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Short communication

A demonstration of the use of ultra-performance liquid chromatography–mass spectrometry [UPLC/MS] in the determination of amphetamine-type substances and ketamine for forensic and toxicological analysis ☆

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Abstract

We have recently seen the emergence of ultra-performance liquid chromatography (UPLC) coupled to mass spectrometry as an alternative to traditional high-performance liquid chromatography techniques. The strengths of UPLC technology promote the ability to separate and identify drug compounds with significant gains in resolution and sensitivity and marked reductions in the overall time of analysis. As increased throughput is the desire of the practical toxicology laboratory, the aim of this study was to trial commercially available technology by assessment of the separation of several commonly encountered amphetamine-type substances. From injection of a poly-drug reference standard and whole blood extract, we successfully separated and identified amphetamine, methamphetamine, ephedrine, pseudoephedrine, phentermine, MDA, MDMA, MDEA and ketamine in less than 3 min using the Acquity UPLC-Micromass Quattro Micro API MS instrumentation (Waters Corporation, USA). In addition to this significant reduction in overall run time, all peaks exhibited acceptable resolution using selected ion recording (SIR), with analysis indicating the capability to separate 5–11 peaks in 1.75 min using the current system parameters. From this introductory data, it is therefore indicated that the technological advancements defining ultra-performance liquid chromatography will allow it to serve as a powerful analytical tool for rapid throughput analysis.
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1. Introduction

It is only within the last decade that the use of traditional high-performance liquid chromatography–mass spectrometry (HPLC/MS) has been readily accepted by the practical working toxicology laboratory for high throughput analysis. Relatively recent advances, notably the use of atmospheric pressure ionization (API) techniques such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), have now promoted the widespread use of LC/MS as an analytical tool [1,2]. Since review articles by Maurer [3] and Marquet and Lachatre [4], the number of publications directly relevant to applications in forensic and clinical toxicology has increased, mainly with a focus on improvements in separation and identification relative to gas chromatography–mass spectrometry (GC/MS). Hoja et al. noted that nearly 70% of everyday samples encountered in toxicological laboratories can be handled using LC [5], and the advantages of LC have been well described, particularly the capabilities in analyzing polar or thermolabile compounds without requiring derivatization [6–9].

The use of LC/MS is now commonplace, therefore it was only logical to push the technology closer to the theoretical capacities of the instrumental components. Much

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more recently, the desire for significantly reduced analysis times with increased sample throughput, sensitivity, and resolution has resulted in the development of ultrafast separations and identification using LC/MS techniques [10,11]. Termed ultra-performance liquid chromatography (UPLC) or ultrafast liquid chromatography, the improvements in such parameters are largely due to advancements in the particle size and bridging structure of the column packing, though complemented by additional instrumental modifications [12,13].

The Acquity UPLC instrument (Waters Corporation, USA) is an example of a commercially available UPLC system with full instrument modifications: advancements in the solvent delivery module, in combination with the column technology, allow the Acquity to run routinely at pressures up to 15,000 psi; the Acquity sample manager has been modified to inject down to 1 μl using a needle-in-needle probe; and, the UPLC photodiode array and tunable UV–vis detectors have been modified accordingly in terms of increased sampling rate and detector cell dispersion [14]. At the center of the advancements, however, is the column, which utilizes pressure-tolerant 1.7 μm hybrid particles containing a bridged ethylsiloxane/silica structure (BEH) [15]. It is the combination of these technological developments that produce increased sensitivities and improved peak resolution, while significant reductions in analysis times provide the rapid throughput desirable to the working laboratory. Recent publications examining its application in drug discovery and metabolism have been produced demonstrating the improvements in sensitivity, resolution, and analysis time [16–19], and have also included the separation of the common *Ephedra* alkaloids ephedrine and pseudoephedrine [20]. However, few studies to date have examined the separation of common and novel illicit amphetamine-type substances and designer analogues often encountered in the forensic laboratory in solid-state form or in biological specimens.

Therefore, as this technology should be of significant practical advantage to the forensic/post-mortem toxicology laboratory, we initially tested the system with a poly-drug reference standard containing eight amphetamine-type substances: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethamphetamine (MDEA), ephedrine, pseudoephedrine, and phentermine. This poly-drug standard also contained phenylethylamine, a commonly encountered putrefactive amine indicative of decomposition [21–24]. In addition to the standard mix, we also evaluated a whole blood extract that was reflective of the high toxic range observed in forensic/post-mortem toxicology. This sample contained amphetamine, methamphetamine, MDA, MDMA, phenylethylamine, and ketamine, the latter of which was included due to its increasing occurrence in club- or designer drug street samples [25]. From analysis of these two reference solutions, we provide a demonstration of the new UPLC technology and gain an indication of any potential in improvements in analysis time, peak separation, and identification power when coupled to mass spectrometry.

2. Experimental

Amphetamine, methamphetamine, MDA, MDMA, MDEA, ephedrine, pseudoephedrine, phentermine, and phenylethylamine (hydrochloride salts) were obtained from the National Measurement Institute (Sydney, NSW), and a methanolic stock solution was prepared in a concentration of 10 μg/mL. Four microlitres of this stock was then injected on the Acquity system (approximating 40 ng of each drug of interest injected). In addition, a whole blood sample was spiked with amphetamine, methamphetamine, MDA, MDMA, phenylethylamine, and ketamine for a final concentration of 2 μg/mL. Extraction of this sample was then performed using an Oasis MCX solid phase extraction column (60 mg, Waters Corporation, USA) by the following procedure. Two millilitres of 100 mM sodium phosphate buffer (pH 6) was added to the 1 mL of spiked blood, followed by vortex-mixing and centrifugation. The column was conditioned with 3 mL methanol, 3 mL deionized water, and 1 mL phosphate buffer (pH 6). The sample was introduced to the column, allowed to pass, then followed by 3 mL deionized water, 1 mL 0.1 M acetic acid, and 3 mL methanol. The column was then dried under vacuum at approximately 5 psi for 10 min, then elution performed using two 1 mL fractions of 10% ammonium hydroxide in methanol. Five microlitres of this was injected on the Acquity system. No concentration step was performed, leading to approximately 5 ng of each drug injected assuming 100% recovery.

2.1. UPLC conditions

The column used was Waters UPLC BEH C18 (2.1 mm × 50 mm), with the target temperature set at 40 °C. The Waters Acquity TUV single wavelength detector was programmed for analysis at 254 nm. The mobile phase used for the Waters Acquity UPLC-UV and MS system was aqueous pyrrolidine (0.5 mL glacial acetic acid and 1.0 mL pyrrolidine in 500 mL reagent grade water) and methanol under isocratic conditions (flow rate 0.4 mL/min; 50:50 for methanolic standard; 52:48 for whole blood extract).

2.2. MS conditions

A Waters Micromass Quattro Micro API mass spectrometer instrument (data analysis software MassLynx V4.0) was used in positive electrospray ionization mode. Nitrogen was used as the drying gas. Desolvation gas flow was 648 L/h, and cone gas flow was maintained at 58 L/h. Desolvation temperature was 445 °C and source temperature was 119 °C. Observed capillary and cone voltages were 3510 V and 22.83 V, respectively (note: improved response was observed for MDA using a cone voltage of 50 V, therefore this parameter was included for MDA in the range for the whole blood extraction analysis program; similarly, 15 V was used for amphetamine).

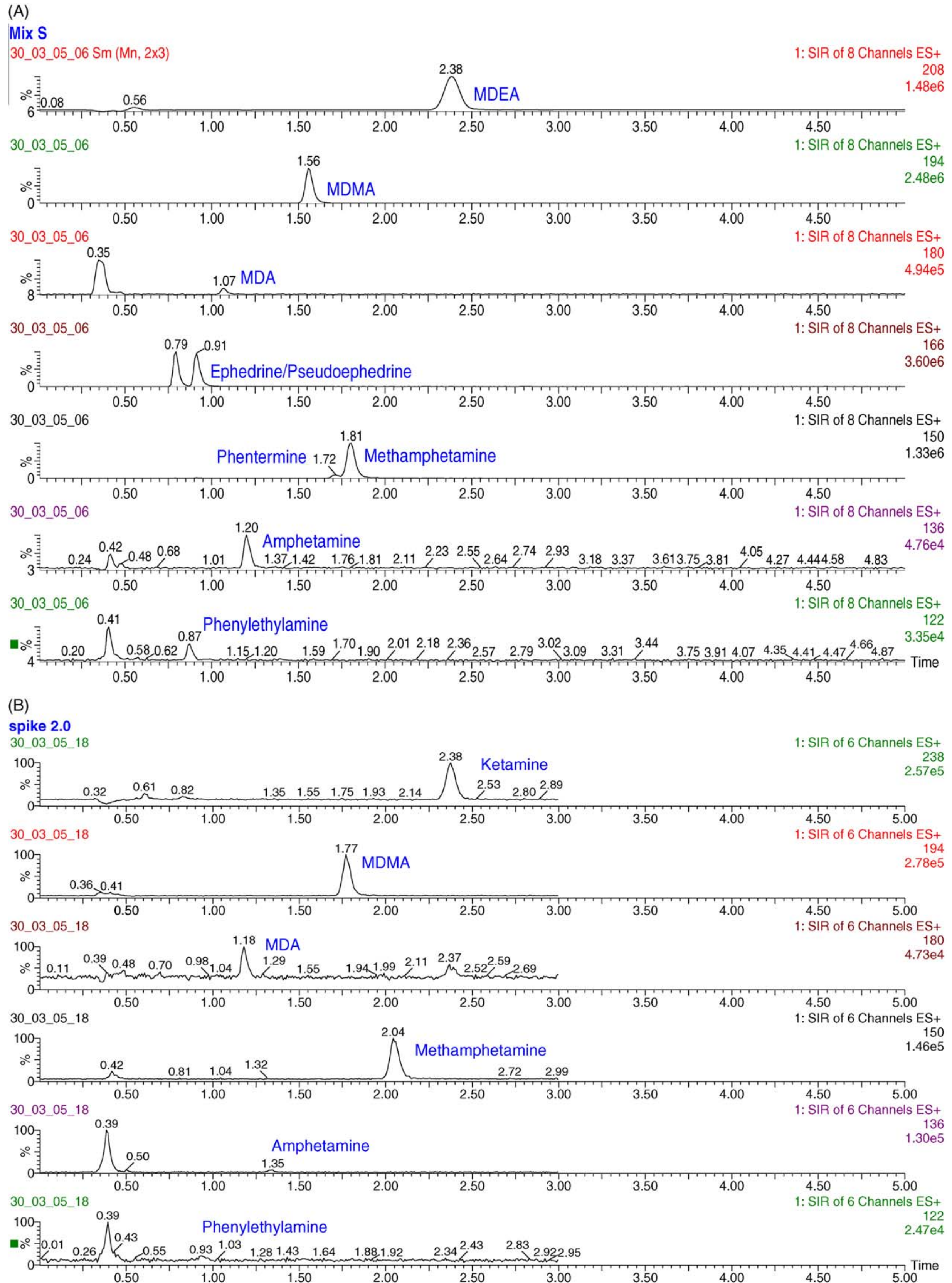

Fig. 1. (A) Selected ion recording (SIR) chromatograms of a methanolic drug poly-standard containing eight amphetamine-type substances with phenylethylamine putrefactive marker ($[M+H]^+$ ions monitored are in right hand margin; isocratic 50:50 aq. pyrrolidine:methanol at 0.4 mL/min); (B) Selected ion recording (SIR) chromatograms of four amphetamine-type substances, phenylethylamine, and ketamine from extracted whole blood (baseline at zero, non-linked vertical axes; isocratic 52:48 aq. pyrrolidine:methanol at 0.4 mL/min).

3. Results and discussion

The injection of the polydrug reference standard showed favorable separation and acceptable peak resolution with regard to the concentration injected (Fig. 1A), and it was observed that all eight amphetamine-type substances separated in less than 2.5 min under the 50:50 isocratic conditions. While analytical methods using MS and MS/MS have been described for such substances with individual peak retention times ranging from 1.5 min to 13 min [6,8,9,26] none of these exhibit both the peak separation and total analysis time we observed from a drug mixture by both tunable UV detection and selected ion recording (SIR). This is strong indication of the potential of UPLC to significantly reduce analysis time while promoting baseline separation for these licit/prohibited drug compounds.

The individual SIRs for each of the eight substances are shown in Fig. 1A, in addition to that of the decomposition marker, phenylethylamine. Note that the protonated molecular ions ($[M+H]^+$) monitored are indicated along the right margin of the SIR chromatograms. From the elution order and approximate peak-to-peak resolution (Table 1), the observed potential for coelution interference appears minimal under the applied instrument conditions and sample concentration. Approximate baseline peak-to-peak resolution was calculated from $R=(t_{rb}-t_{ra})/((1/2)(W_{ba}+W_{bb}))$, where t_r is retention time and W_b is peak width at base; the value of 1.0 indicates 10% overlap, and 1.5 illustrates complete resolution of two peaks of equal size [27]. From our results (Table 1), the resolution of the phenylethylamine putrefactive base (0.87 min) indicates potential concentration-dependent coelution with either ephedrine (0.79 min) or pseudoephedrine (0.91 min), but it is unlikely this would interfere with mass spectral identification in a forensic toxicological case with the use of selected ion recording. Similarly, there is partial coelution of phentermine (1.72 min) and methamphetamine (1.81 min) observable at m/z 150. This potential quantitation interference could be alleviated with the use of an optimized gradient in the circumstance that these two compounds are encountered in a toxicological specimen.

In the analysis of the extracted whole blood sample (Fig. 1B), the change in isocratic conditions (from 50% to 52% aq. pyrrolidine) caused an expected shift in the separation of the five compounds (phenylethylamine, MDA, amphetamine, MDMA, methamphetamine). Again, there was favorable separation and resolution taking into account concentration considerations, as indicated by the SIRs (Table 1, Fig. 1B). Note that ketamine is observable in less than 2.5 min as well. Thus, while there is potential for ketamine to coelute with MDEA when using UV detection, there are markedly discernible fragmentation patterns in the mass spectral data that would allow positive identification and quantitation of both. Should coelution of ketamine and MDEA be observed in a toxicological or forensic chemistry case utilizing only UV detection (e.g. at 254 nm), altering the isocratic conditions or employing an optimized gradient too would likely correct this. Also, it should be noted that a major fragment from ketamine occurs at m/z 180, the ion used for the identification and quantitation of MDA. This is observable as the peak at 2.37 min in the MDA SIR of Fig. 1B. From the separation using the stated conditions, this ketamine artefact does not interfere with the identification of MDA in any way, but could possibly be used as a secondary confirmation for the presence of ketamine. Therefore, from the data for the extracted whole blood specimen, the UPLC conditions again indicate favorable separation, sensitivity, and resolution when challenged with the task of toxicological interpretations of therapeutic to toxic drug levels.

With peak widths (W_p) ranging from 0.1 min to 0.2 min in both the methanolic poly-drug standard and the whole blood extract, the improved resolving power of UPLC is evident in reference to the total run time. The initial peak to final peak analysis time in the case of the standard solution is 1.75 min, providing a separation range of 5–11 peaks based on a resolution factor of 1.5 for complete baseline separation (calculated as $1.75/(1.5 \times W_P)$). Resolving power for the whole blood extract is similar at 7–10 peaks in 1.60 min. One could then imagine the further power of the ultra-performance instrumentation when considering adjustments to column length and run time.

Table 1
Retention times and peak-to-peak (PtP) resolution of ephedrine, phenylethylamine, pseudoephedrine, MDA, amphetamine, MDMA, phentermine, methamphetamine, MDEA, and ketamine (by elution order)

Peak	Retention time (min)	Next peak	Approximate PtP resolution
Methanolic poly-drug standard (isocratic 50:50 aq. pyrrolidine:methanol at 0.4 mL/min)			
Ephedrine	0.79	Phenylethylamine	1.0
Phenylethylamine	0.87	Pseudoephedrine	0.5
Pseudoephedrine	0.91	MDA	2.1
MDA	1.07	Amphetamine	1.7
Amphetamine	1.20	MDMA	3.6
MDMA	1.56	Phentermine	2.1
Phentermine	1.72	Methamphetamine	1.2
Methamphetamine	1.81	MDEA (2.38 min)	3.8
Whole blood extract (isocratic 52:48 aq. pyrrolidine:methanol at 0.4 mL/min)			
Phenylethylamine	0.93	MDA	3.3
MDA	1.18	Amphetamine	2.2
Amphetamine	1.35	MDMA	5.6
MDMA	1.77	Methamphetamine	2.7
Methamphetamine	2.04	Ketamine (2.38 min)	3.4

4. Conclusion

These introductory trials of the ultra-performance technology have demonstrated the potential of rapid separation and identification of illicit drugs such as amphetamine-type substances. While more research and system optimization are required before reporting the full validation of the instrumental method, analysis has indicated prospective gains in sensitivity and resolution, with a strong illustration of the reduction in time of analysis. The ability to separate and identify eight amphetamine-type substances and ketamine in less than 3 min with acceptable baseline resolution is a powerful tool providing opportunity to increase the throughput of the working toxicology laboratory without sacrificing the quality of the analysis.

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