Determination of L-arginine and N^G,N^G- and $N^G,N^{G'}$-dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor^TM fluorescent derivatives

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Abstract

A new HPLC assay for the detection of L-arginine, N^G,N^G-dimethyl-L-arginine (ADMA) and $N^G,N^{G'}$-dimethyl-L-arginine (SDMA) in plasma using the derivatisation reagent AccQ-Fluor^TM (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) is described. The fluorescent derivatives produced are extremely stable enabling routine processing of large numbers of samples. Arginine and its metabolites are extracted from plasma on strong cation exchange (SCX) cartridges with N^G-monomethyl-L-arginine (NMMA) as internal standard, derivatised and separated on a C18 column with acetonitrile in 0.1 M sodium acetate buffer pH 6. Separation of the stereoisomers ADMA and SDMA was excellent and improvements to the solid phase extraction (SPE) procedure enabled good recovery (>80%) of arginine, ADMA and SDMA. The utility of the method is exemplified by comparison of plasma concentrations of ADMA, SDMA and arginine in healthy volunteers and diabetic/ischaemic patients.
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1. Introduction

Measurement of N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine or ADMA) in plasma has been of considerable interest since Vallance et al. [1] demonstrated inhibition of nitric oxide synthase (NOS) by endogenous ADMA in vitro and in vivo. These authors were the first to link elevated plasma ADMA levels in patients with renal failure to the development of hypertension and possibly immune dysfunction associated with these patients. Since then, a body of work has emerged on ADMA in conditions including renal disease [2–5], atherosclerosis [6,7], hypertension [8–10], hypercholesterolaemia [11], syndrome X and diabetes [12–14]. Increased levels of ADMA in human plasma [1,15] are thought to contribute to unwanted cardiovascular effects such as platelet hyperaggregability, monocyte adhesion and vascular smooth muscle cell proliferation by inhibiting NOS. The other NOS inhibitor present

in plasma, N^G-monomethyl-L-arginine (NMMA) occurs at approximately one-tenth the concentration of ADMA [15].

Methylated arginine residues are present in proteins as a consequence of the action of protein methyl transferases. During proteolysis, the asymmetrically methylated ADMA and NMMA, and the symmetrical $N^G,N^{G'}$-dimethyl-L-arginine (symmetric dimethylarginine or SDMA) are released into the cytoplasm [16]. In addition to clearance by the kidneys, ADMA and NMMA can be metabolised by dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine, and this enzyme may be a critical regulator of these two compounds. SDMA is not a NOS inhibitor, therefore an assay for ADMA must be able to distinguish between the two isomers.

Measurement of ADMA and SDMA faces problems of low concentration in plasma, difficulty in chromatographic separation, and lack of simple and reliable detection methods. Current assays for arginine, ADMA and SDMA generally involve ion exchange extraction from plasma followed by HPLC separation with fluorescence detection [8,10,13,14,17–21], although capillary electrophoresis [1] and liquid chromatography (LC) mass spectrometry

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[22] have also been described. Derivatisation with *ortho*-phthaldialdehyde (OPA) and 2-mercaptoethanol is widely used but a major drawback is the instability of the fluorescent derivative necessitating manual or automated derivatisation just prior to analysis by HPLC. Recent publications describe modified assays that attempt to improve the stability of the fluorescent derivative by using 3-mercaptopropionic acid instead of mercaptoethanol in conjunction with OPA [20] or by using another derivatising agent naphthalene-2,3-dicarboxaldehyde [21]. Both these assays significantly reduce the rate of degradation of derivatised mixtures held at 4–7 °C. Besides the loss of fluorescence during HPLC analysis, other limitations are that ADMA and SDMA are not well resolved under certain chromatographic conditions, some assays do not recover arginine, and rapid deterioration of the LC column was encountered in this laboratory with OPA derivatives separated with citrate or phosphate buffered mobile phases.

In the present communication, we describe a method for ensuring a highly stable fluorescent derivative which also overcomes the problems of poor baseline resolution and interferences in the chromatogram. For this purpose we adapted the extraction procedure of Pettersson et al. [17] based on strong cation exchange (SCX). The detection method is based on derivatisation with AccQ-Fluor[TM] reagent [23] to produce fluorescent derivatives stable for 1 week at room temperature, allowing routine processing by HPLC of large numbers of samples without the need for precise injection times after derivatisation. By conducting two extractions of different volumes of plasma in parallel, good recovery of arginine as well as ADMA and SDMA is achieved. ADMA and SDMA are chromatographically well separated, and small numbers of endogenous or reagent interferences are situated away from peaks of interest. Details of method validation in plasma matrix are described, and a number of plasma samples from healthy volunteers and diabetic patients have been analysed for arginine, ADMA and SDMA.

2. Experimental

2.1. Chemicals and reagents

L-Arginine HCl, L-homoarginine HCl, ADMA HCl and L-NMMA acetate salt were purchased from Sigma (St. Louis, MO, USA). SDMA di-HCl was obtained from Calbiochem (La Jolla, CA, USA). The AccQ-Fluor[TM] kit from Waters (Milford, MA, USA) contained the fluorescent reagent powder 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, a vial of acetonitrile diluent for reconstituting this reagent to approximately 10 mM, and a vial of aqueous borate buffer (0.2 M, pH 8.8) for the derivatisation reaction. Several grades of triethylamine (TEA) were found to contain significant levels of impurities that interfered with the assay even though they were of >99.5% purity. Acceptable grades

(all ≥99.5% purity) were from Aldrich (cat. no. 471283, MI, USA) and Fluka (cat. nos. 90335 and 90337, Buchs, Switzerland). Solid phase extraction (SPE) cartridges for plasma extraction were Bond Elut SCX, 1 cc/100 mg from Varian (Harbor City, CA, USA). Cartridges with ion exchange capacity at least 0.8 meq/g were purchased as arginine was less efficiently recovered from batches with lower ion exchange capacity (see Section 3). All other reagents and solvents were of analytical or HPLC grade.

2.2. Plasma samples

Blood was collected from 12 healthy volunteers (5 male, 7 female) into heparinised tubes, centrifuged (+4 °C) at 2700 × *g* for 20 min and the plasma stored at −80 °C until extraction. Plasma samples from 53 patients (18 female, 35 male) admitted to the coronary care unit with acute coronary syndromes (ACS) and blood sugar levels (BSL) >10 mmol/l consistent with known or newly diagnosed diabetes mellitus were also collected. Mean serum creatinine for the patient cohort was 0.11 ± 0.04 mmol/l; only one patient had a creatinine level >0.20 mmol/l. The relationship between serum creatinine and ADMA concentration was non-significant ($r = 0.03$, $P = 0.85$).

2.3. Extraction procedure

ADMA and SDMA were determined from 0.5 ml of plasma combined with 0.05 ml of internal standard NMMA (20 µg/ml), 0.2 ml of 10% 5-sulfosalicylic acid and made up to 1.6 ml with water. A parallel extraction of 0.1 ml plasma was prepared for arginine quantitation due to low recoveries of arginine from SCX cartridges (see Section 3). The plasma solutions were vortexed, kept on ice for 10 min to allow complete protein precipitation and centrifuged at 9000 × *g* for 2 min. The supernatant was loaded onto Bond Elut SCX 100 mg cartridges conditioned with 1 ml methanol followed by 2 ml of freshly prepared 2% (w/v) trichloroacetic acid. The plasma solutions were passed through the SCX cartridges at about 3–4 ml/min and unwanted plasma components rinsed off with 3 ml of 0.1 M sodium phosphate buffer (pH 6) followed by 1 ml methanol. Arginine and ADMA/SDMA were eluted from their respective cartridges with 2 ml of freshly prepared 2% TEA in 65% methanol/water. The eluents were dried under a stream of nitrogen at 55 °C. The residues were redissolved in distilled water (0.2 ml) and centrifuged at 9000 × *g* for 2 min to remove particulates prior to derivatisation for HPLC.

2.4. Derivatisation

Plasma extract (50 µl) and 50 µl borate buffer were combined with 10 µl of fluorescent derivatising reagent (AccQ-Fluor[TM]) in low volume HPLC vial inserts under vigorous vortexing for several seconds. The reaction was

complete after 1 min and the resulting derivatives were stable for at least 1 week at room temperature.

2.5. Chromatographic conditions

The fluorescent derivatives were separated on a 5 μ Luna C18(2) column (150 mm × 3.0 mm) with SecurityGuard^{TM} C18 cartridge (Phenomenex, Torrance, CA, USA) at 30 °C with an acetonitrile gradient in sodium acetate buffer at a flow rate of 1 ml/min. Mobile phase solutions A and B were 4 and 30% acetonitrile in 0.1 M sodium acetate pH 6, respectively. The gradient conditions were A:B = 94:6 (20 min), then 84:16 over 0.2 min, hold until 35 min, flush 15:85 (3 min), return to initial conditions 94:6 over 0.5 min, allow column to re-equilibrate for 6 min. Conditions were maintained by two Waters 510 pumps with an automated gradient controller, and extracts (20 μl) were injected with a Waters 717plus autosampler. Analytes were detected by a Perkin-Elmer (Melbourne, Australia) LS40 fluorescence detector at λ_{ex} 250 nm, λ_{em} 395 nm. Data was collected with Laura LabLogic (v. 1.2) HPLC software for windows (Daintree Scientific, St. Helens, Tasmania, Australia).

2.6. Standard curves and recovery

Calibration standards for ADMA and SDMA (0.05, 0.25, 0.5, 1, 1.5 μg/ml) and arginine (5, 10, 15, 20, 25 μg/ml) were prepared from stock solutions (0.4 mg/ml in 0.1 M HCl) diluted in water and stored at −20 °C. Standard curves for ADMA and SDMA were prepared by combining 0.5 ml of calibration standard with 0.5 ml of plasma and extracting by the procedure detailed in Section 2.3. For arginine quantitation 0.1 ml of standard was combined with 0.1 ml plasma. Plasma from healthy volunteers was used for preparation of calibration curves and controls as larger volumes were easily obtained. Calibration curves were calculated by graphing the ratio of peak area (arginine, ADMA or SDMA) to area of the internal standard versus concentration. Analyte recovery for accuracy and precision was determined by spiking "healthy" plasma with known quantities of arginine and dimethylarginines, extracting and quantitating spiked components from the standard curve.

2.7. Statistical analysis

Validation data are expressed as mean concentration and coefficient of variation in brackets. Plasma data are presented as mean ± standard deviation and are compared by the non-parametric Mann–Whitney U-test.

3. Results and discussion

The sample preparation component of this assay involves parallel plasma extractions for arginine and ADMA/SDMA. Arginine is quantified from 0.1 ml plasma,
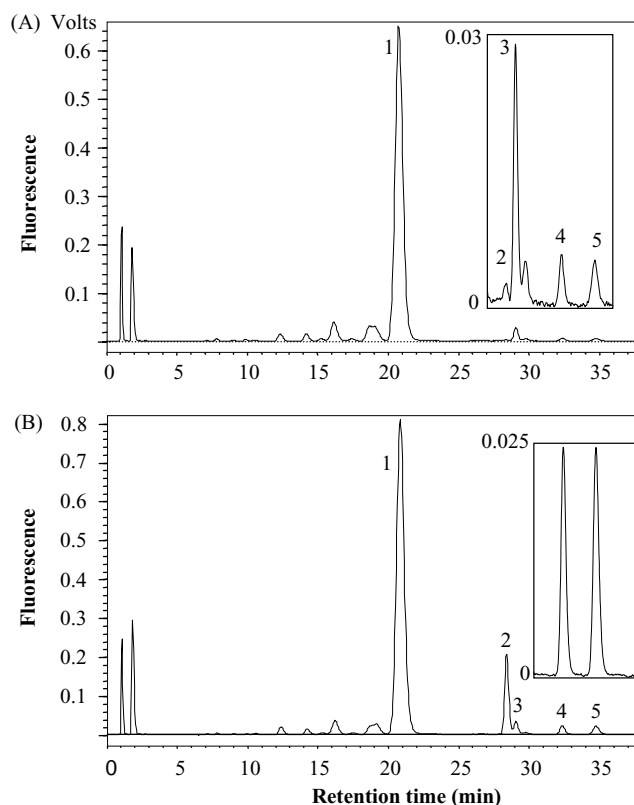

Fig. 1. Typical chromatogram of normal plasma (A) without internal standard, and plasma spiked (B) with internal standard NMMA and a calibration standard containing arginine (5 μg/ml), ADMA and SDMA (0.25 μg/ml each). Peak identity: (1) arginine; (2) NMMA; (3) homoarginine; (4) ADMA; (5) SDMA (see Section 2 for chromatographic conditions). Inset A: region 27–36 min magnified 20×; inset B: region 30–38 min magnified 25×.

and ADMA/SDMA are quantified from 0.5 ml plasma. All other aspects of the extraction and chromatographic analysis are identical. 0.5 ml of plasma for ADMA/SDMA quantitation produces satisfactory sensitivity to accurately determine low levels in "healthy" subjects. Sensitivity is not an issue with arginine so 0.1 ml plasma is ample for quantitation, but eliminates the problem of analyte breakthrough on the SPE cartridges. Two things contribute to cartridge breakthrough: sorbent capacity is exceeded and/or the analyte is weakly retained. In the case of arginine, it appears to be a combination of these two factors. Fig. 1 illustrates the separation of the analytes in plasma (0.5 ml) from a healthy volunteer and the same plasma spiked with standard. The level of arginine in these extracts is qualitative only. The retention times are as follows: arginine (20.8 min), NMMA (28.4 min), homoarginine (29 min), ADMA (32.3 min), SDMA (34.7 min). The peaks at 14.2, 18.7 and 29.7 min are due to reagent interference (TEA). Other basic amino acids such as citrulline, lysine and histidine do not interfere with the assay. ADMA and SDMA are well separated, and homoarginine and NMMA are sufficiently resolved to allow accurate integration of the internal standard peak. NMMA was chosen as internal standard in preference to homoarginine
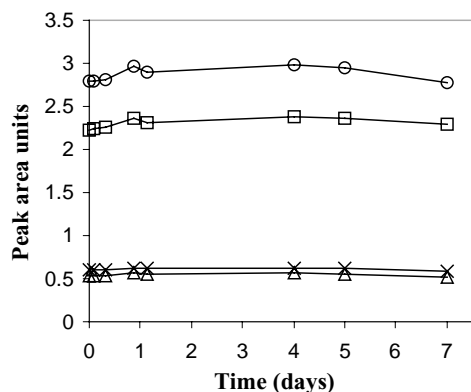
Fig. 2. Stability of derivatised standard mixture of arginine ($\square$, 2 μg/ml), NMMA ($\bigcirc$, 2 μg/ml), ADMA ($\triangle$, 0.5 μg/ml), SDMA ($\times$, 0.5 μg/ml) over 7 days at room temperature.

as its peak area in plasma is <0.5% of the amount of NMMA added.

The stability of AccQ-Fluor[TM] derivatives of a standard mixture and an extracted plasma were tested by measuring fluorescence over 7 days. Fig. 2 graphs the results for a standard mixture of arginine, NMMA, ADMA and SDMA stored in the autosampler at room temperature ($\sim$20 °C). Peak areas of the four components in both distilled water and plasma extract after 7 days were within 5% of the initial value. Previous attempts to improve the stability of fluorescent ADMA derivatives necessitate the extracts to be stored at low temperature if large numbers of samples are to be processed. Teerlink et al. [20] substituted 3-mercaptopropionic acid for mercaptoethanol in the derivatisation reaction to produce derivatives whose peak areas decrease by 35% over 72 h at 7 °C. Marra et al. [21] use naphthalene-2,3-dicarboxaldehyde to produce highly fluorescent isoindoles which decrease in fluorescence by 11–16% over 72 h at 4 °C. Good precision was reported for these assays because the internal standard adjusts for the loss in fluorescence and the sensitivity of the assay is not compromised by the decreased peak areas.

Plasma analyte concentrations were calculated from a calibration curve prepared in plasma from a healthy volunteer, extracted and chromatographed under the same conditions. Typical linear regression equations from standard curves for each analyte were: arginine ($y = 0.0817x - 0.039$), ADMA ($y = 0.4032x - 0.003$) and SDMA ($y = 0.4808x - 0.002$)

Table 2
Interassay variability of QC plasma, mean in μM ($n = 5$) and CV (%)

| | Mean (CV) | | |
	L-Arginine	ADMA	SDMA
Low QC	132.5 (5.7)	0.48 (6)	0.37 (4.5)
High QC	158.4 (8.1)	2.67 (1.5)	2.88 (1.5)

Low QC is plasma from healthy volunteer; high QC is same plasma spiked with arginine (28.7 μM), ADMA and SDMA (2.47 μM).

Table 3
Comparison of arginine and its metabolites (mean ± S.D., μM) in healthy subjects and diabetic/ischaemic patients

	Healthy ($n = 12$)	Patients ($n = 53$)	P-value[a]
ADMA	0.44 ± 0.08	0.53 ± 0.11	0.0068
SDMA	0.42 ± 0.09	0.59 ± 0.28	0.0408
Arginine	84.5 ± 19.8	64.4 ± 21.7	0.0038
Arginine/ADMA	196.7 ± 54.6	123.5 ± 37.1	0.0002
ADMA/SDMA	1.08 ± 0.17	1.06 ± 0.44	0.7289

[a] Mann–Whitney U-test.

where y is the peak area ratio of analyte to internal standard and x is the analyte concentration in μg/ml. The correlation coefficients for ADMA and SDMA standards in plasma were ≥0.999; and ≥0.977 for arginine. Interassay variability of the slopes of the calibration curves ($n = 5$) was 9.5% (arginine), 2.7% (ADMA) and 2.8% (SDMA). Metabolite concentrations in plasma were converted from μg/ml to μM by the factors 5.74 (arginine), and 4.94 (ADMA, SDMA) based on their respective molecular weights.

Table 1 summarises the accuracy and precision of the assay for plasma ($n = 5$) containing endogenous levels of arginine, ADMA and SDMA as well as two spiked concentrations. All coefficients of variation (CV) were less than or equal to 5%. ADMA and SDMA recoveries at both low and high spike concentrations were within the range (71–100%) quoted in these papers [11,13,14,17,19–21]. Arginine was recovered at >80% which agrees well with the few papers that provide detail of arginine recovery [19–22]. Interassay CVs for 2 quality control (QC) plasma samples analysed for arginine, ADMA and SDMA in consecutive sample sets are shown in Table 2. The limit of detection in plasma for ADMA and SDMA was 0.1 μM based on a signal/noise ratio of 3.

A typical application for this assay is illustrated with Table 3 summarising the concentration of arginine, ADMA

Table 1
Intraassay variability of arginine, ADMA and SDMA (μM) in plasma ($n = 5$) with CV (%)

| | L-arginine | | ADMA | | SDMA | |
	Mean (CV)	Recovery (%)	Mean (CV)	Recovery (%)	Mean (CV)	Recovery (%)
Healthy plasma	87.4 (1.8)		0.38 (2.3)		0.39 (5)	
Low spike	110.5 (1.9)	81	0.86 (1.7)	97	0.85 (3)	94
High spike	137.4 (2.0)	87	2.65 (1.9)	92	2.69 (1.5)	93

Low spike (added arginine 28.7 μM, ADMA and SDMA 0.49 μM); high spike (added arginine 57.4 μM, ADMA and SDMA 2.47 μM). Recovery data (%) are included.

and SDMA in 12 healthy volunteers and 53 diabetic patients with unstable angina pectoris. The mean ADMA level in healthy individuals agrees well with literature values [5,7,8,12,17,19,20] and ADMA concentration is significantly higher in the patient group which agrees with previous reports [12–14] for diabetic and insulin resistant patients. Arginine and SDMA concentrations fall within the range of published levels for healthy individuals and patients [1,3,5,6,17,19,20,22].

The Luna C18(2) column and sodium acetate mobile phase detailed in Section 2 has been successfully used for over 1000 injections in this laboratory. Modifications to the SPE assay of Pettersson et al. [17] such as decreasing the pH and ionic strength of the wash solution improved arginine recovery from >20 to >80%. Batches of SCX cartridges at the lower end of the manufacturer's specificity range (0.6–0.9 meq/g) for ion exchange capacity did not efficiently retain arginine from samples such as the more concentrated calibration standards in plasma. Testing larger cartridges introduced other limitations. It was therefore decided to reduce the volume of plasma extracted for arginine quantitation to 0.1 ml to avoid cartridge breakthrough, however, it was essential that cartridges with ion exchange capacity of ≥ 0.8 meq/g were purchased.

In conclusion, this paper describes a new HPLC method for arginine, ADMA and SDMA which incorporates a simple derivatisation procedure to produce highly stable fluorescent derivatives. The HPLC column and mobile phase conditions produce excellent separation of the arginine metabolites from the internal standard and endogenous plasma components. Extraction of plasma on SCX cartridges has been improved with particular emphasis on recovery of arginine. The assay can be automated for routine analysis and its sensitivity and precision is similar to derivatisation with OPA.

Acknowledgements

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