJC research project technical lead J.H. Data interpretation and manuscript preparation were by R.S.P., P.D.R., L.A.G., and I.D.W. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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