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Quantitative determination of underivatized polyamines by using isotope dilution RP-LC–ESI-MS/MS

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ABSTRACT

A rapid, sensitive and selective method using LC–MS/MS was developed and validated for the simultaneous quantitative determination of five polyamines N^1,N^{12}-diethylspermine (DESpm), *N*-ethylspermine (EtSpm), N^1-ethylspermidine (EtSpd), spermidine (Spd) and N^1-ethyl-1,3-diaminopropane (EtDAP) without any derivatization steps. The LC–MS/MS system was operated using the positive electrospray ionization (ESI) mode. The chromatographic separation only took 10 min and was performed on a reversed phase C18 column with 0.1% heptafluorobutyric acid as the ion-pairing agent and acetonitrile gradient. Stable, deuterium labelled internal reference compounds of the five analytes were included in the quantification. The lower limit of quantification for all of the five analytes was 0.03 μM and the method was linear for DESpm, EtSpd, Spd and EtDAP over the range of 0.03–60 μM and for EtSpm over the range of 0.03–30 μM. Correlation coefficients (R^2) were always >0.995 for all the analytes. The precision of the overall method ranged from 0.2 to 9.7% as intra-day variability and from 0.9 to 6.8% as inter-day variability. The intra-day and inter-day accuracy of the assay ranged between 87.6–109.8% and 89.6–106.6%, respectively. The method has been applied successfully to quantify metabolites of DESpm as a substrate for recombinant human polyamine oxidase.

1. Introduction

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is a widely used method for analysis of drugs and endogenous compounds. When compared to conventional detection methods, the selectivity of MS/MS eliminates possible interfering peaks and improves both analytical sensitivity and specificity when analyzing low molecular weight compounds in complex matrices. In this study, LC–MS/MS was used to assay the metabolism of polyamine analogue N^1,N^{12}-diethylspermine (DESpm) by human recombinant polyamine oxidase (hPAO).

Polyamines are polycationic aliphatic amines, which are widely distributed in nearly every prokaryotic and eukaryotic cell type. The tetramine, spermine (Spm), the triamine spermidine (Spd) and their diamine precursor, putrescine (Put) have been shown to be essential in the regulation of mammalian cell growth and differentiation [1]. Their total intracellular concentration lies in the millimolar range [2]. Polyamine analogues are actively transported into the cells through the same transport system as the natural polyamines. However, they are incapable of fulfilling the crucial cellular functions of the natural polyamines and this leads to cell growth inhibition [3,4]. Some of the analogues, e.g. N^1,N^{11}-diethylnorspermine (DENSpm) and DESpm, display cytotoxic activity and are promising chemotherapeutic agents. They may also serve as tools for a novel form of antiproliferative and antiparasitic intervention, and hence their metabolism needs to be clarified [2,4–8].

Polyamines have been studied by using several analytical methods [9–12]. The techniques used for quantification are mainly based on chromatographic separations using high-performance liquid chromatography (HPLC) with pre- or post-column derivatization. Derivatization is needed to increase the sensitivity of the method when using traditional UV or fluorescence detection. The main drawbacks associated with derivatization are the elongated analysis times, low reproducibility, interference problems and derivatization instability [10,13]. Nonetheless, there are a few methods for polyamine analysis where a derivatization procedure is not needed, these being mainly based on capillary electrophoresis. [9,10,14–18].

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Reversed phase separation of underivatized polyamines is challenging due to their low column retention and susceptibility to undergo severe tailing [8,19]. Traditionally underivatized polyamines have been separated using alkyl sulfonates as ion-pairing agents [10], but these are not suitable for electrospray ionization mass spectrometry due to their low volatility. Recently, three LC methods based on MS/MS detection have been published which utilize the separation and detection of underivatized biogenic amines with good sensitivity from cheeses [20,21] and wines [22]. The first method uses ammonium acetate as the mobile phase additive and a 150 mm × 2 mm Luna C18 column for separations of cadaverine (CAD), histamine (HIS), Spd, Spm, tyramine (TYR) and tryptamine (TRP) [20]. The values of limit of quantification (LOQ) for standard solutions ranged from 5.6 to 68.2 μg L^{-1}. The second method concerns the separation and analysis of HIS, 2-phenylethylamine (PEA) and TYR using trifluoroacetic acid (TFA) as a mobile phase additive [21]. Good separation was achieved for these three amines, but the method is not suitable for more polar polyamines which are not sufficiently retained on a reversed phase column using TFA containing mobile phases [8,19]. The third method utilizes ammonium acetate and perfluoroheptanoic acid mixture as an ion-pairing agent and 150 mm × 4.6 mm ZORBAX Eclipse XDB-C8 column for separation of TYR, TRP, PEA, HIS, CAD, Put, Spd and Spm [22]. Heptylamine was used as an internal standard. This method achieved the separation of these amines except for HIS, CAD and Put, which co-eluted at the same retention time. Detection limits (LOD) in synthetic wine ranged from 0.47 to 40.1 μg L^{-1}.

We have previously shown that polyamines can be separated and qualitatively analyzed by LC–MS/MS using volatile HFBA as ion-pairing agent [8]. An excellent chromatographic separation of 12 polyamines with good symmetrical peak shapes was achieved in 10 min and even the closely related N^1-acetylspermidine (N^1AcSpd) and N^8-acetylspermidine could be separated from each other and analyzed. Qualitative identification of metabolites formed from DESpm in the reaction catalyzed by hPAO include *N*-ethylspermine (EtSpm), N^1-ethylspermidine (EtSpd), Spd and N^1-ethyl-1,3-diaminopropane (EtDAP) [8]. However, further studies are needed to elucidate the exact origin of the formed Spd and EtDAP, and the properties of EtSpm and EtSpd as the substrates of hPAO. Schematic presentation of the possible catabolic pathways of DESpm and its metabolites formed in the reaction catalyzed by hPAO is shown in Fig. 8 [8].

In this study, a fast and sensitive quantitative method was developed and validated to separate and analyze five polyamines (DESpm, EtSpm, EtSpd, Spd and EtDAP) by LC–MS/MS using HFBA as ion-pairing reagent in chromatographic separation and stable, deuterium labelled reference compounds as internal standards in quantification. Unlike conventional methods of polyamine analysis, separation and quantitative analysis were carried out without any derivatization and after simple sample preparation. The developed method was successfully applied to the quantification of metabolites of DESpm as a substrate for hPAO. The main catabolic pathway of DESpm with hPAO was proved to be similar to the catabolism of N^1,N^{12}-diacetylated spermine [6], with minor pathways being the de-ethylation [5], and the previously demonstrated endo-cleavage producing EtDAP [8]. EtSpm was most likely further metabolized to Spd, mimicking the catabolism of N^1AcSpd by PAO [6].

2. Experimental

2.1. Reagents

N^1-(3-amino-propyl)-butane-1,4-diamine trihydrochloride (Spd) was from Aldrich. The publication describing the efficient preparation methods for N^1-ethyl-propane-1,3-diamine dihydrochloride (EtDAP), N^1-ethyl-3,3-$^2H_2$-propane-1,3-diamine dihydrochloride (EtDAP-2D), N^1-(1,1-$^2H_2$-(3-amino)propyl)-butane-1,4-diamine trihydrochloride (Spd-2D), N^1-(3-ethylamino-propyl)-butane-1,4-diamine trihydrochloride (EtSpd), N^1-(1,1-$^2H_2$-(3-ethylamino)propyl)-butane-1,4-diamine trihydrochloride (EtSpd-2D), *N*-(3-amino-propyl)-*N*′-(3-ethylamino-propyl)-butane-1,4-diamine tetrahydrochloride (EtSpm), *N*-(3-amino-propyl)-*N*′-(1,1-$^2H_2$-(3-ethylamino)propyl)-butane-1,4-diamine tetrahydrochloride (EtSpm-2D), *N*, *N*′-bis-(3-ethylamino-propyl)-butane-1,4-diamine (DESpm) and *N*, *N*′-bis-(1,1-$^2H_2$-(3-ethylamino)propyl)-butane-1,4-diamine (DESpm-4D) is in preparation. Ultra gradient HPLC-grade acetonitrile (ACN) was purchased from J.T. Baker, and heptafluorobutyric acid (HFBA, >99%) from Fluka. Sterile filtered deuterium oxide (>99%) was from Spectra Stable Isotopes, USA. Sodium 3-(trimethylsilyl)-1-propionic acid (TSP, 99.8% atom D) was from Euriso-top, France. All other reagents (formic acid, glycine, NaOH) were from Sigma. Ultrapure water was prepared using a Milli-Q Gradient system (Millipore, Milford, MA, USA). Recombinant human polyamine oxidase was produced as described earlier [23].

2.2. LC–MS/MS instrumentation and analytical conditions

LC separations, MS/MS detection and analysis of the compounds were achieved with Agilent 6410 Triple Quad LC/MS equipped with Agilent 1200 Series Binary Pump SL pumping system and Agilent 1200 Autosampler. Data acquisition and analysis were performed using an Agilent MassHunter Workstation software (Agilent Corporation, MA, USA).

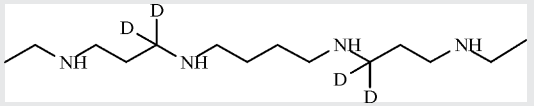
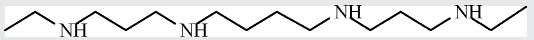
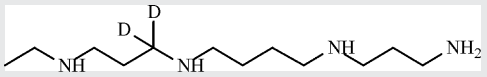
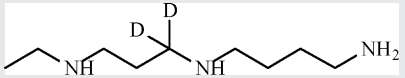
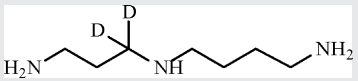
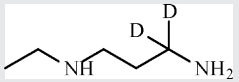
The chromatographic separations were carried out using a Phenomenex Gemini reversed phase C18 column (3 μm, 30 mm × 2 mm, 110 Å) protected with a Phenomenex C18 guard column (4 mm × 2 mm). The column was thermostated to 25 °C and the autosampler tray temperature was set at 10 °C. The injection volume was 10 μL and the injection was performed using 10 s needle wash with 50% ACN. A gradient solvent system consisting of 0.1% (v/v) HFBA in water (solvent A) and 0.1% (v/v) HFBA in ACN (solvent B) was used and the gradient was increased from 2 to 50% B over 10 min at a flow rate of 0.2 mL min^{-1}.

Ionization was achieved using electrospray ionization (ESI) in the positive mode with the capillary voltage 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 40 psi. Drying gas (nitrogen) temperature was 300 °C and gas flow was 8 L min^{-1}. Fragmentor voltage value was set to 90 V for all analytes to obtain the highest precursor ion abundance. MS/MS experiments were based on selected reaction monitoring (SRM) analysis, with high purity nitrogen as collision gas. The product spectra of each polyamine were recorded similarly as described earlier [8]. Precursor ions, selected product ions for quantification and qualification, and collision energy values for all analytes used in the following quantitative SRM analysis are given in Table 1. Both resolving quadrupoles were maintained at unit resolution (0.7 amu) during SRM analysis. Dwell time for all was adjusted to 50 ms, and electron multiplier voltage was 1400 V.

2.3. Preparation of standards

Stock solutions of five calibration standards and five internal standards were prepared by dissolving an appropriate amount in 0.5 or 1 mL D_2O to yield a concentration of approximately 100 mM. Weighing of the analytes was done with a calibrated analytical balance (Ohaus GA 200 D, England). All solutions were also analyzed by NMR (Bruker Avance (Bruker, Rheinstetter, Germany) spectrom-

Table 1
Structures and mass spectra properties of polyamines used in this study

	Structure	Calculated mass (amu)	Measured precursor ion $[M+H]^+$	Quantifier ion MS/MS Q1>Q3 (m/z)	CID (eV)	Qualifier ion (m/z)	CID (eV)	Other product ions, not included in the quantification assay	Retention time (R.S.D.% $n=175$)
DESpm-4D		262.30	263.3	263 > 159.1	10			114.1, 88.0	7.42 (0.01)
DESpm		258.28	259.2	259 > 157.1	10	259 > 112.0	20	86.0	7.43 (0.02)
EtSpm-2D		232.26	233.2	233 > 159.1	10			129.1, 114.0, 112.0	7.22 (0.01)
EtSpm		230.25	231.2	231 > 157.1	10	231 > 129.1	10	112.0	7.23 (0.01)
EtSpd-2D		175.20	176.1	176 > 88.0	15			114.0, 72.0	6.15 (0.01)
EtSpd		173.19	174.1	174 > 86.0	15	174 > 72.0	15	112.0	6.15 (0.01)
Spd-2D		147.17	148.0	148 > 114.0	10			72.0	5.57 (0.02)
Spd		145.16	146.1	146 > 112.0	10	146 > 72.0	15		5.57 (0.01)
EtDAP-2D		104.13	105.1	105 > 88.0	5			60.0	3.08 (0.12)
EtDAP		102.12	103.1	103 > 86.0	5	103 > 58.0	15		3.08 (0.09)

CID, collision energy.

eter operating at 500.13 MHz to ensure the actual concentration of the stock solutions and the purity of the analytes. Quantitative NMR spectra were measured from each stock solution diluted to 1:1 TSP (100 mM) as an internal reference and collecting 32 scans using a 90° pulse angle, and a 60 s relaxation time. Stock solutions were stored in a refrigerator at a temperature between 1 and 9 °C.

One millimolar stock solution of five polyamines in 90 mM Gly–NaOH–FA buffer was prepared by pipetting 100 μL of 10 mM mixture of five polyamines in water, 100 μL 50% formic acid, 90 μL 1 M glycine–NaOH buffer pH 9.5 and 710 μL water. Standard working solutions (STD) were then prepared by diluting this 1 mM stock solution with 90 mM Gly–NaOH–FA buffer (90 mM glycine–NaOH buffer, pH 9.5, to which 50% FA has been added to yield 5%, v/v FA) to achieve STD concentrations of 0.03, 0.1, 0.3, 1, 3, 10, 30 and 60 μM. Working solutions for quality control (QC) samples were prepared from a separate stock solution by diluting with the same 90 mM Gly–NaOH–FA buffer to achieve concentrations of 0.05, 0.2, 2 and 20 μM. Internal standard (IS) working solution containing 1 μM of each five deuterated polyamine analogue was prepared from 100 mM stock solutions by diluting with water. All the dilutions were made daily using volumetric pipettes and flasks.

Each calibration standard was prepared by pipetting 100 μL of each STD working solution, 100 μL of 1 μM IS working solution and 50 μL 0.5% HFBA in a polypropylene tube. Calibration curves were drawn by having accurate concentration of working solution in *x*-axis, and peak-area ratio sample vs. internal standard in *y*-axis. Final concentrations in the sample vials were 0.012, 0.04, 0.12, 0.4, 1.2, 4, 12 and 24 μM, and an IS concentration of 0.4 μM. After vortexing, samples were transferred into Agilent glass vial inserts (Borosilicate glass, part number 5181-3377) for the LC–MS/MS analysis.

2.4. Metabolic studies of DESpm

Enzymatic degradation of DESpm was performed with hPAO. Reactions were carried out in a total volume of 360 μL including 100 mM glycine–NaOH buffer pH 9.5, 1 mM DESpm and 1 μg hPAO. The reactions were allowed to proceed for the indicated time at +37 °C and then stopped by the addition of 40 μL of 50% formic acid in water. The incubation at time 0 min was stopped immediately, with the second incubation lasting 30 min and the third one for 60 min. In addition, a sample containing only hPAO and no DESpm was included to ensure that no interference in the IS or STD MS/MS channels was arising from the enzyme addition. Parallel samples not containing hPAO were also studied to ensure that all metabolites were arising only from the reaction with hPAO. All reactions were carried out in triplicate and stored at −20 °C.

2.5. Sample preparation

Prior to LC–MS/MS analysis, samples were diluted to 1:10 with 90 mM Gly–NaOH–FA buffer and passed through a 0.22 μm filter. Dilution with the buffer did not alter the sample matrix. Samples were prepared by pipetting 100 μL of diluted sample solution, 100 μL of IS working solution and 50 μL 0.5% HFBA in a polypropylene tube. After vortexing, samples were moved in Agilent glass vial inserts for the LC–MS/MS analysis.

QC samples were prepared similarly as the standards and the incubation samples by pipetting 100 μL of each QC working solution, 100 μL of 1 μM IS working solution and 50 μL 0.5% HFBA in a polypropylene tube. Final QC concentrations in the sample vials were 0.02, 0.08, 0.8 and 8 μM, and IS concentration of 0.4 μM. The calibration curve included a blank sample (100 μL of 90 mM Gly–NaOH–FA buffer, 100 μL of water and 50 μL 0.5% HFBA) and a "zero" sample of 100 μL of 90 mM Gly–NaOH–FA buffer, 100 μL of 1 μM IS working solution and 50 μL 0.5% HFBA).

2.6. Assay validation

Assay validation was performed according to the FDA guideline for bioanalytical method validation [24], with two exceptions. Recovery was not determined as the sample preparation did not include sample extraction or processing steps. Inter-laboratory precision was not studied as all the samples were analyzed in the same laboratory using the same instruments.

Calibration standards were analyzed before the samples within each analysis batch. Calibration curves, ranging from 0.03 to 60 μM (in the original sample, 0.012–24 μM in the sample vial) of the five analytes, were run on four separate days. Calibration included a blank sample and a "zero" sample. Calibration curves were constructed from the peak-area ratios of each analyte to their deuterated analogues as an IS using a $1/x$ weighted linear least-squares regression model.

Five replicates of QC samples at four concentrations (0.05, 0.2, 2 and 20 μM in the original QC sample, 0.02, 0.08, 0.8 and 8 μM in the QC sample vial) were included in each run to determine the intra-day and inter-day precision and accuracy of the assay. Accurate concentrations of QC working solutions were used in the method validation. R.S.D. of the concentrations was used as an index of precision. Accuracy was calculated by comparing the mean experimental concentrations of assayed QC samples with their nominal values, and percentage values were used as the index.

The lower limit of quantification (LLOQ) for the method was defined as the lowest working solution concentration analyzed with accuracy within 80–120% and precision better than 20% R.S.D. [24]. The LLOQ was determined by calculating precision and accuracy for six samples that were independent of the calibration curve.

The system suitability was checked by performing three replicate injections of 2 μM QC sample (0.8 μM in the sample vial). R.S.D. of peak areas of three injections was below 1.5%.

The stability of five deuterated polyamine analogues in water and the level of interference to STD MS/MS channels were assessed by analyzing 100 μM IS stock solution after 1 week of storage in a refrigerator before sample preparation. Final sample contained 50 μM IS in 0.1% HFBA. The interference arising from the STD solution to IS MS/MS channels, was assessed by analyzing the sample containing 24 μM STD and 0.1% HFBA in 90 mM Gly–NaOH–FA buffer. SRM analysis included transitions to qualifier and quantifier product ions and also transitions to other product ions described in Table 1. The percentage level of interferences to STD MS/MS channels from IS solution was calculated from the peak-area ratios of each analyte to their deuterated analogues using the equation $A_{std}/A_{IS} \times 100\%$, and the interference to IS MS/MS channels from STD solution was calculated similarly ($A_{IS}/A_{std} \times 100\%$).

The stability of five analytes in samples was assessed by analyzing 2 μM QC working solutions stored for 18 h at room temperature (short-term temperature stability), stored for 1 week in a refrigerator at a temperature between 1 and 9 °C (long-term stability) and after going through three cycles of freezing at −20 °C before sample preparation and analysis. For the post-preparative stability study, the same sample was analyzed after the sample preparation and again after 20 h storage in the instrument autosampler.

For the stability studies of working solutions in glass containers, 2 μM QC working solution was stored in a glass container at 10 °C for 24 h before the sample preparation and analysis. Results were compared to 2 μM QC working solution stored in polypropylene tube at the same conditions.

Three different container systems for the samples were evaluated by analyzing three different samples; one which was transferred after sample preparation into Agilent glass vial inserts (part number 5181-3377), the other in Agilent deactivated vial insert (part number 5183-2086) and the third in Agilent polypropy-

lene insert (part number 5182-0549) for the LC–MS/MS analysis. These three post-preparative samples were analyzed after the sample preparation and again after 20 h storage in the instrument autosampler.

The effect of the filtration through 0.22 μm filtter to the sample concentration was evaluated by analyzing 2 μM QC working solution sample filtered through 0.22 μm filter before sample preparation and comparing the results to the unfiltered samples.

A post-column infusion experiment was performed to evaluate the matrix effect after sample injection. The infusion setup consisted of a post-column PEEK mixing tee (VICI Jour Research AB, Sweden) and syringe pump (801 Syringe pump, Univentor, Malta) as previously reported [25]. A constant flow of 1 μM stock solution of all five standards and five IS in water containing 0.1% HFBA was delivered via the PEEK mixing tee to the mobile phase at flow rate 6 μL min^{-1}. All quantifier and qualifier product ions listed in Table 1 were monitored after the injection and gradient elution of the blank sample.

3. Results and discussion

3.1. LC–MS/MS optimization

Analyses were performed by RP-LC followed by ESI in the positive ion mode and MS/MS detection. The structures, molecular masses, retention times, measured precursor ions and MS/MS data for quantification of the compounds studied are summarized in Table 1. All studied polyamines produced protonated $[M+H]^+$ molecules and no adduct formation was observed [8]. In order to determine the optimal conditions for SRM analysis, the positive ion product spectra of each polyamine were recorded as described previously [8]. The fragment ions in the IS and STD MS/MS spectra are presumably similar type ring structures as described before [19,26–28], although product ions with linear structures are also possible [20,29].

An excellent chromatographic separation of the quantified analytes with good symmetrical peak shapes was achieved in 10 min using volatile HFBA as the ion-pairing agent essentially as reported before [8], with few exceptions. The gradient was increased from 2 to 50% organic and no post-column addition of propionic acid (PrA) was used to prevent suppression. HFBA is known to suppress ionization in the MS, and the addition of PrA has been demonstrated to compensate for this suppression to some extent [8,30–32]. Post-column addition of IPA/PrA was shown to increase polyamine signal in electrospray by 4–10-fold [8]. However, addition of IPA/PrA results in severe background in certain studied MS/MS channels, especially concerning EtSpm ion transition m/z 231–157. Since the PrA addition was not beneficial for all analytes, and to keep the chromatography as simple as possible, no post-column addition was used in this study.

In the experimental conditions, the retention times for each compound did not exhibit any significant changes (R.S.D. <0.12%, $n = 175$) as shown in Table 1 for each compound. However, a high carry-over (approx. 0.1%) after concentrated samples was noted during chromatographic method validation. In particular, traces of DESpm and EtSpm remained in the system after several blank injections. One possible source for the contamination is the injector valve. Standards and QC samples were analyzed before the samples within each analysis batch, and reasonable numbers of blank injections were performed before the samples to minimize any carry-over effects.

The interference arising from IS in the STD MS/MS channels, and from the STD solution in the IS MS/MS channels, was studied as described in Section 2. The interference arising from IS in the STD MS/MS channels ranged from 0 to 0.2%, and the interference from the STD solution in the IS MS/MS channels ranged from 0.04 to 0.5%.

The matrix effect time window after sample injection was studied as described in Section 2. No ion suppression arising from the matrix was found to affect to the sensitivity of the method.

3.2. Sample preparation

The solvent used for sample dilution can influence the chromatographic separations, since the compounds are not retained completely by the column without using 0.1% HFBA for dilution, probably because of the insufficient replacement of the polyamine chloride ions with solvent acid ions [8]. For this reason all the samples, standards and QC samples were prepared to a final concentration of 0.1% HFBA.

Metabolic studies of DESpm were carried out using 100 mM Gly–NaOH buffer pH 9.5, and incubations were stopped with 50% FA resulting in the sample matrix solution to be 90 mM in concentration of Gly–NaOH and 5% of FA. All the standards and QC samples were prepared to have the same matrix to ensure that accuracy, precision, selectivity and sensitivity would not be affected when any samples originally above the upper limit of the standard curve were diluted with the 90 mM Gly–NaOH–FA buffer. To compensate for any possible bias in accuracy originating from the sample preparation, all the samples, standards and QC samples were prepared similarly by adding IS solution and HFBA solution to the sample.

3.3. Linearity of calibration curves and lower limit of quantification (LLOQ)

The method was linear for DESpm, EtSpd, Spd and EtDAP over the range 0.03–60 μM (0.012–24 μM in the injected solution) and for EtSpm over the range 0.03–30 μM (0.012–12 μM in the injected solution). The mean equations of the calibration curves ($n = 4$) with standard deviations are shown in Table 2. X is the concentration of each analyte in the sample before sample preparation (μM) and Y is the peak-area ratios of each analyte to its deuterated analogue. The correlation coefficients (R^2) were always >0.995 for all analytes. The mean correlation coefficients are shown in Table 2.

The LLOQ for determination of DESpm, EtSpm, EtSpd, Spd and EtDAP, was 0.03 μM (0.012 μM in the injected solution) for all, which is equivalent to 49 pg of DESpm, 45 pg of EtSpm, 34 pg of EtSpd, 31 pg of Spd and 21 pg of EtDAP as their hydrochloride salts injected on-column. LC–MS/MS SRM chromatograms of Spd (the common endogenous polyamine) in the zero, LLOQ and 60 min incubation sample as an example, are shown in Fig. 1a–c.

3.4. Assay precision and accuracy

For all QC levels the intra-day precision ranged from 0.2 to 9.7% (R.S.D.) and accuracy ranged between 87.6 and 109.8%. The inter-day precision of the overall method ranged between 0.9 and 6.8%,

Table 2
The mean equations of the calibration curves ($n = 4$) with standard deviations shown in parentheses

Compound	Regression equation	R^2
DESpm	$Y = 1.2738\ (0.0339)X + 0.0012\ (0.0061)$	0.9998 (0.0001)
EtSpm	$Y = 1.2555\ (0.0346)X + 0.0027\ (0.0008)$	0.9980 (0.0007)
EtSpd	$Y = 0.9628\ (0.0133)X + 0.0008\ (0.0010)$	0.9994 (0.0002)
Spd	$Y = 1.1087\ (0.0186)X + 0.0032\ (0.0013)$	0.9966 (0.0013)
EtDAP	$Y = 1.1863\ (0.0391)X + 0.0191\ (0.0021)$	0.9993 (0.0006)

X is the concentration of each analyte in the sample (μM), Y is the peak-area ratios of each analyte to its deuterated analogue, and R^2 is the mean correlation coefficient.

Table 3
Precision and accuracy for the method. Accurate LLOQ and QC working solution concentrations were used in method validation

	Concentration (μM)	Measured concentration (μM)[a]				Precision (R.S.D. %)		Accuracy (%)	
		Day 1	Day 2	Day 3	Day 4	Intra-day[b]	Inter-day[c]	Intra-day [b]	Inter-day[c]
DESpm	0.0285	0.0310 ± 0.0004*	0.0302 ± 0.0014*	0.0298 ± 0.0004*	0.0299 ± 0.0019*	1.3*	3.8 (24)	104.5*	106.1 (24)
	0.0475	0.0470 ± 0.0008*	0.0446 ± 0.0012	0.0453 ± 0.0015	0.0423 ± 0.0014	2.7	4.4 (21)	95.4	94.6 (21)
	0.1900	0.1737 ± 0.0032	0.1665 ± 0.0036	0.1726 ± 0.0015	0.1679 ± 0.0025	0.7	2.2 (20)	90.8	89.6 (20)
	1.9000	1.7253 ± 0.0687	1.7917 ± 0.0217	1.8272 ± 0.0223	1.7993 ± 0.0237	1.0	2.7 (20)	96.2	94.0 (20)
	19.0000	18.9772 ± 0.3832**	18.9351 ± 0.1055	18.8975 ± 0.2665	19.0345 ± 0.1467	1.1	0.9 (19)	99.5	99.8 (19)
EtSpm	0.0293	0.0282 ± 0.0020*	0.0290 ± 0.0020*	0.0288 ± 0.0017*	0.0284 ± 0.0018*	5.6*	5.9 (24)	98.4*	97.8 (24)
	0.0488	0.0472 ± 0.0009*	0.0467 ± 0.0020	0.0473 ± 0.0028	0.0467 ± 0.0018	4.8	3.2 (21)	97.0	96.3 (21)
	0.1951	0.1917 ± 0.0065	0.1896 ± 0.0038	0.1899 ± 0.0057	0.1946 ± 0.0030	2.4	2.2 (20)	97.3	98.1 (20)
	1.9510	1.9925 ± 0.0545	2.0508 ± 0.0435	2.0354 ± 0.0159	2.0265 ± 0.0277	0.6	1.8 (20)	104.3	103.9 (20)
	19.5100	19.8600 ± 0.3410**	19.5803 ± 0.2071	19.5680 ± 0.2591	19.5976 ± 0.2157	1.1	1.1 (19)	100.3	100.7 (19)
EtSpd	0.0285	0.0291 ± 0.0013*	0.0290 ± 0.0008*	0.0289 ± 0.0009*	0.0288 ± 0.0015*	2.8*	3.5 (24)	101.4*	101.5 (24)
	0.0475	0.0481 ± 0.0019*	0.0472 ± 0.0018	0.0460 ± 0.0019	0.0459 ± 0.0040	3.3	4.6 (21)	96.9	98.6 (21)
	0.1901	0.1843 ± 0.0059	0.1822 ± 0.0057	0.1831 ± 0.0043	0.1780 ± 0.0035	1.9	2.4 (20)	96.3	95.7 (20)
	1.9014	1.8300 ± 0.0697	1.8879 ± 0.0472	1.9052 ± 0.0328	1.8735 ± 0.0178	1.4	2.4 (20)	100.2	98.6 (20)
	19.0140	19.5120 ± 0.4093**	19.5534 ± 0.3746	19.4347 ± 0.0439	19.2874 ± 0.1849	0.2	1.1 (19)	102.2	102.3 (19)
Spd	0.0302	0.0285 ± 0.0027*	0.0285 ± 0.0029*	0.0289 ± 0.0016*	0.0282 ± 0.0011*	5.3*	6.8 (24)	95.5*	94.4 (24)
	0.0504	0.0487 ± 0.0009*	0.0511 ± 0.0036	0.0496 ± 0.0018	0.0500 ± 0.0029	2.9	4.1 (21)	98.4	98.8 (21)
	0.2015	0.2073 ± 0.0131	0.2100 ± 0.0053	0.1989 ± 0.0087	0.2091 ± 0.0034	3.5	3.7 (20)	98.8	102.4 (20)
	2.0146	2.1011 ± 0.0327	2.1631 ± 0.0258	2.1181 ± 0.0286	2.1648 ± 0.0200	1.1	1.6 (20)	105.1	106.1 (20)
	20.1460	21.5265 ± 0.5214**	21.6929 ± 0.3505	21.1469 ± 0.5069	21.5169 ± 0.4361	1.9	1.8 (19)	105.0	106.6 (19)
EtDAP	0.0293	0.0322 ± 0.0013*	0.0296 ± 0.0016*	0.0302 ± 0.0010*	0.0293 ± 0.0019*	3.0*	5.7 (24)	103.0*	103.4 (24)
	0.0489	0.0477 ± 0.0017*	0.0489 ± 0.0031	0.0488 ± 0.0027	0.0474 ± 0.0028	4.4	4.3 (21)	99.7	98.5 (21)
	0.1956	0.1787 ± 0.0037	0.1833 ± 0.0084	0.1766 ± 0.0035	0.1839 ± 0.0011	1.6	2.7 (20)	90.3	92.4 (20)
	1.9560	1.8077 ± 0.0559	1.9526 ± 0.0292	1.8825 ± 0.0483	1.9382 ± 0.0730	2.0	3.7 (20)	96.2	96.9 (20)
	19.5600	19.2735 ± 0.4312**	20.0995 ± 0.5752	19.4990 ± 0.5199	20.2537 ± 0.5777	2.1	2.8 (19)	99.7	101.3 (19)

[a] Mean values ± 95% confidence intervals ($n = 5$), * $n = 6$, ** $n = 4$
[b] Values obtained from runs on day 3 ($n = 5$), * $n = 6$.
[c] Values obtained from all runs on four separate days. Number of replicates in parenthesis.

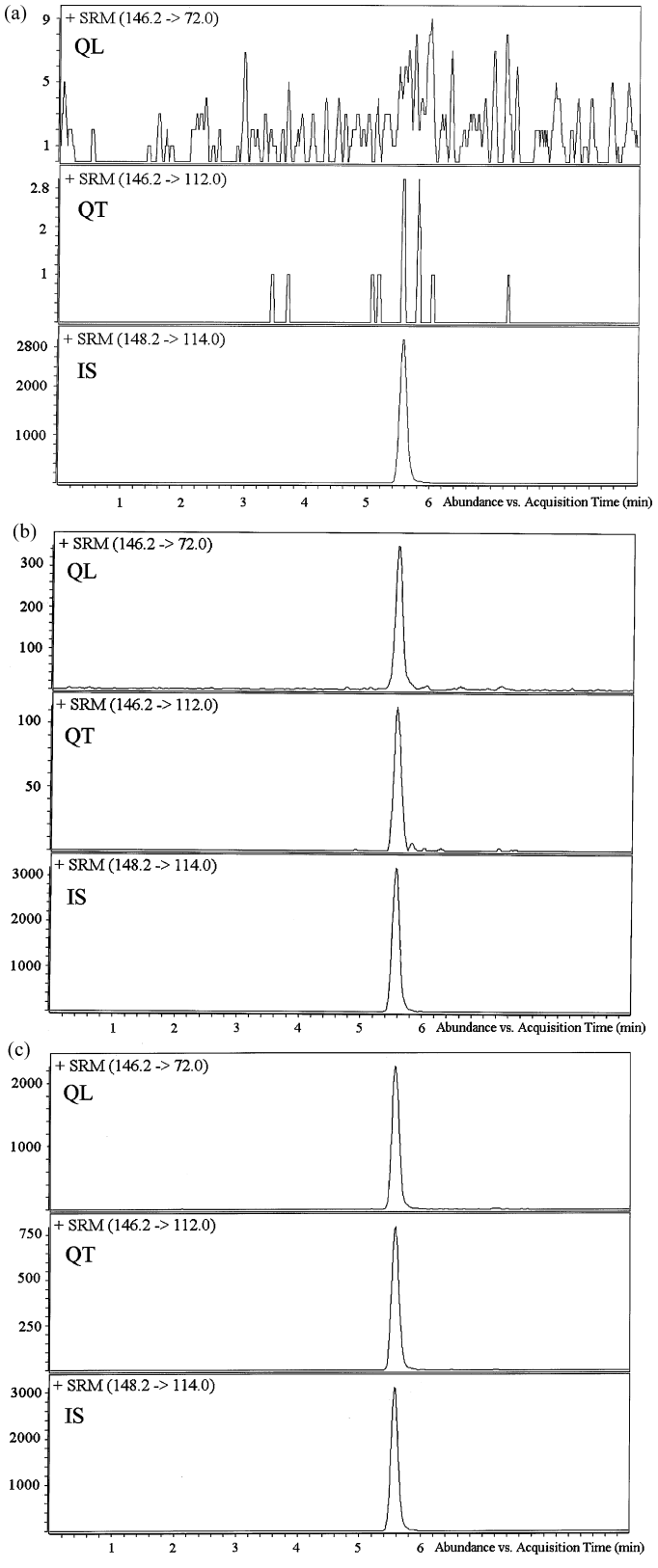


Fig. 1. Positive ion SRM chromatograms of Spd in the mixture of five studied polyamines and their deuterated analogues as an IS. (a) Zero sample, corresponding to buffer, 0.4 μM IS and 0.1% HFBA in the sample vial with injection volume of 10 μL; (b) LLOQ (0.03 μM) sample, corresponding to 0.012 μM STD, 0.4 μM IS and 0.1% HFBA in the sample vial with injection volume of 10 μL; (c) 60 min incubation sample. QT, quantifier transition; QL, qualifier transition; IS, deuterated analogue as an internal standard.

and the inter-day accuracy of the assay ranged between 89.6 and 106.6%. The results for LLOQ and QC are shown in Table 3.

3.5. Stability of the analytes

Stock solutions of polyamines and their deuterated analogues were stable for at least 18 h at room temperature, 1 week in a refrigerator, and at least 1 week in a freezer. Working standard solutions were stable in polypropylene tubes for at least 1 week. Analytes were also found to be stable after three freeze–thaw cycles and no degradation was seen during post-preparative storage in the instrument autosampler.

Glass tubes were noted to adsorb the analytes in low concentration samples when stored at 10 °C for 24 h, so all the solutions should be stored in polypropylene containers. However, analytes were stable and no significant adsorption was observed when the samples containing 0.1% HFBA were stored in Agilent glass vial inserts, Agilent deactivated glass vial inserts or Agilent polypropylene vial inserts in the instrument autosampler at 10 °C for 24 h.

3.6. Application of the method to metabolic studies of DESpm

Enzymatic degradation of DESpm was performed with hPAO and quantification of DESpm metabolites was carried out using the conditions described in detail in Section 2. Positive ion LC–MS/MS SRM chromatograms of a 60 min incubation experiment, as an example, are shown in Fig. 2. Quantitative results for measured samples, with 95% confidence intervals ($n = 3$) in parenthesis, are shown in Table 4. No interference was arising from the enzyme addition to the sample in the IS or STD MS/MS channels (data not shown). Metabolites formed in the catabolism of DESpm included EtSpm, EtSpd, Spd and EtDAP as already reported previously [8]. Unlike before, in this study DESpm, used as an enzyme substrate, contained no traces of EtSpm. During the studies, concentrations of four DESpm metabolites were 0.03–60 μM, after dilution of the samples to 1:10 with the buffer, which did not alter the matrix composition.

When assessing the results shown in Table 4, it can be concluded the major metabolite was EtSpd (82%), indicating that DESpm is catabolized in a similar manner as diacetylated spermine [6]. How-

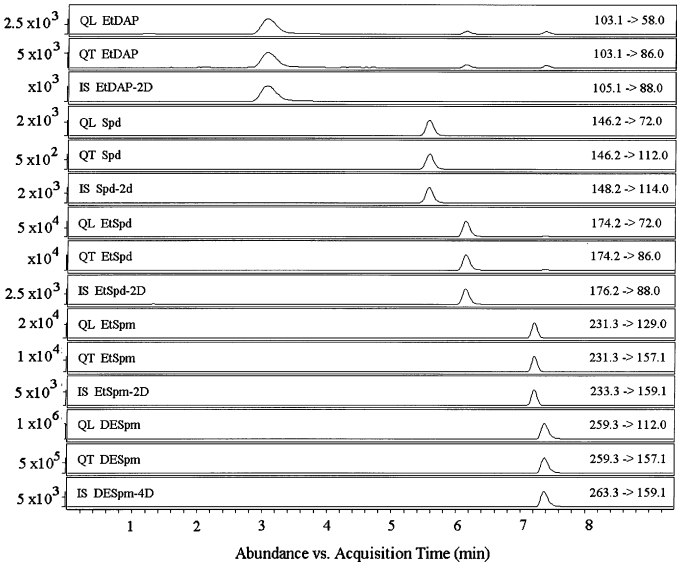


Fig. 2. Positive ion SRM chromatograms of a 60 min incubation experiment carried out with DESpm as the substrate of hPAO. QT, quantifier transition; QL, qualifier transition; IS, deuterated analogue as an internal standard.

Table 4
Quantitative results (μM) of DESpm reactions with hPAO

Sample	DESpm	EtSpm	EtSpd	Spd	EtDAP
Without hPaO	94.24 (4.76)	ND	ND	ND	ND
0 min	94.29 (4.96)	0.03 (0.01)	0.07 (0.02)	ND	ND
30 min	86.64 (1.18)	1.13 (0.03)	7.79 (0.51)	0.072 (0.003)	0.56 (0.06)
60 min	78.49 (3.48)	1.87 (0.08)	14.56 (3.47)	0.254 (0.030)	1.03 (0.18)

Mean values from triplicate incubation samples, with 95% confidence intervals ($n = 3$) in parenthesis. ND, not detected.

ever, also EtSpm (11%) and EtDAP (6%) were detected, pointing to minor catabolic pathways such as de-ethylation [5], and the previously shown endo-cleavage [8]. Comparing the 30 min and 60 min incubation results, it can be seen that after 60 min incubation, the amounts of EtSpd and EtDAP are 1.87 and 1.84 times greater than the amounts resulting after the 30 min incubation, respectively. Thus, the formation of EtSpd and EtDAP is linear in time within 95% confidence intervals. However, the amounts of EtSpm and Spd after 60 min incubation were 1.66 and 3.53 times greater than the amounts resulting after the 30 min incubation, respectively. In conclusion, EtSpm appears to be further metabolized to Spd, mimicking the catabolism of N^1AcSpd by PAO [6].

4. Conclusions

All polyamines in the study (DESpm, EtSpm, EtSpd, Spd and EtDAP) were separated and quantified without any derivatization using the developed LC–MS/MS method. Stable isotope reference compounds of the studied molecules were included as internal standards to the method. HFBA as an additive in the LC separations has two important functions in the analysis of polyamines. It enables the chromatographic separation of the highly polar analytes, giving them good symmetrical peak shapes and prevents the unwanted interaction of these basic analytes to free silanol groups in the column and to capillaries in the instrument. The sensitivity of the LC–MS/MS method was about 30 times better than the previously used LC fluorescense system [33]. Moreover, the analysis time could be further decreased by using higher eluent flow rates.

The developed LC–MS/MS method described here can be recommended for use in quantitative polyamine analysis. In comparison to conventional methods of polyamine analysis, the developed LC–MS/MS is easier to use and is substantially faster due to minimal sample pre-treatment and rapid chromatographic separation. Moreover, the method allows absolute identification of the products by highly sensitive MS and the possibility to eliminate interfering peaks arising from the matrix by selective MS/MS. The developed method was successfully applied to quantify metabolites of DESpm as a substrate for hPAO. During the studies, concentrations of four DESpm metabolites were between 0.03 and 60 μM after dilution of the samples to 1:10 with the buffer, which did not alter the matrix composition. Quantitative analysis of the DESpm metabolites indicate that hPAO mediated degradation is more complicated than believed earlier.

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