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Rapid and reliable analysis of underivatized amino acids in urine using tandem mass spectrometry

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ABSTRACT

Nowadays there is a great interest in the development of fast and reliable methods for the determination of amino acids (AAs) in biological samples due to their biological importance. In the present work, a method based on the use of a guard column (gC) prior to tandem mass spectrometry (gC-MS/MS) is proposed for the determination of proteinogenic AAs in urine. Heptafluorobutyric acid (HFBA) is used as ion pairing reagent. Comparison of the gC-MS/MS method versus tandem mass spectrometry (MS/MS) in stand-alone mode showed improved sensitivity and peak shape, and solved some problems related to interfering compounds, with a total analysis time of 2.8 min. All the proteinogenic AAs were adequately determined using the gC-MS/MS method, except glutamic acid (Glu).

To confirm quantitative results obtained with gC-MS/MS for individual AAs, an ion-pair liquid chromatography tandem mass spectrometry method (LC-MS/MS) has also been developed. Both methods (gC-MS/MS and LC-MS/MS) were validated using synthetic urine. For the gC-MS/MS method, LODs and LOQs values were found to be between 0.004 and 0.425 mg/L and 0.01 and 1.40 mg/L, respectively. Aspartic acid (Asp) showed the highest LOD and LOQ values (3 mg/L and 9 mg/L, respectively). A one-point standard addition method and internal standard normalization were used for the quantification because matrix effects were observed. L-alanine-1-^{13}C (Ala-^{13}C) and L-leucine-1-^{13}C (Leu-^{13}C) were used as isotopically labeled internal standards.

To demonstrate the applicability of the gC-MS/MS method in the reliable determination of AAs in real samples, urine from eighteen healthy volunteers were analyzed using both gC-MS/MS and LC-MS/MS methods. Similar quantitative results were obtained for individual AAs with both of them. In addition, possible differences in AAs concentrations related to sex were checked, but the results did not show significant differences for the evaluated compounds.

1. Introduction

Amino acids (AAs) are important compounds involved in a great number of biological processes. There are two types of AAs: proteinogenic, that are encoded by genome and participate in the synthesis of proteins, and non-proteinogenic, that perform other functions such as neurotransmitters or metabolic intermediates. Alterations in the levels of AAs have been associated with different pathologies such as cancer [1,2], inborn errors of metabolism [3] and diabetes [4], among others.

Considering the important role of AAs in the organism, there is a great interest in the development of fast and reliable methods that allow their determination in biological samples. This fact is manifested in the large amount of literature referring to their determination in biological matrices, where interesting review articles have been reported [5–7]. Biological samples can be taken from patients using invasive or non-invasive sampling procedures. Samples whose collection is invasive, such as blood, suppose an added stress to the patient and are more difficult to obtain. In contrast, non-invasive samples, such as urine and saliva, do not necessarily entail stress for the patient and their collection is simple. For this reason, the analysis of the latter is of particular interest. Urine, compared to other biofluids, has a large number of metabolites and compounds of clinical interest [8]. In particular, it has been shown that the concentration of amino acids in urine can be used for the diagnosis and monitoring of different diseases [2,3].

Liquid chromatography-mass spectrometry (LC-MS) has been the most widely used technique for AAs analysis in urine samples [2,3,5,6],

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although gas chromatography and capillary electrophoresis coupled to MS have also been described [9,10]. When reversed-phase liquid chromatography (RPLC) is proposed, separation of AAs is not easily achieved and it is necessary to apply a previous derivatization reaction [2,11–13] or the addition of an ion pairing reagent in the mobile phase [3,14,15]. An alternative to RPLC-MS is the use of hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC-MS) [16–19]. Special columns for AAs, proteins and peptides separation have also been described [20,21].

Recently there has been a great interest in the development of stand-alone mass spectrometric methods, mainly due to its high speed of analysis and instrumental simplicity [22]. For the determination of AAs in urine, several applications can be found [23–25], although its use is still scarce. These methods proposed the use of unconventional equipment [24], laborious sample treatments [25] or the derivatization of the AAs [23].

In this work, a fast and reliable MS method is proposed for the determination of the proteinogenic AAs in urine. A guard column (gC) is placed prior to mass spectrometry detection (gC-MS/MS) to perform a short chromatographic separation step. The use of the gC enhances sensitivity and improves peak shape compared to the use of stand-alone tandem mass spectrometry (MS/MS) and reduces the analysis time in relation to conventional LC methods. The method proposed here involves a simpler sample treatment procedure than the already stand-alone MS methods proposed for the determination of AA in urine, without the need of a derivatization step [23–25]. To confirm the results obtained with the gC-MS/MS methodology, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method is also developed. The results obtained with these two methodologies were compared to demonstrate the applicability of the gC-MS/MS methodology. To the best of our knowledge, this is the first time that this approach (gC-MS/MS) has been applied to the determination of AAs in urine.

2. Material and methods

2.1. Chemical

Methanol (MeOH), creatinine (Cre) and heptafluorobutyric acid (HFBA) were purchased from Sigma Aldrich (Steinheim, Germany). The AAs: alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cystine (Cys-Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ille), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val) were supplied by Sigma-Aldrich. Isotopically labelled internal standards (ILIS) L-alanine-1-^{13}C (Ala-^{13}C) and L-leucine-1-^{13}C (Leu-^{13}C) were also obtained from Sigma-Aldrich.

Acetylglycine, creatine, guanidinoacetic acid, N-acetylasparagine, N-acetylornithine, ornithine and sarcosine were used for isobaric interference evaluation and were obtained from Sigma Aldrich.

Synthetic urine was prepared according to [26] using sodium chloride, magnesium sulfate, ammonia and hydrochloric acid supplied by Scharlau (Barcelona, Spain) and potassium chloride, calcium chloride and urea from Panreac (Barcelona, Spain).

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

2.2. Standard solutions

Stock solutions of AAs, ILIS and creatinine were prepared in UHQ water at a concentration of 1000 mg/L. For Cys-Cys and Tyr, solutions were prepared in HCl 0.1 M at the same concentration as the other AAs. All of them were stored in the dark at 4 °C. These solutions were used to prepare standard solutions and to spike the synthetic and natural urine samples.

2.3. Sample preparation

First morning urine samples were collected from apparently healthy individuals of both sexes in disposable sterile specimen collection cups and were frozen and stored at −20 °C in the dark. For analysis, samples were thawed at room temperature and centrifuged at 1815g during 10 min. The supernatant was filtered with nylon filters (0.45 μm, 17 mm i.d.). Then, a 1/10 dilution of the filtered supernatant was done, adding 100 μL to a vial and mixing it with 900 μL of UHQ water (0.1% HFBA, v/v). ILIS were added to all the samples at a concentration of 2 mg/L. An aliquot (5 μL) was injected into the system. The method used for the measurement of creatinine content was the Jaffé method [27].

2.4. LC-MS/MS analysis

LC-MS/MS analysis was carried out 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 equipped with an electrospray ionization (ESI) source, all from Agilent Technologies (Waldbronn, Germany). 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. Multiple Reaction Monitoring (MRM) mode (unit mass resolution) was used for analyte quantification.

The chromatographic column used was a Kinetex EVO-C18 column 100 Å (2.1 × 150 mm) from Phenomenex (Torrance, CA, USA) packed with 2.6 μm core–shell particles. The mobile phase was composed of a UHQ water with 0.1% HFBA (v/v) (Solvent A) and MeOH with 0.1% HFBA (v/v) (Solvent B) mixture. The mobile phase flow rate was 150 μL/min and the injection volume used was 5 μL. The gradient elution used was as follows: 2% B for 5 min, then from 2% to 50% B from 5 min to 10 min, after from 50% to 70% B from 10 min to 12 min, hold at 70% B during 10 min, and then returning to 2% B from 22 to 23 min and holding conditions during 12 min. A suitable separation was achieved in less than 22 min and 13 min were required to re-equilibrate the column. The total chromatographic run time was 35 min. This analysis mode was used for sample treatment optimization.

All the parameters related to the experimental conditions used are shown in Table S1 (Supplementary material).

2.5. MS/MS and gC-MS/MS analysis

Stand-alone MS/MS and gC-MS/MS analyses were performed using the instrumental configuration described for LC-MS/MS. A six-port valve was used to switch from MS/MS or gC-MS/MS analysis to LC-MS/MS analysis without any instrumental modification.

When MS/MS analysis was performed, the six-port valve was directly connected to the mass spectrometer with a peek tube. The mobile phase used was 100% MeOH (0.1% HFBA, v/v) under isocratic conditions at a flow rate of 500 μL/min. Total analysis runtime was 1.4 min. Taking account the time required for injection, each sample was analyzed in 2.2 min.

The gC-MS/MS analysis was carried out using a XBridge® C18 guard column (2.1 × 10 mm, 3.5 μm). Isocratic conditions were used, with a mobile phase composition of 80% UHQ water (Solvent A) and 20% MeOH (Solvent B). In both solvents, 0.1% HFBA (v/v) was added. The mobile phase flow rate was 500 μL/min. 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 300 μL/min). Total analysis runtime was 1.5 min. Each sample was analyzed in 2.8 min.

The conditions of the mass spectrometer were the same described for LC-MS/MS analysis and MRM mode was also used for analyte quantification.

2.6. Method validation

Methods were validated using synthetic urine. The following parameters were evaluated: calibration curves, limits of detection (LODs) and quantification (LOQs), repeatability (intra-day precision), reproducibility (inter-day precision) and accuracy.

Matrix effect evaluation was carried out using real and synthetic urine. The slopes of the calibration curves obtained in the two matrices were compared using a Student́s *t*-test (significance level, 0.05%).

3. Results and discussion

3.1. Sample treatment

Sample treatment optimization was performed using the LC-MS/MS method. After protein precipitation and filtration of urine samples (see Section 2.3.), UHQ water with 0.1% HFBA (v/v) was used for supernatant dilution. Different urine dilution ratios were tested: 1/1, 1/5, 1/10 and 1/20 (v/v). Deficient peak shape for Lys, Arg and His was obtained for 1/1 and 1/5 dilution ratios. The dilution ratio 1/10 was selected because of its optimum results.

3.2. gC-MS/MS method optimization

Initially, stand-alone MS/MS was considered for AAs determination in urine. The experimental conditions used were similar to those reported previously [28]. It was observed that when using this methodology, sensitivity was not enough for the reliable quantification of some of target compounds, such as Asp and Met. Also, peak morphology was not acceptable, as can be seen in Fig. 1.A.

Thus, a guard column (gC) was incorporated into the instrumental configuration setup prior to MS detection to carry out a short chromatographic separation step that minimized the shortcomings of the stand-alone MS/MS determination. Synthetic urine spiked with 1 mg/L of all AAs and 2 mg/L of ILIS was used for gC-MS/MS method optimization. A XBridge® C18 guard column (2.1 × 10 mm, 3.5 μm) was proposed. Isocratic conditions were used with a mobile phase composed of UHQ water with 0.1% HFBA (Solvent A) and MeOH with 0.1% HFBA (Solvent B). Different percentages of water content in the mobile phase were considered. The best results were obtained with 80% UHQ water and 20% MeOH, both with 0.1% HFBA (v/v). The flow rate used was 500 μL/min and the injection volume was set at 5 μL. Under these conditions, a slight separation of the compounds was achieved and an increment on the analytical signals was observed, as well as a peak shape improvement (Fig. 1.B). All these enhancements were accomplished without a significative increase in analysis time: total analysis runtime was 1.5 min, with a total time of 2.8 min, considering the time required for injection (21 samples per hour).

To avoid carry-over, a MeOH injection was included after sample injection, using sequential injection mode (5 μL of sample plus 50 μL of MeOH). Different MeOH injection speeds were considered: 100, 200, 300 and 400 μL/min. The best results were obtained with an injection speed of 300 μL/min. Under these conditions, retention time reproducibility was evaluated using real urine. RSD values (%) were below 6 % (five replicates, three different days).

3.3. LC-MS/MS method optimization

The optimization of the LC-MS/MS method was performed using synthetic urine spiked with 1 mg/L of all AAs and 2 mg/L of ILIS. A Kinetex EVO-C18 column 100 Å (150 mm × 2.1 mm, 2.6 μm) was used for the chromatographic separation of the AAs. After injection volume, flow rate and separation gradient optimization, the instrumental conditions were set as described in Section 2.4. Retention time reproducibility was also evaluated using real urine and RSD values (%) were found to be below 2 % for all AAs (five replicates, three different days).

3.4. MS optimization and evaluation of isobaric interferences

Multiple Reaction Monitoring (MRM) acquisition parameters were optimized using solutions of the target compounds in UHQ water at a concentration of 1 mg/L. Different collision energies were evaluated in 1–60 eV range. Different dwell times were also tested for all the evaluated methodologies (LC-MS/MS, gC-MS/MS and stand-alone MS/MS): 20, 50 and 100 ms. With high dwell time values, peaks showed poor definition, especially when stand-alone MS/MS and gC-MS/MS methods were used due to the high speed of analysis. On the contrary, with a dwell time of 20 ms, peaks were more clearly defined (Figure S1). Thus, this value was chosen as optimum. The MS conditions selected for each analyte are shown in Table S1. Cysteine (Cys) was determined as Cys-Cys. This one is more stable than Cys, which is easily oxidized and dimerized [13].

An exhaustive evaluation of the possible existence of interfering compounds was performed by an in-depth bibliographic research using Human Metabolome Database and Metlin data search. Also, six urine samples from both sexes were analyzed using the LC-MS/MS method [29], to check if the target AAs presented interfering compounds at their selected transitions (Table S1). From these studies, it was concluded that only Val, Arg, and Leu displayed interferences with a significant contribution to their analytical signal. These were: guanidinoacetic acid

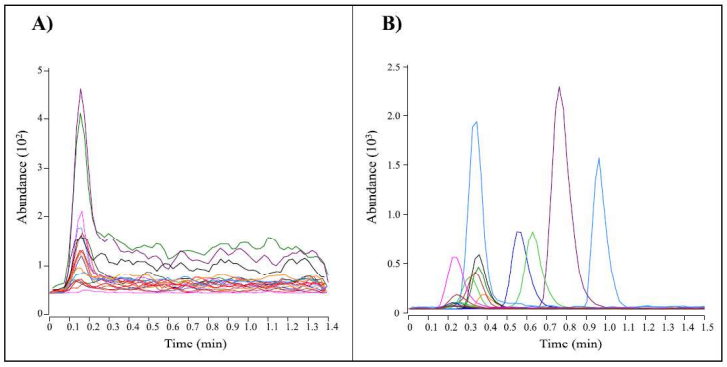

Fig. 1. MRM signals of synthetic urine spiked with AAs at a concentration of 1 mg/L (except Asp, at 6 mg/L) obtained with A) the MS/MS method and B) the gC-MS/MS method.

for Val (for transition 118.1 → 72.1), N-acetylasparagine for Arg (for transition 175.1 → 70.1) and creatine for Leu (for transition 132.1 → 43.1). Identity of the interfering compounds was confirmed injecting pure standards.

Fig. 2.A shows the MRM signals obtained for transition 132.1 → 43.1 when MS/MS and gC-MS/MS methods were used. As it is shown, when the first one was used, it was not possible to distinguish between Leu and creatine, which hinders Leu quantification. However, due to the slight separation obtained with the gC, Leu and creatinine peaks were perfectly resolved. Similar behavior was obtained for guanidinoacetic acid and Val, and N-Acetylasparagine and Arg.

Interferences of isobaric compound from the matrix was not the only issue for AAs determination in urine. Transitions 147.1 → 84.1 and 147.1 → 130.0 were shared by Lys and Gln. For Glu, it was also observed that at its selected transitions (148.1 → 84.0 and 148.1 → 56.0), Gln and Lys also appeared because of their ^{13}C contribution.

As can be seen in Fig. 2.B, even with the inclusion of the gC, the peaks obtained for Gln and Lys were not resolved and it was difficult to integrate each peak separately. Quantification of Lys was not problem because Lys could be quantified using the transition 147.1 → 67.0, specific for this compound. For Gln, the signal obtained for the transition 147.1 → 84.0 was integrated and the concentration was assigned to Gln and Lys altogether. Then, due to the concentration of Lys could be determined as previously mentioned, Gln concentration could be

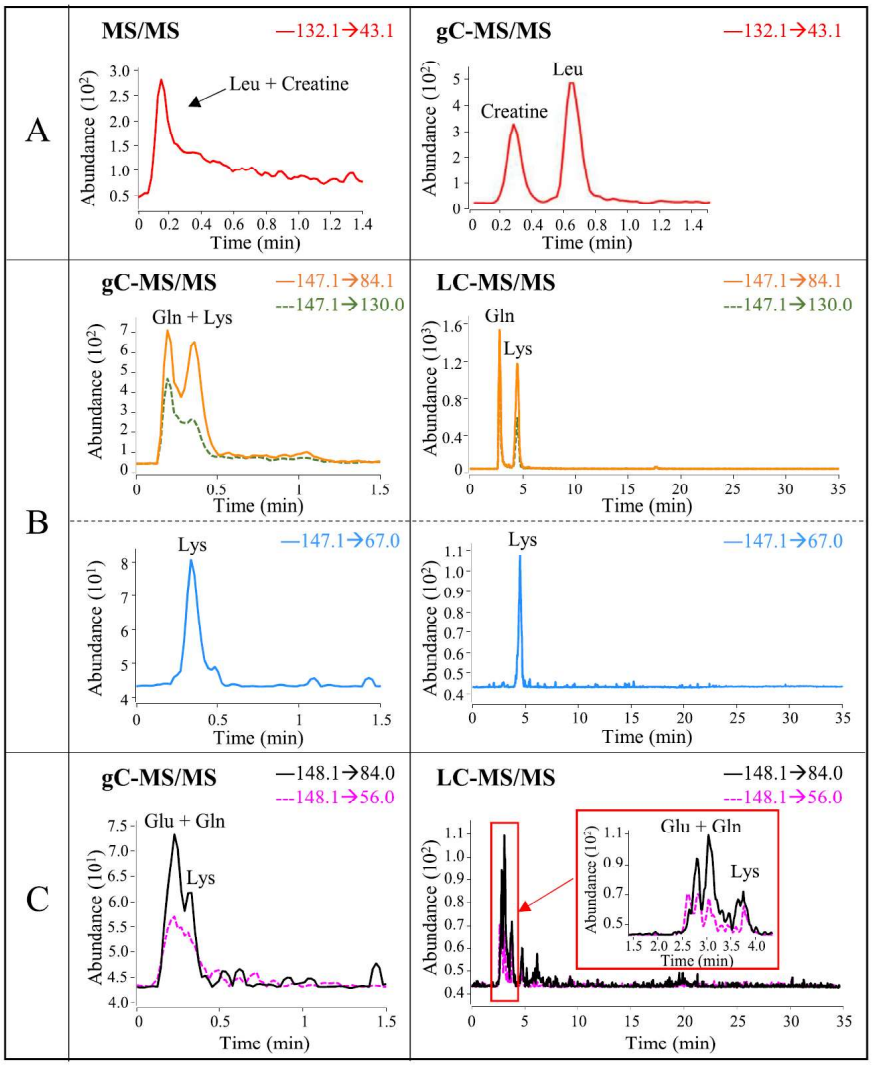

Fig. 2. MRM signals obtained for a urine sample for A) transition 132.1 → 43.1 using the MS/MS and gC-MS/MS methods, B) Gln and Lys transitions using the gC-MS/MS and LC-MS/MS methods and C) Glu transitions using the gC-MS/MS and LC-MS/MS methods.

calculated by the difference.

For Glu (148.1 → 84.0), two unresolved peaks were observed when the gC was used (Fig. 2.C). The first one corresponded to the signals obtained for Glu and Gln and the second one to Lys. Under these conditions, it was difficult to integrate each signal separately. A similar situation was found when the LC-MS/MS method was used (Fig. 2.C). For these reasons, Glu was eliminated from the analysis because its correct quantification was not possible.

3.5. Analytical characteristics of gC-MS/MS vs LC-MS/MS

Synthetic urine spiked with different concentrations of the 19 AAs (five concentration levels) were used to evaluate the analytical characteristics of both methodologies (gC-MS/MS and LC-MS/MS). Ala-^{13}C and Leu-^{13}C were selected as isotopically labeled internal standards and they were added at a concentration of 2 mg/L. They were used as follows: Ala-^{13}C for Asn, Ser, Asp, Gly, Gln, Cys-Cys, Thr, Ala, Glu, Pro, His and Lys area normalization and Leu-^{13}C for Val, Arg, Met, Ile, Leu, Tyr, Phe and Trp area normalization. This selection was performed based on LC-MS/MS method retention time proximity. Each concentration level was analyzed in triplicate. All calibration curves showed good linear behavior with satisfactory coefficients of determination (R^2) for all the compounds using both methods (Table 1, Table 2). Their validity was checked using ANOVA analysis and it was observed that they did not exhibit any lack of fit. The slopes obtained for gC-MS/MS and LC-MS/MS methods were compared using a Student *t*-test (significance level, 0.05%). These slopes were demonstrated significantly different ($p < 0.05$).

The limits of detection (LODs) and the limits of quantification (LOQs) were determined as the analyte concentration corresponding to a signal-to-noise ratio (S/N) of 3 and 10, respectively [30]. For the gC-MS/MS method, LODs values were found to be between 0.004 and 0.425 mg/L and LOQs values between 0.01 and 1.40 mg/L. For Asp, the highest LODs (3 mg/L) and LOQs (9 mg/L) were obtained. Asp sensitivity differences could be related to the ion suppression caused by the rest of the components of the matrix when this method was used, being this compound the first to elute from de gC and one of the most polar analytes [31]. For the LC-MS/MS method, LODs and LOQs were in the 0.002–0.160 and 0.007–0.528 mg/L ranges, respectively (Table 1, Table 2).

Repeatability (intra-day precision) and reproducibility (inter-day precision) were studied using synthetic urine spiked at LOQ, 1.5 times and 2 times the LOQ. The values, expressed as relative standard deviations (RSD, %), are shown in Table1 and Table 2. These values were highly satisfactory for both methods.

Accuracy was also evaluated as apparent recoveries (expressed as percentages). These values were calculated as the ratio of the measured concentration to the spiked concentration. Acceptable recovery values were found in the range 80–133 % for both methodologies (Table 1, Table2).

3.6. Analysis of urine samples

3.6.1. Matrix effect

To evaluate matrix effect, calibration curves (five levels) were obtained in two different matrices (synthetic and real urine) using both gC-MS/MS and LC-MS/MS methodologies. Concentration levels were selected based on the expected concentrations of the AAs in urine and each one was analyzed in triplicate. The calibration curves exhibited linear behavior and did not exhibit any lack of fit. The slopes of the calibration curves were compared using a Students *t*-test (significance level, 0.05%). The slopes differed significantly ($p < 0.05$), as shown in the Table S2. These results showed the existence of matrix effect. For this reason, quantification was performed using a one-point standard addition and normalization to IS protocol [32]. Internal standards were added at a concentration of 2 mg/L.

Fig. 3 shows the concentration of AAs (normalized to creatinine content) predicted in one urine using the quantification protocol previously described with both LC-MS/MS and gC-MS/MS methodologies. Similar results were obtained with both methods, which demonstrated the reliability of the second one for screening purposes. The only disparity was observed for Asp, which was not detected and quantified in urine with the gC-MS/MS method, because of the high LOD found for this compound.

3.6.2. Determination of AAs in urine

Urine samples from eighteen healthy volunteers with ages between 11 and 68 years were analyzed using both gC-MS/MS and LC-MS/MS methods. Table S3 shows the mean values (including the lowest and highest ones) of the normalized concentrations of the target compounds

Table 1
Analytical characteristics of the gC-MS/MS method obtained with synthetic urine.

Compounds	R^2	Slope [a]	LOD (mg/L)	LOQ (mg/L)	Repeatability (%)[b] LOQ	1.5 LOQ	2LOQ	Reproducibility (%)[b] LOQ	1.5 LOQ	2 LOQ	Recovery (%)[c]
Asn	0.9934	0.052 ± 0.003	0.122	0.401	5.1	3.1	4.8	5.7	5.3	5.7	80
Ser	0.9988	0.190 ± 0.004	0.211	0.695	5.9	7.7	6.6	8.4	8.7	6.6	87
Asp	0.995	0.061 ⊥ 0.003	2.730	9.009	4.1	6.0	6.7	7.4	5.3	3.7	133
Gly	0.9962	0.098 ± 0.004	0.326	1.076	6.7	11	9.2	9.4	8.9	9.2	100
Gln − Lys	0.9989	0.310 ± 0.007	0.085	0.281	3.3	4.2	3.5	4.4	4.8	4.4	104[d]
Cys-Cys	0.9945	0.081 ± 0.004	0.051	0.167	5.8	4.5	8.7	8.3	9.6	7.6	130
Thr	0.9967	0.203 ± 0.009	0.425	1.401	5.6	6.6	6.8	11	5.9	4.7	85
Ala	0.9952	0.474 + 0.019	0.236	0.777	5.3	7.2	3.5	6.1	6.3	5.2	100
Pro	0.9904	1.651 + 0.101	0.019	0.063	6.8	5.5	7.6	11	6.9	7.7	100
His	0.9988	1.115 ± 0.024	0.010	0.034	8.9	2.7	7.7	9.9	7.8	8.0	90
Lys	0.9939	0.071 ± 0.004	0.040	0.132	6.9	5.7	3.8	8.6	5.9	7.6	80
Val	0.9933	1.139 ⊥ 0.071	0.014	0.048	4.9	4.8	5.4	6.2	8.4	7.6	123
Arg	0.9978	0.212 ± 0.007	0.031	0.101	7.9	5.4	5.8	11	11	7.8	96
Met	0.9972	0.381 ± 0.013	0.006	0.018	6.9	5.4	5.4	5.0	7.4	6.7	90
Ile	0.9992	0.535 ± 0.010	0.015	0.050	6.1	6.2	9.7	7.0	6.4	7.3	88
Leu	0.9980	0.675 ± 0.017	0.019	0.061	9.4	6.5	5.5	8.4	6.0	7.0	100
Tyr	0.9966	0.098 ± 0.003	0.018	0.061	11	5.9	9.0	12	13	11	90
Phe	0.9985	1.551 + 0.038	0.005	0.017	9.5	9.0	4.7	12	7.5	8.4	92
Trp	0.9932	0.652 ± 0.044	0.004	0.013	9.7	10	10	15	15	16	107

[a] Expressed in arb. units vs. concentration in mg/L.
[b] Calculated for ten replicates on the same day (repeatability) and for five replicates on three different days (reproducibility).
[c] Obtained at the first level of the calibration curve for each compound.
[d] Gln recovery, calculated as the difference of Gln + Lys concentration minus Lys concentration.

Table 2
Analytical characteristics of the LC-MS/MS method obtained with synthetic urine.

Compounds	R^2	Slope [a]	LOD (mg/L)	LOQ (mg/L)	Repeatability (%)[b]			Reproducibility (%)[b]			Recovery (%)[c]
					LOQ	1.5 LOQ	2LOQ	LOQ	1.5 LOQ	2 LOQ	
Asn	0.9929	0.206 ± 0.014	0.024	0.079	4.9	7.6	5.5	11	4.7	5.9	120
Ser	0.9779	0.439 ⊥ 0.046	0.022	0.071	6.8	4.2	4.4	5.8	6.8	2.5	110
Asp	0.9924	0.122 ⊥ 0.008	0.133	0.439	4.9	3.4	3.0	4.3	4.2	3.8	80
Gly	0.9919	0.144 ⊥ 0.010	0.068	0.224	5.2	2.8	3.6	8.6	7.9	3.5	120
Gln	0.9973	0.142 ± 0.004	0.134	0.443	5.8	4.0	2.4	5.1	6.7	5.9	100
Cys-Cys	0.9946	0.049 ± 0.002	0.046	0.152	9.3	9.0	10	11	9.1	12	100
Thr	0.9969	0.133 ± 0.004	0.160	0.528	3.9	1.9	2.3	4.7	5.7	4.8	95
Ala	0.9984	0.465 + 0.011	0.149	0.492	3.4	2.3	2.8	4.9	4.4	4.9	100
Pro	0.9983	6.232 ± 0.156	0.004	0.012	2.8	3.2	2.5	3.2	6.8	4.8	107
His	0.9904	1.380 ± 0.096	0.007	0.023	6.3	3.2	6.7	4.9	7.2	3.5	110
Lys	0.9913	0.046 ⊥ 0.003	0.052	0.170	5.7	4.5	7.8	8.7	6.7	7.4	120
Val	0.997	3.577 ⊥ 0.139	0.006	0.021	1.7	3.0	3.3	3.8	2.8	3.2	93
Arg	0.9988	0.432 ± 0.011	0.018	0.061	3.0	2.8	2.0	4.8	3.7	2.9	89
Met	0.999	1.228 + 0.023	0.005	0.015	4.6	4.6	3.1	5.3	5.7	3.8	94
Ile	0.9984	0.629 ± 0.015	0.011	0.035	4.5	2.2	2.9	2.9	2.6	3.7	96
Leu	0.9991	0.661 ± 0.014	0.011	0.036	2.7	3.6	2.7	3.7	2.4	2.3	98
Tyr	0.9992	0.413 + 0.007	0.013	0.043	5.8	3.6	4.0	5.5	5.0	5.0	83
Phe	0.9992	1.584 ⊥ 0.027	0.002	0.007	3.9	5.1	3.2	8.9	5.2	4.9	96
Trp	0.9992	0.813 ± 0.014	0.006	0.018	4.4	2.5	1.9	5.8	3.2	3.2	100

[a] Expressed in arb. units vs. concentration in mg/L.
[b] Calculated for ten replicates on the same day (repeatability) and for five replicates on three different days (reproducibility).
[c] Obtained at the first level of the calibration curve for each compound.

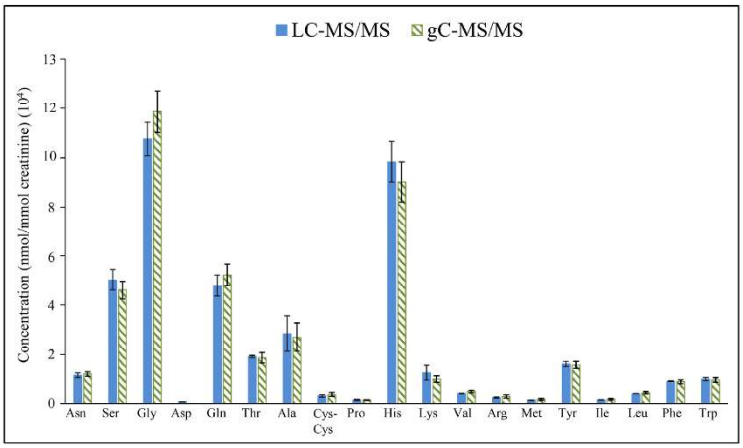

Fig. 3. Comparison of AAs concentrations (normalized to creatinine content) in one urine sample obtained with the LC-MS/MS and gC-MS/MS methods.

obtained with both methodologies. A Mann-Whitney *U* test was implemented in order to know whether there were significant differences between groups (gC-MS/MS and LC-MS/MS). No significant differences ($p > 0.05$) were observed, demonstrating again the applicability of the gC-MS/MS method for the reliable determination of proteinogenic AAs in urine.

AAs concentration differences related to sex were studied (Fig. 4). Results obtained with the gC-MS/MS were evaluated using a Mann-Whitney *U* test (significance level, 0.05%). Results showed that there were not significant differences between groups for the target compounds.

3.7. Comparison with other proposed methods

Table S4 summarizes the methodologies already proposed for the determination of proteinogenic AAs in urine samples based on LC and/or MS analysis. Regarding methodologies based on LC-MS, research from the last 5 years was considered due to the huge number of publications found. However, for stand-alone MS methods, research was expanded to the last 10 years because just one publication was found in the last 5 years.

Comparing the gC-MS/MS method proposed in the present manuscript with the already published MS methodologies, only one work was found with lower times of analysis [24]. Authors proposed the use of an extractive electrospray ionization (EESI) ion source, which implied the use of unconventional equipment which may have a higher cost. The other works that proposed MS methods used a derivatization step of AAs for their determination [23,33]. Our method involves a simpler sample treatment procedure with no derivatization of the compounds required.

Regarding LC-MS analysis, most of the publications found required the derivatization of the AAs or the use of HILIC columns (references in supplementary material). Other methods used ion pairing reagents in reversed-phase mode or special column (references in supplementary material). Times of analysis in all these methods were higher than the

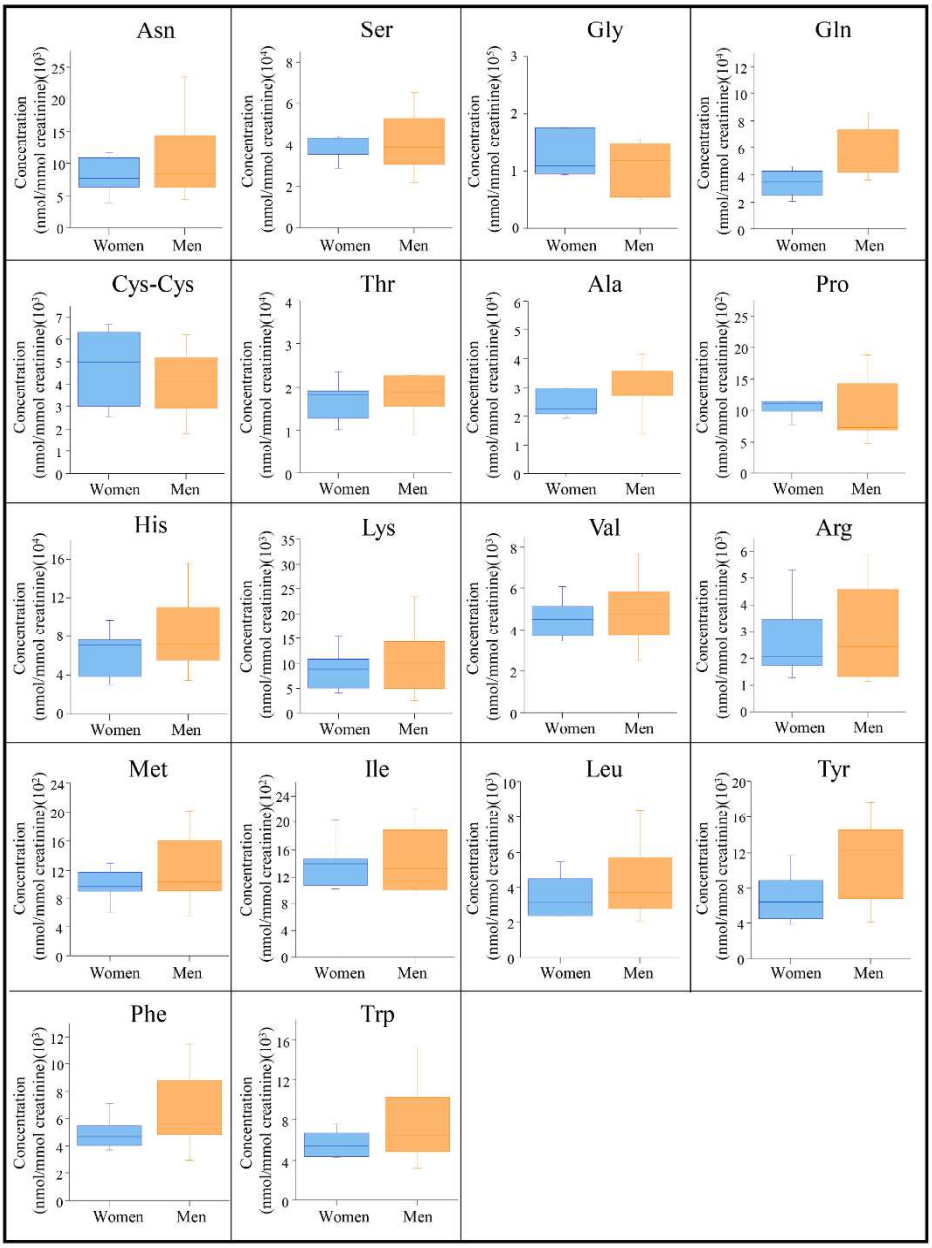

Fig. 4. Box plots representing the concentration of the AAs (expressed in nmol/mmol creatinine) found in urine samples from women (8) and men (10), using the proposed gC-MS/MS methodology.

one obtained with the gC-MS/MS methodology.

LODs and LOQs reported in some of the aforementioned publications were lower than the values obtained here, as can be observed in Table S3. However, the LODs and LOQs obtained with the proposed methodology were highly satisfactory and adequate for the determination of AAs in urine (except for Asp), as we have demonstrated analyzing different samples.

The gC-MS/MS method presented here used conventional instrumentation and a simple sample treatment without derivatization. The time of analysis was lower than most of the other methods reported in bibliography and the LODs and LOQs obtained were suitable for the determination of proteinogenic AAs, except for Asp, in urine samples. Therefore, the proposed gC-MS/MS method is a novel, fast and reliable alternative for the determination of proteinogenic AAs in urine.

4. Conclusions

In this work, we have successfully applied for the first time the use of a guard column prior to tandem mass spectrometry (gC-MS/MS) for the determination proteinogenic AAs in urine samples with a simple sample treatment step. The use of the gC implied a short chromatographic separation step, which allowed to obtain improved analytical signals, as well as better sensitivity, compared with the results obtained when stand-alone MS/MS was used, without a significative increase in analysis time. Moreover, the use of the gC allowed us to solve some of the problems related to isobaric compounds.

Results obtained with the gC-MS/MS methodology were confirmed using a LC-MS/MS method. Both methods were validated using synthetic urine and satisfactory results were obtained. Matrix effect was evaluated and confirmed. Thus, a one-point standard addition and normalization to IS protocol was used for AAs quantification.

Urine samples from eighteen healthy volunteers were analyzed with both methodologies. The results obtained with gC-MS/MS were similar to those obtained with the chromatographic method. Asp was not quantified in urine samples due to the high LOD found for this compound. AAs concentration differences related to sex were also studied. Results showed that there were not significant differences between groups.

The aforementioned results demonstrate that the gC-MS/MS method proposed here is a fast and reliable option for the determination of AAs in urine. It involves a simple sample treatment step without any derivatization and uses conventional instrumentation, advantages over other MS/MS methods already reported in bibliography for the determination of AAs in urine. Besides the method proposed here reduces the analysis time compared to conventional LC-MS/MS methods that involve higher times of analysis.

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, Funding acquisition, Project administration. **Encarnación Rodríguez-Gonzalo:** Conceptualization, Supervision, Writing – review & editing, Visualization. **Bernardo Moreno-Cordero:** Writing – review & editing. **José Luis Pérez-Pavón:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2021.106914.

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