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# HPLC analysis of asymmetric dimethylarginine (ADMA) and related arginine metabolites in human plasma using a novel non-endogenous internal standard

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ABSTRACT

*Background:* Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthesis which has been implicated in the endothelial dysfunction. Methods for ADMA measurement often yield widely differing results, and few methods simultaneously offer satisfactory accuracy and precision. We describe a fully validated HPLC method for analysis of arginine and its methylated derivatives in human plasma using a novel internal standard.

*Methods:* Arginine and related metabolites are extracted from plasma by solid phase extraction (SPE), derivatised with ortho-phthaldialdehyde and separated by isocratic reverse phase chromatography. Monoethylarginine (MEA), which is not endogenously present in human plasma was used as internal standard. SPE and chromatographic procedures are optimised and recovery, precision, linearity and sensitivity of the assay established. The suitability and performance of MEA is compared with that of monomethylarginine (MMA), the internal standard most commonly used in HPLC methods.

*Results:* SPE yields high and reproducible recoveries (>90%). The analytes of interest are chromatographically well resolved. The method has high sensitivity (LOD, 0.01 μmol/L for arginine and 0.001 μmol/L for ADMA, SDMA and homoarginine) and good precision (CV, 2.5% for ADMA). The data obtained with the internal standards MEA and MMA is comparable in terms of assay precision and population reference intervals.

*Conclusions:* We describe an optimised isocratic HPLC method for the simultaneous measurement of arginine and related metabolites in plasma which exhibits satisfactory precision and is suitable for routine use. Its main advantage over other published HPLC methods is the use of the novel internal standard, MEA, which unlike other commonly used internal standards is not inherent in human plasma.



## 1. Introduction

Measurement of $N^G$, $N^G$-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) in plasma is of considerable interest, as its increased concentration is believed to be a cause of endothelial dysfunction in a number of pathological conditions, such as hypertension, hyperlipidaemia, renal failure, diabetes mellitus, hyperhomocystinaemia and ischaemic heart disease [1,2]. ADMA is an endogenous and potent competitive inhibitor of the enzyme nitric oxide synthase (NOS), which synthesises nitric oxide (NO) from arginine in the vascular endothelium [3]. It is derived in vivo from degradation of proteins containing post-translationally methylated arginine residues. Through this pathway the constitutional isomer $N^G$, $N'^G$-dimethyl-L-arginine (symmetric dimethyl-arginine, SDMA), and monomethyl-L-arginine (MMA) are also produced, the latter also having an inhibitory effect on NOS, but not the former [4]. However, SDMA is known to inhibit arginine uptake by endothelial cells, so may still have a role to play in endothelial dysfunction [5]. SDMA is also known to be a sensitive indicator of renal function, correlating closely with glomerular filtration rate [6], and its measurement may therefore be of importance in certain contexts [7].

As well as being the main regulator of vascular tone, NO has a number of other effects generally considered to be "anti-atherogenic", including inhibition of both smooth muscle cell proliferation and platelet adhesion and aggregation. These effects, it is proposed, explain how ADMA exerts its detrimental effects on the vascular endothelium [8,9].

Measurement of ADMA is hampered by its low concentration in plasma and difficulty in chromatographic separation of the two isomers ADMA and SDMA. Whilst many methods for measurement of ADMA have been described, there are few which simultaneously exhibit

*Abbreviations:* ADMA, NG, NG-dimethyl-L-arginine, asymmetric dimethylarginine; CV, Coefficient of variation; CVA, Analytical variation; CVI, Intra-individual biological variation; ELISA, Enzyme linked immuno-sorbent assay; GC-MS, Gas chromatography-mass spectrometry; HArg, Homoarginine; HPLC, High performance liquid chromatography; LC-MS, Liquid chromatography-mass spectrometry; MEA, Monoethylarginine; MMA, Monomethylarginine; NO, Nitric oxide; NPA, N-propyl-L-arginine; OPA, Ortho-phthaldialdehyde; QC, Quality control; SD, Standard deviation; SDMA, NG, N'G-dimethyl-L-arginine, symmetric dimethylarginine; SPE, Solid phase extraction.

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acceptable accuracy and precision and a high capacity for sample throughput [10]. Current methods for determination of ADMA in plasma include HPLC [11–18], mass spectrometric methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) [19–23] and gas chromatography-tandem mass spectrometry (GC-MS) [24], capillary electrophoresis [25] and ELISA [26]. Although mass spectrometric methods easily allow separation of ADMA and SDMA, the technique is very expensive and HPLC remains the most widely used method for measurement of ADMA in plasma. HPLC methods generally involve solid phase extraction of ADMA from plasma, pre-column derivatisation, followed by reverse phase separation with fluorescence detection. Gradient elution is often required to achieve baseline separation of the isomers ADMA and SDMA [12,13,17,18].

In the present study, we describe a simple and reliable procedure for extraction of arginine, and its methylated derivatives, ADMA, SDMA, MMA and homoarginine (HArg), from plasma and simultaneous separation of these compounds by isocratic reverse phase HPLC. All analytes, particularly, ADMA and SDMA, were chromatographically well resolved. Sample clean-up is by solid phase extraction (SPE) on cation exchange columns, yielding high recoveries. Fluorescent derivatization is with ortho-phthaldialdehyde (OPA) and 3-mercaptopropionic acid. We use the novel internal standard monoethylarginine (MEA), which is clearly separated from the analytes of interest, and unlike other commonly used internal standards is not inherent in human plasma. The suitability and performance of MEA is compared with that of monomethylarginine (MMA) which is the internal standard most commonly used in HPLC methods for measurement of ADMA in plasma.

## 2. Materials and methods

### 2.1. Chemical and reagents

ADMA, SDMA, and MEA were obtained from Calbiochem (Nottingham, UK). Homoarginine, MMA, arginine, OPA and 3-mercaptopropionic acid were obtained from Sigma Chemical Company (Poole, UK). The analytical C18 column (Symmetry, 4 μm, 4.6×150 mm, protected by a 3×4 mm guard column) and solid phase extraction cartridges (Isolute PRS columns, 1 mL/50 mg) were obtained from Kinesis Ltd (Epping, UK).

### 2.2. Preparation of standards

Individual standards of arginine, HArg, ADMA and SDMA (1 mM) were prepared in 10 mM hydrochloric acid (HCl) and stored at −70 °C. Stability of these stocks was checked by chromatography. A combined calibration standard containing 20 μM arginine and 1 μM HArg, ADMA and SDMA was prepared by diluting the stock standards in distilled water, and stored in aliquots at −70 °C. A stock solution of the internal standard MEA and MMA (1 mM) was prepared in 10 mM HCl and diluted to obtain a working solution of 5 μM which was aliquoted and stored at −70 °C.

### 2.3. Sample extraction with MEA as internal standard

Blood was collected into tubes containing EDTA. Arginine, SDMA, ADMA and HArg were extracted from plasma using Isolute PRS cation exchange SPE columns. The extraction columns were placed on Vac Elut extraction system, activated and equilibrated with 2 mL methanol followed by 2 mL of 50 mM borate buffer, pH 8.5. In a glass tube, 0.2 mL of plasma sample, QC or aqueous calibrator was mixed with 80 μL of internal standard (MEA, 5 μM) and 720 μL of borate buffer, which was then loaded on to the equilibrated SPE column. The column was consecutively washed with borate buffer (1 mL), water (3 mL) and methanol (3 mL). These steps were performed under gravity, with no vacuum suction required (flow rate was <0.5 mL/min). Analytes were eluted with 3 mL of a solution containing 50% methanol and 10% concentrated ammonia in water. The eluent was then evaporated to dryness at 80 °C under air.

Using the Vac Elut system, 40 samples could be extracted within 2 h for overnight analysis by HPLC.

### 2.4. Sample extraction with MMA as internal standard

In order to compare the performance and suitability of the two internal standards, plasma samples were also extracted using MMA as the internal standard. The samples were extracted as described above, but using 80 μL of MMA (5 μM) as internal standard instead of MEA.

### 2.5. Derivatisation

The dried extract was dissolved in 0.1 mL water and 0.1 mL of the derivatizing agent was then added and the samples thoroughly mixed and equilibrated for 15 min. The derivatization reagent was freshly prepared each week as described by Teerlink [11] with minor modifications. Briefly, 10 mg of OPA was dissolved in 0.2 mL of methanol, followed by the addition of 1.8 mL of 200 mM borate buffer (pH 8.5), and finally 10 μL of 3-mercaptopropionic acid. The reagent was stable for one week when stored at 4 °C, provided mercaptopropionic acid (5 μL) was added every 48 h to the stock solution. Shortly before use, the stock solution was diluted five fold in the same borate buffer.

The derivatised samples were transferred to autosampler vials, maintained at 10 °C, and 20 μL injected onto the HPLC analytical column for chromatography.

### 2.6. Chromatographic conditions

The HPLC system consisted of a solvent delivery system and fluorimeter (Waters, Watford, UK). The isocratic mobile phase for chromatography was 50 mM acetate buffer (pH 6.3) containing 9% (v/v) acetonitrile. The mobile phase was filtered through a 0.45 μm nylon filter and pumped to waste through the analytical column at a flow rate of 1.5 mL/min. The mobile phase was optimized with respect to separation, signal response, reproducibility of results, column stability and analysis time by varying the pH and concentration of the acetonitrile and buffer.

### 2.7. Detection

Following separation, Arginine, MMA, HArg, ADMA and SDMA were measured fluorimetrically using a programmable fluorescence detector (Waters 2475). Excitation and Emission wavelengths were 340 and 455 nm respectively. After elution of arginine, the sensitivity was changed, at 14 min, from 32,000 to 800 EUFS (Emission Units Full Scale), as the concentration of arginine is 100–200 times greater than that of ADMA and SDMA in plasma. Signals from the detector were collected by a data management system (Millennium 2010, Waters, Watford, UK).

### 2.8. Quantification

Quantification was done by the method of internal standardisation using a single level calibration. Arginine, HArg, ADMA and SDMA concentrations (μmol/L) in plasma were calculated by multiplying the peak height ratio of the analyte to internal standard in the sample and combined standard chromatograms by the concentration of the analyte in the combined standard. Although present in low concentrations (approximately 20–100 nmol/L), MMA could also be quantified. However, under the assay conditions described, its quantification was imprecise and therefore unreliable at this low concentration, and for this reason we refrained from determining its reference interval; current research has almost entirely focused on ADMA, given that it is the predominant NOS inhibitor in plasma.

### 2.9. Accuracy and precision

Accuracy was determined from the recovery of exogenously added analytes from plasma. Plasma samples were spiked with varying concentrations of arginine, HArg, ADMA and SDMA as shown in Table 1. These samples were prepared and analysed as described, and recovery calculated as the percentage recovered in the spiked sample after subtraction of the basal concentration. Accuracy is often expressed as the bias from the expected concentration of a reference material; however, such material does not currently exist for these analytes.

Between batch CV was determined using plasma, from a single donor, obtained from the blood transfusion service, which we found to be a good source of plasma in sufficient volumes for this purpose. Plasma was stored in aliquots at −70 °C and used as

**Table 1**
Mean (SD) relative recovery of the method using spiked plasma ($n$=3)

| Analyte added (μmol/L) | Mean (SD) relative recovery, %[a] | | | |
|---|---|---|---|---|
| | Arginine | HArg | ADMA | SDMA |
| 0.5 | | | 100.0 (6.1) | 99.5 (4.2) |
| 1.0 | | | 100.7 (2.6) | 99.0 (3.6) |
| 2.0 | | 93.4 (8.3) | 105.4 (0.6) | 99.6 (2.5) |
| 4.0 | | | 103.6 (1.9) | 98.0 (1.3) |
| 5.0 | | 92.1 (1.8) | | |
| 10.0 | | 93.5 (0.5) | | |
| 50.0 | 103.4 (0.6) | | | |
| 100.0 | 99.5 (0.2) | | | |

The mean concentrations of Arginine, HArg, ADMA and SDMA in the unspiked plasma pool sample were 79.5, 1.57, 0.34 and 0.27 μmol/L respectively.

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