Research article

Biomedical Chromatography

Received 6 August 2010, Accepted 23 August 2010 Published online in Wiley Online Library: 06 November 2010

(wileyonlinelibrary.com) DOI 10.1002/bmc.1530

Basic drug analysis by strong cation-exchange liquid chromatography–tandem mass spectrometry: simultaneous analysis of amisulpride, and of metamfetamine and amfetamine in serum/plasma

L. Couchman,* P. E. Morgan and R. J. Flanagan

ABSTRACT: In the HPLC of basic drugs and metabolites, good efficiency and peak shape can often be attained using strong cation-exchange packings with isocratic 100% methanol eluents containing an ionic modifier at an appropriate pH* and ionic strength. Solvent extracts can be analysed directly, and use of ammonium acetate as modifier facilitates the use of atmospheric pressure chemical ionization (APCI)–tandem mass spectrometry, selected reaction monitoring mode. For the analysis of amisulpride and of metamfetamine/amfetamine in plasma (200 μL) after single oral doses in man, a column packed with Waters Spherisorb S5SCX (5 μm average particle size, 100 × 2.1 mm i.d.) was used with methanolic ammonium acetate (40 mmol/L, pH* 6.0, flow rate 0.5 mL/min) as eluent (35°C). Deuterated internal standards were used for each analyte. Detection was by positive-mode APCI. Responses for all analytes were linear over the calibration ranges. Intra-assay precision (RSD) was 2–18%, and inter-assay precision was 2–12%. The limit of detection was 0.5 μg/L for all analytes. No significant matrix effects or isobaric interferences were noted. The total analysis time was 7 min. Similar methodology can be applied to a wide range of basic analytes using MS/MS detection. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: HPLC-MS/MS basic drugs; HPLC-MS/MS amfetamine/metamfetamine; HPLC-MS/MS amisulpride

Introduction

Propylsulfonic acid-modified silica (strong cation exchange, SCX) HPLC packings give excellent selectivity and peak shapes in the analysis of many basic drugs and their *N*-dealkylated metabolites using non-aqueous (100% methanol) ionic eluents at appropriate apparent pH (pH*) and ionic strength (Croes *et al*., 1995; Morgan *et al*., 2010). Such systems allow the direct injection of organic solvent sample extracts, and have been applied successfully to the routine achiral analysis of a number of basic drugs and their metabolites in human serum/plasma by HPLC-UV (Flanagan *et al*., 2001).

With UV detection, ammonium perchlorate or ammonium nitrate provide useful ionic modifiers, having adequate solubility in methanol and minimal absorption at lower wavelengths. In order to move to mass spectrometric (MS) detection, however, an alternative modifier had to be considered. Ammonium acetate (40 mmol/L, pH* 6.0) was found to give very similar results to the modifiers used with UV detection, but had the advantage of compatibility with atmospheric pressure chemical ionization (APCI)–tandem mass spectrometric (MS/MS) detection. In order to illustrate the application of this technique, the analysis of amisulpride, and of metamfetamine and its metabolite amfetamine, in plasma after single dose administration to man (Murray *et al*., unpublished) is described. Limits of accurate measurement of 0.5 μg/L were achieved routinely using 200 μL of sample.

Materials and methods

Chemicals and reagents

Amfetamine, metamfetamine, amfetamine-D_5, metamfetamine-D_{11} and amisulpride-D_5 (certified reference solutions in methanol, all >99% purity) were from LGC Standards (Teddington, UK). Amisulpride (98%), aqueous sodium hydroxide solution (4 mol/L), and 1-butanol (HPLC grade) were from Sigma Aldrich (Poole, UK). Ammonium acetate (MS grade) was from Fluka (Poole, UK), and methanol (HPLC grade) and butyl acetate (glass distilled) were from Rathburn (Walkerburn, UK). NIST-traceable aqueous pH reference buffer solutions were from Merck (Beeston, UK), and were used to calibrate a combination pH electrode (Liq-Glass,

* Correspondence to: L. Couchman, Toxicology Unit, Department of Clinical Biochemistry, Third Floor, Bessemer Wing, King's College Hospital, Denmark Hill, London SE5 9RS, UK. E-mail: lewis.couchman@nhs.net

Toxicology Unit, Department of Clinical Biochemistry, King's College Hospital NHS Foundation Trust, London SE5 9RS, UK

Abbreviations used: APCI, atmospheric pressure chemical ionization; EQA, external quality assurance; EQC, external quality control; IQC, internal quality control; SCX, strong cation exchange; SRM, selected reaction monitoring.

Hamilton). Coarsely filtered, human plasma (pooled, and from individual donors) was from Sera Laboratories International (Haywards Heath, UK). Hydrochloric and acetic acids (both AnalaR grade) were from BDH (Poole, UK). Water was deionized (18.2 MΩ, Elga PureLab, Marlow, UK).

An internal standard solution containing amfetamine-D_5, metamfetamine-D_{11} and amisulpride-D_5 (each 0.5 mg/L) was prepared by appropriate dilution of stock solutions of amphetamine-D_5 (100 mg/L free-base in methanol), metamfetamine-D_{11} (100 mg/L free-base in methanol) and amisulpride-D_5 (100 mg/L in 0.1 mol/L hydrochloric acid) with deionized water and stored at 2–8°C.

The HPLC eluent was prepared by diluting an ammonium acetate stock solution (100 mmol/L in methanol) to 40 mmol/L with methanol, vacuum-filtration (0.45 µm, nylon 66; Phenomenex, Macclesfield, UK) and adjusting to pH* 6.0 with glacial acetic acid.

Apparatus

Glass test tubes (60 × 7 mm, Dreyer tubes) were from Esslab (Benfleet, UK). Sample/calibration solution/internal quality control (IQC) solutions were prepared using variable volume air-displacement pipettes (BioHit, Finland), and internal standard added using a repeating volume dispensing pipette (Multipette®, Hamilton). Extended fine-tipped pastettes (Alpha Laboratories, UK) were used to transfer sample extracts into 0.5 mL polypropylene micro tubes with lids (Sarstedt, UK). Other equipment included vortex mixers (VWR, UK), 2 mL polypropylene screw-top tubes (Alpha Laboratories, UK) and a micro-centrifuge (Mikro 200, Hettich). Weighings were performed using a five-place analytical balance (Genius ME225D, Sartorius).

Calibration and internal quality control solutions

Methanolic stock solutions containing metamfetamine (1000 mg/L free base), amfetamine (100 mg/L free base) and amisulpride (500 mg/L) were diluted with methanol to give working solutions A (10 mg/L each analyte) and B (1 mg/L each analyte). Separate stock and working solutions were used for calibration and IQC preparation.

Appropriate volumes of working solution were evaporated to dryness under a gentle stream of air, and reconstituted with analyte-free pooled human plasma to prepare calibration solutions ($n = 7$) over the following concentration ranges: amfetamine 0.5–50 µg/L; metamfetamine 1–400 µg/L; and amisulpride 1–400 µg/L. IQC solutions ($n = 3$) were similarly prepared at 1.5, 8 and 30 µg/L for amfetamine, and 4, 20 and 150 µg/L for amisulpride and for metamfetamine. After thorough mixing and equilibration (24 h, 2–8°C), calibration and IQC solutions were stored in *ca* 1 mL portions in 2 mL polypropylene screw-top tubes at −20°C until required.

External quality cntrol/quality assurance

For amfetamine and metamfetamine, external quality control (EQC) material in human plasma/serum was not available. Instead, urine EQC materials (Biorad Liquichek® Urine Toxicology C2 and C4, 375 and 1500 µg/L both analytes, respectively) were diluted in analyte-free pooled human plasma (final concentrations: 3 and 45 µg/L both analytes for levels C2 and C4, respectively) and included within each analysis batch. For amisulpride, serum samples from the UKNEQAS Heathcontrol Psychoactives external quality assessment (EQA) scheme (Cardiff Bioanalytical Services, UK) were assayed as part of the method validation procedure.

Sample preparation

Sample, calibration or IQC solution (200 µL), internal standard solution (25 µL) and 4 mol/L sodium hydroxide solution (50 µL) were vortex-mixed (5 s) in a Dreyer tube. After 5 min, extraction solvent (butyl acetate : butanol, 9 + 1; 100 µL) was added and the tube contents vortex-mixed (30 s) and centrifuged (14,000***g***, 4 min). A portion (>40 µL) of the organic (upper) layer was transferred to an autosampler vial and capped prior to analysis. Study samples were analysed in triplicate. Samples that were found to contain analyte concentrations above a calibration range were diluted as appropriate in analyte-free human plasma and reanalysed.

HPLC-MS/MS

The HPLC system consisted of an autosampler (3059AS), pump (3185PU) and column oven (CO2067; all Jasco, Great Dunmow, UK). The stainless steel HPLC column (100 × 2.1 mm i.d) was packed with Waters Spherisorb S5SCX, and protected by a guard column (10 × 2.1 mm i.d) packed with the same material (HiChrom, Reading, UK). The column oven temperature was maintained at 35°C, and the autosampler tray at 5°C. The eluent flow-rate was 0.5 mL/min, and the injection volume was 20 µL.

MS/MS (Thermo Quantum Access TSQ, ThermoFisher Scientific, Hemel Hempstead, UK) was carried out in positive ionization mode using APCI [needle discharge current 4 µA; temperatures: vaporizer 391°C; capillary 207°C; auxiliary, sheath and sweep gases 10, 10 and 0 (arbitrary units) respectively]. Data were collected in high-resolution (0.40 FWHM), selected reaction monitoring (SRM) mode, with two *m/z* transitions per analyte. Instrument control and data acquisition and processing were performed using Xcalibur (version 2.0, ThermoFisher Scientific). Analyte-specific MS parameters are detailed in Table 1. The first 0.75 mL of eluent from each injection was diverted to waste.

Method validation

The peak area ratio of analyte to internal standard was plotted against analyte concentration to produce a calibration curve, and a line fitted by least squares regression. The line was weighted 1/concentration. Calibration and IQC solutions were assayed at the beginning and end of each analytical sequence, with all IQCs repeated after every 10 injections. Assay acceptance criteria were (i) correlation coefficient (R^2) > 0.98 for each analyte, and (ii) IQC and if appropriate EQC values within ±15% of their nominal values. Intra- and inter-assay precision (% RSD) and accuracy were measured by replicate analysis ($n = 10$) of the IQC solutions on the same day, and duplicate analyses (mean of duplicates) on different days ($n = 10$), respectively. The stability of the analytes in plasma was evaluated by analysis of IQC samples: (i) through three freeze–thaw cycles, the assay being calibrated each time with standard solutions that had not been thawed previously; and (ii) before and after standing for 24 h at room temperature. Analyte recovery was investigated by comparison of the mean peak area of each analyte from extracted IQC solutions ($n = 6$ at each concentration) with the mean peak area obtained from a non-extracted solution at the equivalent concentration in methanol ($n = 6$ at each concentration).

Table 1. Retention time data and SRM parameters for amfetamine, metamfetamine, amisulpride and their deuterated analogues, and for a range of other basic drugs and metabolites

Analyte	Retention time (min)	Precursor ion (*m/z*)	Fragment ions (*m/z*)	Collision energy (V)	Tube lens voltage (V)
Quetiapine	1.56	384	253	20	139
			221	36	
Aripiprazole	1.92	448	285	27	161
			176	34	
Dehydroaripiprazole	2.12	446	285	22	184
			98	41	
Phentermine	2.16	150	91	20	70
			65	37	
Amfetamine	2.18	136	91	17	82
			65	37	
Amfetamine-D_5	2.19	141	93	18	92
			92	16	
Methylenedioxyamfetamine	2.31	180	135	16	72
			77	42	
Clozapine	2.32	327	270	23	111
			192	44	
9-Hydroxy risperidone	2.44	427	207	27	137
			110	44	
Mephedrone	2.45	178	145	20	75
			91	36	
Cathinone	2.62	150	117	22	70
			105	19	
Ephedrine (and pseudoephedrine)	2.62	166	115	27	72
			91	33	
Methylenedioxyethylamfetamine	2.65	208	105	17	76
			77	42	
Metamfetamine	2.76	150	91	20	82
			65	40	
Risperidone	2.76	411	191	28	125
			110	47	
Metamfetamine-D_{11}	2.78	161	97	19	92
			96	26	
Norephedrine (and norpseudoephedrine)	2.85	152	115	22	72
			91	31	
Methylenedioxymetamfetamine	2.98	194	135	17	74
			77	37	
Norclozapine	3.01	313	270	23	111
			192	37	
Olanzapine	4.94	313	256	22	108
			198	39	
Sulpiride	5.42	342	214	34	108
			112	27	
Amisulpride	5.52	370	242	27	101
			196	40	
Amisulpride-D_5	5.54	375	242	28	111
			196	40	

Biomed. Chromatogr. 2011; **25**: 867–872

wileyonlinelibrary.com/journal/bmc

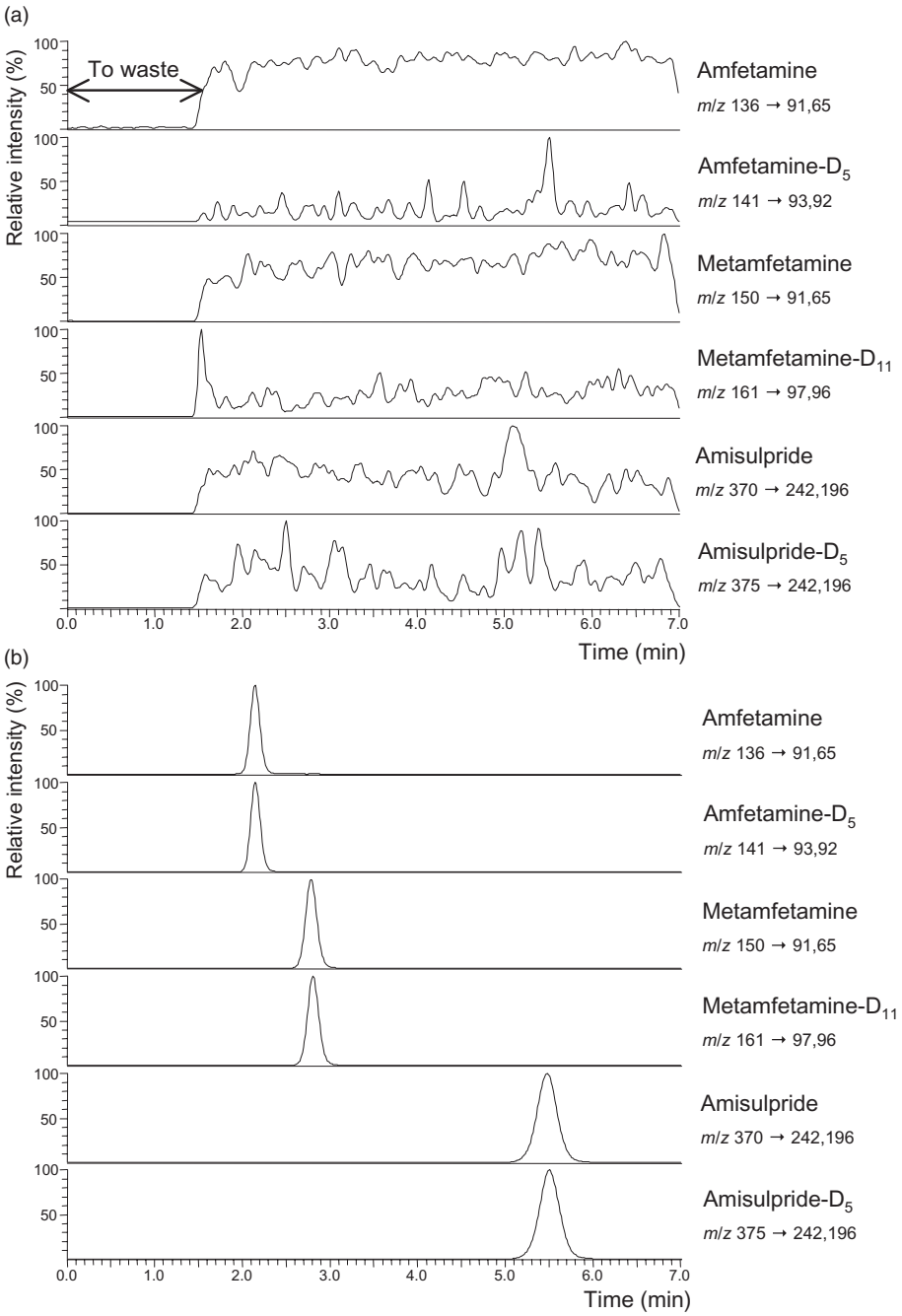

Figure 1. Representative chromatograms: (a) extracted blank plasma; (b) an extracted IQC sample (amfetamine, 30 µg/L; metamfetamine and amisulpride, both 150 µg/L); and (c) an extract of a plasma sample (concentrations, amisulpride 261 µg/L, amfetamine 2.5 µg/L, metamfetamine 61 µg/L).

To investigate potential ion suppression, analyte-free plasma from six independent sources was analysed without addition of internal standard solution. The detector response for each analyte transition was monitored whilst a solution containing all analytes was infused post-column (Bonfiglio *et al.*, 1999).

Results

Typical chromatograms are shown in Fig. 1. The response was linear over the calibration ranges used. Intra- and inter-assay precision and accuracy data are summarized in Table 2. The limit of accurate measurement, taken as a peak at least 5 times the average height of the standard deviation of the baseline noise, was 0.5 µg/L for each analyte (200 µL sample). Recoveries of amfetamine, metamfetamine and amisulpride from human plasma were 52.0 ± 0.4, 50.0 ± 1.2 and 66.0 ± 1.7%, respectively. No significant matrix effects were observed.

EQA samples for amisulpride ($n = 5$) were assayed in duplicate, and the results showed accuracy in the range 82–94% compared with consensus mean. Samples not containing amisulpride ($n = 2$) were correctly identified as such. EQC samples for amfetamine and metamfetamine (diluted C2 and C4) were within ±15%

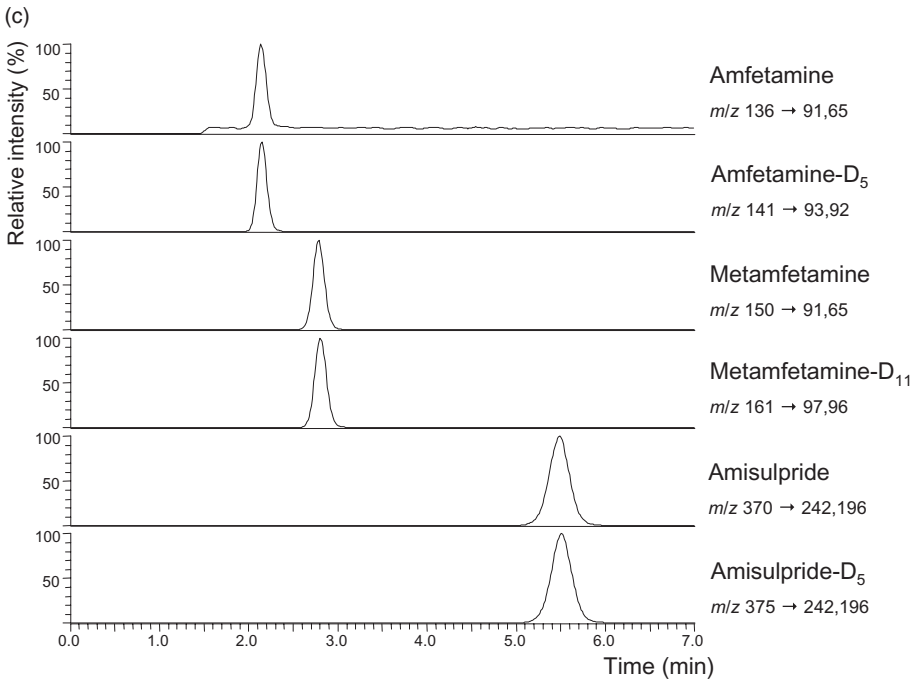

Figure 1. *Continued.*

Table 2. Intra- and inter-assay accuracy and precision

		Nominal concentration (µg/L)	Mean of measured concentrations (µg/L)	RSD (%)	Accuracy (%)
Intra-assay (n = 10 each concentration)					
Low	Amfetamine	1.5	1.7	18.3	110
	Metamfetamine	4.0	4.6	5.8	116
	Amisulpride	4.0	4.7	12.3	117
Medium	Amfetamine	8.0	8.5	3.2	107
	Metamfetamine	20	22	3.3	112
	Amisulpride	20	20	3.1	98
High	Amfetamine	30	32	3.6	107
	Metamfetamine	150	171	3.2	114
	Amisulpride	150	152	2.1	101
Inter-assay (n = 6 each concentration)					
Low	Amfetamine	1.5	1.7	6.5	112
	Metamfetamine	4.0	5.0	9.0	123
	Amisulpride	4.0	4.0	11.7	109
Medium	Amfetamine	8.0	8.0	3.4	104
	Metamfetamine	20	22	2.3	111
	Amisulpride	20	20	2.2	101
High	Amfetamine	30	34	6.0	112
	Metamfetamine	150	175	3.4	116
	Amisulpride	150	152	2.0	101

(RSD < 10% from all batch analyses, $n = 20$) of the nominal concentrations for both analytes.

Analysis of freshly prepared EQC solutions, diluted with plasma as described above, using calibration solutions prepared 6 months previously, gave the following results [found (nominal) concentration, µg/L]: amfetamine 2.4 (3.0); 47.4 (45.0); metamfetamine 2.9 (3.0); 46.5 (45.0). Data on the stability of amisulpride in human plasma have been reported (Péhourcq *et al*., 2003).

Potential interference from other phenethylamines, including methylenedioxyamfetamine, methylenedioxymetamfetamine ('ecstasy'), methylenedioxyethylamfetamine, ephedrine/pseudoephedrine, norephedrine/norpseudoephedrine, mephedrone and cathinone was excluded by analysis of methanolic solutions (10 mg/L) of each compound. SRM transition and retention time data for these compounds and for some other basic drugs and metabolites are given in Table 1. Note that cathinone

and phentermine are isobaric with metamfetamine, but cathinone has unique fragment ions, and both compounds were resolved from metamfetamine. No interferences were encountered in the study samples analysed (n = 107 from 18 subjects).

Discussion

The use of SCX-modified HPLC packings with a 100% methanol eluent offers a number of advantages compared with traditional reversed-phase HPLC, including selectivity for protonated analytes and direct injection of organic extracts. Firstly elution is isocratic. Secondly, high methanol content eluents are ideal for use with MS/MS detection, due to more efficient in-source desolvation when compared with aqueous eluents (Kostiainen and Kauppila, 2009). When combined with SCX-HPLC and MS/MS, selectivity and sensitivity for basic drugs and metabolites is enhanced. Liquid–liquid extraction of the plasma/serum samples reduces the potential for interference from endogenous compounds and is simple, amenable to batch analysis, and is relatively inexpensive (Flanagan *et al*., 2006). Moreover, MS maintenance requirements are reduced since the extracts analysed are relatively clean, especially given that the initial 0.75 mL of eluent flow were diverted to waste. The absence of an extract evaporation step is an additional advantage when analysis of volatile analytes such as amfetamine is contemplated. The method developed showed no ion suppression or enhancement effects from extracted plasma, and no isobaric interferences were observed.

Conclusions

The method developed was simple, robust and selective. Sensitivity was adequate for the measurement of plasma metamfetamine and amfetamine, and amisulpride, after single doses. With little modification, the method can easily be applied to the analysis of a wide range of basic drugs and metabolites in plasma.

Acknowledgements

The authors wish to thank Dr Graham Murray (Department of Psychiatry, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK) for supplying the study samples used in this work.

References

Bonfiglio R, King RC, Olah TV and Merkle K. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Communications in Mass Spectrometry* 1999; **13**: 1175–1185.

Croes K, McCarthy PT and Flanagan RJ. HPLC of basic drugs and quaternary ammonium compounds on microparticulate strong cation-exchange materials using methanolic or aqueous methanol eluents containing an ionic modifier. *Journal of Chromatography A* 1995; **693**: 289–306.

Flanagan RJ, Harvey EJ and Spencer EP. HPLC of basic drugs on microparticulate strong cation-exchange materials—a review. *Forensic Science International* 2001; **121**: 97–102.

Flanagan RJ, Morgan PE, Spencer EP and Whelpton R. Micro-extraction techniques in analytical toxicology: short review. *Biomedical Chromatography* 2006; **20**: 530–538.

Kostiainen R and Kauppila TJ. Effect of eluent on the ionisation process in liquid chromatography–mass spectrometry. *Journal of Chromatography A* 2009; **1216**: 685–699.

Morgan PE, Manwaring V and Flanagan RJ. HPLC of basic drugs using non-aqueous ionic eluents: evaluation of a 3 µm strong cation-exchange material. *Biomedical Chromatography* 2010; **24**: 318–323.

Péhourcq F, Ouariki S and Bégaud B. Rapid high-performance liquid chromatographic measurement of amisulpride in human plasma: application to manage acute intoxication. *Journal of Chromatography B* 2003; **789**: 101–105.