COMPARISON BETWEEN ONE AND TWO-DIMENSIONAL LIQUID CHROMATOGRAPHIC APPROACHES FOR THE DETERMINATION OF PLASMATIC STROKE BIOMARKERS BY ISOTOPE DILUTION AND TANDEM MASS SPECTROMETRY

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ABSTRACT

This work presents the evaluation of one and two dimensional liquid chromatography for the quantification of three stroke outcome predictors in plasma. Isotopically labelled analogues of L-arginine (L-Arg), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are used to quantify the three analytes by isotope dilution and tandem mass spectrometry. Chromatographic isotope effects were not observed between natural L-Arg and its ^{15}N-labelled analogue but they were observed between natural ADMA and SDMA and their multiple deuterated analogues. Under these conditions bidimensional chromatography through the use of an automated multiple heart cutting mode provided unsatisfactory results for ADMA and SDMA due to the different amount of natural and labelled compound transferred from the first to the second chromatographic dimension. In contrast, using one dimensional liquid chromatography after a derivatization step to esterify carboxylic groups, chromatographic isotope effects did not alter the initial mass balance as full coelution of natural and labelled analogues or baseline resolution between the analytes were not required. This method was successfully validated following the Clinical & Laboratory Standards Institute guidelines and applied to the analysis of plasma samples from patients who had suffered an intraparenchymal haemorrhagic stroke.

INTRODUCTION

L-arginine (L-Arg) methylation is an important post-translational modification leading to asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)[1]. These molecules are involved in the metabolic pathway of nitric oxide and have a primary role in the physiopathology of vascular endothelial dysfunction and arteriosclerosis[2][3][4][5][6]. Several studies have shown the interest of ADMA and SDMA as biomarkers in pathologies with a high prevalence and economic impact, such as diabetes, chronic kidney disease or cardiovascular diseases [7][8][9]. Indeed, recent works indicated the relationship between the contents of ADMA and SDMA in plasma with the outcome after acute cerebrovascular events [10][11][12].

Despite these evidences, the determination of both methylarginines is still restricted to the scope of scientific research. The transition to the clinical laboratory requires the development of analytical methods, providing a satisfactory performance for a routine basis, as well as reference methods and certified reference materials. The determination of L-Arg, ADMA and SDMA by immunoassays or chromatography with fluorescence detection has been previously reported in the literature [13][14][15]. Liquid chromatography coupled to electrospray ionization and tandem mass spectrometry (HPLC-ESI-MS/MS) is the gold standard technique as it provides the required specificity, sensitivity and robustness for this clinical problem [16][17][18]. Nevertheless, when working with complex bio-matrices, ionization suppression effects in ESI significantly affect the sensitivity, selectivity, accuracy and precision of the developed methodologies [19]. As methylarginines in plasma are in the ng g^{-1} range [20][21], ESI ionization is affected due to the high matrix to analyte concentration ratio [19].

The use of surrogate internal standards is a widely adopted strategy to correct for matrix effects [22][23]. Surrogate internal standards of similar chemical structure than the analytes are commonly added at the beginning of the sample preparation to correct for such errors. However, they may present a different behaviour during the sample preparation and/or chromatographic separation. Thus, the use of stable-isotope labelled analogues as surrogate internal standards is the preferred strategy to achieve an efficient internal standardization [24][19]. In this way, isotope dilution mass spectrometry (IDMS) can be applied to improve robustness, accuracy, precision and sample throughput [25][26].

L-Arg, ADMA and SDMA are polar compounds that show poor retention in reversed phase chromatography. The addition of organic modifiers such as fluorinated acids increases the hydrophobicity of molecules by forming ion pairs with their charged groups. The interaction with the stationary phase is enhanced resulting in sharper and more symmetrical peaks [27][28][29]. However, fluorinated acids are not suitable for ESI-MS measurements as they causes an important signal suppression in the ESI source [30]. L-Arg, ADMA and SDMA, have been determined by hydrophilic interaction chromatography (HILIC) coupled to MS/MS but the use of small amounts of fluorinated acids was also required [27, 31]. Previous publications reporting the quantification of LArg, ADMA and SDMA make use of non-specific internal standards for at least one of the three compounds. Only one previous work reported the quantification of the three L-Arg, ADMA and SDMA with three compound-specific labelled internal standards [27]. However, they make use of TFA and propionic acid in aqueous mobile phases and they perform the quantification of the underivatized compounds. So, their method showed detection limits between 10 to 50 higher than those obtained in this work.

Two-dimensional liquid chromatography (2D-HPLC) in the multiple heart-cutting (MHC) mode allows the use of mobile phases in the first dimension which are not compatible with the ESI source [32-34]. MHC also enables a purification of the sample while increasing the chromatographic resolution between analytes and interfering matrix compounds as demonstrated previously in our laboratory [35]. However, 2D separations are more time consuming and require a more sophisticated instrumentation. Alternatively, the retention of polar compounds in reverse phase separations can be enhanced applying a derivatization step which, at the same time, may provide a significant improvement in the signal-to-noise ratio, alleviating the effect of complex matrices in ESI [36][37]. We compare in this work 2D-HPLC and chemical derivatization to develop a reference method for the accurate and precise determination of L-Arg, ADMA and SDMA in plasma by IDMS. The validation of the final methodology was performed according to the Clinical & Laboratory Standards Institute (CLSI) criteria, aimed at a future implementation in a clinical laboratory. The method is now being applied to the analysis of plasma samples of patients with haemorrhagic stroke.

EXPERIMENTAL

Instrumentation

Chromatographic separations were performed on an Agilent 1290 Infinity 2D-HPLC system. Measurements were carried out with a triple quadrupole mass spectrometer Agilent 6460 equipped with an electrospray source with a jet stream. OpenLab CDS Chemstation and MassHunter Acquisition software (Agilent Technologies) were used to control the 2D-HPLC system and the triple quadrupole respectively. The first dimension incorporated a 1290 Infinity binary pump connected to an autosampler, thermostated column compartment, and a 1260 Infinity variable wavelength detector. A 2-pos/4-port duo valve connects the two dimensions and is coupled to selector valves that include six 80 µL sampling loops. The same system was used for 1D-HPLC separation by connecting the 1D column directly to the MS system. All standard solutions and mixtures were prepared gravimetrically using an analytical balance model MS205DU (Mettler Toledo, Zurich, Switzerland). A CEP 2000 Benchtop Centrifuge (Capricorn Labs, Ringwood, UK) and a Micro Star 17 microcentrifuge (VWR International, Radnor, USA) were used for the centrifugation of plasma samples. A FB 15024 vortex mixer (Fisher Scientific, Hampton, NH, USA) was used for the homogenization of samples and working solutions. Ultra-pure water was obtained from a Purelab Flex 3 water purification system (Elga Labwater, Lane End, UK). For the derivatization step a Thermomixer compact (Eppendorf, Hamburg, Germany) was used. A centrifugal vacuum concentrator (Genevac, Suffolk, UK) was used to remove solvents. For the calculation of analytes concentration by IDMS Microsoft Excel© and IsoPatrn© software[38] were used.

Reagents and Materials

L-Arg, ADMA and SDMA as hydrochloride salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). Surrogate internal standards $^{15}N_4$-arginine (as its hydrochloride salt), $^{2}H_7$-ADMA and $^{2}H_6$-SDMA were purchased from Sigma-Aldrich, Cambridge Isotope Laboratory (Andover, MA, USA) and Toronto Research Chemicals (Toronto, Canada), respectively. Acetonitrile (Optima ^{TM} LC-MS Grade) was purchased from Fisher Scientific (Waltham, MA, USA), formic acid (>98%) was purchased from Sigma-Aldrich and heptafluorobutyric acid (99.5%) from Scharlab (Barcelona, Spain). The derivatizing reagent (3M solution of hydrogen chloride in 1-butanol) was purchased from Sigma-Aldrich.

Exemption of the informed consent was given from the Drug Research Ethics Committee of the Principality of Asturias, for using anonymized remaining plasma-EDTA samples from patients attended at the reference clinical laboratory of the HUCA (Central University Hospital of Asturias). These plasma samples were pooled, aliquoted and stored at -80 °C until the

analyses carried out for all the experimental development and validation procedures described in this paper.

Procedures

Sample treatment

A gravimetrically controlled amount of sample and isotopically labelled analogues was mixed in an Eppendorf tube. The resulting mixture was vortexed and poured into a Vivaspin™ 500 centrifugal concentrator (Sartorius, Goettingen, Germany) of 5000 molecular weight cut-off to remove proteins from biological fluids. The filters were centrifuged for 45 min at 16200 *g* and the filtered solution was taken and evaporated to dryness.

For 2D-HPLC-ESI-MS/MS using the MHC mode the dried sample was reconstituted in the 1D mobile phase and injected into the system. For 1D-HPLC-ESI-MS/MS measurements, 50 µL of 3M hydrogen chloride in 1-butanol solution was added to the dried sample as derivatizing reagent and the mixture was incubated at 60ºC for 30 min on a thermostatic shaker. Then, the derivatized sample was taken back to dryness, reconstituted in the 1D mobile phase and injected in the HPLC-system.

Chromatographic conditions by 2D-HPLC-ESI-MS/MS

The 1D-column was a ZORBAX 300 Å SB-C18 rapid resolution HD 2.1 x 100 mm 1.8 µm (Agilent Technologies) held at 30 ˚C during the chromatographic separation. Mobile Phases A and B were 0.1% heptafluorobutyric acid in water and acetonitrile, respectively. The 2D-column was a ZORBAX Eclipse plus C18 2.1 x 50 mm 1.8 µm (Agilent Technologies) held also at 30 ˚C. Mobile Phases A and B were 0.1 % formic acid in water and 0.1% formic acid in acetonitrile respectively. The injection volume of the prepared samples into the HPLC system was 2 µL. The chromatographic separation of the analytes was accomplished by gradient elution in the 1D-column. The flow rate in the first dimension was established at 0.4 mL min^{-1} and the gradient went from 2% B to 8% B in 6 min then, a linear gradient to 80% B up to 8 min was applied. A post-analysis time of 2 min was applied for column equilibration. The MHC was performed storing fractions of 80 µL of the mobile phase from the first dimension in three sampling loops at 2.17, 3.80 and 4.06 min for L-Arg, ADMA and SDMA respectively. In the second dimension an isocratic elution with 2% B as mobile phase was used at a flow rate of 0.4 mL min^{-1}. The overall analysis stop time was set to 25 min.

Chromatographic conditions by 1D-HPLC-ESI-MS/MS

A ZORBAX 300 Å SB-C18 rapid resolution HD 2.1 x 100 mm 1.8 µm (Agilent Technologies) held at 30 ˚C was use. The injection volume was 1 µL. Mobile Phases A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. The chromatographic separation of the analytes was accomplished by gradient elution. The established flow was 0.4 mL min^{-1} and the gradient went from 1% B to 10 % B in 6,5 min. Then, a linear gradient to 80% B up to 8 min was applied for column cleaning. A post-analysis time of 2 min was applied for column equilibration. L-Arg, ADMA and SDMA eluted at 2.6, 5.3 and 5.5 minutes, respectively. The overall analysis stop time was set to 12 min. In this case, the HPLC flow from the column was directly connected to the ESI source.

Measurement of the samples by tandem mass spectrometry

The HPLC system was coupled to a triple quadrupole mass spectrometer equipped with an electrospray source working in positive mode. The ionization source working conditions for 1D and 2D separations are given in **Table 1**. The parameters of the ion source are different when applying the 1D and 2D approaches for two main reasons. First, the mobile phase composition entering in the ion source are slightly different in both approaches and secondly, and more importantly, when applying the 2D approach underivatised compounds are ionized whereas when applying the 1D approach the derivatised compounds (esterification of the carboxylic group) are determined. In both approaches, the three analytes the precursor ion selected was a single charged ion $[M+1H]^+$ and the samples were measured using the Selected Reaction Monitoring mode (SRM). Fragmentation of the precursor ions was carried out with nitrogen as collision gas. A divert valve directed the HPLC flow of the chromatographic run to the ion source from 2 to 20 min in MHC-HPLC-MS/MS method and from 2.1 to 4.9 min in 1D-HPLC-MS/MS method, otherwise the eluent flow was directed to the waste. In both methods, two SRM transitions per analyte and two SRM transitions per surrogate internal standard were measured for quantification. The SRM transitions, collision energy and fragmentor voltage are given in **Table 2**. The fragmentation of L-Arg by Collision Induced Dissociation (CID) corresponded to loss of $[-NH_3]$ leading to the product ion $[C_{10}H_{20}N_3O_2]^+$. The fragmentation of ADMA corresponded to the loss of $[-C_2NH_7]$ leading to the product ion $[C_{10}H_{20}N_3O_2]^+$ whereas SDMA fragmentation lead to the loss of $[-CNH_5]$ and hence the product ion $[C_{11}H_{22}N_3O_2]^+$.

Quantification by isotope dilution and multiple linear regression.

For both 1D- and 2D- HPLC-MS/MS the concentration of the analytes was determined by isotope dilution tandem mass spectrometry and multiple linear regression. To apply this

strategy, the isotope distribution of the natural and labelled fragment ions are theoretically calculated using the software IsoPatrn© developed by Ramaley and Cubero Herrera [38]. For validation purposes, isotopic distributions can be also experimentally measured and compared with the theoretical values. Then, the isotopologue distribution of a given fragment ion in the isotope-diluted sample ($A_{mixture}$), can be expressed as a linear combination of the isotopologue distribution of natural abundance ($A_{natural}$) and isotopically labelled analogue (A_{tracer}). The relative contribution of both isotope patterns in the experimental mass spectrum are the molar fractions ($x_{natural}$ and x_{tracer}) that can be calculated by multiple linear regression solving equation (1) written for n isotopologues:

$$\begin{bmatrix} A^1_{mixture} \\ \vdots \\ A^n_{mixture} \end{bmatrix} = \begin{bmatrix} A^1_{natural} & A^1_{tracer} \\ \vdots & \vdots \\ A^n_{natural} & A^n_{tracer} \end{bmatrix} \cdot \begin{bmatrix} x_{natural} \\ x_{tracer} \end{bmatrix} + \begin{bmatrix} e^1 \\ \vdots \\ e^n \end{bmatrix} \quad (1)$$

The concentration of the analytes in the sample ($C_{natural}$), is then calculated by applying equation (2):

$$\mathrm{C_{natural}} = \mathrm{C_{tracer}} \times \frac{\mathrm{x_{natural}}}{\mathrm{x_{tracer}}} \times \frac{\mathrm{m_{tracer}}}{\mathrm{m_{natural}}} \times \frac{\mathrm{w_{natural}}}{\mathrm{w_{tracer}}} \quad (2)$$

Where C_{tracer} is the concentration of the analogues, m_{tracer} and $m_{natural}$ the weights taken from the labelled standard and sample and $w_{natural}$ and w_{tracer} the molecular weights of natural abundance and labelled analytes.

RESULTS AND DISCUSSION

Characterization of the internal standards

The application of IDMS and multiple linear regression avoids the use of a calibration graph for the quantification of the analytes in the sample. It provides the direct quantification of the analyte in the sample applying equation (2) but requires the previous knowledge of the isotopic enrichment and the amount of the labelled analogues added to the sample [25]. First, the isotopologue distribution of natural and labelled analogues must be measured and compared with the theoretical values that can be obtained using the software IsoPatrn© [38]. **Figure S1** shows the comparison of the theoretical and experimental values for the three analytes and their labelled analogues. As can be observed, the experimental isotopologue distributions obtained for both natural and labelled analogues modes agreed well with the theoretical values. Then, concentration of the working solutions of labelled compounds was calculated by reverse isotope

dilution using natural abundance standards. The isotopic enrichment of the labelled analogues was calculated as described in a previous publication [39]. The average enrichment of 5 independent replicates and the associated standard deviation obtained were 98.85 ± 0.02 %, 98.01 ± 0.01 % and 99.84 ± 0.00 % for $^{15}N_4$-arginine, $^{2}H_7$-ADMA and $^{2}H_6$-SDMA respectively.

2D-HPLC-MS/MS measurements

2D-HPLC-MS/MS was initially evaluated for the determination of plasmatic L-Arg, ADMA and SDMA combining two reversed phase separations. 2D-HPLC using the MHC mode allows the use of mobile phases in the first dimension which are not compatible with the ESI source as low volumes of the 1D mobile phase (tipically 40-80 µL) are transferred and diluted with the 2D mobile phase flow before entering the ESI source. Therefore, in the first dimension we could evaluate the use of HFBA as organic modifier. HFBA is not compatible with the ESI source due to the formation of non-volatile ion pairs that increase signal suppression. In contrast, its use enhances the hydrophobicity of the analytes by forming ion pairs with their charged groups. In this way, the interaction of the molecules with the hydrophobic stationary phase is increased providing a higher retention with sharper and more symmetrical peaks. Also, MHC enables the purification of the sample while increasing the chromatographic resolution between analytes and interfering matrix compounds. **Figure S2** shows HPLC-UV chromatograms of a standard solution containing 400 µg g^{-1} of L-Arg, 300 µg g^{-1} of ADMA and 200 µg g^{-1} of SDMA using 0.1 % HFBA as organic modifier. As can be observed, the three analytes show a significant retention in the C18 column, enabling its separation from the elution of other interfering polar compounds. After fraction collection, a reversed phase separation in which formic acid is used as organic modifier was applied in the second dimension and coupled to the ESI source. **Figure 1** shows the 1D-LC-UV and 2D-LC-MS/MS chromatograms of a standard solution containing 400 µg g-1 of L-Arg, 300 µg g-1 of ADMA and 200 µg g-1 of SDMA. The fractions transferred to the second dimension are subjected to a second chromatography in which specific SRM transitions for the analytes and the labelled analogues are measured by LC-MSMS

Once the MHC cutting conditions were optimized, we carried out the optimization of the injection volume to obtain the highest sensitivity for each analyte in real samples without enhancing ionization suppression due to matrix effects. A pooled plasma was measured by 2D-MHC-HPLC-MS/MS at different injection volumes (from 0.5 to 5 µL). **Figure S3** shows that 2 µL provided the highest sensitivity for the analytes with lower signal (ADMA and SDMA) so it was selected for further experiments. Then, the accuracy of the methodology was evaluated

analysing a fortified pooled plasma. In each measurement session, the endogenous concentration of the target compounds in the pooled plasma was determined analysing n=5 independent replicates injected in triplicate into the 2D-HPLC-MS/MS system. Then, fortified samples were prepared adding to 0.25 mL of the pooled plasma known amounts of the natural abundance analytes to perform recovery experiments at three different levels. To all fortified samples, a known amount of the three labelled analogues was also added for IDMS quantification. Three independent replicates of each concentration level were measured by 2D-HPLC-MS/MS. The whole recovery experiment was repeated in a second measurement session. **Tables S1**, **S2** and **S3** of the Supporting information shows the individual results of both measurement days. Although L-Arg recoveries ranged from 93 to 99 %, recoveries for ADMA ranged from 103 to 268 % and for SDMA from 143 to 185%. **Table 3** shows the Slope x 100 (%Recovery), intercept (endogenous concentration) and square of the correlation coefficient obtained when plotting the added concentration vs the experimental concentration of all the individual experiments. As can be observed, a satisfactory global recovery was obtained for L-Arg (96.3 ± 0.5%). However global recovery values of 163±7.8 % and 170±12.4 % were obtained for ADMA and SDMA respectively.

These results may be a consequence of chromatographic isotope effects occurring when deuterated compounds are used as surrogate internal standards for quantification by IDMS. When using deuterated compounds as internal standards, isotope effects may lead to a different retention time between the analytes and the labelled analogues. Such difference increases with the number of deuterium atoms present in the labelled molecule. When using the MHC mode, a different retention time between natural and labelled analogue may lead to a different amount of natural and labelled compound transferred from the first to the second chromatographic dimension. Thus, the molar ratio of natural and labelled analogue initially present in the sample is altered leading to a wrong determination of concentration of the analyte in the sample. If the natural abundance compound is preferentially transferred to the second dimension compared to the labelled analogue an overestimation of the recovery values is observed. This would explain the anomalous results observed for ADMA and SDMA, since seven and six deuterium atoms are present in their labelled analogues, respectively. This is not the case of L-Arg, as a $^{15}N_4$ labelled analogue was used and no isotope effects have been reported for ^{14}N and ^{13}C labelled analogues.

1D-HPLC-ESI-MS/MS measurements

Chromatographic separation by 1D HPLC-ESI-MS/MS

Due to the unsatisfactory results obtained when using the 2D-HPLC-ESI-MS/MS strategy with the MHC mode, we tried an alternative approach based on the application of a derivatization step to increase the retention of the analytes in the C18 column. For this purpose, the esterification of the carboxylic groups of L-Arg, SDMA and ADMA was carried out with 3M hydrogen chloride in 1-butanol. **Figure 2** shows a 1D-HPLC-ESI-MS/MS chromatogram of an isotope-diluted human plasma containing 6.35, 0.17 and 0.17 µg g^{-1} of L-Arg, ADMA and SDMA, respectively. A shift in the retention time between natural and deuterated ADMA and SDMA is observed but no shift is not observed between L-Arg and $^{15}N_4$-labelled L-Arg. As no fractionation of the sample is carried out before ESI-MS/MS measurement, the molar ratio is not altered. Also, different SRM transitions are measured for ADMA and SDMA (see **Table 2**). So, the initial mass balance is not altered as the proper integration of the natural and labelled peak areas does not require baseline resolution between compounds or a full coelution of natural and labelled analogues.

Sensitivity comparison between 2D-HPLC-MS/MS and 1D-HPLC-MS/MS

The analysis of the same pooled plasma was performed by 2D-HPLC-MS/MS and 1D-HPLC-MS/MS. **Figure S4** shows the comparison of the peak area obtained for the most abundant SRM transition for natural abundance L-Arginine, ADMA and SDMA by 1D-HPLC-MS/MS and 2D-HPLC-MS/MS. The peak areas obtained by 1D-HPLC-MS/MS were 6.18, 4.08 and 18.41 times higher than those obtained by 2D-HPLC-MS/MS for L-Arg, ADMA and SDMA, respectively. This is probably due not only to the absence of the fractionation process involved in 2D-HPLC-MS/MS but, more importantly, to a higher ionization efficiency of the derivatized analytes. According to these results, a full validation of the 1D-HPLC-MS/MS method was carried out following the guidelines of the CLSI.

Trueness of the 1D-HPLC-MS/MS methodology

The trueness of the 1D-HPLC-MS/MS methodology was evaluated following the CLSI C62 guideline. Due to the absence of reference materials and methods we performed spike and recovery analysis of a fortified pooled plasma. In each measurement session, the endogenous concentration of the target compounds in the pooled plasma was determined analysing n=3 independent aliquots injected in triplicate into the 1D-HPLC-MS/MS system hence resulting in

9 replicates per concentration level. Spiked samples were prepared adding, to 0.25 mL of the pooled plasma, known amounts of the natural abundance analytes to perform recovery experiments at three different levels. To all fortified samples, a known amount of the three labelled analogues was added for IDMS quantification. The whole experiment was repeated in four different measurement sessions to evaluate the reproducibility of the recoveries. **Tables S4**, **S5** and **S6** of the Supporting information shows the individual results obtained for L-Arg, ADMA and SDMA, respectively. **Table 3** shows the Slope x 100 (%Recovery), intercept (endogenous concentration) and square of the correlation coefficient obtained when plotting the added concentration vs the experimental concentration of all the individual experiments carried out. Satisfactory results were obtained for L-Arg, ADMA and SDMA with global recoveries of 103.5 ± 0.4 %, 105.7 ±0.5 % and 93.2 ±0.6 %, respectively.

Intraday and interday variability in the measurement of plasmatic L-Arg, ADMA and SDMA by 1D-HPLC-ESI-MS/MS.

The intraday (several independent sample preparations and injections in the same day) and the interday variability (repeating the experiment on different days) was evaluated following the CLSI EP15-A2 guideline. A pooled plasma was fortified with known amounts of natural standard to achieve four increasing concentration levels. The experiment was carried out in four different days. In each day, at least 3 independent replicates of an unspiked and spiked pool plasma were measured by 1D-HPLC-MS/MS. The results are given in **Table 4**. The repeatability values obtained in four different measurement sessions at four different concentration levels ranged, depending on the concentration level, from 0.4 to 4.0, from 0.7 to 7.3 and from 0.7 to 6.2 (expressed as % CV) for L-Arg, ADMA and SDMA respectively. The reproducibility values obtained from the measurements of all sessions ranged, depending on the concentration level, from 1.3 to 3.6, from 2.9 to 8.1 and from 2.0 to 5.2 (expressed as % CV) for L-Arg, ADMA and SDMA respectively.

Linearity assessment of plasmatic L-Arg, ADMA and SDMA determination by 1D-HPLC-ESI-MS/MS.

Linearity assessment of plasmatic L-Arg, ADMA and SDMA determination by 1D-HPLC-ESI-MS/MS was verified according to the CLSI EP06-A guidelines. Seven concentration levels of each analyte in the same pool of plasma were analysed. For all concentration levels, the IDMS quantification was carried adding the same amount of labelled compounds. **Table S7** shows,

for each level, the analyte concentration and the added amount of labelled compounds. The endogenous level was obtained from the direct measurement of the plasma pool whereas higher concentration levels were prepared by adding known amounts of natural standard to the plasma pool. Concentrations below the endogenous level were obtained by diluting the plasma pool with phosphate buffered saline (PBS). For each concentration level n=3 independent replicates were analysed and each replicate was injected in triplicate into the 1D-HPLC-ESI-MS/MS. **Figure 3** shows the results obtained when plotting the theoretical concentration versus the experimental concentration for the three compounds. As can be observed in **Table S7**, adding 3.5 µg of $^{15}N_4$-arginine, 0.2 µg of $^{2}H_7$-ADMA and 0.3 µg of $^{2}H_6$-SDMA, a satisfactory linearity is obtained from 1.3 to 73.5 µg L-Arg g^{-1}, from 0.03 to 1.7 µg ADMA g^{-1} and from 0.05 to 4.8 µg SDMA g^{-1}.

Carryover evaluation

In all measurement sessions carryover was evaluated injecting the same volume of the initial mobile phase after each sample and after every fourth sample the injection of the mobile phase was duplicated. We did not observe in any mobile phase injection a detectable signal of the analytes for the concentration range of the samples analysed in this work: from 3.8 to 29.3 µg g^{-1} for L-Arg, from 0.09 to 0.32 µg g^{-1} for ADMA and from 0.08 to 0.57 µg g^{-1} for SDMA.

Method limits of detection and quantification by 1D-HPLC-ESI-MS/MS.

Detection and quantification limits were calculated according to the EP17-A CLSI guideline. First, 3 independent replicates of a 1:5 dilution of a pooled plasma with PBS were analysed performing n=3 injections per diluted pooled plasma. The results obtained are shown in **Table 5**. Detection limits and quantification limits were calculated as 3 and 10 times the standard deviation of the measurements, respectively. Secondly, we analysed 6 independent replicates of PBS performing n=10 injections per blank. **Table 6** shows that detection and quantification limits were significantly higher when analysing the diluted pooled plasma than PBS for the three analytes due to matrix effects. For L-ARG and ADMA the limits increased about 20 times but for SDMA detection and quantification limits increased about 200 times as shown in **Table 5**. For the method application in human plasma samples, the detection and quantification limits obtained from diluted plasma should be chosen. **Figure S7** of the supporting information shows a representative 1D-HPLC-ESI-MS/MS chromatogram of the 1:5 dilution of the pooled plasma.

Determination of L-Arg, ADMA and SDMA in plasma of patients with haemorrhagic stroke.

The 1D-HPLC-ESI-MS/MS method is currently applied to plasma samples from a clinical study of 40 patients who had suffered an intraparenchymal haemorrhagic stroke in the last 12 hours. The median (interquartile range) obtained expressed as µg g^{-1} were 10.46 (8.29-13.03), 0.16 (0.14-1.18) and 0.14 (0.12-1.18) for L-Arg, ADMA and SDMA respectively. For the three compounds, the imprecision and detection limits of the method is adequate. The median plasma levels obtained for the target compounds in these patients were comparable to data published papers by other authors [11], and the potential prognosis value of the markers is now being studied (manuscript in progress).

CONCLUSIONS

HPLC-MS/MS is the method of choice for the determination of L-Arg, ADMA and SDMA in human plasma. However, due to the low retention of these polar compounds in reversed phase separations we evaluated two alternative strategies. First, 2D-HPLC-MS/MS using the MHC mode, which allows the use of mobile phases not compatible with ESI and secondly, the combination of a derivatization step and conventional 1D-HPLC-MS/MS.

The 2D-HPLC-MS/MS method using 0.1% heptafluorobutyric acid in water as aqueous mobile phase in the first dimension increases the retention of the analytes in the chromatographic column, providing good peak shape and chromatographic resolution while avoiding the derivatization step prior MS/MS measurements. However, recovery experiments demonstrated that important errors in the determination of ADMA and SDMA by IDMS in fortified samples occurred. This was attributed to a different chromatographic behaviour of the analytes and their deuterated analogues rather than potential H-D exchanges during sample preparation. It would be thus recommended the use of ^{15}N- or ^{13}C-labelled analogues to avoid chromatographic isotope effects when the multiple heart cutting mode is applied. Due to the lack of commercially available ^{15}N- or ^{13}C-labelled ADMA and SDMA the full validation of the 2D-HPLC-MS/MS strategy could not be carried out.

In contrast, esterification of the carboxylic groups prior 1D-HPLC-MS/MS provided a satisfactory retention of the analytes in the chromatographic column using ESI compatible mobile phases, a faster chromatographic separation than 2D-HPLC-MS/MS and an increase of sensitivity. Following the CLSI recommendations we have demonstrated that the method could be considered as a candidate reference method for the determination of plasmatic L-Arginine, ADMA and SDMA as it offers satisfactory results in terms of precision, accuracy, linearity and

limits of detection and quantification for the three analytes. According to the values obtained in samples from patients who had suffered an intraparenchymal haemorrhagic stroke in the last 12 hours the analytical characteristics of the method were found to be satisfactory to continue with our clinical study.

AUTHOR CONTRIBUTIONS

Adela Cortés Giménez-Coral: Investigation, validation, methodology, writing original draft. **Pablo Rodríguez-González**: supervision, writing-review and editing, methodology, project administration and funding acquisition. Adriana González Gago: supervision, writing-review and editing, methodology **Eva Cernuda Morollón** and **Elena Lopez-Cancio**: Resources. **Belén Prieto**: supervision, writing-review and editing, methodology and resources. **J. Ignacio García Alonso**: supervision, writing-review and editing and funding acquisition.

CONFLICTS OF INTEREST

There are no conflicts to declare.

ACKNOWLEDGEMENTS

Adela Cortés Giménez-Coral acknowledges financial support from the Jose Luis Castaño-SEQC foundation through post residencia scholarship. Financial support from the Spanish Ministry of Science and Innovation through Project PGC2018-097961-B-I00 is acknowledged.

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FIGURES AND TABLES

Figure 1. 1D-LC-UV and 2D-LC-MS/MS chromatograms of a standard solution containing 400 µg g-1 of L-Arg, 300 µg g-1 of ADMA and 200 µg g-1 of SDMA. The fractions transferred to the second dimension are indicated in yellow in the 1D-LC-UV chromatogram. The SRM transitions measured for the three analytes in the second dimension are included in the 2D-LC-MSMS chromatograms

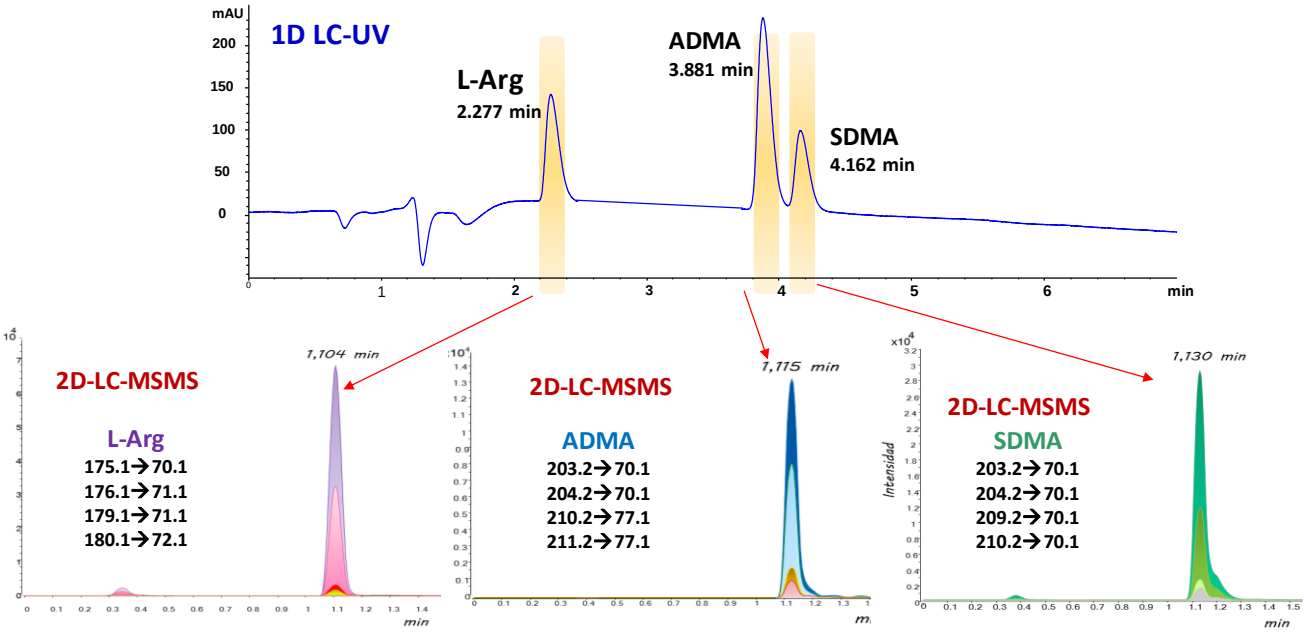

Figure 2. 1D-HPLC-MS/MS chromatogram of an isotope diluted human plasma sample containing 6.35,0.17 and 0.17 µg g-1 of L-Arg, ADMA and SDMA respectively.

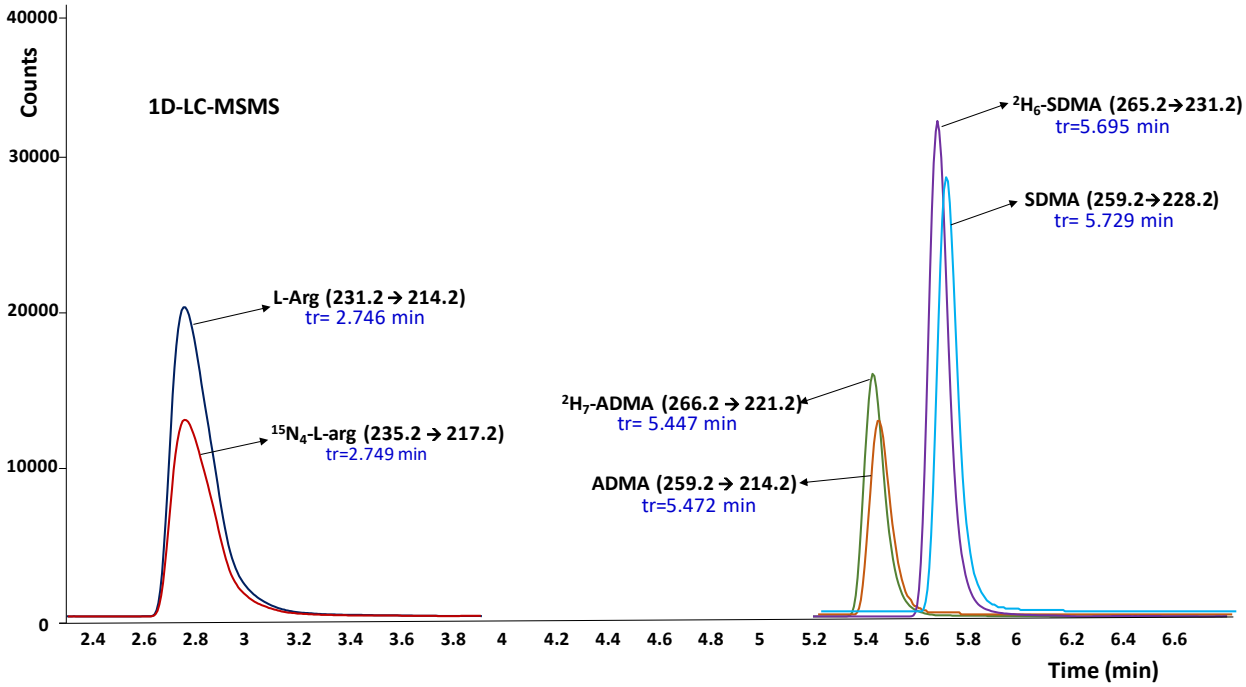

Figure 3. Comparison of the theoretical and experimental concentration obtained at seven concentration levels of L-Arg, ADMA and SDMA.

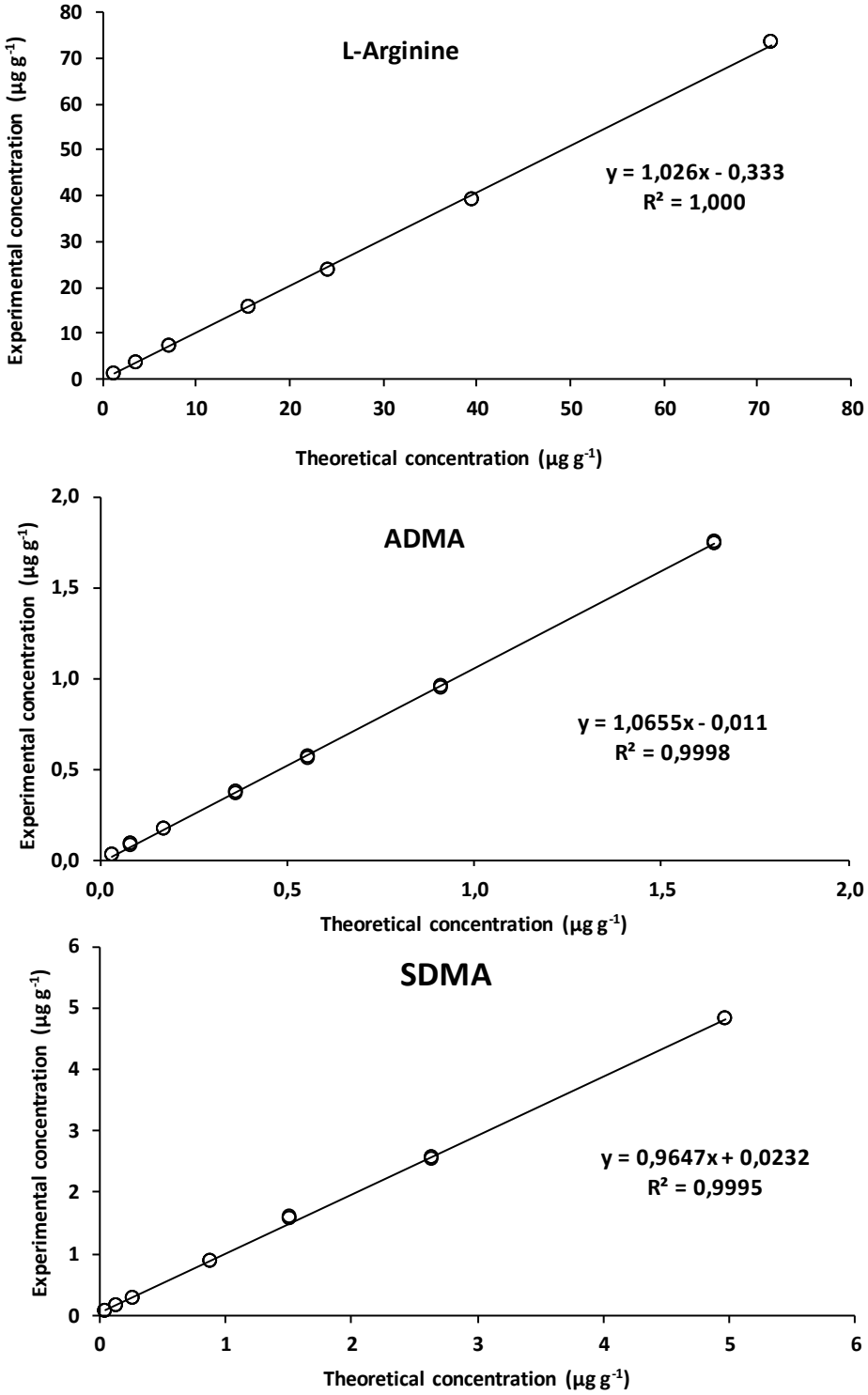

Table 1. Experimental conditions of the ionization source applied in the 1D-HPLC-MS/MS and 2D-HPLC-MS/MS methods developed in this work.

	1D-HPLC-MS/MS	2D-HPLC-MS/MS
Mass spectrometer	Agilent 6460	
Ion source	Electrospray jet stream	
Ionization mode	Positive	
Gas temperature	350 ˚C	300 ˚C
Gas flow	5 L min^{-1}	9 L min^{-1}
Sheath gas temperature	400 ˚C	400 ˚C
Sheath gas flow	12 L min^{-1}	12 L min^{-1}
Nebulizer pressure	35 psi	40 psi
Capillary voltage	2500 V	3000 V
Nozzle voltage	0 V	0 V

Table 2. Optimum values of SRM transitions, fragmentor voltage and collision energy for analytes and labelled analogues applied in the 1D-HPLC-MS/MS and 2D-HPLC-MS/MS methods developed in this work.

	1D-HPLC-MS/MS			2D-HPLC-MS/MS		
	SRM transition	Fragmentor (V)	Collision Energy (eV)	SRM transition	Fragmentor (V)	Collision Energy (eV)
L-Arginine	231.2→214.2	80	10	175.1→70.1	135	24
	232.2→215.2	80	10	176.1→71.1	135	24
$^{15}N_4$-Arginine	235.2→217.2	80	10	179.1→70.1	135	24
	236.2→218.2	80	10	180.1→71.1	135	24
ADMA	259.2→214.2	100	12	203.2→70.1	135	28
	260.2→215.2	100	12	204.2→70.1	135	28
$^{2}H_7$-ADMA	265.2→220.2	100	12	210.2→77.1	135	28
	266.2→221.2	100	12	211.2→77.1	135	28
SDMA	259.2→228.2	100	12	203.2→70.1	135	24
	260.2→229.2	100	12	204.2→70.1	135	24
$^{2}H_6$-SDMA	265.2→231.2	100	12	209.2→70.1	135	24
	266.2→232.2	100	12	210.2→70.1	135	24

Table 3. Slope x100 (% recovery), intercept (endogenous concentration) and correlation coefficient when plotting the added concentration versus the experimentally obtained concentration obtained for L-Arg, SDMA and ADMA in a pooled plasma by 2D- and 1D-HPLC-ESI-MS/MS. Uncertainty of the values correspond to the standard deviation obtained from

n=2 and n=4 independent recovery experiments carried out in different days by 2D- and 1D-HPLC-ESI-MS/MS, respectively. Each recovery experiment was carried out at three different concentration levels in which n=3 independent replicates were analysed.

	L-Arginine		ADMA		SDMA	
	2D	1D	2D	1D	2D	1D
Added concentration ($\mu g\ g^{-1}$) (Level 1, 2 and 3)	7.9, 15.7, 31.6	8.1, 15.9, 30.6	0.2, 0.4, 0.7	0.2, 0.4, 0.8	0.7, 1.3, 2.7	0.6,1.23, 2.5
Correlation coefficient R^2	1.00	1.00	0.84	1.00	0.69	0.99
Endogenous concentration ($\mu g\ g^{-1}$) (Intercept)	7.19 ± 0.08	7.29 ± 0.08	0.18 ± 0.03	0.19 ± 0.00	0.44 ± 0.17	0.31 ± 0.01
% Recovery (Slope x100)	96.3 ± 0.5	103.5 ± 0.4	163.4 ± 7.8	105.7 ± 0.5	170.1 ± 0.1	93.2 ± 0.6

Table 4. Number of injections, concentration ($\mu g\ g^{-1}$) and % CV of L-Arg, ADMA and SDMA at four concentration levels. Uncertainty of the values correspond to the standard deviation of the concentration obtained for the replicates indicated.

Level	Day	Injections			Concentration ($\mu g\ g^{-1}$)			CV (%)		
		L-Arg	ADMA	SDMA	L-Arg	ADMA	SDMA	L-Arg	ADMA	SDMA
0	1	18	18	18	7.25 ± 0.03	0.20 ± 0.00	0.27 ± 0.00	0.4	1.8	0.7
	2	18	18	18	7.27 ± 0.12	0.17 ± 0.00	0.27 ± 0.00	1.7	1.7	1.6
	3	9	9	9	7.36 ± 0.02	0.20 ± 0.01	0.28 ± 0.00	0.4	5.3	0.9
	4	9	9	9	7.38 ± 0.05	0.19 ± 0.00	0.26 ± 0.00	0.6	0.7	0.9
	Average (54 injections)				**7.30 ± 0.09**	**0.19 ± 0.02**	**0.27 ± 0.01**	**1.3**	**8.1**	**2.0**
1	1	9	9	9	15.32 ± 0.23	0.40 ± 0.01	0.91 ± 0.01	1.5	1.8	1.6
	2	9	9	9	15.17 ± 0.42	0.38 ± 0.03	0.87 ± 0.05	2.8	7.3	6.2
	3	9	9	9	15.98 ± 0.28	0.41 ± 0.01	0.92 ± 0.02	1.7	2.8	2.0
	4	9	9	9	15.57 ± 0.42	0.38 ± 0.01	0.91 ± 0.01	2.7	2.9	0.8
	Average (36 injections)				**15.48 ± 0.51**	**0.39 ± 0.02**	**0.89 ± 0.05**	**3.3**	**6.4**	**5.2**
2	1	9	9	9	25.51 ± 0.54	0.61 ± 0.01	1.55 ± 0.02	2.3	1.3	1.0
	2	9	9	9	23.71 ± 0.44	0.59 ± 0.02	1.55 ± 0.01	1.8	3.7	0.9
	3	9	9	9	23.27 ± 0.93	0.58 ± 0.02	1.49 ± 0.06	4.0	3.1	3.8
	4	9	9	9	24.70 ± 0.81	0.61 ± 0.01	1.57 ± 0.05	3.3	1.8	3.5
	Average (36 injections)				**23.85 ± 0.85**	**0.59 ± 0.02**	**1.54 ± 0.05**	**3.6**	**3.6**	**3.2**
3	1	9	9	9	40.03 ± 0.20	1.01 ± 0.01	2.68 ± 0.06	0.5	1.0	2.1
	2	9	9	9	39.97 ± 0.35	0.99 ± 0.03	2.58 ± 0.12	0.9	2.9	4.8
	3	9	9	9	42.64 ± 1.51	1.02 ± 0.02	2.62 ± 0.14	3.5	2.2	5.4
	4	9	9	9	41.75 ± 0.41	1.01 ± 0.02	2.65 ± 0.12	0.4	2.0	4.4
	Average (36 injections)				**41.08 ± 1.40**	**1.00 ± 0.03**	**2.60 ± 0.13**	**3.4**	**2.9**	**4.9**

Table 5. Average concentration, standard deviation, detection limit and limit of quantification expressed as three and ten times the standard deviation of the measurements of blank (B) and low concentration samples (LCS) for L-Arg, ADMA and SDMA.

		Replicates	Average ($ng\ g^{-1}$)	SD	LOD ($ng\ g^{-1}$) 3 SD	LOQ ($ng\ g^{-1}$) 10 SD
L-Arginine	**B**	60	37.8	1.80	5.41	18.04
	LCS	9	1368.2	32.3	96.7	322.9
ADMA	**B**	60	0.07	0.03	0.09	0.29
	LCS	9	30.73	0.48	1.43	4.76
SDMA	**B**	60	0.02	0.01	0.02	0.08
	LCS	9	50.31	1.58	4.75	15.83