LC-MS/MS Chiral Separation of "*d*" and "*l*" Enantiomers of Amphetamine and Methamphetamine

Enantiomeric Separation

Ian Moore[1], Takeo Sakuma[1], Michael J. Herrera[2], David Kuntz[2]
[1]AB SCIEX; Concord, Canada; [2]Clinical Reference Laboratory, Kansas, USA

Amphetamine and methamphetamine are psycho-stimulant drugs of the phenethylamine and amphetamine class of psychoactive drugs. Both compounds occur in two enantiomers, dextrorotary and levorotary.

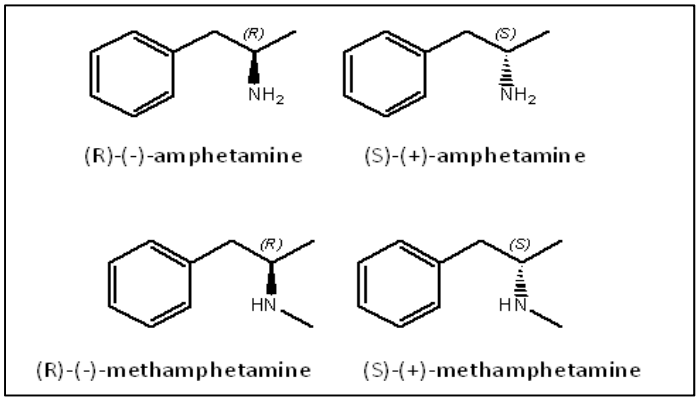
In the case of the methamphetamine, the dextromethamphetamine [*d*-isomer; (S)-(+)-methamphetamine] is pharmacologically more active than the levomethamphetamine [*l*-isomer; (R)-(-)-methamphetamine] and therefore has a higher potential for abuse; typically being found in illicit preparations. The *l*-methamphetamine has less activity towards the central nervous system and is often used in nasal decongestant pharmaceutical preparations that are sold over-the-counter (OTC) and is therefore legal. Levomethamphetamine is also a metabolite of various drugs, for example selegiline which is used for treatment of Parkinson's and dementia.

(±)-Amphetamine, marketed as *Benzedrine*, has traditionally been used for the treatment of asthma and congestion. The *d*-enantiomer is pharmacologically more active and *d*-amphetamine, marketed as *Dexedrine,* is used as treatment for narcolepsy and as a diet pill. Adderall is a blend of *d* and *l*-enantiomers of amphetamine used for the treatment of attention deficit-hyperactivity disorder (ADHD). Due to widespread abuse, amphetamine was made a Schedule II drug in the USA.

Amphetamine was first synthesized in 1887[1] and is a semi-synthetic form of ephedrine. Ephedrine is a natural product from *Ephedra sinica* and extracts are traditionally used for the treatment for asthma, hay fever and colds. (±)-Ephedrine and its diastereomer (±)-pseudoephedrine are the primary interfering compounds in an analysis of amphetamine and methamphetamine. This is made more significant by the fact that pseudoephedrine (Sudafed) is found in OTC formulations and supplements.

Chiral analysis in drug detection has become increasingly important over the past decade. Traditional screening methods for the detection of amphetamines include immunoassays and GC-MS.

Figure 1. Structures of amphetamine and methamphetamine enantiomers



Immunoassays that have been designed to cross react with one or the other enantiomer often have problems with cross reactivity with the wrong enantiomer. GC-MS has a few disadvantages when compared to other analytical techniques. One being the extra step required in the sample preparation involving derivitization of the analytes, commonly using 1-(trifluoroacetyl)-L-prolyl chloride (L-TPC). The purity of such derivitization reagents is often not 100 %, leading to result bias. LC-MS/MS utilization in forensic toxicology screening for drugs and metabolites has become increasingly popular due to the selectivity, sensitivity and speed of LC-MS/MS. For the analysis of the amphetamines, LC-MS/MS eliminates the need to derivitize and allows direct, 100 % detection. Mass spectrometry alone, however, cannot distinguish between stereoisomers, since it characterizes compounds solely in terms of mass. Separations are required up front of the mass spectrometer. Diastereomers were traditionally separated by cellulose or cyclodextrin-based normal phase chromatography, which were not always compatible with electrospray ionization (ESI). In the last several years a new generation of LC phases were developed that can be used in more ESI- compatible reversed phase chromatography. Enantiomers however require a chiral column. Chiral columns have a single enantiomer of a chiral compound bonded to a solid support and in this chiral environment two enantiomers have a different affinity to the stationary phase and can be successfully separated. Here we present a chiral LC-MS analysis that uses a macrocyclic

glycopeptide-based chiral LC column for the separation of the amphetamine and methamphetamine enantiomers.

Experimental

Urine was spiked with racemic amphetamine and methamphetamine at concentrations of 1, 2, 4, 8, 40, 200, 1000, 5000, 25 000 ng/mL in order to prepare the calibrators. Urine was also spiked at concentrations 6, 20 and 15 000 ng/mL for QCs.

Sample Preparation

Phenomenex Strata-X Drug B strong cation exchange with mixed-mode sorbent was used for the solid phase extraction (SPE) procedure. 25 µL of internal standard solution (1000 ng/mL racemic amphetamine-D_8 and 200 ng/mL racemic methamphetamine-D_{14}) was added to 250 µL of sample. 250 µL 50 mM phosphate buffer pH 6 was then added to the sample before loading onto the SPE column. No equilibration of the SPE cartridges was necessary. The cartridge was then washed with 1 mL of 100 mM sodium acetate pH 5 followed by a further wash with 1 mL methanol. The SPE cartridge was dried under full vacuum for 10 minutes and the analytes eluted using 500 µL ethyl acetate, isopropyl alcohol, ammonium hydroxide 70:20:10. 25 µL 0.5 M methanolic-HCl was added prior to drying for 15 minutes at 35 °C under nitrogen. The sample was then reconstituted in 250 µL mobile phase.

Liquid Chromatography

Separation was carried out using a Shimadzu Prominence HPLC system and Supelco Astec Chirobiotic V2 25 cm x 2.1 mm, 5 µm column held at 20 °C. Mobile phase was methanol, 0.1 % (v/v) glacial acetic acid and 0.02 % (v/v) ammonium hydroxide, 250 µL/min flow rate.

Mass Spectrometry

A 3200 QTRAP® System operating in Multiple Reaction Monitoring (MRM) mode was used for detection. Each analyte and internal standard was monitored using two transitions and the system ran in positive TurboIonSpray® probe mode. A two-position; six-port diverter value was used to direct water, delivered from pump A, into the ionization source for the first seven minutes of the LC run whilst directing the mobile phase to waste. At seven minutes the diverter value was switched to direct the mobile phase into the mass spectrometer, at 14 minutes the diverter valve was switched again to direct water into the source.

Table 1. MRM transitions for amphetamine and methamphetamine and their respective internal standards

Compound	Q1	Q3
Amphetamine 1	136	91
Amphetamine 2	136	119
Amphetamine-D_8 1	144	97
Amphetamine-D_8 2	144	127
Methamphetamine 1	150	91
Methamphetamine 2	150	119
Methamphetamine-D_{14} 1	*164*	98
Methamphetamine-D_{14} 2	*164*	130

Results and Discussion

Figure 3 shows a representative chromatogram of urine spiked at 40 ng/mL of each analyte, extracted and analyzed. The LLOQs for methamphetamine and amphetamine are 1 ng/mL and 4 ng/mL, respectively. Data at these levels are shown in figures 4 and 5. How well the separation of the enantiomeric forms of both amphetamine and methamphetamine was achieved in relation to concentration is shown in Figure 2. Adequate separation is achieved for all concentrations with baseline resolution up to the 1000 ng/mL for methamphetamine enantiomer separation. Baseline separation is achievable up to a concentration of 25 000 ng/mL, for both racemic amphetamine and methamphetamine, if 25 fold less sample is loaded on the column.

Figure 2. Chromatographic separation of racemic amphetamine and methamphetamine

Concentration (ng/mL)	Average Resolution	
	Amphetamine	Methamphetamine
1	N/A	1.699
2	N/A	1.730
4	1.409	1.647
8	1.411	1.722
40	1.278	1.596
200	1.338	1.544
1000	1.346	1.505
5000	1.221	1.246
25000	1.093	1.064

Resolution calculated using width at 50% height

Figure 3. Extracted ion chromatograms for a 40 ng/mL extracted calibrator

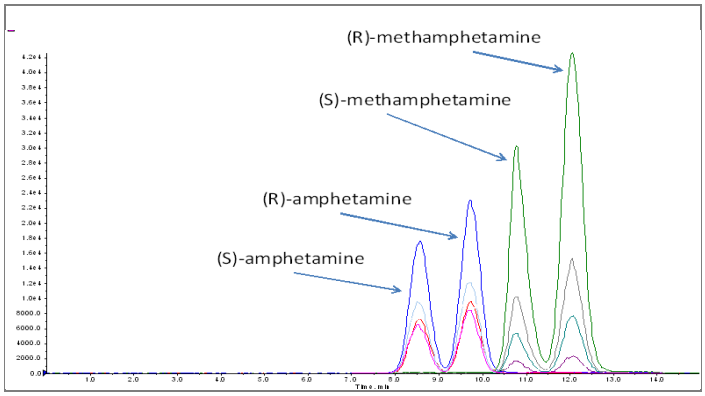
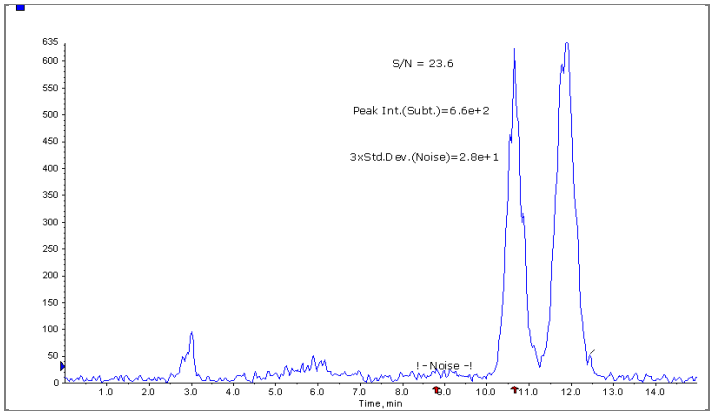
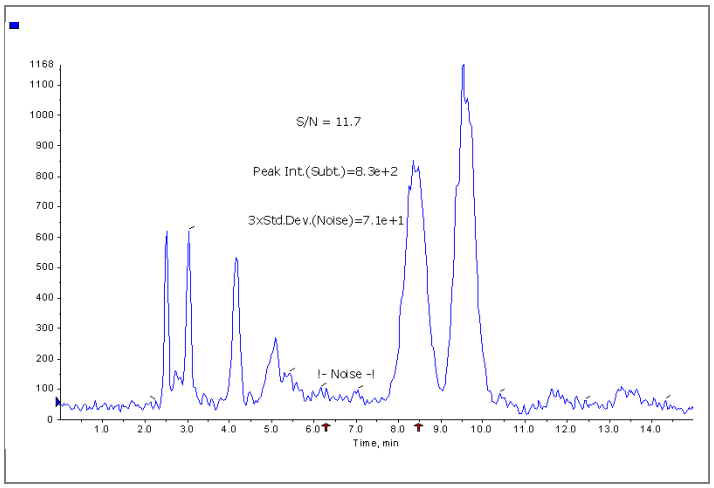


Figure 4. Chromatogram of methamphetamine at LLOQ, 1 ng/mL



Signal to Noise obtained at three standard deviations

Figure 5. Chromatogram of amphetamine at LLOQ, 4 ng/mL

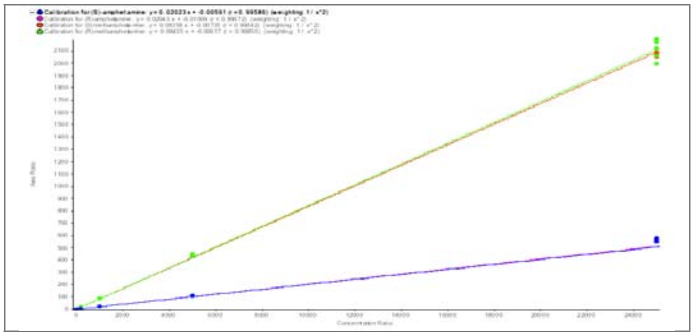


Signal to Noise obtained at three standard deviations

Linearity of the method covered the range from LLOQ to 25000 ng/mL. Precision and accuracy were typically better than 10%. Calibration curves demonstrating the precision, accuracy and linearity for each analyte are shown in Figure 6. Figure 11 shows the linearity statistics over three batches with a minimum correlation coefficient (r) of 0.9953 and coefficient of determination (r^2) of 0.9906 and a maximum r value of 0.9988 and r^2 of 0.9978. The cutoff value of 20 ng/mL is typically used and with LLOQ of ≤ 4 ng/mL, this method has more than sufficient sensitivity. Both intra-day and inter-day accuracy and precision of the assay were determined and are summarized in Figures 7, 8, 9 and 10. All intra-day and inter-day % accuracy and % CVs for all calibrators and QCs were below 15 %.

Recoveries from urine using the SPE method were evaluated and determined to be >95% for all compounds. Matrix effects were evaluated at 20 ng/mL using three different lots of urine. Average % accuracy differences obtained for all four compounds from lot A, B and C were 4.3 %, 24.3 % and -3.3 % respectively. Interferences from over-the-counter-drugs on a 20 ng/mL racemic sample of amphetamine and methamphetamine were also evaluated. The drugs investigated in the study ranged from 50 to 625 µg/mL and included those shown in Table 2. Of the 20 compounds listed (including enantiomers) only phenylephrine and norephedrine showed any interference with the analysis of either amphetamine or methamphetamine enantiomers. Phenylephrine present at 10 µg/mL, leads to a 10 times overestimation of the (S)-methamphetamine concentration present (219 ng/mL *versus* 20 ng/mL). Norephedrine present at 100 µg/mL leads to ~3.5 times overestimation of the (S)-amphetamine concentration present.

Figure 6. Calibration curves for amphetamine and methamphetamine



Precision (%CV) and accuracy were typically within 5% and 10%, respectively, across the analytical range.

Figure 7. Inter-day and intra-day accuracy and precision for analysis of amphetamine calibrators

(S)-Amphetamine Standards (ng/mL)

Intra-Day	Accuracy	Precision
MIN	88.20	1.13
MAX	110.63	10.43

Inter-Day	Accuracy	Precision
MIN	89.96	2.74
MAX	108.92	7.94

		C	D	E	F	G	H	I
		4.00	8.00	40.0	200	1000	5000	25000
Batch #1	Mean, N=5	4.23	7.24	36.68	192.1	992.51	5311.03	27657
	%CV	3.32	4.85	4.41	2.25	1.45	1.48	2.01
	Accuracy	105.67	90.47	91.71	96.05	99.25	106.22	110.63
Batch #2	Mean, N=5	4.17	7.49	35.28	207.97	985.51	5052.72	27187.74
	%CV	6.76	10.43	1.23	7.37	1.54	1.13	3.5
	Accuracy	104.28	93.59	88.2	103.98	98.55	101.05	108.75
Batch #3	Mean, N=5	4.01	N/A	N/A	185.29	931.65	5332.09	26845.76
	%CV	5.59	N/A	N/A	1.28	1.35	1.84	2.13
	Accuracy	100.17	N/A	N/A	92.64	93.16	106.64	107.38
Inter-Day	Mean, N=15	4.13	7.36	35.98	196.63	969.89	5231.94	27230.17
	%CV	5.55	7.94	3.72	7.14	3.20	2.88	2.74
	Accuracy	103.37	92.04	89.96	98.31	96.99	104.64	108.92

*N=10

(R)-Amphetamine Standards (ng/mL)

Intra-Day	Accuracy	Precision
MIN	88.71	0.62
MAX	109.76	6.78

Inter-Day	Accuracy	Precision
MIN	91.17	2.18
MAX	109.30	6.22

		C	D	E	F	G	H	I
		4.00	8.00	40.0	200	1000	5000	25000
Batch #1	Mean, N=5	4.21	7.27	37.45	191.40	991.84	5319.43	27236.92
	%CV	4.47	3.32	1.60	1.05	1.70	1.20	0.62
	Accuracy	105.29	90.85	93.64	95.70	99.18	106.39	108.95
Batch #2	Mean, N=5	4.15	7.57	35.48	202.82	988.74	5115.33	27440.54
	%CV	3.75	4.43	1.25	6.78	1.93	1.53	3.10
	Accuracy	103.80	94.58	88.71	101.41	98.87	102.31	109.76
Batch #3	Mean, N=5	4.01	N/A	N/A	183.23	929.58	5302.88	27295.45
	%CV	3.34	N/A	N/A	1.17	0.68	1.12	2.65
	Accuracy	100.19	N/A	N/A	91.61	92.96	106.06	109.18
Inter-Day	Mean, N=15	4.12	7.42	36.47	193.70	970.05	5245.88	27324.30
	%CV	4.22	4.30	3.16	6.22	3.38	2.18	2.23
	Accuracy	103.12	92.71	91.17	96.85	97.01	104.92	109.30

*N=10

% Accuracy and % CV are shown for differing concentration amphetamine calibrators

Figure 8. Inter-day and intra-day accuracy and precision for analysis of methamphetamine calibrators

(S)-Methamphetamine Standards (ng/mL)

Intra-Day	Accuracy	Precision
MIN	91.61	0.74
MAX	108.58	12.53

Inter-Day	Accuracy	Precision
MIN	93.14	2.23
MAX	104.01	7.59

		A	B	C	D	E	F	G	H	I
		1.00	2.00	4.00	8.00	40.00	200.00	1000.00	5000.00	25000.00
Batch #1	Mean, N=5	1.04	1.94	3.84	7.33	39.67	206.73	1056.95	5216.03	24845.22
	%CV	1.35	3.90	2.52	1.73	1.32	1.68	1.10	0.78	0.74
	Accuracy	103.59	96.96	95.90	91.61	99.18	103.36	105.69	104.32	99.38
Batch #2	Mean, N=5	1.03	1.95	3.79	7.57	39.88	217.16	1009.59	5000.94	24335.09
	%CV	11.24	3.63	3.02	1.59	3.03	1.66	1.05	0.96	2.40
	Accuracy	103.27	97.33	94.73	94.63	99.71	108.58	100.96	100.02	97.34
Batch #3	Mean, N=5	1.00*	2.01	3.94	N/A	N/A	196.49	986.41	5303.76	24455.73
	%CV	9.69	12.53	7.13	N/A	N/A	0.80	1.26	2.71	3.15
	Accuracy	100.23	100.55	98.57	N/A	N/A	98.25	98.64	106.08	97.82
Inter-Day	Mean, N=15	1.03	1.97	3.86	7.45	39.78	208.01	1017.65	5173.58	24545.35
	%CV	7.59	7.56	4.75	2.32	2.23	4.49	3.17	3.02	2.33
	Accuracy	102.83	98.27	96.40	93.14	99.44	104.01	101.77	103.47	98.18
		*N=2, **N=12			*N=10					

(R)-Methamphetamine Standards (ng/mL)

Intra-Day	Accuracy	Precision
MIN	91.92	0.76
MAX	108.93	5.69

Inter-Day	Accuracy	Precision
MIN	93.29	1.54
MAX	104.21	4.40

		A	B	C	D	E	F	G	H	I
		1.00	2.00	4.00	8.00	40.00	200.00	1000.00	5000.00	25000.00
Batch #1	Mean, N=5	1.03	1.95	3.87	7.35	39.28	204.84	1052.36	5251.72	24976.46
	%CV	2.78	2.08	1.98	2.52	1.17	1.26	1.02	1.15	3.88
	Accuracy	103.16	97.39	96.74	91.92	98.20	102.42	105.24	105.03	99.91
Batch #2	Mean, N=5	1.05	1.88	3.75	7.57	39.59	217.86	1017.00	4985.53	24851.16
	%CV	5.69	3.03	1.29	1.28	1.89	2.34	1.52	1.82	2.39
	Accuracy	105.07	94.23	93.77	94.64	98.97	108.93	101.70	99.71	99.40
Batch #3	Mean, N=5	1.03*	2.00	3.80	N/A	N/A	198.78	999.39	5272.40	24737.89
	%CV	2.20	4.86	4.47	N/A	N/A	0.76	1.42	1.71	2.88
	Accuracy	103.08	100.04	95.00	N/A	N/A	99.39	99.94	105.45	98.95
Inter-Day	Mean, N=15	1.04	1.94	3.81	7.46	39.44	208.42	1022.91	5169.88	24855.17
	%CV	3.86	4.19	3.01	2.41	1.54	4.40	2.55	3.00	2.91
	Accuracy	103.92	97.23	95.18	93.29	98.59	104.21	102.29	103.40	99.42
		*N=2, **N=12			*N=10					

% Accuracy and % CV are shown for differing concentration amphetamine calibrators

Figure 9. Inter-day and intra-day accuracy and precision for analysis of amphetamine QCs

(S)-Amphetamine QCs (ng/mL)

Intra-Day	Accuracy	Precision
MIN	90.50	1.32
MAX	110.00	11.81

Inter-Day	Accuracy	Precision
MIN	98.67	2.16
MAX	105.73	9.44

		QC-L	QC-M	QC-H
		6.00	20.0	15000
Batch #1	Mean, N=6	5.72	18.80	16161.87
	%CV	2.04	3.20	1.32
	Accuracy	95.41	93.99	107.75
Batch #2	Mean, N=6	6.60	19.39	15777.28
	%CV	2.81	3.45	1.66
	Accuracy	110.00	96.95	105.18
Batch #3	Mean, N=6	5.43	21.24	15640.69
	%CV	6.45	11.81	2.13
	Accuracy	90.50	106.20	104.27
Inter-Day	Mean, N=18	5.92	19.81	15859.95
	%CV	9.44	9.08	2.16
	Accuracy	98.67	99.04	105.73

(R)-Amphetamine QCs (ng/mL)

Intra-Day	Accuracy	Precision
MIN	93.46	1.66
MAX	107.97	12.19

Inter-Day	Accuracy	Precision
MIN	94.64	2.71
MAX	107.15	9.02

		QC-L	QC-M	QC-H
		6.00	20.0	15000
Batch #1	Mean, N=6	5.86	18.69	16195.71
	%CV	2.60	3.13	1.67
	Accuracy	97.74	93.46	107.97
Batch #2	Mean, N=6	6.27	19.42	15900.73
	%CV	2.67	2.14	1.66
	Accuracy	104.50	97.10	106.00
Batch #3	Mean, N=6	5.76	21.10	15530.22
	%CV	4.40	12.19	3.16
	Accuracy	95.93	105.50	103.53
Inter-Day	Mean, N=18	5.68	19.62	16073.05
	%CV	5.93	9.02	2.71
	Accuracy	94.64	98.12	107.15

% Accuracy and % CV are shown for differing concentration amphetamine calibrators

Figure 11. Linearity statistics

(S)-Amphetamine	r	Slope	Intercept
Batch #1	0.9959	0.0202	-5.91E-03
Batch #2	0.9953	0.0201	-1.16E-02
Batch #3	0.9971	0.0205	5.09E-03
%CV		1.05	

(S)-Methamphetamine	r	Slope	Intercept
Batch #1	0.9984	0.0836	-7.35E-03
Batch #2	0.9975	0.0826	-1.21E-02
Batch #3	0.9973	0.0849	8.71E-04
%CV		1.33	

(R)-Amphetamine	r	Slope	Intercept
Batch #1	0.9967	0.0204	-1.01E-02
Batch #2	0.9965	0.0204	-1.31E-02
Batch #3	0.9967	0.0211	-4.41E-03
%CV		1.88	

(R)-Methamphetamine	r	Slope	Intercept
Batch #1	0.9985	0.0844	-6.17E-03
Batch #2	0.9978	0.0837	-2.76E-03
Batch #3	0.9988	0.0852	2.95E-02
%CV		0.90	

Figure 10. Inter-day and intra-day accuracy and precision for analysis of methamphetamine QCs

Intra-Day	Accuracy	Precision
MIN	93.10	1.47
MAX	108.81	13.24

Inter-Day	Accuracy	Precision
MIN	95.84	3.30
MAX	102.16	9.15

Intra-Day	Accuracy	Precision
MIN	96.23	1.33
MAX	111.13	12.53

Inter-Day	Accuracy	Precision
MIN	98.65	3.09
MAX	103.64	9.24

(S)-Methamphetamine QCs (ng/mL)		QC-L 6.00	QC-M 20.0	QC-H 15000
Batch #1	Mean, N=6	5.59	19.67	14985.31
	%CV	1.47	1.85	3.49
	Accuracy	93.10	98.34	99.90
Batch #2	Mean, N=6	5.90	19.86	14666.68
	%CV	5.00	2.42	1.59
	Accuracy	98.36	99.32	97.78
Batch #3	Mean, N=6	5.76	21.76	14597.92
	%CV	13.13	13.24	4.19
	Accuracy	96.05	108.81	97.32
Inter-Day	Mean, N=18	5.75	20.43	14749.97
	%CV	8.05	9.15	3.30
	Accuracy	95.84	102.16	98.33

(R)-Methamphetamine QCs (ng/mL)		QC-L 6.00	QC-M 20.0	QC-H 15000
Batch #1	Mean, N=6	5.77	19.60	15019.08
	%CV	1.82	1.59	2.25
	Accuracy	96.23	98.02	100.13
Batch #2	Mean, N=6	6.12	20.33	14470.07
	%CV	2.69	1.33	1.66
	Accuracy	102.07	101.63	96.47
Batch #3	Mean, N=6	5.87	22.23	14952.38
	%CV	4.25	12.53	3.72
	Accuracy	97.89	111.13	99.68
Inter-Day	Mean, N=18	5.92	20.73	14826.66
	%CV	3.89	9.24	3.09
	Accuracy	98.65	103.64	98.84

% Accuracy and % CV are shown for differing concentration amphetamine calibrators

Table 2. Compounds used in the evaluation of the over-the - counter drug interferences

	Concentration (µg/mL)
(-)-pseudoephedrine	125
(+)-pseudoephedrine	*125*
(-)-ephedrine	*100*
acetaminophen	*10*
aspirin	*5*
(±)-chlorpheniramine	*5*
caffeine	*5*
diphenhydramine	*5*
dextromethorphan	*5*
ibuprofen	*5*
(±)-MDA	*10*
(±)-MDMA	*10*
(±)-MDEA	*10*
phentermine	*10*
phenylephrine	*10*
norephedrine	*100*

Summary

An LC-MS/MS method has been developed that allows the separation of racemic amphetamine and methamphetamine to allow the accurate quantification of the individual enantiomeric forms. The linear quantifiable range is from ≤4 ng/mL to 25 000 ng/mL. Precision and accuracy were better than 5 % for most concentrations and better than 15 % for all concentration levels.

References

Edeleano, L. (1887). *Chem. Ber.* 20:616-622.

Publication number: 7400113-01, Document number: RUO-MKT-02-0403-A

AB SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
www.absciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
www.absciex.com/offices