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Development and validation of an LC–MS/MS method for the quantification of ephedrines in urine

K. Deventer*, O.J. Pozo, P. Van Eenoo, F.T. Delbeke

DoCoLab, UGent, Department of Clinical Chemistry, Microbiology and Immunology, Technologiepark 30, B-9052 Zwijnaarde, Belgium

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ABSTRACT

The objective of this study was to develop a simple and robust LC–MS/MS method for the quantification of ephedrine type substances in urine. Sample preparation consisted of a 10-fold dilution step of the samples into the internal standard solution (ephedrine-d_3, 4 μg/mL in water). Baseline separation of the diastereoisomers norpseudoephedrine–norephedrine and ephedrine–pseudoephedrine was performed on a C8-column using isocratic conditions followed by positive electrospray ionisation and tandem mass spectrometric detection. The mobile phase consisted of 98/2 (H_2O/ACN) containing 0.1% HAc and 0.01% TFA. Calibration curves were constructed between 2.5 and 10 μg/mL for norephedrine and norpseudoephedrine and 5 and 20 μg/mL for ephedrine, pseudoephedrine and methylephedrine. The bias ranged from −5.5 to 12% for norephedrine, −4.1 to 8.0 % for norpseudoephedrine, 0.3 to 2.1 % for ephedrine, 1.6 to 2.6 % for pseudoephedrine and 2.9 to 5.0 % for methylephedrine. Precision of the method varied between 2.8 and 10.4% for all compounds and the matrix effect was less than 15%. The applicability of the method has been checked by the analysis of 40 urine samples. The results were compared with those obtained with the common GC–NPD method. Results show a good correlation between both methods with correlation coefficients higher than 0.95 for all analytes.

1. Introduction

Stimulants are synthetic derivatives of the endogenous stimulant adrenaline and have similar pharmacological effect on mental function and behaviour, producing excitement and euphoria and increase motor activity. One of the oldest therapeutically applied stimulants are the group of the ephedrines. These substances can be found as natural source in Ephedra or Ma Huang (*Ephedra sinica*). Extracts of these plants are still used nowadays in nutritional supplements [1]. The major active compounds are five optical active compounds including ephedrine (EP), pseudoephedrine (PEP), norephedrine (NEP), methylephedrine (MEP) and norpseudoephedrine (NPEP) also known as cathine (Table 1). PEP is frequently applied therapeutically for the treatment of allergic rhinitis. Preparations containing EP are used for the treatment of cough. Because of the (frequent) therapeutical use of ephedrines the regulations of the World Anti-Doping Agency (WADA) [2] are complicated: PEP and NEP are not on the list and can be used unrestricted. For NPEP a threshold of 5 μg/mL and for EP and MEP a threshold of 10 μg/mL is applied. In anti-doping analysis ephedrines used to be quantified in urine by GC–NPD, GC–MS or HPLC-UV after preconcentrating the sample by liquid–liquid extraction (LLE) [3–5] or after an online clean-up [6]. The sensitivity of liquid chromatography coupled to mass spectrometry (LC–MS) and the compatibility of the system with the aqueous matrix of urine has allowed quantitative analysis of ephedrines without preconcentration step by direct injection [7,8]. The aim of this work is to develop and validate a quantitative method for the direct urinary detection of ephedrines by LC–MS/MS. Several chromatographic as well as mass spectrometrical problems encountered during method development are discussed. Finally, the applicability of the developed methods is tested by comparing results with those obtained by the GC–NPD method routinely applied in our laboratory.

2. Experimental

2.1. Standards and reagentia

EP, PEP, NEP, MEP, ephedrine-d_3 (EP-d_3) and trifluoroacetic acid (TFA) (LC–MS grade) were purchased from Sigma–Aldrich (Bornem, Belgium). NPEP was purchased from National Measurement Institute (Sydney, Australia). Methanol (MeOH) (HPLC-grade) was purchased from Acros-Organics (Geel, Belgium), acetonitrile (ACN) (HPLC-grade) from Biosolve (Valkenswaard, The Netherlands),

* Corresponding author. Tel.: +32 9 3313295; fax: +32 9 3313299.
E-mail address: Koen.Deventer@UGent.be (K. Deventer).
URL: http://www.docolab.UGent.be (K. Deventer).

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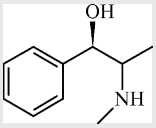
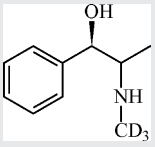
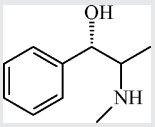
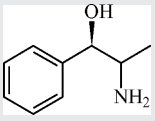
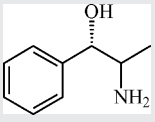
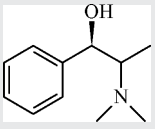
Table 1
Detection settings for the investigated substances.

Compound	Structure	[M+H]+	CE	DI
Ephedrine		166	27, 19, 19	115, 117, 133
Ephedrine-d_3 (IS)		169	20	117
Pseudoephedrine		166	27, 19, 19	115, 117, 133
Norephedrine		152	30, 24, 17	91, 115, 117
Norpseudoephedrine		152	30, 24, 17	91, 115, 117
Methylephedrine		180	28, 31, 14	115, 117, 135

CE: collision energy, DI: diagnostic ion, The tube lens voltage was for all substances set to zero.

acetic acid (HAc) and HPLC-grade water from Fischer Scientific (Loughborough, United Kingdom). Gases used for the mass spectrometer were helium (Alfagaz-grade) and nitrogen (LASAL2001-grade) both from Air-Liquide (Desteldonk, Belgium).

2.2. Apparatus

The HPLC system consisted of a Surveyor MS-pump and Surveyor autosampler with a 25 μL sample loop (all from Thermo Separation Products, Thermo, San Jose, CA, USA). During method validation three different columns were evaluated: a Zorbax RX C8 column (150 mm × 2 mm, 5 μm) (Agilent, Diegem, Belgium), a Pursuit XRS C8 (150 mm × 2 mm, 5 μm) and a Polaris C8 (150 mm × 2 mm, 5 μm) both from Varian (Sint-Katelijne-Waver, Belgium). The columns were maintained at 35 °C during analysis. The mobile phase consisted of 98/2 H_2O/ACN containing 0.1% HAc and 0.01% TFA. Isocratic elution at a flow rate of 0.4 mL/min was performed. The total run time was 16 min per sample. The LC effluent was pumped to a Quantum Discovery mass spectrometer (Thermo, San Jose, CA, USA) equipped with an ESI source, operated in the positive ionisation mode. The capillary temperature was 300 °C. The sheath gas flow rate was set at 50 arbitrary units. No auxilliary gas was used. The mass spectrometer was operated in the SRM mode and three transitions were monitored per compound (Table 1). The tube lens voltage was set to zero for all compounds. The isolation width was set 0.7 FWHM. The scan speed and scan width were maintained at 100 ms and 0.01 amu, respectively. The collision gas pressure was 1.5 mTorr for all compounds.

2.3. Sample preparation

Twenty microliter was pipetted into an autosampler vial containing 180 μL of internal standard solution (4 μg/mL EP-d_3 in H_2O), 1 μL was injected into the chromatographic system.

2.4. Validation

A five-point calibration curve was generated (covering 0.5× threshold and 2× threshold) by spiking blank urine with the ephedrines in triplicate at 2.5, 3.75, 5, 7.5 and 10 μg/mL for NPEP and NEP and 5, 7.5, 10, 15 and 20 μg/mL for EP, PEP and MEP.

The area ratios (ARs) of the total ion chromatogram (TIC) of the three product ions and the product ion of the IS were plotted versus the concentration. The precision and bias of the method were tested by the analysis of spiked samples at three

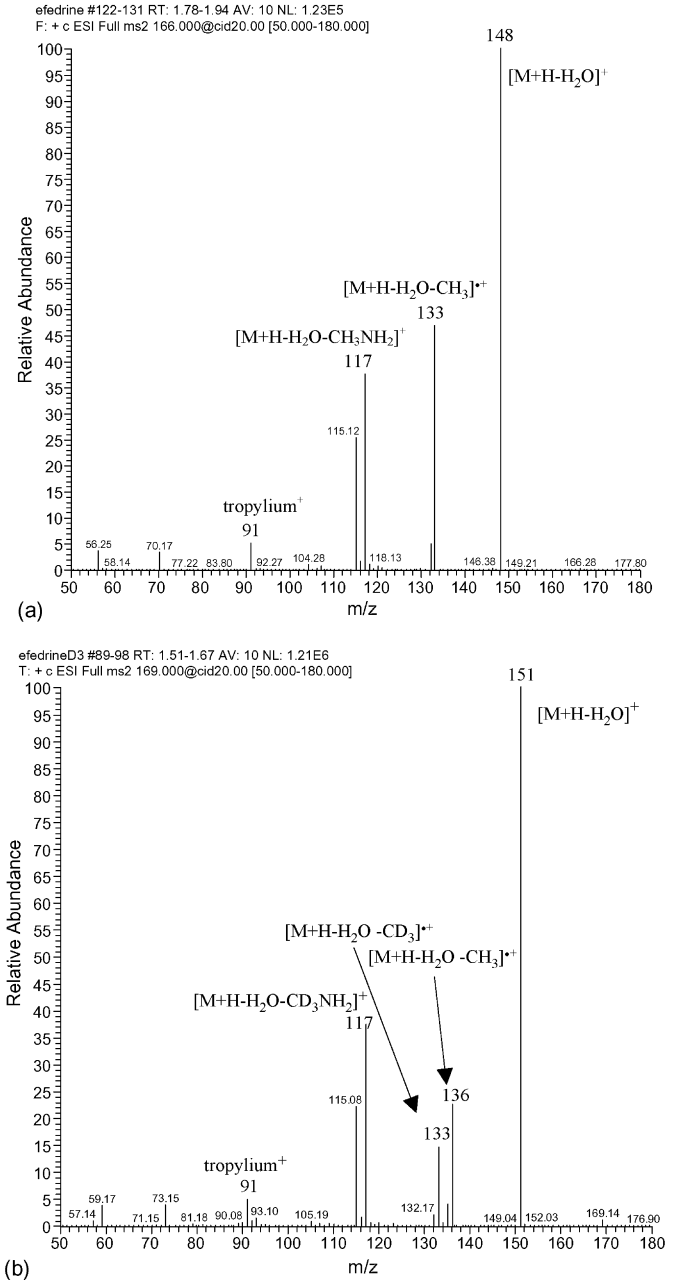


Fig. 1. Full scan MS/MS spectrum (10 eV) and suggested product ions for EP (a) and EP-d_3 (b).

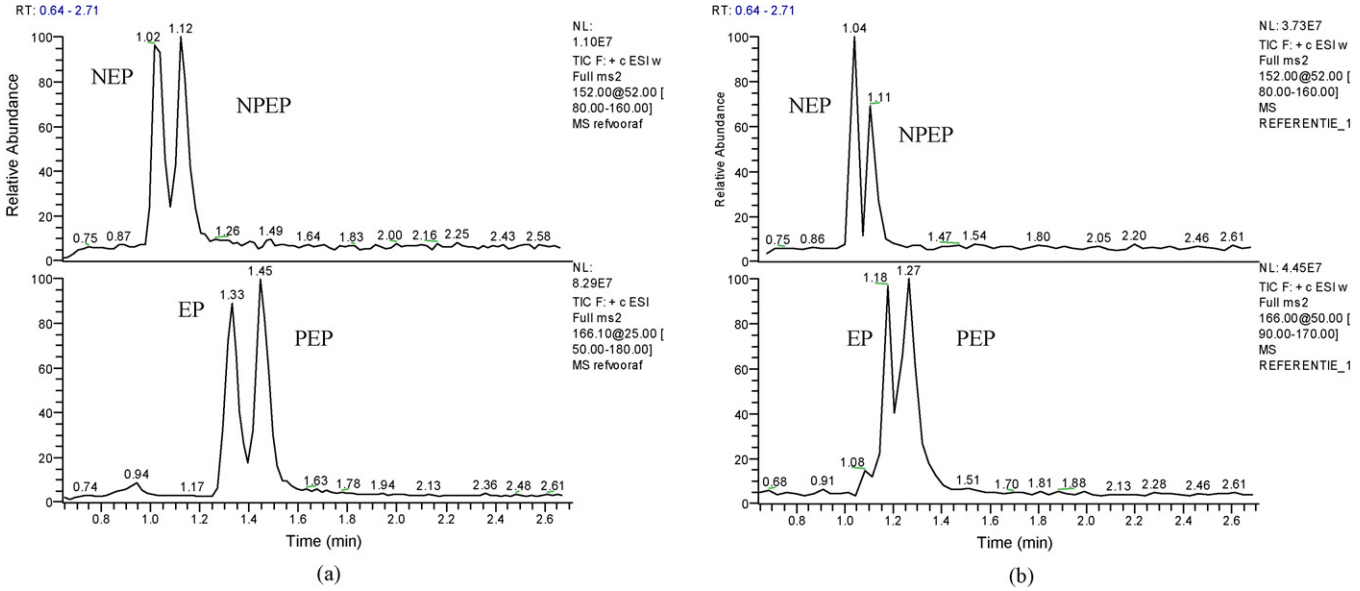


Fig. 2. Separation of the isobaric compounds on Pursuit XRS C8 (a) and on Polaris C8 (b) using isocratic 98/2 H_2O/ACN (0.1% HAc).

levels (2.5, 5 and 10 μg/mL for NPEP and NEP and 5, 10 and 20 μg/mL for EP, PEP and MEP). Precision was assessed as the percentage relative standard deviation (%RSD) of both repeatability (within-day) and reproducibility (between-day and different analysts) for a selected compound and level. Maximum allowed tolerances for reproducibility and repeatability can be calculated from the Horwitz-equation $RSD_{max} = 2^{(1-0.5\log C)}$ (C = concentration (μg/mL) × 10^{-6}). The maximum allowed tolerances for repeatability and reproducibility are 2/3 RSD_{max} and RSD_{max}, respectively [9].

Bias was defined as the difference between the calculated amount and the specified amount for the selected compound and expressed as a percentage [10]. The limit of quantification (LOQ) of the method was defined as the lowest concentration where acceptable reproducibility and bias could be guaranteed. Selectivity was tested by analysing several structurally related and other routinely screened doping agents, including other stimulants, diuretics, corticosteroids and anabolic steroids. Concentrations in these mixtures were 1 μg/mL. Specificity was tested by analysing 10 blank urines as described above to evaluate the presence of matrix interference. To evaluate the matrix effect water and 6 other urines were spiked in sixfold at the threshold-level. The matrix effect was determined by comparing the areas in water with this obtained in the spiked urine samples.

2.5. Application to real samples

In order to evaluate the applicability of the method for routine purposes, 40 samples previously analysed by the GC–NPD routinely applied in our laboratory were analysed using the LC–MS/MS methods. A comparison of the results was performed.

3. Results and discussion

3.1. Method development

3.1.1. Mass spectrometry

Amphetamines contain an amine function which can be easily protonated. Hence, very abundant protonated molecular ions $[M+H]^+$ were observed for all compounds with both APCI and ESI. Deprotonated molecular ions were not detected in negative ionization mode. ESI is routinely used at our laboratory and was further preferred as interface. Because tandem mass spectrometry often results in improved sensitivity this technique was applied.

Table 2
Bias, repeatability, reproducibility and tolerance limits of the LC–MS/MS method at 1/2 threshold, threshold and 2×x treshold including the lowest point of the calibration curves.

	Concentration (μg/mL)	Bias (%)	Repeatability RSD (%)	Reproducibility RSD (%)	RSD_{max} (%)	2/3 RSD_{max} (%)
NPEP	2.5	−8.00	1.85	10.38	13.9	9.29
	5	−6.84	1.84	7.49	12.6	8.37
	10	−4.13	1.64	6.62	11.3	7.88
NEP	2.5	5.54	4.18	7.64	13.9	9.29
	5	12.03	5.39	7.01	12.6	8.37
	10	10.03	4.82	5.96	11.3	7.88
EP	5	1.25	1.40	3.83	12.6	7.54
	10	0.30	1.45	3.65	11.3	7.10
	20	2.14	1.42	2.92	10.2	6.80
PEP	5	1.62	1.49	3.43	12.6	7.88
	10	0.91	2.10	3.77	11.3	7.54
	20	2.57	2.65	2.87	10.2	7.10
MEP	5	5.07	3.37	6.99	12.6	7.88
	10	2.91	3.15	4.81	11.3	7.54
	20	3.86	1.91	3.24	10.2	7.10

All substances show similar fragmentation behaviour starting with the loss of H_2O from the protonated molecular ion (Fig. 1) [8]. Because losses of water are less specific and generally show higher noise levels [11], this product ion was not used in the quantitation method. Further fragmentation of the ephedrines could be partially explained by the availability of the deuterated IS (EP-d_3). The loss of the amine moiety (ammonia for NEP and NPEP, CH_3NH_2 for EP and PEP, $(CH_3)_2NH$ for MEP and CD_3NH_2 for EP-d_3) from the dehydrated precursor molecule could be assigned to the product ion *m/z* 117. This amine loss is typical for amphetamine type stimulants [12,13]. Besides, loss of 15 Da from the dehydrated precursor ion was also found. This fragment could be assigned to the loss of the radical $^{\bullet}CH_3$ resulting for EP in a product ion with *m/z* 133 and for EP-d_3 in two product ions, *m/z* 133 and *m/z* 136 (Fig. 1). Losses of a radical

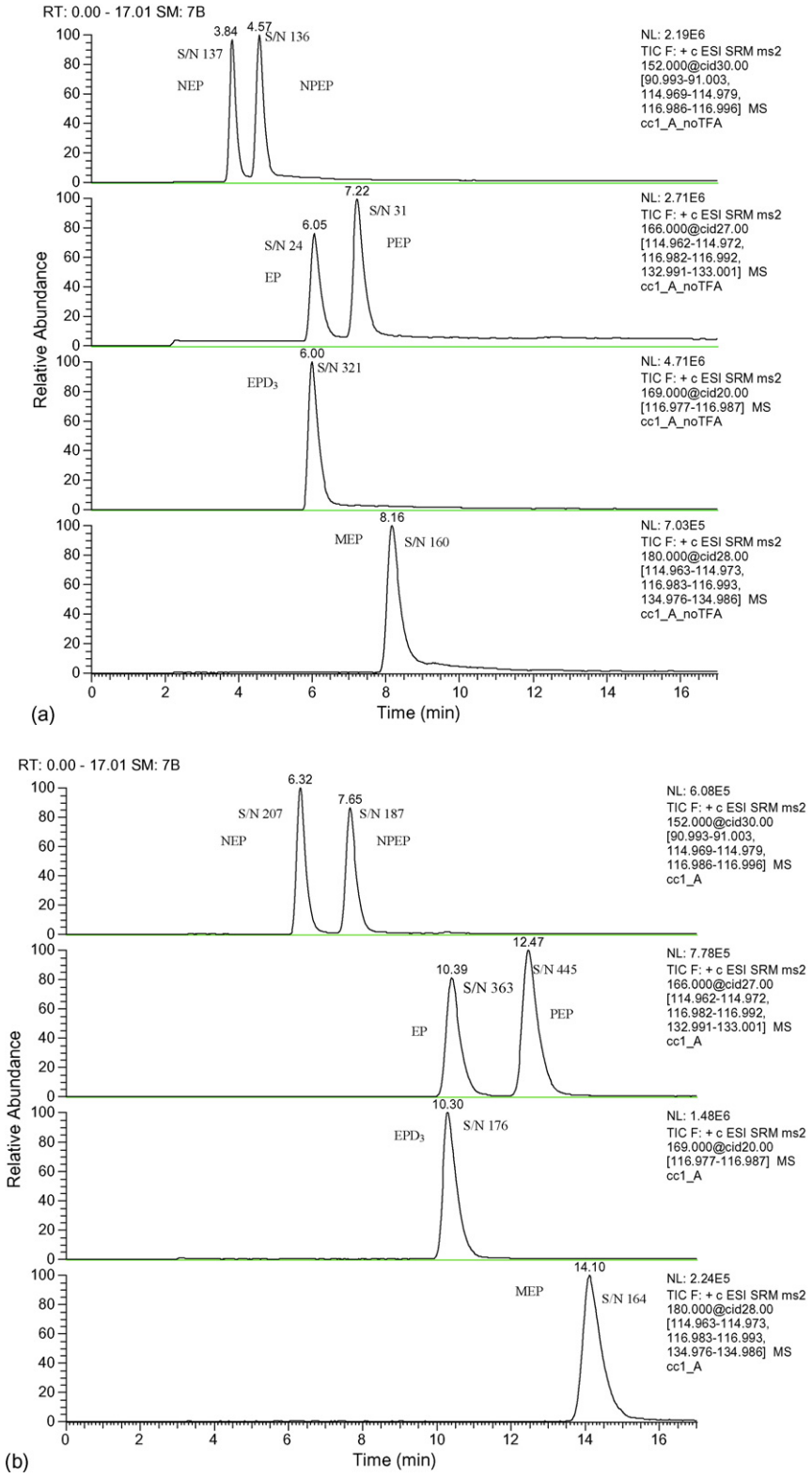


Fig. 3. Total ion chromatogram for the lowest calibrator (1/2 threshold), analysed using isocratic 98/2 H_2O/ACN with 0.1% HAc (a) and 0.1% HAc/0.01% TFA (b).

is not very common in collision-induced fragmentation, but is possible due to the stabilization of the remaining radical product ion by its conjugated system. The ion at *m/z* 91 was also observed for all compounds and has previously been assigned to the tropylium ion [13]. Further fragmentation and other product ions could not be assigned directly and accurate mass experiments should be performed to explain them. Despite the selection of specific product ions for all substances, noise levels were fairly high when real samples were analysed. This observation was assigned to the low *m/z* values of both the precursor and product ions. To reduce this noise the tube lens voltage was set to zero for all compounds minimizing in source fragmentation of coeluting matrix substances.

3.1.2. Chromatography

The presence of a hydroxyl and amine moiety, an aromatic ring and few saturated C–H bonds (Table 1) make ephedrines highly polar and difficult to retain on alkyl-based reversed phases.

The poor retention also results in difficulties in separation of the isobaric compounds NEP–NPEP and EP–PEP. Baseline separation is mandatory because NEP and PEP are not on the prohibited list [14]. As a consequence, their retention and separation on alkyl-based reversed phase columns is mostly achieved using high amounts of aqueous phase and low amounts of organic modifier. Additionally, depending on the type of alkyl-phase, mobile phases with high ionic strength and tri-or tetra-alkylated additives are recommended to obtain satisfactory peak shape [15]. Unfortunately such chromatographic conditions are not compatible with LC–MS.

Recently the separation of ephedrines on a Zorbax RX C8 was described using 98/2 H_2O/ACN with only 0.1% HAc as additive and ESI-MS^2 as detector [8]. Transferring these chromatographic conditions onto a Pursuit XRS C8 column at our laboratory yielded poor separation (Fig. 2a). Moreover after a few injections the ephedrines shifted into the solvent front. However, after rinsing the column with 100% ACN, retention and partial separation was observed for several injections. This observation was assigned to dewetting of the C8-phase caused by the low amount of ACN (2%) in the mobile phase. The retention shift could be explained by the gradual expulsion of ACN, used for the initial rinse of the column, from the interior of the pores preventing the ephedrines to be retained to the column.

In a second approach a Polaris C8 was evaluated. This type of column can be used with 100% aqueous mobile phase. Using this column chromatography was reproducible but still no baseline resolution, between the isobaric compounds, was observed (Fig. 2b).

Ultimately, the previously suggested Zorbax RX C8 column [8] was also evaluated. Although this column is not specially designed for use in high aqueous media dewetting was not observed and good reproducibility of RTs was found (Fig. 3a). Consequently, this column was further used. Despite the good performance tailing was still dominant, in particular for the late eluting MEP (Fig. 3a). In peptide and protein separation, ion-pairing with TFA often results in improved chromatography [16] and 0.01% TFA was therefore added to the mobile phase resulting in an increased retention due to ion pairing. Both resolution and peak shape improved significantly for all investigated substances (Fig. 3b). The use of TFA can cause ion suppression, resulting in a lower signal. Indeed, the absolute signal decreased, however due to the improved chromatography no problematic loss in S/N was observed for the target compounds. Because the mobile phase in this method has a low eluting strength, strong retained compounds can affect subsequent injections when they start eluting. Therefore, after every batch of samples, the column was rinsed with 100% ACN.

3.1.3. Sample preparation

Initially, as described by Spyridaki et al. [8], the samples were spiked with IS, filtrated and subsequent injected. During initial experiments we observed that the signal for the IS (EP-d_3) was suppressed by EP in the urine samples fortified at 15 and 20 μg/mL. Because the IS is also used for the quantification of NPEP and MEP this could lead to an erroneous quantification. Another problem was observed when real samples were analysed. Some of these samples had shifted RTs. The common feature of these samples were found by GC–NPD to contain high amounts of PEP (>100 μg/mL).

To overcome these problems, samples were diluted. Although a fourfold dilution already solved both problems for suppression and retention time instability, finally a 10-fold dilution was used without compromising sensitivity (Fig. 3). Besides the improvements regarding in ion suppression and retention time stability this 10-fold dilution and the injection of only 1 μL significantly reduces the amount of urinary matrix loaded onto the column. Hence a sample filtration step was not needed. This approach was supported by the analysis of 300 research samples which did not show analytical problems which could be attributed to the omission of the filtration step.

3.2. Method validation

Using a least square fit, good linearity ($r^2 \geq 0.98$) was observed for all substances. None of the calibration curves was forced through the origin and for the regression calculation a weighing factor of $1/x$ was used for all data points. The results for precision and bias are summarised in Table 2 and did not exceed 2/3 RSD_{max} neither for repeatability nor reproducibility. Deviation of the mean measured concentration from the theoretical concentration (bias) for all compounds was below the acceptable threshold of 15 and 20% for all levels in the range of calibration curve [10]. As expected, ephedrine which coelutes with its deuterated analogue had the smallest RSD and lowest bias. Using a deuterated IS for every analyte could further improve the results for other compounds as well. Regarding the selectivity, interference from other monitored doping agents could not be found. In addition analysis of 10 different blank control urine samples did not result in the detection of interfering substances, proving the specificity of the method. The limit of quantification (LOQ) of the method was 2.5 μg/mL for NEP and NPEP and 5 μg/mL for EP, PEP and MEP. Matrix effect on LC–MS(MS) should not be underestimated and can hamper correct quantification. Hence, evaluating its effect should be part of developing quantitative methods [17]. Only for urine (Table 3), with high turbidity, the highest effect was observed for the early eluting NEP and NPEP. For all other urine samples the effect was lower than 8.3%. The effect on storage as well as freeze–thaw cycles has not been investigated in this study but have been previously performed at our laboratory [18].

3.3. Application to real samples

Ephedrines are routinely screened for by GC–MS [19]. Semi-quantitative results are generated from this method to decide whether a sample goes to the quantitative confirmation procedure

Table 3
Matrix effect for the investigated ephedrines.

	Matrix-effect ($n = 42$) (%)						
	Urine 1	Urine 2	Urine 3	Urine 4	Urine 5	Urine 6	Water
NPEP	−14.99	+3.1	+1.95	+3.73	+5,00	+3.47	0
NEP	−0.85	+5.8	+1.94	+4.3	+2.58	+2.69	0
EP	−0.28	+4.88	+3.42	+5.84	+6.37	+6.19	0
PEP	−0.47	+5.84	+3.32	+6.42	+6.25	+6.38	0
MEP	−5.28	−2.9	−4.05	+2.58	+8.31	+4.75	0
EP-d_3	+1.82	+6.79	+6.11	+5.86	+7.49	+6.6	0

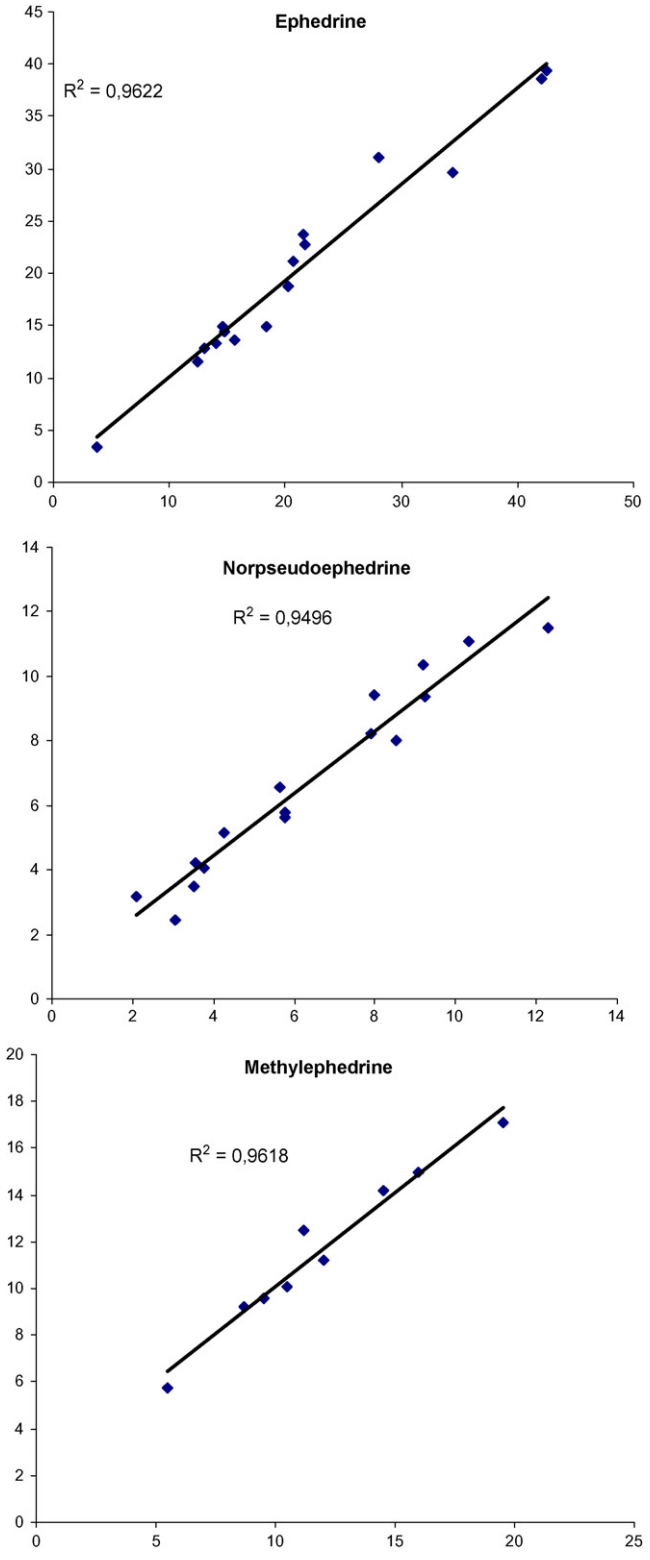


Fig. 4. Comparison of the concentrations calculated by GC–NPD and the LC–MS/MS method for NPEP, EP and MEP.

or not. NPEP and EP are most often detected and sufficient samples ($n = 30$) were available to compare GC–NPD and LC–MS/MS quantification. MEP is not very often detected. Hence, for this compound four proficiency tests and six additional spiked samples were analysed. The results are presented in Fig. 4 and correlations were better than 0.95 for all substances showing good agreement between GC–NPD and LC–MS/MS.

4. Conclusions

A simple and sensitive method was developed and validated for the quantification of five ephedrine type stimulants. Chromatography was investigated using several C8 type columns.

The Zorbax RX C8 exhibited excellent selectivity and specificity for the investigated substances. Adding TFA to the mobile phase showed to be a useful strategy to improve both peak shape as well as resolution. Sample preparation was minimised by a direct 10-fold dilution of the urine directly into the autosampler vial without filtration. Hence, the omission of the filtration step makes the sample preparation time shorter and more economic. Besides, the current approach involves less steps to be incorporated in the uncertainty budget.

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