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☐ Immediately ☐ Within 3-9 months ☐ Not planned

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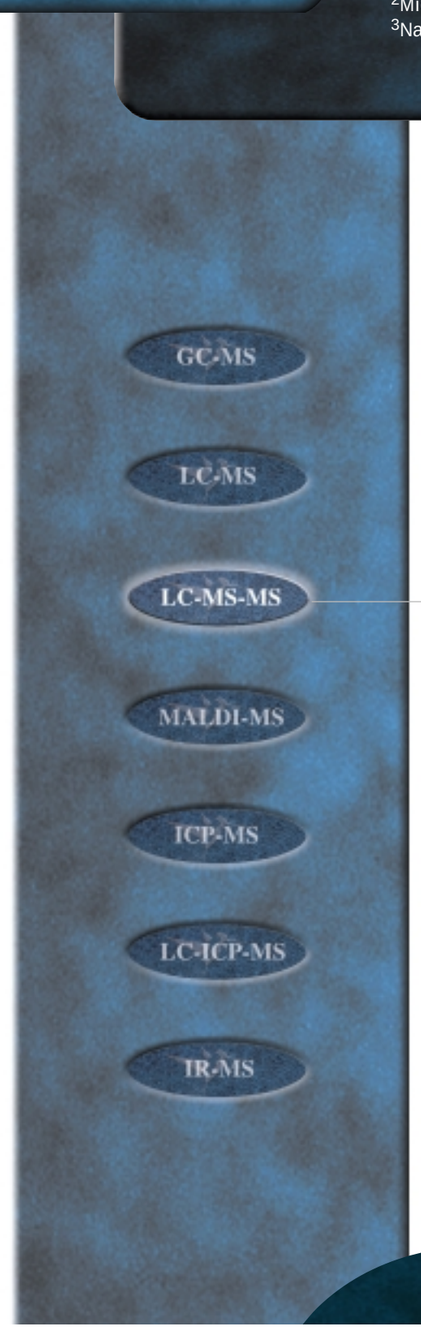

DEVELOPMENT OF A RAPID AND SENSITIVE METHOD FOR THE QUANTITATION OF AMPHETAMINES IN HUMAN PLASMA

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Outline

To develop a rapid sensitive LC-MS/MS method for the simultaneous quantitation of amphetamines in plasma. The method should require minimal sample preparation.

Introduction

- 'Ecstasy' (MDMA), 'EVE' (MDEA) and MDA are amongst the most frequently used recreational drugs.
- Acute toxicity problems include hyperthermia, convulsions and arrythmias. In addition, there are growing concerns of the long-term effects particularly regarding neurotoxicity.
- Target analysis of these drugs and other amphetamines in biological samples is of great importance for clinical and forensic toxicologists alike. The challenge is to analyse such compounds and their metabolites in a limited volume physiological sample.
- At present, most laboratories use GC-MS for this purpose. However, this procedure is very labour-intensive and time-consuming, particularly as solid-phase extraction and derivatization is unavoidable.
- Here we describe the development of an alternative method. Amphetamines were isolated from plasma using a simple methanol extraction procedure and subsequently analysed using HPLC-MS-MS. The developed method has a total analysis time (including sample preparation) of less than 15 minutes and allows the simultaneous analysis of several amphetamines in plasma. Limits of detection of 1ng/ml plasma (or better) were achieved.

Experimental Conditions

LC conditions

HPLC System:		Waters Alliance 2690
Column:		Hypersil BDS C_{18} (100mm x 2.1mm, 3.5µm)
Mobile phase:	(A) =	10mM ammonium acetate
	(B) =	95% acetonitrile :5% 10mM ammonium acetate
		Isocratic elution (85:15)
Flow rate:		0.3mL/min
Injection volume:		10µL

MS conditions

Mass spectrometer:	Micromass Quattro *Ultima* (**Figure 1**)
Ionisation mode:	ES positive ion
Capillary voltage:	3kV
MS/MS:	Collision gas: Argon at 2.5 x 10^{-3} mbar

The Quattro *Ultima* triple quadrupole mass spectrometer incorporating 'Z' Spray source

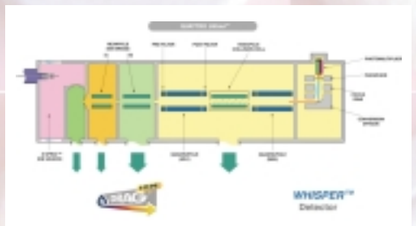

Figure 1

Results and Discussion

Figure 2 shows the resulting MS and MS-MS spectra for 'Ecstasy' (MDMA), 'Eve' (MDEA), MDA, Amphetamine, Methamphetamine and Ephedrine.

MS and MS-MS spectra of several amphetamines and their respective structures

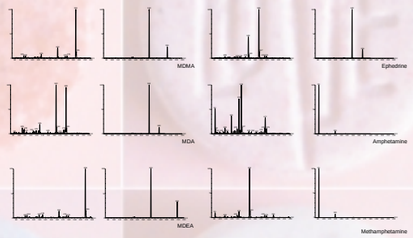

Figure 2

Isotopically labelled internal standards (Radian International) were used for quantification. **Table 1** summarises the multiple reaction monitoring (MRM) transitions and conditions used in the analysis of the amphetamines and their deuterated analogues.

Compound	Precursor (m/z)	Product (m/z)	Cone Voltage (V)	Collision energy (eV)
MDEA	208	163	50	12
MDEA *D5*	213	163	50	12
Methamphetamine	150	91	50	15
Methamphetamine *D14*	164	98	50	18
Amphetamine	136	91	60	17
Amphetamine *D11*	147	98	60	16
Ephedrine	166	148	30	12
Ephedrine *D3*	169	151	40	12
MDA	180	163	30	8
MDA *D5*	185	168	50	10
MDMA	194	163	60	12
MDMA *D5*	199	165	60	13

Table 1

MRM transitions and conditions for the measurement of several amphetamines and their internal standards

Figure 3 shows the MRM chromatograms acquired simultaneously during a single injection (6 out of 12 shown). The typical linearity of response (over 4 orders of magnitude) is demonstrated in **Figures 4a** and **4b**.

MRM chromatograms obtained for a single injection of a mixture of amphetamines (100ng/ml) in mobile phase (respective internal standards are not shown)

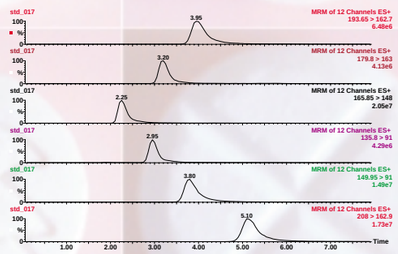

Figure 3

Typical linearity of response, obtained over 4 orders of magnitude, for MDMA (diluted in mobile phase)

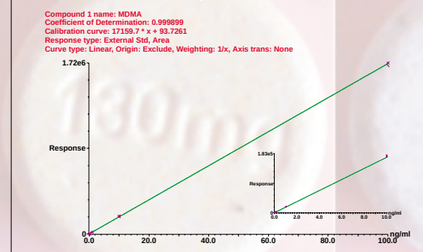

Figure 4a

Typical linearity of response, obtained over 4 orders of magnitude, for MDEA (diluted in mobile phase)

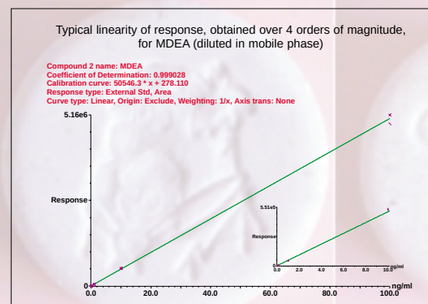

Figure 4b

The experiment was extended to include the determination in 'real' biological matrices by isolating the amphetamines from plasma (methanol extraction) prior to analysis using HPLC/MRM. This simple isolation step takes a fraction of the time needed to prepare samples for typical GC-MS analysis (see **Figure 5**). Once again responses were linear over the four orders of magnitude investigated (Coefficient of Determination > 0.99). Spiked 'Test' plasmas were successfully identified and quantified using the developed method (see **Figure 6**).

Method comparison chart

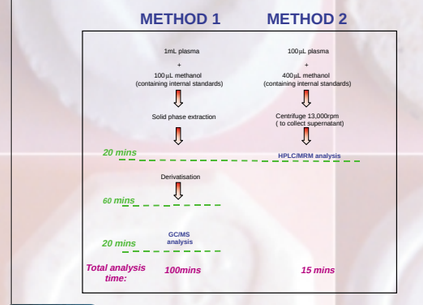

Figure 5

HPLC/MRM analysis of 'Test plasma 1' and 'Test plasma 2', Samples were correctly identified and quantified as MDA and MDMA respectively

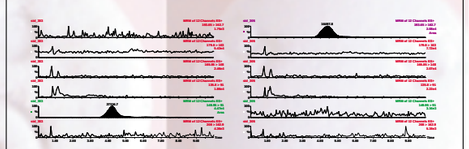

Figure 6

In an aim to further increase the sensitivity of analysis of this group of amphetamines, the potential use of a simple concentration step was investigated. Methanol extracts were dried using a GyroVap™ vacuum concentrator and then reconstituted in 100µL mobile phase prior to HPLC/MRM analysis. The amphetamines were quantified in reference to their respective internal standards. The results (**Figure 7**) demonstrate that the use of internal standards provides a highly successful method of quantifying the amphetamines in extracted plasma and offers increased 'real' sample throughput by negating the need for full standard curves. In addition, the vacuum concentration step is a successful technique by which the sensitivity of analysis may be increased.

Typical linearity of reponse for plasmas containing MDEA and Ephedrine. Amphetamine spiked plasma was firstly extracted using methanol. Following centrifugation, supernatants were dried using a vacuum concentrator before reconstitution in mobile phase and HPLC/MRM analysis. Amphetamines were quantified by reference to their internal standards

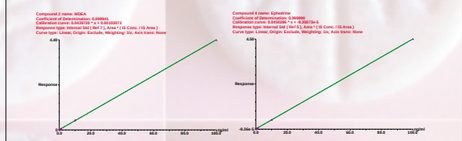

Figure 7

Conclusion

We have developed a simple, rapid method which allows the simultaneous quantitation of several amphetamines in plasma during a single chromatographic run. The procedure which involves the extraction of amphetamines from plasma followed by HPLC/MRM analysis is less time consuming and labour-intensive than the existing method. It has been successfully applied to 'Test' plasma samples.

Future aims

- To extend these investigations in order to fully validate the method against the current GC/MS methodology.
- To apply the validated method to other biological fluids such as saliva and urine.