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HPLC analysis of asymmetric dimethylarginine, symmetric dimethylarginine, homoarginine and arginine in small plasma volumes using a Gemini-NX column at high pH

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ABSTRACT

There is increasing recognition of the clinical importance of endogenous nitric oxide synthase inhibitors in critical illness. This has highlighted the need for an accurate high performance liquid chromatography (HPLC) method for detection of asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) in small volumes of blood. Here, the validation of an accurate, precise HPLC method for the determination of ADMA, SDMA, homoarginine and arginine concentrations in plasma is described. Solid phase extraction is followed by derivatisation with AccQ-Fluor™ and reversed phase separation on a Gemini-NX column at pH 9. Simultaneous detection by both UV–vis and fluorescence detectors affords extra validation. This solid phase extraction method gives absolute recoveries of more than 85% for ADMA and SDMA and relative recoveries of 102% for ADMA and 101% for SDMA. The intra-assay relative standard deviations are 2.1% and 2.3% for ADMA and SDMA, respectively, with inter-assay relative standard deviations of 2.7% and 3.1%, respectively. Advantages of this method include improved recovery of all analytes using isopropanol in the solid phase extraction; sharp, well-resolved chromatographic peaks using a high pH mobile phase; a non-endogenous internal standard, n-propyl L-arginine; and accurate and precise determination of methylated arginine concentrations from only 100 μL of plasma.

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1. Introduction

The clinical importance of endogenous nitric oxide synthase (NOS) inhibitors has long been recognised in chronic disease [1]. Nitric oxide (NO) is important in the maintenance of normal endothelial function [2] and the prevention of platelet aggregation [3]. NO synthesis from L-arginine is reduced in the presence of asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), which are products of methylated protein degradation.

ADMA and homoarginine compete with arginine for specific binding sites on NOS. Homoarginine is an alternative but less efficient substrate for NOS [4] whereas ADMA directly inhibits nitric oxide synthases. ADMA, SDMA and homoarginine each compete with arginine for transport into the cell [5] and may therefore, also limit the amount of arginine available to NOS [6,7]. High concentrations of methylated arginines have been associated with a broad range of chronic diseases, including hypertension [8], renal failure [1], hypercholesterolemia [9] and diabetes [10]. Indeed, elevated ADMA is an independent risk factor for both cardiovascular disease [11] and all-cause mortality [12].

In addition to the importance of ADMA in chronic disease, there is increasing recognition of its important role in acute critical illness [13,14] and acute inflammatory conditions such septic shock [15]. As limited blood is available from critically ill patients, there is a need for an accurate high performance liquid chromatography (HPLC) method for detection of ADMA and SDMA in small volumes of blood.

This paper describes a reversed phase HPLC method for the measurement of arginine, ADMA, SDMA and homoarginine from 100 μL of plasma. The chromatography utilised a Gemini-NX column with a novel, high pH borate buffer-acetonitrile gradient, and the non-endogenous internal standard n-propyl L-arginine (NPLA). Sample preparation utilised solid phase extraction (SPE) and fluorescent derivatisation. The extraction procedure and HPLC method give accurate and precise results from a small volume of plasma.

2. Experimental

2.1. Materials

L-Arginine-HCl, L-homo-arginine-HCl, N^G,N^G di-methyl-L-arginine and $N^G,N^{G'}$ di-methyl-L-arginine were purchased from

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Calbiochem (La Jolla, CA, USA). n-Propyl L-arginine was a product of Cayman Chemicals (Ann Arbor, MI, USA). Sodium tetra borate decahydrate and boric acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Oasis Mixed Mode Cation Exchange (MCX) cartridges (1 mL, 30 cm^3) were purchased from Waters (Milford, MA USA). Isopropanol and ammonia solution 28–30% were purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskego, MI, USA). High purity water was used to prepare all aqueous solutions (Milli-Q water system, Milli-Pore, Billerica, MA, USA). The AccQ-Fluor™ kit from Waters (Milford, MA, USA) contained the fluorescent reagent 6-aminoquinolyl-N-hydroxysuccinimidyl, a vial of acetonitrile diluent, and a vial of aqueous borate buffer (0.2 M, pH 8.8) for the derivatisation reaction.

2.2. Plasma samples

Venous blood from healthy volunteers or patients was collected into lithium heparin tubes, centrifuged (492 × *g* for 8 min) within 120 min of collection and the plasma were frozen at −80 °C until analysis. A pool of plasma from Australian Red Cross blood donors was used as quality control plasma.

Plasma from 30 apparently healthy volunteers was used to determine healthy concentrations of ADMA and SDMA. 8 of these volunteers were laboratory staff (blood collected as above) and 22 were blood bank donors (blood collected according to standard Australian Red Cross blood bank procedures). Blood from blood bank donors was usually separated the day after collection. The age range of the healthy volunteers was 16–61; 18 were female and 12 were male. The use of this plasma was approved by the Ethics Committees of the Australian Red Cross and the Menzies School of Health Research.

2.3. Extraction

Oasis MCX cartridges were affixed to a vacuum manifold and pre-equilibrated with 1 mL of isopropanol, followed by 1 mL of 50 mM borate buffer (pH 9). 100 μL of plasma or calibrator was mixed with 100 μL 15 μM NPLA and diluted with 800 μL 50 mM borate buffer (pH 9) and then loaded onto the cartridge. Cartridges were then washed with 1 mL of water and then 1 mL of isopropanol. Extracts were eluted from the cartridges into glass collection tubes with 1 mL of eluting solvent (isopropanol:water:28–30% ammonia solution (5:4:1)). Flow rates were controlled by vacuum adjustment. The vacuum manifold pressure was less than 254 mm Hg for the pre-equilibration and wash steps, and less than 127 mm Hg for the loading and eluting steps.

Extracts were dried under nitrogen at 75 °C (for approximately 1 h). Dried eluates were reconstituted in 0.2 mL water and transferred to glass storage vials.

2.4. Derivatisation

Extracts were derivatised with Waters AccQ-Fluor™ kit prior to chromatography. In a 250 μL HPLC vial insert; 20 μL of extract, diluted with 70 μL of Waters' borate buffer, was reacted with 10 μL AccQ-Fluor™ reagent by immediate vortexing for 10 s.

2.5. Chromatography

The Shimadzu VP series HPLC system consisted of a gradient pump, degasser, column oven (42 °C) and UV-vis and fluorescence detectors. The detectors were connected in series for simultaneous detection of UV (absorption wavelength = 250 nm) and fluorescence (excitation wavelength = 250 nm, emission wavelength = 395 nm). Extracts were separated on a C18 Gemini-NX analytical column (150 mm × 4.6 mm, 3 μm) protected by a C18 Gemini-NX security guard cartridge (4.0 mm × 3.0 mm), both from Phenomenex (Lane Cove, NSW, Australia). Mobile phase flow rate was 1 mL min^{-1}.

Table 1
Mobile phase delivery program.

Time (min)	Eluent[a]	Value (%)	Event
0.00–18.00	A:B	93:7	Isocratic
18.01–21.00	A:B	93:7 ≫ 92:8	Gradient 7–8% over 3 min
21.01–29.00	A:B	92:8	Isocratic
29.01–40.00	A:B	87:13	Isocratic
40.01–52.00	B:C	65:35	Wash

[a] Eluents: 20 mM borate buffer pH 9 (A), acetonitrile (B) and water (C).

A 100 mM stock solution of sodium tetra borate/boric acid was prepared and filtered (0.2 μm) into a sterile container. The stock was kept at room temperature. Eluent A was a 1:5 dilution of the borate buffer stock solution.

The mobile phase delivery program of 20 mM borate buffer pH 9 (A), acetonitrile (B) and water (C) is shown in Table 1. All eluents were filtered through 0.45 μm filters before use.

2.6. Calibration and validation

Stock solutions of arginine (2.5 mM), homoarginine (500 μM), ADMA (100 μM), SDMA (100 μM) and NPLA (2.5 mM) were prepared, aliquoted and stored at −80 °C. Seven calibration standards were made to encompass physiological and disease-associated concentration ranges. Arginine covered the range of 7.5–200 μM, homoarginine 0.5–12 μM, ADMA 0.25–6 μM and SDMA 0.25–6 μM. The calibration standards were extracted and derivatised in the same manner as plasma samples. Identification of analytes within plasma samples was based on the retention time of the corresponding standard. A seven level calibration curve for each analyte, using peak area/amount ratios of the analytes to internal standard was constructed from integrated chromatograms.

Analyte recovery during the extraction process was determined by calculating the relative recovery and absolute concentrations recovered after calibration standards were subjected to SPE compared with un-extracted calibrator concentrations. Seven standards were run without undergoing SPE in parallel with aliquots of the same standards subjected to SPE. Absolute recovery was calculated by comparing the area of the extracted peaks to the area of the un-extracted peaks. This ensured no particular analyte was preferentially lost through extraction. Relative recovery was calculated by plotting the extracted calibrators onto the curve of the un-extracted calibrators. The percent recovery was calculated by dividing the measured concentration by the theoretical concentration from the un-extracted curve.

The HPLC method was validated by calculating the intra-assay and inter-assay precision of pooled quality control plasma and by determining the spike recovery of analyte added to control plasma. The intra-assay precision of the HPLC method was determined by running a single extract of control plasma 10 times consecutively and calculating the concentration of the analytes of interest. Inter-assay precision was calculated by extracting and running 30 separate control plasmas over 2 months. In order to determine the accuracy of the HPLC method, the pooled quality control plasma was spiked with known concentrations of arginine, homoarginine, ADMA and SDMA. The percent spike recovery was expressed as the recovery of added analyte from spiked plasma samples. This process was repeated three times in 6 months.

Limit of detection (LOD) was determined by a signal to noise ratio of 2:1 and the limit of quantification (LOQ) was determined by a signal to noise ratio of 10:1.

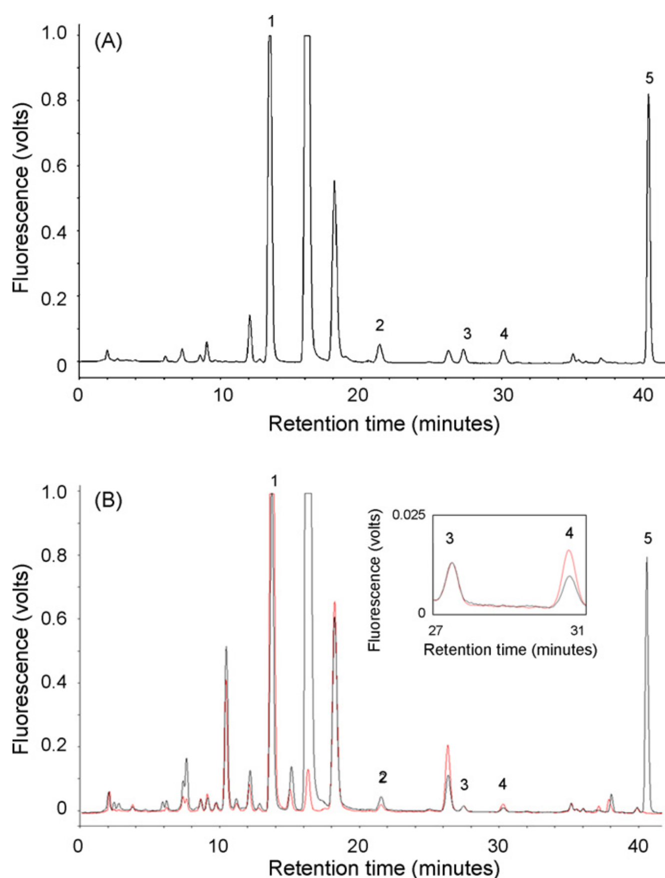

Fig. 1. Fluorescence detection of a calibration standard (A) with 30 μM arginine, 2 μM homoarginine, 1 μM ADMA and 1 μM SDMA and (B) the pooled quality control plasma (black) with 23.68 μM arginine, 1.82 μM homoarginine, 0.48 μM ADMA and 0.39 μM SDMA, overlaid with a chromatogram from a patient with falciparum malaria (red) without internal standard added. Peak identity: (1) arginine; (2) homoarginine, (3) ADMA, (4) SDMA, (5) NPLA. Inset B: region 27–31 min magnified 40×.

3. Results and discussion

3.1. Chromatography

Homoarginine, ADMA and SDMA were detected simultaneously using UV and fluorescence detection. Arginine was out of range of fluorescence detection once above 30 μM and was therefore primarily detected by UV. There was less than 5% deviation between ADMA and SDMA values measured by either fluorescence or UV. Validation data presented in this paper is from the fluorescent detection of ADMA, SDMA and homoarginine and the UV detection of arginine.

This method provided excellent separation of arginine, homoarginine, ADMA, SDMA and NPLA. Fig. 1 shows the separation of analytes in a standard, the pooled quality control plasma and plasma from a malaria patient. Blank samples of water also underwent the extraction and derivatisation processes and were chromatographed to ensure there were no co-eluting peaks originating from the SPE method or the derivatising agent. The pooled quality control plasma and plasma from 2 patients with bacterial sepsis and 2 patients with falciparum malaria were subjected to SPE without the addition of internal standard, to ensure there was a flat baseline under NPLA (see Fig. 1B).

The coefficient of determination (r^2) for each analyte was >0.999. Limit of detection was 0.04 μM for arginine, 0.06 μM for homoarginine, 0.04 μM for ADMA and 0.03 μM for SDMA. The limit of quantification was 0.20 μM for arginine, 0.30 μM for homoarginine, 0.20 μM for ADMA and 0.15 μM for SDMA.

Table 2
Average absolute and relative recovery of analytes from 7 level calibration standards after solid phase extraction ($n = 4$).

Analyte (conc. range)	Absolute recovery mean ± SD %	Relative recovery mean ± SD %
Arginine (7.5–200 μM)	80.9 ± 5.6	98.9 ± 2.5
Homoarginine (0.5–12 μM)	78.1 ± 5.6	94.9 ± 3.2
ADMA (0.25–6 μM)	85.1 ± 6.5	101.6 ± 1.3
SDMA (0.25–6 μM)	86.3 ± 5.2	101.4 ± 2.4
NPLA (15 μM)	83.4 ± 5.5	100.0

Borate was chosen as the mobile phase buffer in this method as it is also the matrix of the derivatised samples and greatest retention time reproducibility is obtained when samples are dissolved in a similar solution to the mobile phase. The borate buffer was prepared to pH 9 as the pK_a of borate buffer is 9.2 and buffers are most effective within 0.5 pH units of their pK_a. The combination of high pH and acetonitrile resulted in sharp, well-resolved chromatographic peaks. The Gemini-NX column was selected for this method as it has a large pH stability range of 1–12.

3.2. Extraction and derivatisation

A number of different extraction solvents and procedures were trialled, including the procedures recommended in the Oasis MCX cartridge literature. Most published methods use methanol in the final eluting solution and/or during the pre-equilibration and wash stages. However, optimal recovery of all analytes, especially NPLA, was obtained by substituting methanol with the slightly less polar alcohol, isopropanol. The cleanest extracts were produced when the cartridges were pre-equilibrated with the sample matrix (50 mM borate pH 9). Water was added to the eluting mixture to increase arginine recovery [16]. The absolute and relative recoveries of the SPE method are shown in Table 2.

As the fluorescent adducts of AccQ-Fluor^{TM} are stable for at least 7 days [17], large batches of samples can be efficiently extracted and derivatised.

3.3. Method validation

Method precision was evaluated using the pooled quality control plasma. The inter-assay percent relative standard deviations (RSDs) ($n = 10$) were less than 2.3% for all analytes. The inter-assay RSDs for ADMA (2.7%) and SDMA (3.1%) compare very well to other HPLC assays using fluorescence detection [16–20] and to HPLC or gas chromatography mass spectrometry methods [21,22]. As ADMA and SDMA have a very narrow concentration range in the general population, high analytical precision is required to produce clinically useful results [23]. Blackwell et al. [24] recently determined the intra-individual variability for ADMA and SDMA to be 7.4% and 5.8%, respectively in healthy European volunteers. The minimum required precision of an assay is defined as 0.75 times the intra-individual variability [24,25]. This definition requires that inter-assay RSDs be ≤5.6% for ADMA and ≤4.4% for SDMA. Desirable imprecision goals are defined as 0.5 times the intra-individual variability [25] which is ≤3.7% for ADMA and ≤2.9% for SDMA [24]. The inter-assay RSDs for ADMA with this method are within the desirable imprecision goals. The inter-assay RSDs for SDMA come close to the desirable imprecision goals and are well within the minimum requirements. As Blackwell et al. note, few published methods for measuring ADMA and SDMA meet these desirable precision goals. Data on the precision of this method are presented in Table 3.

An aliquot of pooled quality control plasma was analysed by HPLC at an independent research laboratory with an established,

Table 3
Intra-assay ($n = 10$) and inter-assay ($n = 30$) precision calculated from pooled quality control plasma.

Analyte	Intra-assay mean (μM) ± SD	Intra-assay RSD (%)	Inter-assay mean (μM) ± SD	Inter-assay RSD (%)
Arginine	21.06 ± 0.2	0.93	23.68 ± 1.86	7.88
Homoarginine	1.87 ± 0.02	1.22	1.88 ± 0.09	4.57
ADMA	0.49 ± 0.01	2.06	0.48 ± 0.01	2.69
SDMA	0.39 ± 0.01	2.26	0.38 ± 0.01	3.07

Table 4
Assay accuracy calculated from spiked plasma ($n = 3$)[a].

Analyte	Concentration (μM)				RSD (%)	Mean spike recovered (μM)	Accuracy/spike recovery (%)
	Mean unspiked plasma	Spike added	Mean spiked plasma	SD			
Arginine	11.70	3.78	15.58	0.41	2.63	3.88	102.8
		7.55	19.86	0.91	4.61	8.16	108.1
		12.60	25.47	1.01	3.95	13.78	109.4
		15.10	26.91	0.82	3.05	15.22	100.8
		25.20	37.50	0.48	1.27	25.80	102.4
		50.50	64.14	1.81	2.82	52.44	103.8
Homoarginine	0.94	0.50	1.35	0.26	18.94	0.41	81.3
		0.75	1.63	0.27	16.34	0.69	92.0
		1.00	1.86	0.29	15.83	0.92	91.7
		1.50	2.42	0.31	12.97	1.48	98.4
		3.00	4.03	0.43	10.65	3.09	103.0
ADMA	0.25	0.13	0.36	0.02	4.81	0.12	92.0
		0.25	0.51	0.03	5.70	0.26	104.7
		0.38	0.62	0.02	2.45	0.38	100.9
		0.50	0.75	0.02	2.05	0.50	100.3
		0.75	1.00	0.06	6.09	0.76	101.1
		1.50	1.78	0.10	5.55	1.53	102.1
SDMA	0.20	0.13	0.32	0.03	7.78	0.13	102.7
		0.25	0.46	0.05	9.96	0.27	106.0
		0.38	0.58	0.03	5.51	0.39	103.6
		0.50	0.70	0.04	5.71	0.51	101.0
		0.75	0.96	0.03	3.13	0.77	102.0
		1.50	1.72	0.07	4.08	1.53	101.9

[a] Calculated as a percentage of spike recovered from spiked plasma after subtraction of the unspiked plasma concentration.

validated method [17]. This laboratory reported mean values of 0.48 μM ADMA and 0.35 μM SDMA, which concurred with the results obtained using this method.

Data on accuracy, expressed as recovery of added analyte from spiked quality control plasma ($n = 3$), are presented in Table 4.

This assay has since been used successfully to measure plasma dimethylarginines in over 194 patients with critical illness. It is important to note that of these patients, only 15 had ADMA more than 1 μM (unpublished data). Hence this assay was optimised to be accurate and precise at low concentrations of ADMA and SDMA.

3.4. Healthy plasma levels

Thirty apparently healthy volunteers provided plasma samples. The mean and standard deviation of each analyte of interest are shown in Table 5. These values were within the healthy range reported by others [24,26], with the exception of L-arginine concentration, which was lower than expected due to the delay in processing blood from blood bank donors [27].

Table 5
Healthy plasma arginine, homoarginine and methylated arginine values ($n = 30$).

	Arginine (μM)	Homoarginine (μM)	ADMA (μM)	SDMA (μM)
Min	23.40	0.86	0.30	0.20
Max	152.92	3.95	0.58	0.54
Mean	66.91	2.15	0.45	0.40
SD	33.46	0.75	0.07	0.09

3.5. Limitations and strengths of the assay

A limitation of this assay is the need to condition new HPLC columns before retention times stabilise, a requirement noted in other methods [28–31]. After conditioning the new column with repeated injections of either standards or the quality control plasma, retention times stabilised and excellent retention times were then obtained for the duration of the column life. This method has been used with three Gemini-NX columns, each lasting approximately 900 injections.

This method is not as short as a number of other published methods because it uses AccQ-Fluor™ derivatisation and a non-endogenous internal standard. AccQ-Fluor™ derivatisation leads to longer chromatography [32,33], however the stable adducts produced by AccQ-Fluor™ give accurate results without requiring on-line derivatisation. Furthermore, the shorter published methods tend to use either monomethylarginine (MMA) or homoarginine as internal standards, concentrations of which may be altered in disease states [20,34]. Using a non-endogenous internal standard gives more accurate results and also allows all analytes to be quantitated in plasma.

This method has several strengths. Firstly, the substitution of methanol with isopropanol in the SPE method gives improved recovery of all analytes. Secondly, a combination of the acetonitrile gradient and borate buffer at pH 9 on the Gemini-NX column produced clearly defined chromatographic peaks. Thirdly, the average accuracy of ADMA was 100.2 ± 4.3% while for SDMA it was 102.9 ± 1.8%. Finally, the inter-assay RSDs for ADMA are within the desirable precision goals set out by Blackwell et

al. [24] while SDMA measurements easily meet the minimum standards and come close to achieving the desirable precision goals.

Importantly, as this method achieves accurate and precise results from small volumes of plasma it is particularly useful for research into critical illness.

Conflict of interest statement

The authors do not have a commercial or other association that might pose a conflict of interest.

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