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A diethylpyrocarbonate-based derivatization method for the LC-MS/MS measurement of plasma arginine and its chemically related metabolites and analogs

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ABSTRACT

Background: Changes in NO metabolism correlate with cardiovascular risk factors and are associated with endothelial dysfunction. NO availability is regulated by nitric oxide synthase (NOS) and arginine and some chemically related metabolites and analogs have the capacity to alter NOS activity. Hence the need for analytical methods for the simultaneous assessment of these analytes.

Methods: Analytes (L-arginine (Arg), N^G-monomethyl-L-arginine (MMA), L-homoarginine (hArg), asymmetric dimethyl-L-arginine (ADMA), symmetric dimethyl-L-arginine (SDMA), and L-citrulline (CIT)) were isolated from human plasma by thermal coagulation of plasma followed by a derivatization with diethylpyrocarbonate. Carbetoxy derivatives were separated on a C18 reversed-phase column in < 10 min using an aqueous solution of 0.4% v/v formic acid and acetonitrile (95:5, v/v) mixture as a mobile phase. Positive electrospray ionization and tandem mass spectrometry in combination with specific multiple reaction monitoring transitions were used for detection of analytes and three deuterated forms of the analytes used as internal standards.

Results: Intra- and inter-day precision %RSD values ranged between 3 and 5.5% and percentage recoveries were close to 100% for all analytes. Plasma concentrations in 20 healthy male volunteers were 58.62 ± 8.81 μmol/L for Arg, 105.08 ± 21.66 nmol/L for MMA, 1.88 ± 0.57 μmol/L for hArg, 0.612 ± 0.140 μmol/L for ADMA, 0.581 ± 0.172 μmol/L for SDMA, and 28.62 ± 11.60 μmol/L for Cit, respectively.

Conclusion: This LC–MS/MS method provides the capacity to quantify the plasma concentrations of arginine and some of its chemically related metabolites. Sample preparation was simple, inexpensive and effortless. Overall, given the short sample preparation and chromatographic run time, the method may be suitable for the fast and reproducible quantitative determination of the analytes in large clinical trials and routine analysis.

1. Introduction

Nitric oxide (NO) is an important regulator of vascular homeostasis. Changes in NO metabolism correlate with cardiovascular risk factors and are associated with endothelial dysfunction [1,2]. In the regulation of NO availability, the semi- or conditionally essential amino acid L-arginine (Arg) plays a key role as rate-limiting substrate for different isoforms of nitric oxide synthase (NOS) that catalyze its oxidation to NO and L-citrulline (Cit) [3,4]. The latter, in turn, is a bioactive by-product as its enzymatic recycling to Arg is essential for NO production in endothelial cells [5]. Moreover, all NOS isoforms can be competitively influenced by arginine analogues such as L-homoarginine (hArg) and methylarginines. The latter are produced by post-translational methylation of Arg moieties in proteins by different arginine methyltransferases (PRMTs) [6]. Upon proteolysis, the methylated arginine residues are released into the cytosol [7] as asymmetric dimethyl-L-arginine (ADMA), with two methyl groups placed on the same terminal nitrogen atom of the guanidino group, ADMA regioisomer with two methyls attached on each of the terminal guanidino nitrogen atoms and commonly referred as symmetric dimethyl-L-arginine (SDMA), and NG-

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monomethyl-L-arginine (MMA), with a single methyl group on the same terminal nitrogen atom which is dimethylated in ADMA. ADMA acts as an endogenous inhibitor of NOS, may serve as a potential source of Cit following degradation catalyzed by dimethylarginine dimethylaminohydrolase (DDAH), and regulates intracellular Arg availability [8–10]. Similar to ADMA, MMA is an endogenously produced NOS inhibitor that undergoes the same degradation route [6,11]. The role of SDMA is less clear although it is considered a weak neuronal NOS inhibitor. Like ADMA, SDMA competes with Arg for cationic amino acid transporters, undergoes renal clearance and is recognized as a valuable marker of renal function [12,13]. hArg is synthesized from Arg by the enzyme L-arginine:glycine-amidinotransferase (AGAT) [14] and compared to Arg has an additional methylene group in its main chain. hArg has been suggested as a weak inhibitor of arginase [15], an enzyme which catalyzes the hydrolysis of Arg to ornithine and urea. Although its lower physiological concentration and affinity to NOS, when compared to Arg, suggest a limited direct NOS-dependent contribution to NO, hArg has recently shown inverse associations with surrogate markers of vascular and cardiac dysfunction [16]. Considerable research has therefore been undertaken to elucidate the roles of Arg and its chemically related metabolites and analogs both as potential targets of therapeutic interventions and as disease markers [17]. Hence the need for analytical methods able to robustly measure their concentrations. The HPLC of arginine, its metabolites and analogs is challenging as Arg and many Arg-like compounds are not well retained in conventional RP-columns [18]. This is further complicated by their structural similarity and the small differences in polarity. Different strategies have been developed to overcome these issues allowing the simultaneous measurement of Arg and different metabolites and analogs in plasma and other biological fluids [19]. They include gas-chromatography [20], ELISA [21], capillary electrophoresis [7], and liquid chromatography on reverse phase or hydrophilic interaction (HILIC) columns and detection by either ultraviolet [11], fluorescence [22], or mass spectrometry with or without derivatization [23]. Each of these approaches, however, has limitations that could hamper their use in practice. For example, HILIC methods usually require longer column equilibration and poor retention and peak shape sometimes can be observed [24]. Derivatization often requires several steps to be carried out at high temperatures and for relatively long periods. On the other hand, derivatization is often required to increase the sensitivity of detection as analytes lack intrinsic chromophores. Furthermore, without derivatization, the lack of separation of the analytes in conventional RP-columns is difficult to overcome even with mass spectrometry because of the same MS and MS/MS profile between the analytes, especially between ADMA and SDMA. Moreover, expensive and/or time-consuming steps, e.g., SPE, lyophilization and/or evaporation, are usually required to adequately prepare the sample either to ensure compatibility with the mass spectrometer, mobile phase, derivatization reaction or column as well as to concentrate it, thus undermining the robustness and reproducibility of the methods [23,25]. With this in mind, we sought to develop a targeted LC-MS/MS metabolomics method with a run time suitable for routine analysis to simultaneously quantify Arg, MMA, hArg, ADMA, SDMA, and Cit in human plasma. The method involved two simple heat treatments to remove the proteins and to extract the analytes, followed by a simple and rapid diethylpyrocarbonate-based derivatization reaction [26,28].

2. Experimental

2.1. Chemicals

Acetonitrile (ACN) HPLC grade, potassium phosphate monobasic (KH_2PO_4), formic acid, potassium hydroxide (KOH), diethylpyrocarbonate (DEPC), L-arginine (Arg), L-homoarginine (hArg), N^G-monomethyl-L-arginine (MMA), asymmetric dimethyl-L-arginine (ADMA), symmetric dimethyl-L-arginine (SDMA), and L-citrulline (Cit) were purchased from Sigma Aldrich (Milan, Italy). Deuterated hArg (d4-hArg, L-homoarginine-d4 dihydrochloride), ADMA (d6-ADMA, N^G,N^G-dimethyl-L-arginine-d6 dihydrochloride), and SDMA (d6-SDMA, N^G,$N^{G'}$-dimethyl-L-arginine-d6) with a mass difference of 4, 6, and 6, respectively, were obtained from DBA Italia (Milan, Italy). High-purity water, obtained from a Millipore Milli-Q system, was used throughout the experiments (Merck Millipore, Italy).

2.2. Solutions

Standard solutions of analytes were prepared in ultrapure water and stored at −80 °C until use. Fresh working standard solutions of the analytes were prepared on the day of the analysis by diluting the stock solutions with Milli-Q water. Thus, the stock solutions of each analyte were mixed together and with water to obtain the highest concentration of the calibration curve: 120 μmol/L for Arg, 5 μmol/L for hArg, ADMA, and SDMA, 500 nmol/L for MMA, and 150 μmol/L for Cit. This solution was further diluted with water to obtain further five scalar concentrations of the analytes. The isotopically labeled internal standards (ISs) solutions were prepared in ultrapure water as a ready-to-use solution and stored at −80 °C until use. Their concentrations were 240, 480, and 189 μmol/L for d6-SDMA and d6-ADMA, and d4-hArg, respectively. Potassium phosphate monobasic, used as a buffer in the derivatization reaction, was prepared in ultrapure water at a concentration of 100 mmol/L and brought to pH 7 with a 2 mmol/L aqueous KOH solution. DEPC, used as a derivatization reagent, was prepared in ultrapure water at a concentration of 33 mmol/L.

2.3. Participants to study and samples collection

Twenty male volunteers with no medical history (age 55 ± 10 years) were randomly selected for this study. After informed written consent was obtained, blood was collected by venipuncture in 5.4 mg K3EDTA vacutainer tubes. Without delay, the blood was centrifuged at 4 °C and 3000 x g for 10 min to separate plasma, which was rapidly stored in 250-μL aliquots at −80 °C until use. The study was performed following the principles outlined in the Declaration of Helsinki and all the procedures were approved by the by the ethics committee of Local Health Unit n° 1 of Sassari, Italy (2262/CE).

2.4. Sample treatment and derivatization

A 200 μL-volume of plasma was spiked with 1 μL of the ISs solution and mixed thoroughly by vigorous vortex-mixing. Tubes were then placed in a block heater for 5 min at 100 °C, cooled to room temperature, and the clot overlaid with 400 μL of ultrapure water. To dislodge the clot from the bottom of the vial, the containers were vortexed vigorously for 10 s, then placed in a block heater for 5 min at 100 °C. After vigorous vortex-mixing, samples were centrifuged at 17,000 x g for 5 min. A 200 μL-volume of clear supernatant was recovered and mixed with a 20 μL-volume of phosphate buffer and 40 μL of DEPC. After vortex-mixing, tubes were left at room temperature for 1 min prior to LC-MS/MS analysis.

2.5. LC equipment, chromatographic and mass spectrometer conditions

The LC apparatus was a Waters system model Acquity UPLC equipped with a Waters Acquity UPLC tandem quadrupole mass spectrometer (TQD) (Waters Italia, Milan, Italy). The separation was achieved on a 100 mm × 4.6 mm Zorbax Eclipse Plus C18 3.5 μm column by using as a mobile phase a mixture of an aqueous solution of 0.4% v/v formic acid and ACN (95:5) isocratically delivered at a flow-rate of 0.8 mL min^{-1}. Chromatographic separation was carried out at 30 °C in an air-conditioned room at about 25 °C with samples held at 10 °C in the autosampler. The amount injected was 20 μL in full loop mode by using a 20 μL sample loop. Column effluents were monitored

Table 1
MRM parameters for the identification of the analytes and internal standards undergoing derivatization with DEPC.

Compound	Parent	Daughter	Retention time	Cone	Collision
	m/z	*m/z*	*min*	*V*	*V*
Arg	247.14	142	3.83	31	20
MMA	261.28	70	5.54	35	29
hArg	261.28	84	7.09	35	26
Cit	248.17	142	7.23	21	15
ADMA	275.33	46	7.58	35	19
SDMA	275.33	70	8.93	35	35
d4-hArg	265.28	88	7.01	31	20
d6-ADMA	281.3	52	7.45	35	22
d6-SDMA	281.3	70	8.82	35	37

by mass spectrometer in multiple reaction monitoring mode (MRM) with a LC-MS/MS run time analysis of 10 min. The capillary voltage in the mass spectrometer was set at 2.5 kV, with ESI source and desolvation temperatures, respectively, of 150 and 500 °C. Nitrogen, used as desolvation gas, was delivered at 600 L h^{-1}, while the argon in the collision cell was delivered at 0.5 mL min^{-1}. Mass spectral parameters for MRM transitions were optimized by IntelliStart in MassLynx V4.1 software by infusing 100 µmol/L of each carbethoxy derivative at a flow rate of 20 µL min^{-1}. Mass detection was accomplished in positive ion mode by MRM of the precursor-product ion transitions *m/z* 247.14 → 142, 248.17 → 142, 261.28 → 70, 261.28 → 84, 275.33 → 46, and 275.33 → 70 for Arg, Cit, MMA, hArg, ADMA, and SDMA, respectively, as well as *m/z* 265.28 → 88, 281.3 → 52, and 281.3 → 70 for d4-hArg, d6-ADMA, and d6-SDMA, respectively. Transitions and optimized values for cone voltage, collision voltage and retention times for each analyte and IS are reported in Table 1.

3. Results

3.1. Study of the derivatization reaction

Concentrations of potassium phosphate monobasic, used as a buffer of derivatization, were trialed in the range of 50–200 mmol/L. As shown in Fig. 1a, the highest derivatization yield was observed for a concentration of 100 mmol/L for all analytes. On average, at any tested buffer concentration, the peak areas for Cit and Arg were about 10 times smaller than the other analytes. The buffer, which has a native pH of 4.5, was then checked for the effect of pH on the derivatization reaction. Thus, a series of 100 mmol/L buffer solutions were brought to pH values ranging from 5 to 11 with a 2 mmol/L aqueous KOH solution. As displayed in Fig. 1b, at the same concentration of 10 µmol/L, all analytes showed an increase in the extent of derivatization as the pH increased. Except for MMA and hArg, derivatization yield reached a maximum at a pH of 7 and plateaued between 7 and 11. Conversely, derivatization yield for MMA and hArg reached a maximum at a pH of 8 and then plateaued at higher pH values. Decreasing sensitivity for MMA and hArg for a pH of derivatization of 7 did not, however, affect their measurability. Therefore, a 100 mmol/L potassium phosphate monobasic solution at pH 7 was used thereafter. This strategy was also based on the closeness of the chosen derivatization pH value of 7 to the native pH of the plasma samples. Regarding the effects of DEPC, its concentration was not directly evaluated in this study, and a tentative value of 33 mmol/L was extrapolated from the literature.

3.2. Chromatographic conditions

ACN and formic acid were tested in the range 1–10% v/vand 0.-0.5% v/v, respectively. For concentrations of ACN above 5% v/v, retention times for all analytes decreased sharply and, as shown in Fig. 2a, the resolution between SDMA and ADMA declined with a consequent reduced accuracy of peak integration for SDMA whose MS/MS transition at *m/z* 275.33 → 70 recognizes also ADMA. Conversely, for concentrations of ACN below 5% v/v, retention time gradually increased (Fig. 2b and c) with, however, a slight loss of sensitivity and peak symmetry. As expected, runtime as well as the distance between SDMA and ADMA increased with an increase in the aqueous content in the mobile phase, reaching a maximum for a ratio aqueous:organic of 99:1 (Fig. 2c). Retention time of derivatized analytes were less affected by formic acid than by ACN. However, as reported in Fig. 1c, the resolution between ADMA and SDMA as well as the peak shape for all analytes improved with the increase of content of formic acid in the mobile phase. Conversely, a general loss of sensitivity, around 5%, was also observed. Overall, a satisfactory balance between retention time, peak area (sensitivity), peak shape, and resolution of adjacent peaks was obtained with a mixture of an aqueous solution of 0.4% v/v formic acid and ACN (95:5) isocratically delivered at a flow-rate of

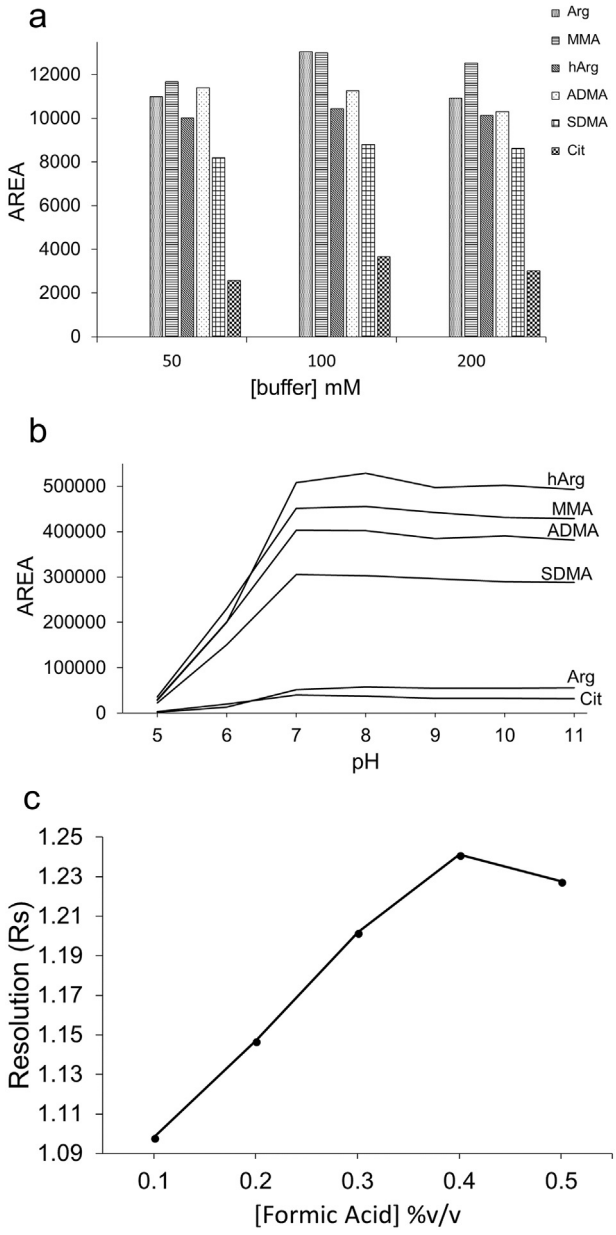

Fig. 1. a) Effect of buffer concentration on the extent of derivatization reaction, b) effect of buffer pH on the yield of derivatization and c) resolution between ADMA and SDMA as a function of the concentration of formic acid in the mobile phase. Analytes were analyzed as 10 µmol/L standard solutions in duplicate.

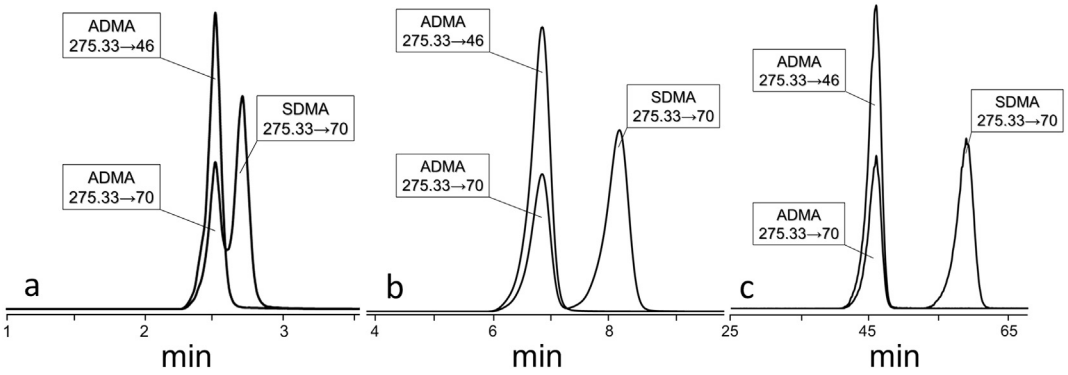

Fig. 2. Effect of ACN amount in the mobile phase on resolution and retention time of ADMA and SDMA for an aqueous:organic ratio of a) 90:10, b) 95:5, and c) 99:1. ADMA and SDMA were at the same concentration of 2.5 μmol/L.

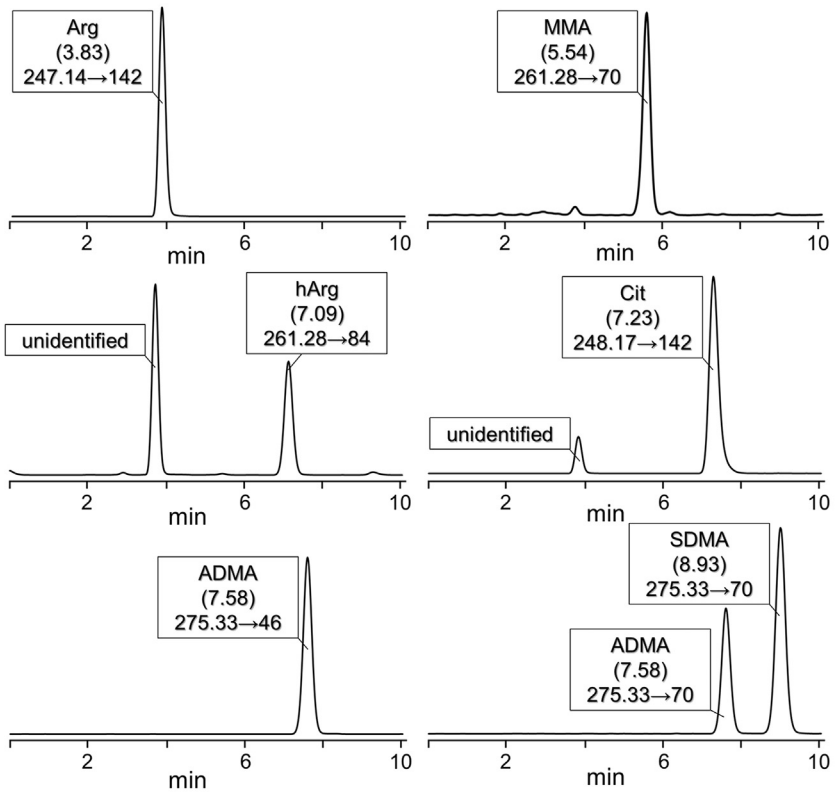

Fig. 3. Representative chromatogram of a real sample obtained under optimized chromatographic conditions.

0.8 mL min^{-1}. Fig. 3 shows a representative chromatogram of a real plasma sample obtained under optimized conditions.

3.3. Assay validation

Table 2 shows the summary results of validation parameters. The calibration curves for each analyte were constructed at six levels by regression of nominal analytes concentration against analyte/ISs peak area ratio of calibration points. d6-ADMA was used as an internal standard to quantify Arg, MMA, and ADMA, d4-hArg was used to quantify Cit and hArg, and d6-SDMA was used to quantify SDMA. The calibration curves were linear and provided a coefficient of determination > 0.99 over the investigated range of concentrations: 3.75–120 μmol/L for ARG, 15.63–500 nmol/L for MMA, 0.31–5 μmol/L for hArg, ADMA, and SDMA, and 3.13–100 μmol/L for Cit. Precision was assessed as intra-day precision and as inter-day precision. For intra-day precision assessment, each analyte in three plasma samples was quantified in 3 individually prepared replicates within a single batch. Inter-day precision was assessed by quantifying three plasma samples in duplicate on 3 separate occasions over a period of 2 weeks. The results, expressed as %RSD of measurements, ranged between 3 and 5.5% for both intra-day and inter-day precision. In particular, %RSDs for intra-day precision were 4.61, 4.01, 3.74, 3.11, 3.12, and 3.34% for Arg, MMA, hArg, ADMA, SDMA, and Cit, respectively. %RSDs for inter-day precision were 5.07, 5.12, 3.97, 3.88, 5.21, and 5.43% for Arg, MMA, hArg, ADMA, SDMA, and Cit, respectively. For the recovery experiments, plasma samples were spiked with 3 different concentrations of the analytes: 100, 75, and 50 μmol/L for Arg, 171.6, 85.8, and 42.9 nmol/L for MMA, 3.65, 1.82, and 0.91 μmol/L for hArg, 1.64, 0.82, and 0.41 μmol/L for ADMA and SDMA, and 90, 50, and 25 μM for Cit. At each analyte concentration, 3 replicates were analyzed in one analytical run, then recovery was computed as recovery% = [(C2 − C0)/C1] × 100, where C2 is the analyte concentration in the final solution after spiking with known concentration of standard, C0 is the original

Table 2
Summary of validation parameters of assay. The mean concentrations of Arg, hArg, MMA, ADMA, SDMA, and Cit in unspiked plasma were 63.4, 1.38, 0.082, 0.510, 0.573, 45.4 μmol/L, respectively.

Analyte	Linear range (μmol/L)	LOD (μmol/L)	LOQ (μmol/L)	Intra-day precision (%RSD)	Inter-day precision (%RSD)	Recovery (%)		Stability (RSD%)
Arg	3.75–120	0.1	0.3	4.61	5.07	Level 1 100 μM	92	1.29
						Level 2 75 μM	96	
						Level 3 50 μM	98	
MMA	0.016–0.5	0.001	0.003	4.01	5.12	Level 1 171.6 nM	102	2.33
						Level 2 85.8 nM	105	
						Level 3 42.9 nM	107	
hArg	0.31–5	0.01	0.03	3.74	3.97	Level 1 3.65 μM	98	1.91
						Level 2 1.82 μM	94	
						Level 3 0.91 μM	93	
ADMA	0.31–5	0.01	0.03	3.11	3.88	Level 1 1.64 μM	105	1.54
						Level 2 0.82 μM	93	
						Level 3 0.41 μM	104	
SDMA	0.31–5	0.01	0.03	3.12	5.21	Level 1 1.64 μM	99	1.71
						Level 2 0.82 μM	102	
						Level 3 0.41 μM	98	
Cit	3.13–100	0.1	0.3	3.34	5.43	Level 1 90 μM	96	2.02
						Level 2 50 μM	97	
						Level 3 25 μM	94	

analyte concentration in the initial solution, and C1 is the added known concentration of standard. As reported in Table 2, recoveries were close to 100% at each concentration for all analytes. As C2 and C0 were calculated by the calibration curves constructed using a stable isotope-labeled version of the analytes, recovery% was also used as a quantitative measure of the extent of matrix effect. A percent recovery close to 100% indicates that matrix effect is negligible. Conversely, values < 100 or > 100 indicate, respectively, suppression or enhancement of ionization. On average, total mean percentage of recoveries for all spiked plasma samples was 99.5% suggesting a limited matrix effect impact that can be minimized by the ISs, thus ensuring the accuracy of the quantitative LC-MS/MS analysis. Similarly, matrix effect (ME%), assessed as $ME\% = B/A \times 100$, where A is the peak area of the analyte (s) recorded for the standard solution, and B the peak area of the analyte(s) recorded for the sample spiked with the target compound(s) after extraction, was close to 100% for all analytes thus supporting the results of recovery. The limits of detection (LODs), evaluated as the concentration that produced a signal-to-noise ratio of about three, were 0.1 μmol/L for Arg and Cit, 0.01 μmol/L for hArg, ADMA and SDMA, and 0.001 μmol/L for MMA. The limits of quantification (LOQs), evaluated as the concentration that produced a signal-to-noise ratio of about ten, were 0.3 μmol/L for Arg and Cit, 0.03 μmol/L for hArg, ADMA and SDMA, and 0.003 μmol/L for MMA. Post-preparative stability was checked by keeping two different prepared samples under autosampler conditions (10 °C) and analyzing them for over 24 h for a total of 24 chromatographic runs each. Stability, expressed as RSD of analyte/ISs peak area ratio, was 1.29, 2.33, 2.02, 1.91, 1.54, and 1.71% for Arg, MMA, Cit, hArg, ADMA, and SDMA, respectively. Potential for cross-talk between MRM functions was also evaluated using a plasma sample without the addition of ISs, a blank solution containing the ISs without the analytes, and a blank solution containing the ISs and the analytes. As displayed in Fig. 4a, which shows the overlap of the transitions of ISs from the blank solution containing both the ISs and analytes vs. a plasma sample without ISs, there was no contribution to the detection of the ISs by endogenous compounds contained in the sample. Similarly, the overlap of the transitions of analytes from a plasma sample added with ISs towards a blank solution containing the ISs without analytes showed no contribution to the detection of the analytes by ISs (Fig. 4b).

4. Discussion

We addressed the analytical challenges related to the measurement of Arg and its metabolites and analogs by developing an original analytical procedure based on derivatization with DEPC. The latter is a well-known, powerful, acylating agent of amino acids in proteins including histidine, lysine, tyrosine, serine, threonine, and cysteine residues [26]. Recently, Sotgia et al. [27,28] used DEPC to successful derivatize hercynine, the betaine of histidine, and to measure its content in commonly consumed beverages and human whole blood. Although modification of Arg by DEPC has also been reported [26], there appear to be no further reports on the reaction with ARG or with its metabolites and analogs. This study confirms that DEPC carbethoxylates Arg and, to our knowledge, shows for the first time that it also reacts with MMA, hArg, ADMA, SDMA, and Cit. The reaction occurs readily in aqueous solution at room temperature within a pH range between 5 and 11 with an optimum value between 7 and 8, depending on the analyte. Mass data reveals that, at the reaction conditions used in

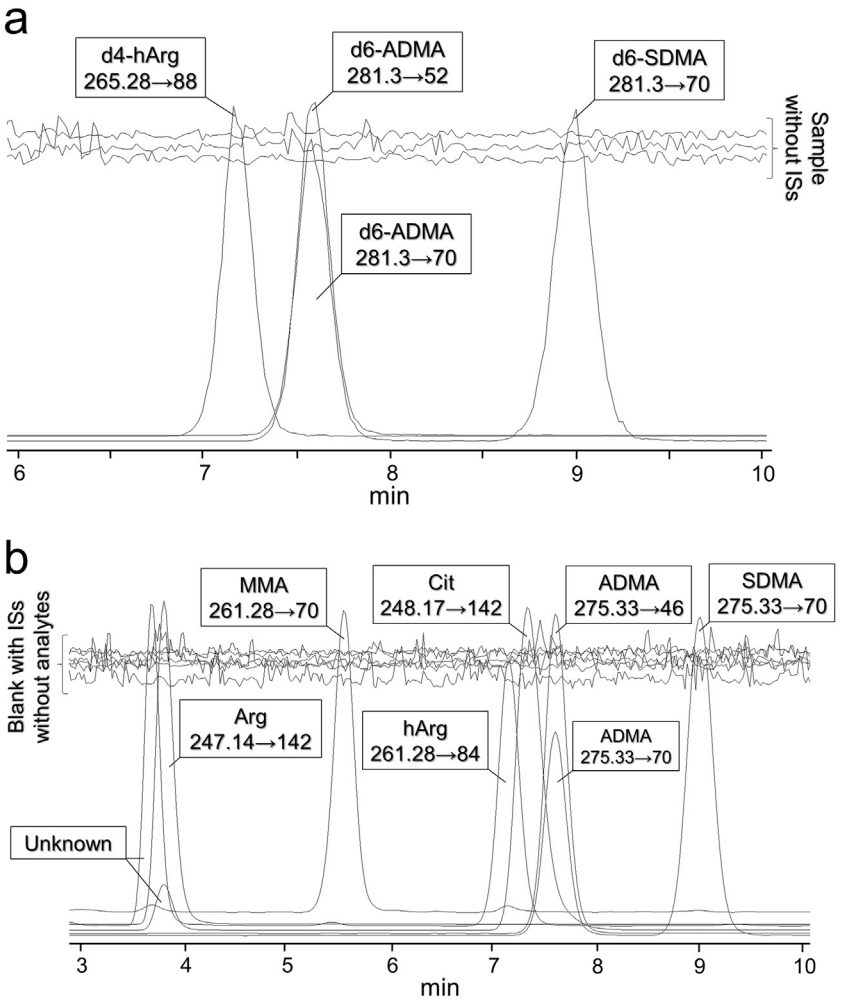

Fig. 4. a) Overlapping of the transitions of the internal standards from a blank solution containing both the ISs and analytes vs. a plasma sample without ISs and b) overlapping of the transitions of analytes from a plasma sample added with ISs towards a blank solution containing the ISs without analytes.

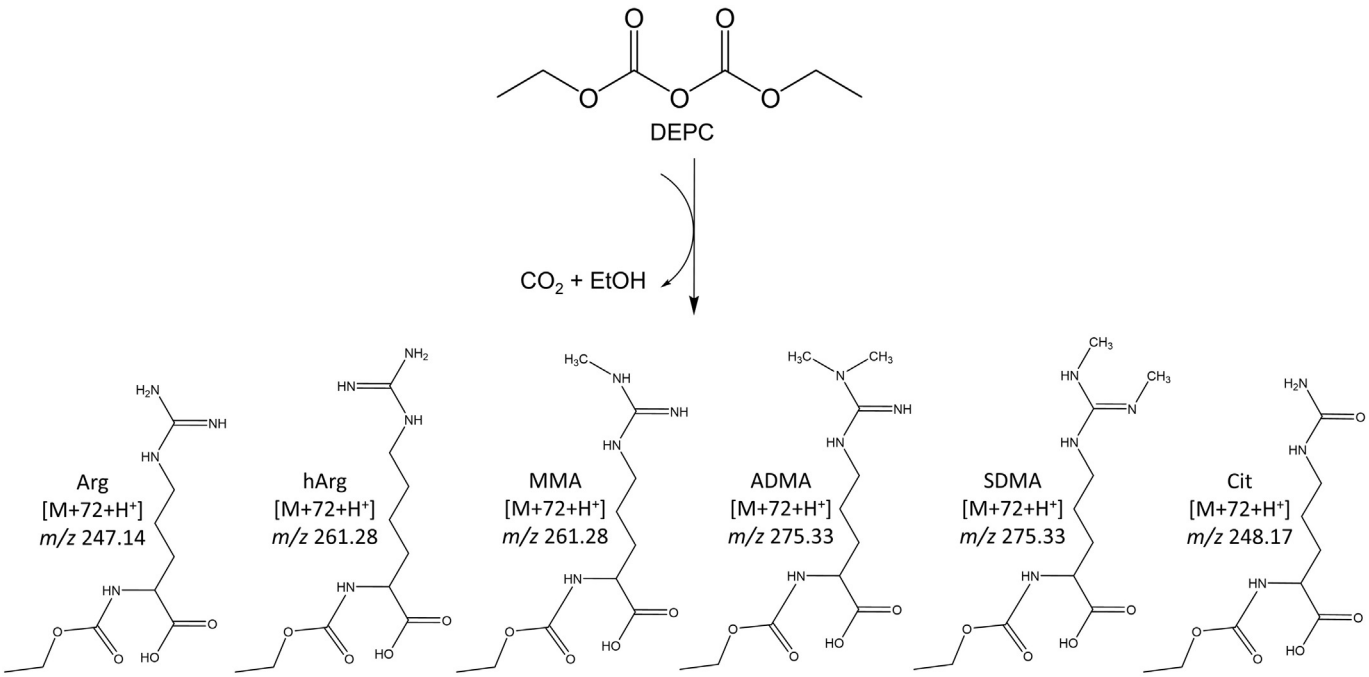

Fig. 5. Proposed reaction for the carbethoxylation of analytes by DEPC.

Table 3
The plasma analytes concentrations in 20 apparently health subjects.

Arg (μM ± SD)	MMA (nM ± SD)	hArg (μM ± SD)	ADMA (μM ± SD)	SDMA (μM ± SD)	Cit (μM ± SD)
58.62 ± 8.81	105.08 ± 21.66	1.88 ± 0.57	0.612 ± 0.140	0.581 ± 0.172	28.62 ± 11.60

this study, a single modification resulting in a mass increase of 72 Da occurs, thus allowing for a straightforward and sensitive detection of the modified analytes as $[(M + 72) + H]^+$ parent ions at *m/z* 247.14, 248.17, 261.28, 261.28, 275.33, and 275.33 for Arg, Cit, MMA, hArg, ADMA, and SDMA, respectively, and at *m/z* 265.28, 281.3, and 281.3 for d4-hArg, d6-ADMA, and d6-SDMA, respectively. Based on the mass increase of molecules following derivatization and on the MS/MS pattern of derivatives, we speculated that acylation does not involve the guanidino group but rather occurs principally at the α-amino moiety for all the analytes. A hypothetical reaction scheme is reported in Fig. 5. Chromatographically, only the separation of SDMA from ADMA was troublesome as it was not possible to identify a univocal MRM transition for derivatized SDMA that also did not recognize ADMA. On the contrary, the MRM transition for derivatized ADMA was unique, therefore, the optimization of the chromatographic conditions mainly concerned SDMA. As shown in Fig. 3, derivatization by DEPC converts the analytes into adducts with increased hydrophobicity, thus making them amenable to separation, under the established chromatographic conditions, in a short time period (< 10 min) by HPLC in RP-mode without using a gradient elution. Despite the short run time of the separation, ADMA and SDMA were well resolved whereby no issues linked to MS/MS cross-talk phenomena, potentially due to the share of the quantification MS/MS transition at *m/z* 281.3 → 70 of SDMA, were observed (see Fig. 4a and b). Similarly, no cross-talk between signals of the ISs and the target analytes were detected. As shown in Fig. 3, besides the analytes, the MS/MS transitions for hArg and Cit, *m/z* 261.28 → 84 and 248.17 → 142, respectively, were also able to detect two unidentified compounds. However, the unknown compounds did not interfere with the quantification of the analytes because of the good chromatographic resolution and their different MS/MS transitions with respect to the analytes with a similar retention time. Although DEPC is not a specific reagent for the analytes, an increase of selectivity by a deep sample clean-up was not needed. In this sense, sample preparation for LC-MS/MS analyses of Arg, its metabolites and analogs usually involves a clean-up step by solid-phase extraction on cation-exchange columns. This involves preventive removal of proteins by precipitation of proteins in acidic medium followed by centrifugation and filtration, or precipitation with organic solvents followed by lyophilization and/or evaporation [23,25]. As a time- and cost-saving alternative, we followed a heat-assisted extraction approach as previously described by Linz and Lunte [29], with some modifications. Two heat treatments at 100 °C in a block heater lasting 5 min each were performed instead of one in boiling water for 2 min. In the first step, heating causes plasma coagulation by inducing protein denaturation and subsequent aggregation. Previous studies showed that at this stage a complete recovery of the analytes from the clot into the water added following heating process occurs either extracting the mixture at 37 °C for 30–60 min or at room temperature overnight [30]. As the extraction rate is a function of temperature and time, a further heating process has been described as useful to speed up the extraction and to increase the recovery of the molecules. Thus, although a full recovery of the analytes was not required for this study as the accuracy of the analyses was ensured by the deuterated internal standards, the aim of the extra heating was to increase the sensitivity maximizing within 5 min the amount of the analytes extracted from the firm gel into the solution. This additional heating step notwithstanding, the entire procedure proved to be time-saving when compared with SPE or other pre-treatments such as evaporation or lyophilization. Overall, considering the time needed to perform the general samples handling such as the centrifugation and vortex-mixing cycles, it required 20 min to process up to 30 samples at a time. Although a specific evaluation was not performed in this study, the results ranging from 93 to 108% of the recovery experiments suggest that analytes are not liable to heat-induced degradation and therefore that the procedure can be reliably implemented. Hence, as displayed in Table 3, the application of the established method for the determination of the analytes in 20 apparently healthy male volunteers provided mean concentrations of 58.62 ± 8.81 μmol/L Arg, 105.08 ± 21.66 nmol/L MMA, 1.88 ± 0.57 μmol/L hArg, 0.612 ± 0.140 μmol/L ADMA, 0.581 ± 0.172 μmol/L SDMA, and 28.62 ± 11.60 μmol/L Cit that, overall, were consistent with that reported in a recent study [31]. The use of specific deuterated internal standards even for Arg, Cit, and MMA would further improve their quantification. ADMA inter-individual variability, 23%, was mildly higher than that, 12%, suggested by other authors [25,32]. However, the latter was computed in a much larger cohort of healthy individuals. Our values are consistent with those reported in studies conducted in a comparable number of individuals [33]. During the measurement of several hundred samples from larger clinical studies (results are pending publication elsewhere), no degradation of the analytical column or the performance of the mass spectrometer was observed thus supporting the usefulness of the method.

5. Conclusion

An attractive approach for the LC-MS/MS analysis of plasma concentrations of arginine and some of its chemically related metabolites and analogs is described herein. Sample preparation was simple, inexpensive and effortless and only required two short heat treatments to remove the proteins and to extract the analytes from the clot. Derivatization with DEPC was likewise fast and simple, enabling the use of conventional reversed-phase columns. Overall, given the short sample preparation and chromatographic run time, the method may be suitable for the fast and reproducible quantitative determination of the analytes in large clinical trials and routine analysis.

Declarations of interest

None.

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