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A simple high pH liquid chromatography–tandem mass spectrometry method for basic compounds: Application to ephedrines in doping control analysis

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ABSTRACT

Solvent systems for use with LC–MS often result in a compromise between chromatographic performance and mass spectrometric detection, exemplified here by a LC–MS/MS method development for the analysis of ephedrines in doping control. Ephedrines, frequently found in therapeutic and nutritional preparations, are among the most commonly administered doping agents in competitive sport. Improved separation of these hydrophilic, basic compounds, some of which are diastereoisomers, is achieved in reversed-phase LC by the use of a high pH mobile phase in order to suppress analyte ionisation, and thus alter their polarity, resulting in reduced peak tailing and enhanced retention. However, when coupled to an ESI-MS detector, this eluent composition generated a non-linear and poorly reproducible signal. APCI yielded greater stability and reproducibility and is here presented as an ion source for the analysis of basic compounds under conditions that suppress their ionisation. Errors as large as 49.3% were observed with ESI, compared with 15.4% generated using APCI, for pseudoephedrine over the calibration range (25–400 μg/mL) in urine with a simple dilution and injection of samples. These data highlight the importance of suitable MS conditions for stable performance, necessary for accurate quantification, without undue compromise to the LC separation.

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1. Introduction

Ephedra compounds, including norephedrine (phenylpropanolamine, PPA), norpseudoephedrine (cathine), ephedrine, pseudoephedrine and methylephedrine, are misused in competitive sport because of their sympathomimetic effects [1]. Frequently found in pharmaceutical preparations and nutritional supplements, their administration is controlled in competition by the World Anti-Doping Agency (WADA) above threshold concentrations in urine [2]. Threshold concentrations in urine are 5 μg/mL for cathine, 10 μg/mL for ephedrine and methylephedrine and 150 μg/mL for pseudoephedrine, while PPA is unrestricted (see Fig. 1 for chemical structures and aqueous pK_a values) [2]. The recent inclusion of pseudoephedrine in the Prohibited List, previously unrestricted by WADA until 2010, necessitates a new validated confirmation method for identification and quantification.

Doping control analysis comprises an initial screening to eliminate negative samples followed by a confirmation procedure to determine the identity of a suspect sample unequivocally. For threshold substances, quantification is also required. Due to the identical elemental composition of the diastereoisomeric pairs (PPA–cathine and ephedrine–pseudoephedrine), they share the same mass spectra and therefore require chromatographic separation for unambiguous identification and accurate quantification. Previously, this separation has been performed by high performance liquid chromatography coupled to ultraviolet detection (HPLC–UV) [3], or gas chromatography–mass spectrometry (GC–MS) which relies upon a sample pre-concentration liquid–liquid extraction followed by complex derivatisation in order to distinguish PPA from cathine and ephedrine from pseudoephedrine [4]. Recently, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has presented an attractive alternative in doping control analysis, claiming simpler sample preparation with no need for derivatisation, faster analysis times and increased sensitivity [5–9]. These are critical factors in doping control analysis where large numbers of samples and restricted analysis time necessitates fast, high-throughput and sensitive detection tools. Furthermore, the sensitivity of LC–MS/MS permits in some cases longer detection times and reduced sample preparation, such as direct dilution and injection of urine [6,10]. The direct dilution and injection approach demonstrates a simple, effective alternative to liquid–liquid or solid phase extraction procedures, omitting labour and time-intensive derivatisation and circumvents an additional potential source of error.

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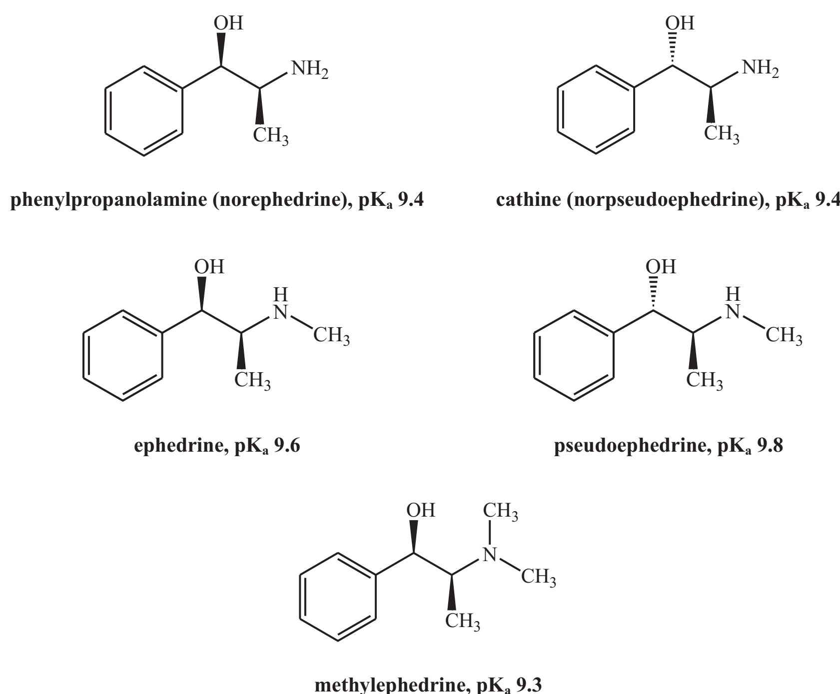

Fig. 1. Chemical structures and pK_a values of the ephedrine compounds considered in this study.

Unfortunately, hydrophilic bases such as ephedrines present inherent challenges for separation by HPLC. Under conventional reversed-phase chromatographic conditions, basic functional groups are ionised and interact undesirably with any residual silanol groups of the stationary phase. The resulting peaks demonstrate poor analyte retention and have large tailing factors, making identification of the peak end difficult, which confounds reliable peak integration and quantification. Previous methods to overcome this problem include the use of acidic mobile phases containing low amounts of organic modifier to retain polar bases or organic additives in order to achieve acceptable peak shapes [11–13]. However, these approaches may suffer disadvantages such as stationary phase dewetting when using highly aqueous mobile phases, causing retention time drifts, or ion suppression from the additives. Organic additives, including trifluoroacetic acid (TFA) and triethylamine (TEA), although frequently employed in LC, are known to cause ion suppression and have a lingering ion-pair effect which is difficult to remove from a LC–MS system and may even require dedicated instrumentation [14].

An alternative approach to separating basic compounds by reversed-phase LC is to use a high pH mobile phase so as to suppress analyte ionisation, and thus polarity, which allows enhanced retention with reduced peak tailing [15–17]. Until recently, the use of high pH mobile phases (above pH 8) has been limited due to the instability of conventional silica based stationary phases. However, the development of chemically stable phases, such as hybrid inorganic/organic materials, facilitate the use of mobile phases of high pH to suppress the ionisation of basic analytes such as ephedrines, with pK_a values ranging from 9.3 to 9.8 (Fig. 1). Improved sample loadability is also demonstrated in reversed-phase chromatography for basic compounds run under pH conditions above their pK_a values [18]. This is an important factor in the current application given the high threshold level of pseudoephedrine which requires an expanded linear dynamic range. This is seen as an additional advantage over low pH separations where column overloading is quickly seen with increasing sample concentration, denoted by characteristic peak tailing and loss of chromatographic efficiency.

However, when coupling LC to MS detection, conditions optimal for chromatographic performance are often not the most favourable for ionisation efficiency and hence a compromise must be made between the two. ESI remains the most popular mode of ionisation for the detection of hydrophilic compounds analysed by LC, even for the detection of basic compounds in high pH eluents [19,20]. Previous studies even report enhanced responses for certain basic compounds when using basic eluents in positive ESI [21,22]. These data do not support the theory of ESI based upon the prior ionisation of analytes in solution and suggests alternative mechanisms of ionisation are dominant, such as gas-phase proton transfer [23]. However, although improvements in sensitivity have been noted, these effects are likely to be compound specific and there is limited knowledge of how pH affects signal stability and repeatability.

This study investigates the use of high pH as an option for the simultaneous quantification of some basic analytes (ephedrines) in diluted urine by LC–MS/MS. Initial experiments showed that the ESI source did not give adequate reproducibility for the quantification of ephedrines in urine over the desired linear dynamic range. Moreover, matrix interferences are a concern, particularly for the direct sample dilution and injection approach adopted herein. Alternative ionisation techniques, such as APCI and APPI, are inherently less affected by interference from co-eluting matrix components compared to ESI. For this reason, here we investigate positive APCI as a comparative mode to determine the most stable and reproducible response for the quantification of basic analytes in diluted urine.

2. Experimental

2.1. Materials

Methanol (HPLC grade), ammonium hydroxide solution (35%) and ammonium bicarbonate were obtained from Fisher Scientific (Loughborough, UK). Formic acid (99–100%) was purchased from VWR (Leicestershire, UK). Ammonium acetate and ammonium formate were from Sigma (Poole, UK). Norephedrine, norpseu-

Table 1
Acquisition segments, SRM transitions and MS parameters, including declustering potential (DP), entrance potential (EP), collision cell exit potential (CXP) and collision energy (CE) for the ephedrines considered in the study. The dwell time of each ion transition was 100 ms.

Compound	Retention window (min)	Ions monitored (m/z)	DP (V)	EP (V)	CXP (V)	CE (V)
Cathine	2.3–2.9	152.1	27	5.0	–	–
		134.1[a]			9	15
		117.2			9	24
		115.2			9	32
Ephedrine	2.9–4.4	166.1	30	6.5	–	–
		148.3[a]			12	16
		133.0			9	30
		117.1			11	26
Pseudoephedrine	2.9–4.4	166.1	30	6.5	–	–
		148.2[a]			12	16
		133.1			9	30
		117.1			11	26
Methylephedrine	4.4–6.5	180.2	35	6.0	–	–
		162.2			14	17
		147.1[a]			12	27
		135.2			10	21
		117.2			10	26
IS (norpehedrine-d_3)	0–2.3	137.2[a]	30	5.0	12	12

[a] Transitions used for quantification.

doephedrine, ephedrine, pseudoephedrine and methylephedrine were purchased as hydrochloride salts from Sigma (Poole, UK) and norephedrine-d_3 (used as internal standard) was purchased as a free base (1 mg/mL in methanol) from LGC Standards (Teddington, UK). Water was purified by an ultra-pure water system (Elga, UK).

2.2. Solutions

2.2.1. Mobile phase

Ammonium formate, ammonium acetate and ammonium bicarbonate were prepared at 5 mM in purified water. Ammonium formate was adjusted to pH 3 with formic acid and ammonium bicarbonate was adjusted to pH 9.8 with ammonium hydroxide solution.

A stock solution of ammonium bicarbonate buffer was prepared at 25 mM in purified water and adjusted to pH 9.8 with ammonium hydroxide solution (35%) for the preparation of the final mobile phase, which consisted of 10 mM ammonium bicarbonate pH 9.8 in water (A) and 10 mM ammonium bicarbonate pH 9.8 in 60% methanol (B). For the preparation of 1 L of mobile phase A, 400 mL of the stock buffer solution was added to 600 mL of water in order to achieve a 10 mM buffer solution. For the preparation of mobile phase B, 200 mL of the stock buffer was added to 300 mL of methanol.

2.2.2. Samples

Stock solutions were prepared at a concentration of 1 mg/mL for norpseudoephedrine, ephedrine and methylephedrine and 10 mg/mL for pseudoephedrine in methanol and stored at −20 °C. A stock solution of norephedrine-d_3, used as an internal standard (IS), was prepared at 10 μg/mL in methanol. Standard working solutions were prepared by diluting stock solutions with water.

A two-step dilution of urine samples was performed before injection: firstly a 45-fold dilution with water was performed, after which samples were vortexed and centrifuged at 76.7 *g*. Aliquots (200 μL) were then mixed with an equal volume of IS solution (norephedrine-d_3, 500 ng/mL diluted with water). Vials were vortexed before being placed in the autosampler.

2.3. LC conditions

Separations were carried out on an Acquity UPLC system (Waters, Milford, MA, USA) with an XBridge 2.5 μm C_{18} 2.1 mm × 50 mm column provided with a 0.2 μm in-line filter. The run time was 6.5 min including re-equilibration time. The mobile phase consisted of 10 mM ammonium bicarbonate pH 9.8 in water (A) and 10 mM ammonium bicarbonate pH 9.8 in 60% methanol (B). The flow rate was 500 μL/min and column temperature set at 45 °C. The weak and strong needle wash lines of the Acquity UPLC system were placed in 90:10 H_2O/CH_3OH 0.2% formic acid and 10:90 H_2O/CH_3OH 0.2% formic acid respectively. The injection volume was 10 μL and was performed in the partial loop with needle overfill mode using a 20 μL sample loop. The gradient conditions started at 16.7% B, increasing to 41.7% over 3.2 min and to 91.7% at 5.2 min, returning to 16.7% for a 1.3 min re-equilibration. Analyst 1.4.2 software from Applied Biosystems was used to control the UPLC.

2.4. Mass spectrometry

Analyte detection was performed using an API 3200 triple quadrupole tandem mass spectrometer (Applied Biosystems) equipped with electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) sources operated in positive ion mode. The MS was operated in selected reaction monitoring (SRM) mode with the inlet conditions optimised for ESI and APCI. The following optimised source conditions were selected for ESI: capillary voltage 5500 V; temperature 450 °C; curtain gas (nitrogen) 10 psi; nebuliser gas (nitrogen) 45 psi and auxiliary gas (nitrogen) 80 psi. The conditions selected for APCI were: nebuliser current 3 μA; temperature 400 °C; curtain gas (nitrogen) 10 psi and auxiliary gas (nitrogen) 45 psi. Data acquisition was divided into four segments based on the expected retention times with a dwell time of 100 ms for each transition giving sufficient data point sampling and sensitivity. Three ion transitions and the precursor ion are all monitored for each analyte in order to satisfy the WADA requirements for identification [24]. Acquisition segments, SRM transitions, dwell times and MS parameters are detailed in Table 1.

2.5. Calibration

A six-point calibration curve including 50–200% of the threshold concentrations was constructed by spiking blank urine at 1, 2.5, 5, 10, 15, 20 μg/mL for cathine, 2.5, 5, 10, 20, 30, 40 μg/mL for ephedrine and methylephedrine and 25, 50, 100, 200, 300, 400 μg/mL for pseudoephedrine. Quality control (QC) samples were prepared at concentrations equal to the WADA threshold levels (cathine 5 μg/mL, ephedrine 10 μg/mL, pseudoephedrine 150 μg/mL and methylephedrine 10 μg/mL).

2.6. Validation

The method was validated for linearity over the dynamic range, selectivity, accuracy, precision, carryover, and ion suppression due to matrix interferences. 10 blank urine samples obtained from different volunteers were analysed as described above to ensure selectivity of the method. Linearity was determined with the six-point calibration and repeatability was assessed by analysing six replicates of a urine sample spiked with each analyte at the threshold concentration. Accuracy and between-assay precision were determined by analysing three replicates of the three different spiked urines on different days.

3. Results and discussion

3.1. Method development

3.1.1. LC optimisation

Mobile phase pH provides a powerful tool in method development for ionisable compounds, although its use has been largely restricted due to the destructive effects on silica-based packing materials. Thus, previous approaches to the analysis of basic analytes have relied upon low pH conditions so as to suppress the ionisation of residual silanol groups or the use of ionic additives and organic modifiers. Unfortunately, such approaches are not without their disadvantages, including reduced retention of hydrophilic bases at low pH and certain mobile phase additives being incompatible with mass spectrometry. However, the chemical stability of hybrid stationary phase materials now facilitates the use of mobile phases of high pH in order to suppress ionisation of basic analytes, without the previously encountered ionic interactions with the packing material, offering distinct advantages for the analysis of basic analytes by reversed-phase LC. Exploitation of pH to render basic compounds in their neutral state results in improved retention, peak shape, and therefore resolution, as demonstrated in this report with the separation of hydrophilic, ionisable ephedrine compounds.

Initial method development investigated the effects of low, mid and high pH mobile phases on peak shape, retention, and hence separation of the ephedrines (phenylpropanolamine, cathine, ephedrine, pseudoephedrine and methylephedrine) in order to determine the best conditions to separate the diastereoisomers. Isocratic elution at 0.4 mL/min was performed in 20% methanol with either ammonium formate (5 mM, pH 3), ammonium acetate (5 mM, pH 7) or ammonium bicarbonate (5 mM, pH 9.8). At pH 3 and 7, retention of the analytes was low, and as such the diastereoisomers co-eluted. For pseudoephedrine, retention times increased from 0.88 and 0.92 min at pH 3 and 7, respectively, to 5.32 min at pH 9.8 (Fig. 2). Suppression of the ionisation of these hydrophilic bases at high pH enhances retention on the reversed-phase column by facilitating hydrophobic interactions between the analyte and C_{18} ligands of the stationary phase. Peak tailing is also greatly improved at pH 9.8 compared to pH 3 or 7. Isocratic elution of pseudoephedrine at pH 3, 7 and 9.8 generated peaks with asymmetry factors of 3.95, 4.75 and 1.96, calculated at 10% peak height. Peak tailing is most severe at pH 7 where both the residual silanol groups of the stationary phase and basic analytes are fully ionised, and thus ionic interactions between the two are greatest. At pH 3, peak tailing is only slightly reduced, since the pH is not low enough to eliminate silanol activity. The benefit of increasing the pH appears at pH 9.8. Even though pH 9.8 is very close to the pK_a of the analytes presented here, this value was shown to provide reproducible retention times and provide an adequate peak shape while preserving the life of the column, which would be shortened at higher pH values, especially with an operating temperature of 45 °C. Although pH 9.8 may not be sufficient to completely eliminate interactions with the sorbent surface, it has been clearly demonstrated to dramatically reduce them, with evident benefit for chromatography. The partial reduction in protonation is sufficient to reduce the degree of ionisation of the analyte, reduce detrimental secondary interactions and notably reduce peak tailing.

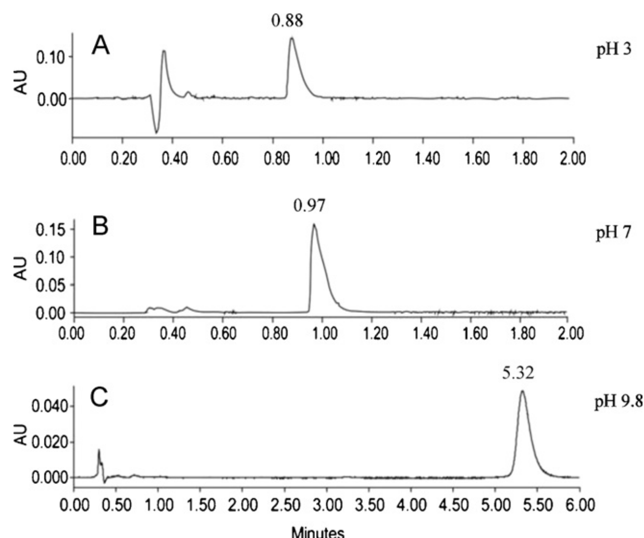

Fig. 2. Effect of pH on retention and peak symmetry for pseudoephedrine (10 μg/mL) using mobile phases at the following pH values: pH 3 (A); pH 7 (B); pH 9.8 (C).

Using a high pH mobile phase also increases sample loadability. Overloading of ionised bases on silica-based phases occurs readily at low pH, a phenomenon that has been widely discussed in the literature [17,18,25–27]. Loading plots for pseudoephedrine at low, mid and high pH illustrate the effects of pH on sample loading capacity (Fig. 3). Increasing the sample mass from 5 to 500 ng demonstrates rapid deterioration of peak shape at pH 3 and 7, whereas at high pH a greater sample mass is tolerated before sample overloading becomes apparent. Reduced protonation, even if not complete, through the use of high pH has previously been documented to improve sample loading capacity, for which several explanations have been proposed. Given the reduced silanol activity of new generation phases, it is likely that reduced analyte ionisation serves to suppress mutual repulsion of protonated species held on the surface of the stationary phase. Alternatively, charged species may be simply not able to fully penetrate the stationary phase, and therefore by reducing analyte protonation the capacity for interaction with the hydrophobic ligands is increased. The ability to tolerate larger sample loading capacities is an additional benefit of operating at high pH with these basic compounds, permitting the simultaneous quantification of high concentrations of pseudoephedrine together with significantly lower levels of the other ephedrines. This is of particular importance considering the different thresholds established by WADA for each specific ephedrine [2]. Since PPA and cathine are commonly present as

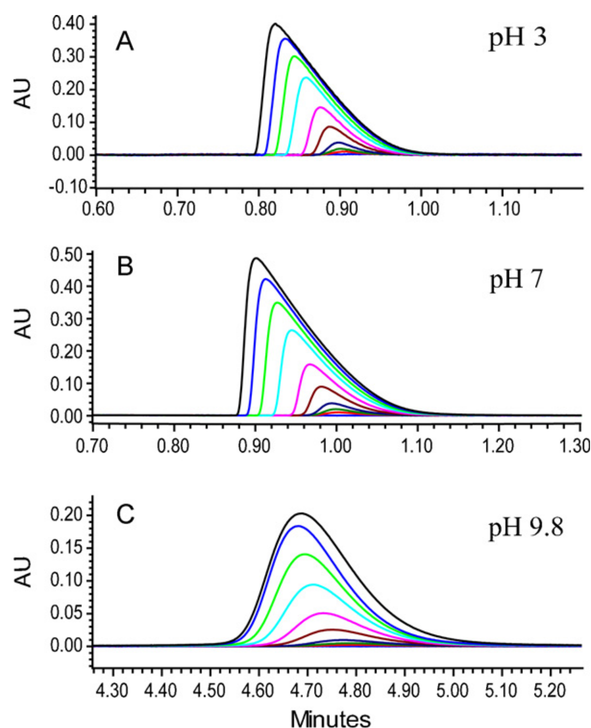

Fig. 3. Overlaid chromatograms of 5–500 ng pseudoephedrine using mobile phases at the following pH values: pH 3.0 (A); pH 7.0 (B); pH 9.8 (C).

metabolites with their respective parent drugs, ephedrine and pseudoephedrine, a large linear range is required for reliable quantification of ephedrine at its threshold level of 10 μg/mL together with its diastereoisomer at the much greater concentration of 150 μg/mL.

These results highlight the importance of mobile phase pH for the analysis of basic compounds, where the best chromatographic performance is achieved under high pH conditions seeking to suppress protonation. Enhanced peak shape and retentivity permit good chromatographic resolution of the diastereoisomers at pH 9.8. Although the stationary phase used is stable up to pH 12, a pH of 9.8 was sufficient to gain the desired separation and was therefore chosen to avoid column damage and preserve column lifetime. An additional important consideration is the effect of the organic modifier on mobile phase pH. Here, the pH of the buffer has been measured before the addition of methanol, which has been reported to increase the pH of aqueous solutions [21]. Thus, it is assumed that although buffered to pH 9.8, with the addition of methanol as an organic modifier the pH of the aqueous ammonium bicarbonate may increase.

Since MS detection is necessary for analyte identification, a compatible eluent composition was carefully selected to avoid undue compromise of the LC separation. Additives and high buffer concentrations, although frequently used to improve chromatographic separations, are incompatible with MS since they often cause ion suppression. Ammonium bicarbonate, at a concentration of 10 mM, was selected for its buffering capacity at high pH and volatility, hence amenable to MS detection. The buffer component was also added to the organic solvent to maintain a uniform concentration throughout the chromatographic gradient.

Gradient conditions, temperature and flow rate were subsequently optimised in order to obtain the best separation within a maximum analysis time of 10 min. The molarity of the ammonium bicarbonate buffer was raised to 10 mM to increase buffering capacity. Fig. 4 illustrates the final chromatographic separation of the four ephedrines of interest at the WADA threshold levels (cathine 5 μg/mL, ephedrine 10 μg/mL, pseudoephedrine 150 μg/mL and methylephedrine 10 μg/mL) under the final conditions.

3.1.2. Mass spectrometry

Although chromatographic optimisation was performed with a MS compatible buffer system, preliminary experiments with positive ESI indicated poor signal stability and repeatability. As a comparative ionisation technique, APCI on the other hand, yields a linear and stable signal for each analyte over the wide calibration range. The high variation in response with ESI compared to APCI is illustrated by overlaid chromatograms from six repeat injections of pseudoephedrine at 150 μg/mL (Fig. 5). Although first experienced with standard solutions without the presence of matrix components, this difference was particularly noticeable for pseudoephedrine in spiked urine, with r^2 values of 0.986 and 0.999 generated with ESI and APCI respectively. The errors between the determined and the actual concentrations were outside of our acceptable limit with ESI, whereas APCI illustrated acceptable errors, with values for pseudoephedrine of 49.3% compared with 15.4% at the lowest calibration point (Table 2). The greater variability with ESI seen with samples in matrix compared to standard

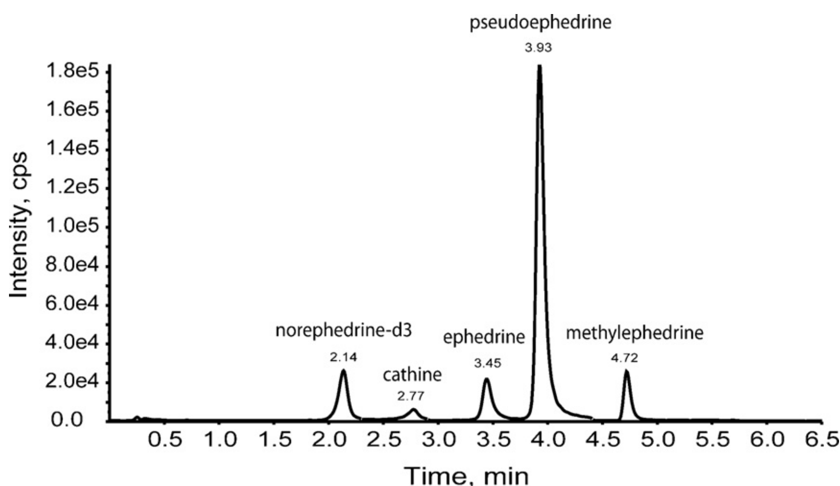

Fig. 4. Total ion chromatogram illustrating the separation of cathine, ephedrine, pseudoephedrine and methylephedrine at the WADA threshold concentrations in spiked urine with the IS norephedrine-d_3 on an XBridge C_{18} 2.5 μm, 2.1 mm × 50 mm column with 10 mM ammonium bicarbonate pH 9.8 in water (A) and 10 mM ammonium bicarbonate pH 9.8 in 60% methanol (B). The gradient conditions started at 16.7% B, increasing to 41.7% over 3.2 min and to 91.7% at 5.2 min, returning to 16.7% for a 1.3 min re-equilibration at 500 μL/min.

Table 2
Comparison of ESI and APCI for the quantification of pseudoephedrine over the range 25–400 μg/mL in urine using the 148 *m/z* ion.

	Conc. (μg/mL)	Slope	Intercept	r^2	Determined conc. (μg/mL)	Error (%)	Average error (%)
ESI	25				12.7	49.3	
	50				45.1	9.82	
	100	0.0132	0.1926	0.9858	112	12.4	15.6
	200				225	12.7	
	300				275	8.19	
	400				404	1.01	
APCI	25				28.8	15.4	
	50				46.7	6.60	
	100	0.0413	−0.4604	0.9992	103	3.29	4.95
	200				195	2.41	
	300				297	1.03	
	400				404	1.02	

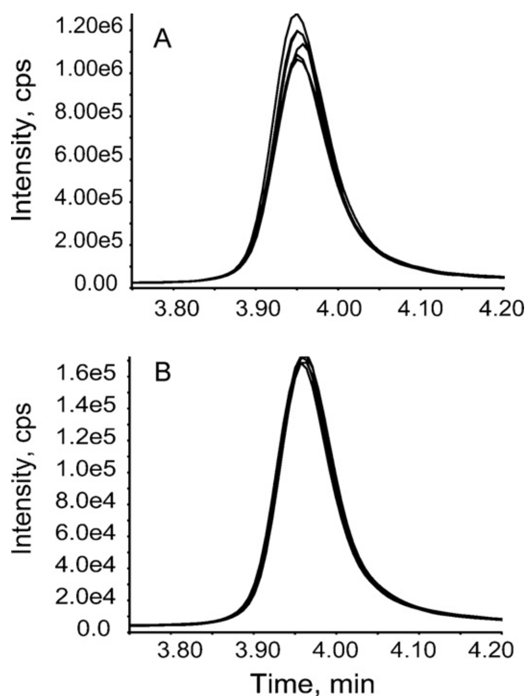

Fig. 5. Overlay of pseudoephedrine peak from six replicate injections of a urine sample spiked at the threshold level (150 μg/mL) using ESI (A) and APCI (B).

solutions suggests some interference from matrix components. Probably the direct dilution and injection approach in this case is not amenable to ESI, whereas APCI offers an alternative in eliminating matrix effects without requiring an extraction procedure. This can be attributed to the inherent ability of APCI to better tolerate salt and matrix effects.

Many contributing factors affecting the complex ESI process have been discussed [23,28]. Among these is the effect of pH on ionisation efficiency, but little has been documented with regard to signal variability. In order to determine whether the effect of pH on the analyte in solution immediately prior to ionisation affects the stability of ESI, post-column infusion to acidify the basic eluent is also being investigated.

3.1.3. Sample preparation

The influence of sample pre-treatment is paramount in the speed and simplicity of analysis, as well as adding potential sources of error. The sensitivity of LC–MS provides the possibility of directly diluting and injecting the sample, without the need for pre-concentration and derivatisation. The dilution factor was determined in order to identify the lowest abundant ion for cathine at the lowest calibration concentration (1 μg/mL) while not saturating the detector with the highest concentration of pseudoephedrine (400 μg/mL). A 90-fold dilution with water was determined suitable in satisfying these criteria.

3.2. Validation

The LC-APCI–MS/MS method was validated in terms of linearity, selectivity, accuracy and precision, carryover and ion suppression due to matrix effects. The suitability of the method is demonstrated by the analysis of two samples previously determined positive for cathine and ephedrine respectively using a validated GC–MS method.

3.2.1. Linearity

A six-point calibration curve including 50–200% of the WADA threshold levels has been generated to confirm linearity over the range for quantification. Correlation coefficients (r^2) were greater than 0.9975 (Table 3). Errors (calculated as the difference between the determined and actual concentration) ranged between −7.5 and 2.1% over a concentration range corresponding to 50–200% of the WADA threshold levels for cathine (5 μg/mL), ephedrine (10 μg/mL), pseudoephedrine (150 μg/mL) and methylephedrine (10 μg/mL).

3.2.2. Selectivity

Selectivity was determined through the analysis of 10 different blank urines with no interference being detected at the expected retention times of the analytes.

3.2.3. Accuracy and precision

The method was tested for accuracy and precision through the analysis of three repeats of urine spiked with each analyte at the WADA threshold level performed on three different days. The results for accuracy, reported as % bias between the estimated and

Table 3
Linearity results for calibration of cathine, ephedrine, pseudoephedrine and methylephedrine.

Compound	Ion (*m/z*)	Range (μg/mL)	Slope	Intercept	r^2	Equation
Cathine	134	1.0–20	0.0445	−0.0045	0.9997	$y = 0.0445x - 0.0045$
Ephedrine	148	2.5–40	0.0658	−0.0206	0.9992	$y = 0.0658x - 0.0206$
Pseudoephedrine	148	25–400	0.0583	−0.5223	0.9995	$y = 0.0583x - 0.5223$
Methylephedrine	147	2.5–40	0.0079	−0.0024	0.9999	$y = 0.0079x - 0.0024$

Table 4
Within- and between-assay precision (% RSD) and accuracy (% bias) for the ephedrines in urine at QC concentrations.

Compound (μg/mL)		Mean conc. (μg/mL)	Accuracy (% bias)	Precision (% RSD)	
				Within-assay	Between-assay
Cathine (5 μg/mL)	Day 1	4.98	−0.47	3.22	4.14
	Day 2	4.64	−7.13	7.54	
	Day 3	5.01	0.13	4.20	
Ephedrine (10 μg/mL)	Day 1	10.4	3.50	3.51	5.20
	Day 2	9.36	−6.37	2.19	
	Day 3	9.64	−3.60	5.08	
Pseudoephedrine (150 μg/mL)	Day 1	155	3.09	1.48	3.65
	Day 2	153	1.90	1.21	
	Day 3	164	9.03	1.65	
Methylephedrine (10 μg/mL)	Day 1	11.0	9.50	3.71	5.77
	Day 2	9.89	−1.07	2.14	
	Day 3	9.96	−0.43	4.01	

actual concentration values, are all less than 10%, and % RSD values for within- and between-assay variation are less than 7.54 and 5.77, respectively (Table 4).

3.2.4. Carryover

Carryover was determined by injecting urine samples spiked with the analytes at a concentration corresponding to five times the WADA threshold [2], followed by injection of blank mobile phase. No peak was detected in the blank sample.

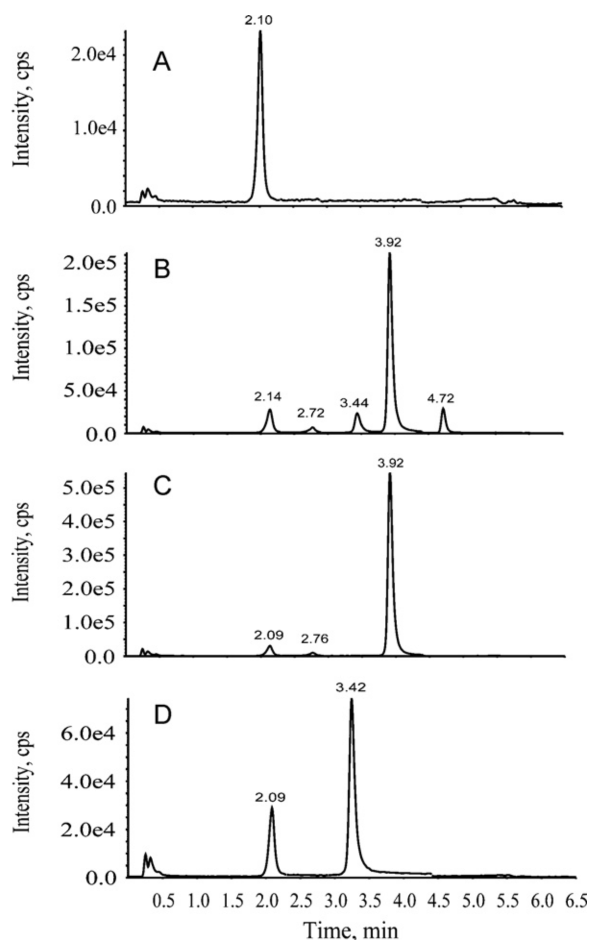

Fig. 6. Extracted ion chromatograms for the analysis of a blank (A), a quality control sample (B) and actual samples previously determined positive for cathine and pseudoephedrine (C) and ephedrine (D).

3.2.5. Matrix effects

The importance of ion suppression due to matrix components from the urine is emphasised since no extraction was performed before injection. This was determined by comparing responses of 10 different spiked urine samples with each analyte at the WADA threshold level with a spiked standard at the same concentration prepared in water. For all compounds the matrix effect was less than 10% apart from one which gave a value of 13%.

3.2.6. Application to real samples

This method has been applied to the analysis of two samples previously determined to be positive for cathine and ephedrine respectively using a GC–MS method. LC–MS/MS chromatograms are reported in Fig. 6. The estimated concentrations are 7.56 μg/mL and 283 μg/mL of cathine and pseudoephedrine in sample C, and 48.3 μg/mL ephedrine in sample D. These mean values are similar to those obtained by GC–MS, estimated at 7.85 μg/mL and 44.7 μg/mL for cathine and ephedrine, respectively, all measured in triplicate. Pseudoephedrine was detected and quantified in sample A while the corresponding GC–MS data are not available since the substance was not prohibited in sport when the original analysis was performed. This illustrates the need for a simultaneous confirmation method since multiple ephedrine compounds are often present in a positive urine sample.

4. Conclusions

A new, simple LC-APCI–MS/MS method has been developed and validated for the identification and quantification of ephedrines in urine for doping control analysis. The use of a high pH mobile phase has allowed for improved chromatographic separation of the basic compounds without undesirable additives or the need for column re-generation. A direct dilution and injection approach circumvents the time and labour-intensive sample preparation associated with current GC–MS methodologies and provides the sensitivity and selectivity required. Moreover, the method requires a small sample volume and permits the accurate quantification of cathine, ephedrine, pseudoephedrine and methylephedrine in a single injection, the results of which compare favourably with the current GC–MS method.

Our findings emphasise the difficulties in selecting eluents optimal for both chromatographic performance and analyte ionisation. In this case, the incompatibility was overcome through the use of APCI, which provided a more reliable and reproducible mode of ionisation compared to ESI. Preliminary results from post-column acidification with ESI confirm the significant effect of pH on signal intensity for ephedrines, although no considerable effect on signal stability is noted. Future work must be performed in order to further

understand the effects of eluent pH on the stability of ionisation of basic compounds with ESI and APCI.

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