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Development of a fast and reliable methodology for the determination of polyamines in urine by using a guard column as a low-resolution fractioning step prior to mass spectrometry. Comparison with flow injection-mass spectrometry analysis

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ABSTRACT

In this paper, we propose a fast and reliable method for the determination of polyamines and related compounds in urine samples using flow injection analysis directly coupled to a triple quadrupole mass spectrometer, with the inclusion of a guard column (XBridge® C18 guard column; 2.1 × 10 mm, 3.5 μm) as a low-resolution fractioning step prior to MS analysis. The use of the guard column resulted in a sensitivity enhancement, a critical issue because of the low concentration of the target compounds found in urine samples. Moreover, it represents an interesting alternative to the use of other sample preparation techniques. Using this instrumental setup, each sample can be analyzed in 2.7 min, which makes this method appropriate for screening analysis. A method including a chromatographic separation step is also proposed, if confirmation of the results is required (samples with abnormal concentrations of the compounds). In this case, the total analysis time was 33.3 min. All the steps involved in sample preparation and instrumental conditions were optimized. In both methods, an ion-pairing reagent was added to the mobile phase and to the diluted sample. For the screening method, the limits of detection were found to be between 0.6 and 21 μg/L and the limits of quantification between 2.0 and 70 μg/L. Influence of the matrix was confirmed; thus quantification was performed using the one-point standard addition method and normalization to IS. Comparable results were obtained with the screening and the confirmatory methods when applied to urine samples of apparently healthy volunteers, showing that the proposed screening methodology is suitable for rapid and reliable analysis of polyamines and related compounds in these samples.

1. Introduction

Nowadays, there is an increasing interest on the development of fast and reliable methodologies that allow the analysis of a great number of samples within the shortest time possible and with instrumental setups common in research laboratories [1,2]. With the use of mass spectrometry (MS), it is possible to perform the analysis with high sensitivity and selectivity and, depending on the coupled technique, with shorter or longer times of analysis. The use of strategies where the sample is introduced directly into the mass spectrometer allows shorter times of analysis compared to those in which there is a previous separation step (chromatographic or electrophoretic). In this way, it is possible to perform high throughput analysis, a very important aspect in routine analysis and when there is a large number of samples to analyze [2]. However, the use of these non separative methodologies based on direct injection into the mass spectrometer presents several disadvantages, such as matrix effects due to the absence of a previous separation step and the presence of isobaric interferences that could hamper analyte quantification if no specific fragmentation patterns are present [1,3]. These problems can be alleviated by the use of different sample treatment techniques, but, in many cases, imply extensive sample manipulation, specifically if the compounds are present at low concentrations in complex matrices [4–8].

Using guard columns is a widespread practice in analytical chemistry with different purposes, mainly as column protection [9–11] or as a sample clean-up step [12,13]. It also has been proposed the use of guard columns as chromatographic columns in order to perform liquid chromatography (LC), with times of analysis from 7 to 14 min [14–16].

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Recently, Couchman et al. [17] have proposed an ultra-fast LC-MS method using a guard column as the chromatographic one for the sub-minute analysis of therapeutic drugs in plasma samples, but it required the use of high performance pumps and double-needle autosamplers.

In the present work, we propose a fast and reliable method for the determination of polyamines and related compounds in urine samples. Two different options were evaluated, i) the use of flow injection analysis coupled to a mass spectrometer with an electrospray ion source and a triple quadrupole (FIA-MS/MS) and ii) the inclusion of a guard column as a low-resolution fractioning step prior to MS quantification (gC-FIA-MS/MS), in order to increase the sensitivity of the analysis. The final objective is the development of a method for screening purposes. A methodology including a chromatographic separation step (LC-MS/MS) is also proposed in order to confirm the results obtained with the screening one. This chromatographic method will be only used when required, that is, to analyze samples with altered concentrations of the compounds.

Polyamines and acetylated polyamines have long been associated with cancer [18] and elevated concentrations of these compounds have been found in urine of patients diagnosed with this disease [19–21]. Ornithine and gamma-amino butyric acid, also included in this study, are involved in the metabolic pathway of polyamines [18,22]. Accordingly, the development of fast and reliable methodologies for their determination is important, especially using samples that do not involve invasive sampling procedures. Several methodologies have already been proposed for the determination of the aforementioned compounds in urine samples, mainly using a chromatographic separation step [21,23–26]. Other non-separative methodologies have also been proposed, but they involve laborious sample preparative steps, such as solid phase extraction or the use of a derivatization reaction [4,5,27]. To the best of our knowledge, this is the first time that this fast approach (gC-FIA-MS/MS) is applied to the determination of these compounds in urine samples.

2. Material and methods

2.1. Chemicals

Putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm), N-acetylputrescine (N-AcPut), N^1-acetylspermine (N-AcSpm), L-ornithine (Orn) and γ-aminobutyric acid (GABA) were supplied by Sigma-Aldrich (Steinheim, Germany). Isotopically labelled internal standards putrescine-2,2,3,3-d_4 (Put-d_4), spermidine-(butyl-$^{13}C_4$) (Spd-$^{13}C_4$) and 4-aminobutyric acid-2,2,3,3,4,4-d_6 (GABA-d_6) were also supplied by Sigma-Aldrich, as well as methanol, heptafluorobutyric acid (HFBA), trifluoroacetic acid (TFA), propionic acid (PrA) and creatinine (Cre).

Sodium chloride, magnesium sulfate, ammonia, hydrochloric acid, formic acid and acetic acid were supplied by Scharlau (Barcelona, Spain). Potassium chloride, calcium chloride and urea were supplied by Panreac (Barcelona, Spain).

For isobaric interference evaluation, 4-acetamidobutyric acid, agmatine, 2-aminoisobutyric acid, D-2-aminobutyric acid, DL-3-aminoisobutyric acid, L-asparagine, choline, N^G,N^G-dimethylarginine, glycylglycine, 4-guanidinobutyric acid, hexylamine, isobutyric acid, isopentylamine, isovaleric acid, morpholine, pyruvic acid, sebacic acid, 3-ureidopropionic acid, uridine and valeric acid were supplied by Sigma-Aldrich.

The ultra-high quality (UHQ) water used throughout the study was obtained with a Wasserlab Ultramatic purification system (Noain, Spain).

2.2. Standard solutions and synthetic urine

Stock solutions of the target compounds were prepared in UHQ water at a concentration of 2500 mg/L. Cre was prepared at concentration of 1000 mg/L, as well as the isotope-labeled internal standards. All the solutions were stored in darkness at 4 °C and were used to spike the water, the synthetic urine and the urine samples at the different concentrations analyzed. Synthetic urine was prepared according to the procedure of N.C. Van de Merbel et al [28].

2.3. Sample preparation

First morning urine samples were collected from apparently healthy individuals of both sexes using disposable sterile containers and were frozen and stored at − 20 °C in the dark until analysis. After thawing at room temperature, samples were centrifuged at 1815*g* during 10 min to precipitate the proteins. The supernatant was filtered with nylon filters (0.45 μm, 17 mm i.d.) and 100 μL were added to a vial and mixed with 900 μL of UHQ water (0.1% HFBA, v/v). An aliquot (5 μL) was injected into the system. Creatinine content was measured using the Jaffé method [29].

2.4. Instrumental configuration

The LC-MS/MS instrumental setup consisted of a 1200 series LC chromatograph equipped with a binary pump, a membrane degasser, an autosampler, two six-port valves and a 6410 LC/MS triple quadrupole (QqQ) mass spectrometer, all from Agilent Technologies (Waldbronn, Germany). The triple quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source. The QqQ nebulizer pressure and voltage were set at 35 psi and + 4000 V, respectively. Nitrogen was used as the drying (12 L/min, 350 °C) and collision gas. Analyte quantification was carried out under Multiple Reaction Monitoring (MRM) mode (unit mass resolution). All the parameters related to the instrumental configuration are listed in Table S1 (Supplementary material).

For LC-MS/MS analyses, a Cortecs C18 reversed-phase analytical column (2.1 × 50 mm, 2.7 μm) from Waters (Milford, MA, USA) was used. The mobile phase consisted of a UHQ water (Solvent A) and MeOH (Solvent B) mixture. In both solvents, 0.1% HFBA (v/v) was added. The solvent gradient used was as follows: 2% B for 12 min, then from 2% to 70% B from 12 min to 14 min, hold at 70% B during 6 min, and then returning to 2% B from 20 to 22 min and holding conditions during 10 min. The flow rate used was 0.15 mL/min. Under these conditions, a suitable separation was achieved in less than 23 min and 9 min were required to re-equilibrate the column. The total chromatographic runtime was 32 min. Taking into account the time required for injection, each sample was analyzed in 33.3 min.

For FIA-MS/MS analyses, a six-port valve was used to switch from the screening to the chromatographic method without any instrumental modification. A peek tube was used to connect the valve to the mass spectrometer. This analysis mode was used for sample treatment optimization. Final conditions implied isocratic conditions, 100% MeOH (0.1% HFBA, v/v), and a flow rate of 0.5 mL/min. Total analysis run time was 1.4 min. Each sample was analyzed in 2.2 min.

In order to improve the analysis, a XBridge® C18 guard column (2.1 × 10 mm, 3.5 μm) was included in the FIA-MS/MS configuration. Isocratic conditions were used, with a mobile phase composition of 80% H_2O (0.1% HFBA, v/v) and 20% MeOH (0.1% HFBA, v/v) and the flow rate used was 0.5 mL/min. Total analysis runtime was 1.4 min. Each sample was analyzed in 2.7 min.

3. Results and discussion

3.1. Urine sample preparation

Protein content is a critical parameter because of ion suppression, especially when the samples are injected directly into the mass spectrometer. Protein precipitation was accomplished by the use of an acidified organic medium with different volatile acids [24,26]. HFBA,

formic acid and acetic acid were tested (0.1%, v/v), using MeOH as organic medium. The precipitation medium (300 μL) was mixed with 250 μL of the thawed urine and the mixture was vortexed (5 min) and centrifuged at 1815*g* for 10 min. The results obtained with the addition of the aforementioned acidified organic media were compared with those obtained when just a centrifugation step (1815*g*, 10 min) was used after thawing the sample at room temperature [21]. No significant differences were observed among procedures. Thus, the simplest one was selected; this was, just a centrifugation step after urine sample thawing.

3.1.1. Influence of the material used for sample filtration

After centrifugation, the supernatant was filtered in order to avoid particles in suspension. It has been described that some polyamines can interact with some materials [30]. For this reason, an evaluation of the kind of filters and syringes used for filtration was performed. Two types of syringes (glass syringes and disposable plastic syringes made of polypropylene and polyisoprene) and three types of filters (nylon, hydrophilic polytetrafluoroethylene and polyvinylidene fluoride, 0.45 μm) were assayed.

Regarding syringe nature, no significant differences were obtained for most of the compounds with both types of syringes, except for Spd, Spm and N-AcSpm with plastic ones, for which a dramatic decrease was observed (Fig. S1). The decrease of the signal was found to be between 25 and 70%. Reproducibility values were also worsened (RSD values between 20 and 57%). Thus, glass syringes were used for further experiments.

With regard to the kind of filters used, similar results were obtained for all of them, except for Orn and GABA. For these two compounds, the analytical signal was slightly superior when nylon filters were used (Fig. S2). Therefore, nylon filters (0.45 μm, 17 mm i.d.) were selected.

3.1.2. Study of sample dilution medium

Firstly, experiments were focused on the study of the stability of the compounds in the injection vial. As stated before, it has been described that the addition of an ion-pairing reagent to the medium avoids the adsorption of some of the target compounds on some material [30]. Thus, an evaluation of the addition of an ion-pairing reagent to the dilution media (UHQ water) was accomplished. TFA was initially selected at a concentration of 0.1% (v/v). The formation of the ion pair improved the stability of the compounds. The reproducibility of the analysis (5 replicates in two different days) was improved, especially for Spd and Spm, with values from 11 and 71% to 5 and 7% when TFA was present. Based on these results, a deeper evaluation of the ion-pairing reagents as well as the organic modifiers used was performed. Reagents were added to the dilution medium (UHQ water), as well as to the carrier phase (MeOH) (Table 1).

As ion-pairing reagents, TFA and HFBA were tested in the 0.01–0.1% (v/v) range. Higher values were not evaluated due to ionization suppression [30,31]. For TFA, the best results were obtained for a concentration of 0.05% v/v (Fig S3). For HFBA, the best results were obtained for a concentration of 0.1% v/v (Fig S4). When the results obtained with the two ion-pairing reagents were compared, an increment on the analytical signal was observed when HFBA 0.1% v/v was used (Fig S5), in good agreement with previous published results [30,32]. Thus, this concentration of ion-pairing reagent was selected for further experiments.

Propionic acid (PrA) was tested as organic modifier (0.1–2%, v/v). Several studies have demonstrated that the addition of PrA, a weak acid, enhance the analytical signal for basic analytes when volatile strong acids are used as ion-pairing reagents (i.e. TFA or HFBA). PrA competes with TFA or HFBA anions for protons and TFA or HFBA, which are more volatile than PrA, will evaporate faster from the droplet during the electrospray ionization process [30,33]. This study was performed with and without HFBA 0.1% (v/v). In both situations, the best results were obtained when 2% v/v PrA was used. However, several issues were observed. These were poor peak shapes and high carryover values (up to 27% when the ion-paring reagent was not used). This behavior could be attributed to the disruption in the formation of the ion pair caused by the PrA. Moreover, it was observed a peak corresponding to an interfering compound (unknown source). This isobaric interference appeared when a blank sample was injected and it shared the transition 203.1 → 129.1 with Spm. We also checked the possibility of adding the PrA (2%, v/v) exclusively to the dilution medium, but the presence of the interfering compound remained.

Finally, we compared the results obtained with the use of HFBA (0.1%, v/v) and HFBA (0.1%, v/v) and PrA (2%, v/v) altogether, this last one added just in the dilution medium. A urine sample spiked with the compounds at a concentration of 1 mg/L was used for this experiment. Results showed that the best analytical response was obtained when HFBA was used alone (Fig. S6). Signal increments ranged between 63% for N-AcPut to 106% for Spm. Thus, it was decided to use HFBA (0.1%, v/v) in both dilution medium and carrier phase for further experiments.

3.1.3. Urine dilution ratio

Different dilution ratios were evaluated (1/1, 1/5, 1/10, 1/20, 1/40, 1/50, 1/75 and 1/100, v/v). Fig. 1 shows the results obtained for GABA and Cad with the FIA-MS/MS and LC-MS/MS methods, respectively. Similar trends were found for the rest of the target compounds. A decrease in the dilution ratio did not imply a proportional increment on the analytical signal due to matrix effects. Moreover, it was observed that for 1/1 and 1/5 dilution ratios, two peaks appeared for Orn (2.0 and 2.9 min). This effect was only present for this compound at its endogenous concentration and it disappeared at dilution ratios higher than 1/10. The same effect was observed when synthetic urine was used as matrix and can be related to the ion content of the matrix, as it has been previously described for bile acids [34] or glyphosate [35]. Thus, based on these results, a 1/10 dilution ratio was selected for further experiments.

3.2. Optimization of the screening analysis

Screening analysis was first optimized using the FIA-MS/MS configuration. Isocratic conditions, i.e. methanol (0.1% HFBA, v/v), were used. Different flow rates (0.2–1.0 mL/min) and injection volumes (1–20 μL) were evaluated. For this experiment, creatinine signal was also recorded (transition 114.1 → 44.1) as an indicator of the passage of

Table 1
Sample treatment: study of ion-pairing reagents and organic modifiers (best results are highlighted in bold).

Sample	Solvent for Sample Dilution (UHQ water)	Carrier/mobile phase (MeOH)
UHQ water (spiked with target compounds at 1 mg/L)	HFBA (0.01–0.1% , v/v)	HFBA (0.01–0.1% ,v/v)
	TFA (0.01–0.1%, v/v)	TFA (0.01–0.1%, v/v)
	PrA (0.1–2%, v/v)	PrA (0.1–2%, v/v)
	PrA (0.1–2%, v/v) + HFBA (0.1%, v/v)	PrA (0.1–2%, v/v) + HFBA (0.1%, v/v)
	PrA (2%, v/v) + HFBA (0.1%, v/v)	HFBA (0.1% , v/v)
Natural urine (spiked with target compounds at 1 mg/L)	**HFBA(0.1% , v/v)**	**HFBA (0.1% , v/v)**
	PrA (2% v/v) + HFBA (0.1%, v/v)	HFBA (0.1% , v/v)

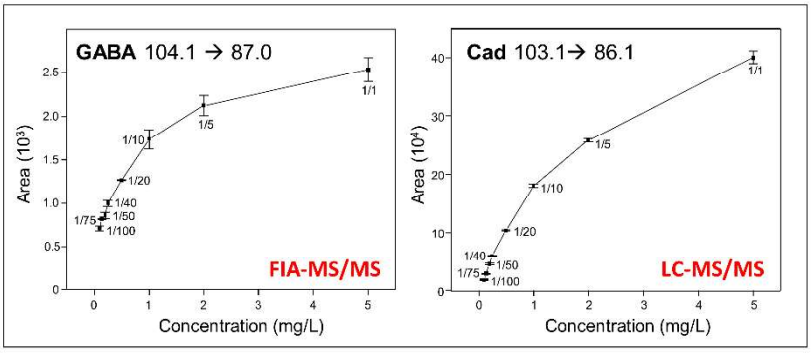

Fig. 1. Evaluation of different dilution ratios (1/1, 1/5, 1/10, 1/20, 1/40, 1/50, 1/75, 1/100, v/v) for GABA and Cad with the FIA-MS/MS and the LC-MS/MS methods, respectively.

the sample through the system. Optimum results were obtained with a flow rate of 0.5 mL/min and an injection volume of 5 μL. Under these conditions, it was possible to analyze each sample in 1.4 min. Although the signal of the compounds appeared in less than 1 min, it was decided to increase the time of analysis up to 1.4 min in order to allow creatinine signal to reach baseline. No re-equilibration time was required since the chromatographic column was removed from the system. Taking into account the time required to inject the next sample, it was possible to analyze 27 samples per hour (2.2 min per sample).

However, when using this configuration, it was observed that the sensitivity of the analysis was not enough for the reliable quantification of some of the target compounds, mainly because of the low concentration found in urine samples and the ion suppression effects caused by the direct introduction of the sample into the mass spectrometer. In order to overcome this issue, a guard column was included in the instrumental setup prior to MS detection. Within this purpose, a XBridge® C18 guard column (2.1 × 10 mm, 3.5 μm) was used. A mobile phase based on a UHQ water:MeOH mixture (both with 0.1% HFBA, v/v) was proposed. Different percentages of water content were considered, up to 98%. The results obtained with and without the presence of the guard column were compared (Fig. 2).

With a high water content in the mobile phase, a low-resolution fractioning of the target compounds was produced when the guard column was used since the weak mobile phase favored analyte retention in reverse phase. Three groups were observed: first group, GABA, Orn and N-AcPut; second group, Put and Cad and third group, Spd, Spm and N-AcSpm. Moreover, an increment on the analytical signals was observed for Put, Cad, N-AcPut, Orn and GABA when compared to FIA-MS/MS signals, as well as a slight peak shape improvement. This behavior could be explained in terms of the clean-up effect from the other components of the matrix and due to the slight separation of the compounds. Optimum results were obtained for a mobile phase composition of 80% UHQ water and 20% MeOH, both with 0.1% HFBA, v/v. However, under these conditions, the third group of compounds exhibited a high retention behavior. In order to decrease the retention of these analytes, a MeOH injection was included after the injection of the sample (sequential injection mode, 5 μL of sample and 50 μL of MeOH at an injection speed of 400 μL/min). Under these conditions, retention time reproducibility was evaluated. RSD values (%) were below 4.5% for all of the compounds (ten replicates at three different days). The inclusion of the guard column in the instrumental setup implied that each sample could also be analyzed in 1.4 min, 2.7 min taking into account the time required for injection (22 samples per hour). Fig. 3a and 3b show the signals obtained under optimum conditions when the FIA-MS/MS method and guard column (gC-FIA-MS/MS) one were used. Based on the obtained results, we decided to include the guard column in the instrumental configuration.

3.3. Confirmatory analysis by LC-MS/MS

The chromatographic separation of the target compounds was accomplished by using a Cortecs C18 reversed-phase analytical column (2.1 × 50 mm, 2.7 μm). Gradient elution was based on a mixture of UHQ water (solvent A) and methanol (solvent B), both with a concentration of 0.1% v/v of HFBA. Seven different gradients were evaluated and the best separation was achieved within the following conditions: 2% B for 12 min, then from 2% to 70% B from 12 min to 14 min, hold at 70% B during 6 min, and then returning to 2% B from 20 to 22 min and holding conditions during 10 min. The flow rate and injection volume were set at 0.15 mL/min and 5 μL, respectively. Under these conditions, a suitable separation was achieved in less than 23 min and 9 min were required to re-equilibrate the column. The total chromatographic run time was 32 min (Fig. 3c). Retention time reproducibility was evaluated for all of the analytes. Four different urine samples were analyzed (3 replicates each) and RSD values (%) were found to be below 1.5% for all of them.

Multiple reaction monitoring (MRM) acquisition parameters were optimized for the target compounds. Different fragmentors and collision energies were evaluated, in the 5 to 200 V and 1 to 60 eV range, respectively. The transitions selected for each analyte are shown in Table S1 (Supplementary material). Dwell time was also evaluated in the 1–100 ms range and a value of 20 ms was chosen as optimum.

3.4. Evaluation of possible MS interfering compounds

The presence of isobaric compounds is one of the main drawbacks when using methods based on direct injection mass spectrometry [1,36], especially when low resolution instruments are used. Therefore, an in-depth evaluation of the possible existence of interfering compounds was performed. An exhaustive bibliographic research, based on previous published articles and Human Metabolome Database and Metlin data search, was performed. A total of 20 possible interferences were found (see Section 2.1. for specific compound information). Aqueous standards of all of them were prepared and injected, and product ion spectra at different collision energies (1–40 eV) were recorded. Furthermore, all of them were also analyzed using the chromatographic method, in order to assign their retention behavior. Finally, six urines from different subjects were analyzed [37].

Four scenarios were found (Fig. 4): (i) no interference due to no similar fragmentation pattern (Fig. 4a), (ii) similar fragmentation pattern but absence of the compound in urine samples (Fig. 4b), (iii) similar fragmentation pattern but no significant contribution because of

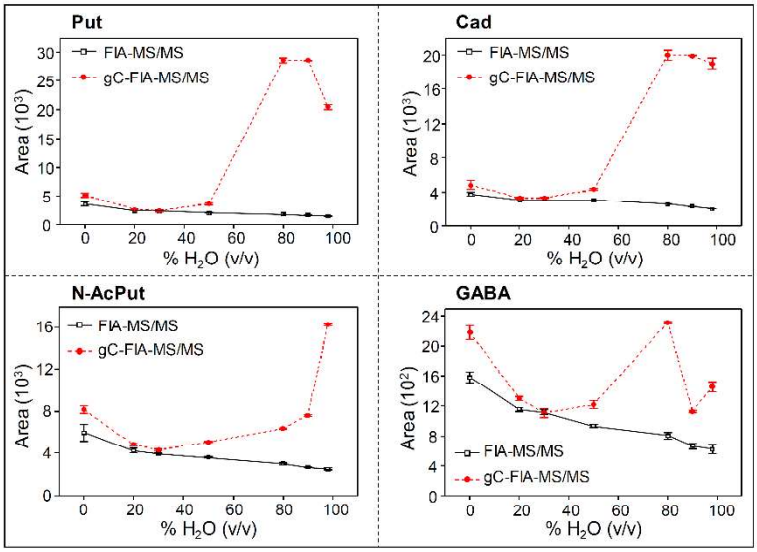

Fig. 2. Analytical signals obtained for putrescine (Put), cadaverine (Cad), N-acetylputrescine (N-AcPut) and gamma aminobutyric acid (GABA) in FIA-MS/MS and gC-FIA-MS/MS analyses with different percentages of UHQ water (v/v) in the carrier phase (MeOH, 0.1% HFBA, v/v).

the low concentration found in urine (Fig. 4c) and (iv) interference confirmed (Fig. 4d). Additional peaks were also observed, corresponding to unknown compounds. However, their contribution was not significant. Only two out of the twenty evaluated compounds were confirmed as interference. This were choline and N^GN^G-dimethylarginine. These compounds interfered with the transition 104.1 → 45.1 of GABA and 203.1 → 112.1 of spermine. Moreover, it was checked that the concentration of these compounds were higher than the concentrations found for GABA and Spm in urine samples. Taking into account those results, it was decided to use the transitions 104.1 → 87.0 and 203.1 → 129.1 for GABA and Spm quantification, respectively.

3.5. Analytical characteristics of the screening and confirmatory methods

The analytical characteristics of both screening and chromatographic methodologies were evaluated for a mixture of the eight target compounds in synthetic urine. Calibration curves were obtained at seven concentration levels (50–1000 μg/L) and each level was analyzed in triplicate. Internal standards were added at a concentration of 300 μg/L. They were used as follows: Put-d_4 was used for Put, Cad and N-AcPut area normalization, GABA-d_6 for Orn and GABA normalization and Spd-$^{13}C_4$ was used for Spd, Spm and N-AcSpm area normalization.

All the calibration curves displayed linear behavior. Their validity was checked using ANOVA analysis and it was observed that they did not exhibit any lack of fit. The values for the determination coefficient (R^2) were higher than 0.98 for every case. The slopes obtained for both methods were demonstrated significantly different, after comparing them using a Student *t*-test (significance level, 0.05). Tables 2 and 3 shows the results obtained for the two methods.

Limits of detection (LODs) and limits of quantification (LOQs) were evaluated. They were determined as the analyte concentration corresponding to a signal-to-noise ratio (S/N) of 3 and 10, respectively [38].

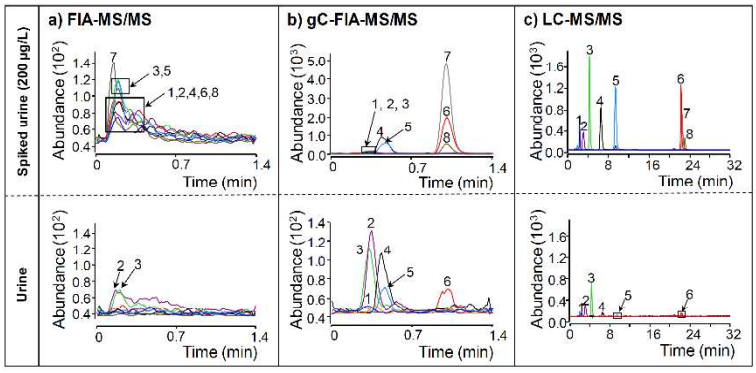

Fig. 3. MRM chromatograms of an unspiked and a spiked urine sample (200 μg/L) obtained with the a) FIA-MS/MS method, b) gC-FIA-MS/MS method and c) LC-MS/MS method. 1, Gamma aminobutyric acid; 2, ornithine; 3, N-acetylputrescine; 4, putrescine; 5, cadaverine; 6, spermidine; 7, N-acetylspermine, 8, spermine.

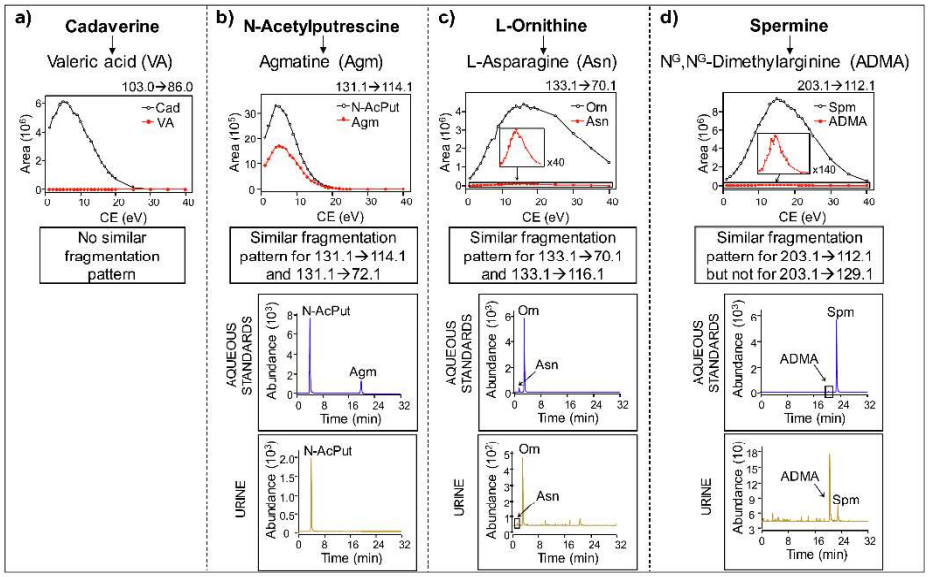

Fig. 4. Scenarios observed in the evaluation of interfering compounds: a) no interference due to no similar fragmentation pattern, b) similar fragmentation pattern but absence of the compound in urine, c) similar fragmentation pattern but no significant contribution because of the low concentration found in urine and d) similar fragmentation pattern and significant contribution.

For the screening method, LODs values were found to be between 0.6 and 21 μg/L and LOQs values between 2.0 and 70 μg/L. For the chromatographic one, LODs and LOQs were in the 0.4–8.2 and 1.2–27 μg/L ranges, respectively. Accuracies of both methods were also checked in terms of apparent recoveries, calculated as the ratio of the measured concentration to the spiked concentration (expressed in percentages). Values were found to be in the range of 80–120% for most compound, except Spm in the chromatographic method, with a recovery value of 69%. Repeatability and reproducibility values, expressed as relative standard deviations (RSD, %), using synthetic urine spiked at three levels of concentration (LOQ, 1.5 times the LOQ and 2 times the LOQ) are also shown in Tables 2 and 3 and values were found highly satisfactory.

3.6. Analysis of urine samples

Urine samples from two apparent healthy volunteers were analyzed. First, matrix effect was evaluated. Different matrices provided significant different slopes (Student *t*-test, significance level: 0.05). Thus, quantification was performed using a one-point standard addition and normalization to IS protocol using the procedure described in ref. [36,39]. Internal standards were added at a concentration of 300 μg/L.

Table 4 shows the concentrations found for the two analyzed urines. Values were normalized to creatinine content. For comparative purposes, results obtained with the screening and chromatographic methods were compared. Similar results were obtained for most of the compounds with both methods, except for Spd. For this compound, lower concentration values were found with the screening method, which results in an underestimation of its concentration. The concentrations found for the rest of the compounds were in good agreement with previous published results [40]. Spd behavior is still under study.

4. Conclusions

Here we have proposed a fast and reliable method for the determination of polyamines and related compounds in urine samples. The inclusion of a guard column in the FIA-MS/MS instrumental configuration resulted in a low-resolution fractioning of the target compounds with an increment on the analytical signals but without an

Table 2
Analytical characteristics of the screening method (gC-FIA-MS/MS) obtained with synthetic urine.

	Calibration range (μg/L)	R^2	b	LOD (μg/L)	LOQ (μg/L)	Repeatability (%)			Reproducibility (%)			Recovery (%)
						LOQ	1.5 LOQ	2 LOQ	LOQ	1.5 LOQ	2 LOQ	
GABA	50–1000	0.9800	0.0058 ± 0.0004	12	40	17	17	15	26	21	17	94
Orn	50–1000	0.9850	0.010 ± 0.001	21	70	8.8	11	16	14	14	14	102
N-AcPut	50–1000	0.9938	0.0020 ± 0.0001	2.3	7.6	9.2	8.4	7.1	9.7	9.1	6.8	96
Put	50–1000	0.9952	0.0034 ± 0.0001	3.3	11	7.1	5.0	8.3	21	13	13	100
Cad	50–1000	0.9926	0.0030 ± 0.0001	3.8	12	11	9.2	9.8	11	11	8.8	106
Spd	50–1000	0.9987	0.0032 ± 0.0001	1.7	5.5	11	5.0	6.3	12	7.9	9.5	102
N-AcSpm	50–1000	0.9957	0.0036 ± 0.0001	0.6	2.0	8.8	18	9.6	7.6	16	9.8	80
Spm	50–1000	0.9885	0.0012 ± 0.0001	2.9	9.6	7.0	10	8.1	9.0	8.9	8.6	98

Table 3
Analytical characteristics of the chromatography method (LC-MS/MS) obtained with synthetic urine.

	Calibration range (μg/L)	R^2	b	LOD (μg/L)	LOQ (μg/L)	Repeatability (%)			Reproducibility (%)			Recovery (%)
						LOQ	1.5 LOQ	2 LOQ	LOQ	1.5 LOQ	2 LOQ	
GABA	50–1000	0.9994	0.0066 ± 0.0001	3.3	11	5.8	5.1	2.1	7.9	6.5	2.2	104
Orn	50–1000	0.9986	0.0041 ± 0.0001	8.2	27	4.3	4.7	3.7	8.5	6.4	6.7	102
N-AcPut	50–1000	0.9981	0.0029 ± 0.0001	0.7	2.3	8.6	5.8	5.5	12	7.8	6.8	119
Put	50–1000	0.9997	0.00352 ± 0.00003	2.8	9.1	4.4	3.0	1.8	5.4	2.9	2.2	104
Cad	50–1000	0.9996	0.0059 ± 0.0001	2.0	6.7	3.3	2.5	2.8	5.2	5.7	5.8	96
Spd	50–1000	0.9995	0.00279 ± 0.00003	1.8	6.0	4.0	4.5	3.4	6.3	6.2	4.0	110
N-AcSpm	50–1000	0.9987	0.00186 ± 0.00003	0.4	1.2	8.6	7.8	6.4	9.4	6.8	5.4	94
Spm	50–1000	0.9988	0.00142 ± 0.00003	2.3	7.6	6.6	3.8	3.9	9.3	6.3	5.1	69

Table 4
Concentration (nmol/mmol creatinine) found of the target compound in two urine samples using the LC-MS/MS and gC-FIA-MS/MS methods.

	Urine 1		Urine 2	
	gC-FIA-MS/MS	LC-MS/MS	gC-FIA-MS/MS	LC-MS/MS
GABA	(8 ± 4) × 10^2	(5 ± 1) × 10^2	< LOD	(24 ± 4) × 10
Orn	(20 ± 6) × 10^2	(23 ± 2) × 10^2	(28 ± 9) × 10^2	(26 ± 2) × 10^2
N-AcPut	(10 ± 2) × 10^2	(11 ± 6) × 10^2	(12 ± 1) × 10^2	(139 ± 7) × 10
Put	(20 ± 6) × 10	(22 ± 5) × 10	(19 ± 4) × 10	(18 ± 1) × 10
Cad	(9 ± 4) × 10	(6 ± 2) × 10	(4 ± 2) × 10	24 ± 6
Spd	44 ± 4	(12 ± 3) × 10	(5 ± 3) × 10	(39 ± 7) × 10
N-AcSpm	< LOD	< LOD	< LOD	< LOD
Spm	< LOD	< LOD	< LOD	< LOD

increase on the time of analysis. All the steps involved in sample preparation and instrumental conditions were optimized. Urine samples were centrifuged and filtered using Nylon filters (0.45 μm) after thawing them at room temperature. Plastic syringes should be avoided in the filtration step due to analyte adsorption. In both methods, an ion-pairing reagent was added to the mobile phase and to the dilution medium of the sample. An in-depth evaluation of possible interfering compounds was also performed and it was only observed that choline and N^G,N^G-dimethylarginine could interfere with the quantification of GABA and Spm, respectively. These interferences were solved using the transitions 104.1 → 87.0 and 203.1 → 129.1 for GABA and Spm quantification, respectively.

The screening (gC-FIA-MS/MS) and chromatographic (LC-MS/MS) methodologies were evaluated in terms of linearity, reproducibility and repeatability, obtaining satisfactory results. The LODs and LOQs obtained was the μg/L range for both methodologies. As matrix effect was observed, a one-point standard addition and normalization to IS protocol was used for quantification. Comparable results were obtained with the screening and confirmatory methods, except for Spd. This proved that the screening methodology could be used as a fast and reliable method for determination of the rest of polyamines and related compounds in urine samples. The chromatographic method would be only used to analyze those samples with altered concentration of the target compounds.

CRediT authorship contribution statement

María Teresa Fernández-del-Campo-García: Investigation, Validation, Formal analysis, Writing - original draft. **Ana María Casas-Ferreira:** Conceptualization, Supervision, Writing - review & editing, Visualization. **Encarnación Rodríguez-Gonzalo:** Conceptualization, Supervision, Writing - review & editing, Visualization. **Bernardo Moreno-Cordero:** Writing - review & editing. **José Luis Pérez-Pavón:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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