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Review

HPLC analysis of ADMA and other methylated L-arginine analogs in biological fluids☆

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Abstract

Post-translational methylation of arginine residues in proteins leads to generation of N^G-monomethylarginine (MMA) and both symmetric and asymmetric dimethylarginine (SDMA and ADMA), that are released into the cytosol upon proteolysis. Both MMA and ADMA are inhibitors of nitric oxide synthase and especially elevated levels of ADMA are associated with endothelial dysfunction and cardiovascular disease. Plasma concentrations of ADMA and SDMA are very low, typically between 0.3 and 0.8 μM, making their quantification by HPLC an analytical challenge. Sample preparation usually involves a cleanup step by solid-phase extraction on cation-exchange columns followed by derivatization of amino acids into fluorescent adducts. Because ADMA and SDMA concentrations in healthy subjects show a very narrow distribution, with a between-subject variability of 13% for ADMA and 19% for SDMA, very low imprecision is an essential assay feature. Procedures for sample cleanup, derivatization, and chromatographic separation of arginine and its methylated analogs are the main topics of this review. In addition, important aspects of method validation, pre-analytical factors, and reference values are discussed.
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Keywords: Reviews; Asymmetric dimethylarginine; Monomethylarginine; Symmetric dimethylarginine; Nitric oxide synthase; Cardiovascular disease; Pre-column derivatization; Solid-phase extraction

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1. Introduction

1.1. Production and elimination of methylated arginine analogs

N-Methylation of the amino acids lysine, histidine, and arginine in proteins is a specific form of post-translational modification allowing the cell to expand the functional repertoire of its proteome. The terminal nitrogen atoms of the guanidino group of arginine can be methylated by a family of protein arginine methyltransferases (PRMTs), of which two classes exist [1]. To date, nine members of the PRMT enzyme family have been identified, most of them belonging to class 1 [2]. Both classes of PRMT catalyze the monomethylation of arginine, but when a second methyl group is attached to monomethylarginine (MMA), the reaction product is PRMT dependent. Class 1 PRMTs catalyze the formation of asymmetric dimethylarginine (ADMA), whereas class 2 PRMTs lead to the formation of symmetric dimethylarginine (SDMA). Structures of these methylated arginine species are shown in Fig. 1. Both classes of PRMT preferentially methylate arginine located in arginine–glycine-rich sequences of proteins [1,3]. Heterogeneous nuclear ribonucleoproteins are a primary target for arginine methylation, but a recent proteomic analysis has identified more than 200 proteins that are putatively arginine-methylated [4]. In general, arginine-methylated proteins show strong interactions with nucleic acids and are involved in the processes of transcription, splicing of pre-mRNA, translation, and nucleocytoplasmic transport [1,3–7]. With a few exceptions [8–10], protein methylation is irreversible and methylated arginine residues remain an integral part of the protein until it is degraded by proteolysis.

Free methylated arginines are released into the cytosol upon proteolysis of methylated proteins. ADMA is actively degraded by the intracellular enzyme dimethylarginine dimethylaminohydrolase (DDAH) into citrulline and dimethylamine (DMA) [11]. Two isoforms of DDAH have been identified that are widely expressed in rat and human tissues [12–14]. It has been estimated that humans generate approximately 300 μmol of ADMA per day, of which more than 80% is metabolized by DDAH to DMA which is excreted in the urine [15]. The remainder escapes degradation by DDAH and enters the plasma compartment after export from the cell. It should be noted that DDAH is also active towards MMA, but cannot metabolize SDMA. This may in part explain why plasma concentrations of SDMA are similar to ADMA concentrations, despite the fact that probably much more ADMA is formed during methylation of arginine. In humans, the kidneys play an important role in the elimination of dimethylarginines from the body, by excreting both ADMA and SDMA into the urine [16,17]. However, net renal extraction from the arterial supply was significantly higher for ADMA compared to SDMA (16.2 versus 10.5%, respectively; $P = 0.001$), indicating that for ADMA not only excretion but also intra-renal metabolism, most likely by DDAH, is involved [18]. For SDMA, which is not degraded by DDAH, renal excretion is the major eliminatory pathway, whereas for ADMA both renal and extra-renal degradation by DDAH constitutes the major route of disposal. It should be noted that there are a striking inter-species differences in the metabolism of methylated arginines (see also Section 2.5). Although rat kidneys extract both ADMA and SDMA from the circulation, urinary excretion of ADMA is almost negligible [19]. Acute total nephrectomy in rats was recently shown to lead to a dramatic increase in plasma levels of SDMA, whereas ADMA levels were hardly influenced, indicating that in this species the kidneys play only a minor role in the elimination of ADMA [20]. Studies in rats have shown that the liver takes up large amounts of ADMA, but not SDMA, from the circulation [21,22], whereas the human liver clears both ADMA and SDMA [23]. Several lines of evidence confirm the important role of the liver in the metabolism of ADMA. Plasma and urine levels of ADMA were shown to be increased in patients with end-stage liver disease [24]. In patients undergoing liver transplantation, preoperative ADMA levels were

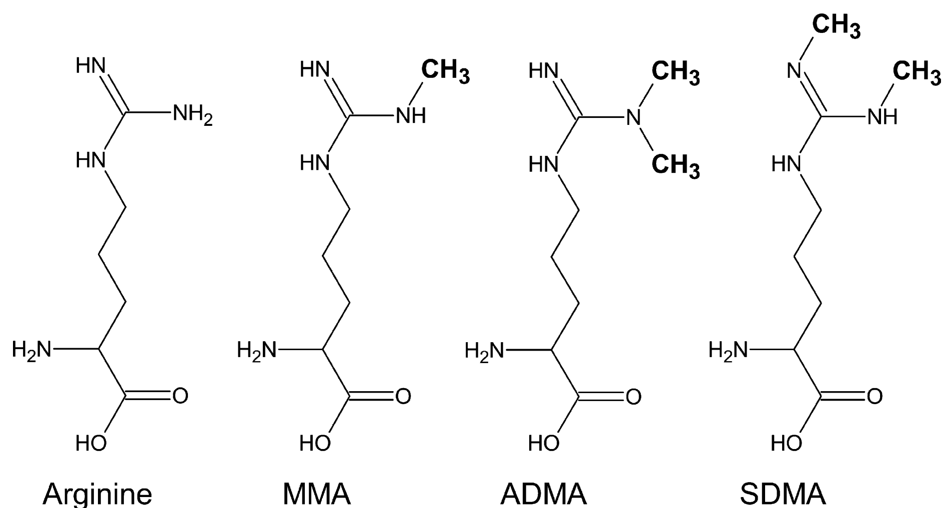

Fig. 1. Structures of arginine, monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA).

highly elevated and decreased significantly after transplantation [25].

1.2. Role of ADMA in cardiovascular disease

Although many clinical conditions are associated with elevated ADMA concentrations (reviewed in [26]), the link between ADMA and cardiovascular disease has been established most firmly. Endothelium-derived nitric oxide (NO) is an important regulator of vascular homeostasis due to its pleiotropic effects on the vessel wall. In addition to its properties as a powerful endogenous vasodilator, NO inhibits the adhesion of inflammatory cells to the vascular wall, the aggregation of platelets, and the proliferation of smooth muscle cells [27]. These effects of NO are generally considered to act as a brake on the process of atherogenesis. Consequently, accumulation of ADMA may lead to endothelial dysfunction [28,29] and accelerate the development of atherosclerosis. Many other risk factors for cardiovascular disease are also associated with a reduced availability of NO and endothelial dysfunction and it has been suggested that ADMA is the ultimate mediator of the adverse effect of these risk factors on the vascular endothelium [30]. ADMA is positively associated with carotid intima-media thickness, a surrogate marker of generalized atherosclerosis, in healthy subjects and patients with renal disease [31–33]. Compelling evidence for the adverse role of ADMA in the cardiovascular system is provided by prospective studies showing an association between ADMA and cardiovascular events and/or mortality. In several high-risk populations, i.e. critically ill patients [34], and patients with end-stage renal disease [35], coronary artery disease [36,37], or idiopathic pulmonary arterial hypertension [38], increased plasma levels of ADMA were shown to predict adverse outcome, independent of traditional risk factors. Recently, elevated ADMA has also been implicated in diseases that are characterized by a high incidence of cardiovascular disease that is not fully accounted for by traditional risk factors, such as systemic lupus erythematosus and sickle cell anemia [39,40].

It is striking that in most prospective studies even slightly raised plasma concentrations of ADMA were found to be associated with increased risk. However, because the between-subject variation of ADMA is very small (see Section 5), even an increase of a few percent may be regarded as moderate in terms of effect size. In addition, it may very well be that ADMA in plasma originates from cellular spillover and that small changes in plasma concentrations of ADMA reflect much larger changes in intracellular concentrations. Measurement of the intracellular concentration of ADMA probably would be more relevant than measurement of its concentration in plasma, because both generation of ADMA and inhibition of NO synthesis are intracellular processes. Unfortunately, it is virtually impossible to measure intracellular ADMA concentrations in large numbers of subjects in epidemiological studies. These considerations notwithstanding, the results of studies published to date convincingly demonstrate that meaningful results can be obtained by measurement of ADMA in plasma.

2. HPLC analysis

2.1. General analytical considerations

Plasma concentrations of methylated arginine analogs are in the submicromolar range in healthy individuals and at most a few micromolar in diseased states. Because the concentrations of other amino acids are two to three orders of magnitude higher, the quantification of methylated arginine analogs in plasma is an analytical challenge. Traditionally, amino acid analysis is performed by ion-exchange chromatography with detection after post-column derivatization. This technique is not only suitable for the analysis of the twenty amino acids occurring in proteins, but also for the analysis of a large number of non-protein amino acids and post-translationally modified amino acids, including ADMA and SDMA [17]. An inherent disadvantage of this technique is that often very long run times are required to obtain adequate resolution. Shorter run times can be achieved by using reversed-phase HPLC separation after pre-column derivatization. Derivatization serves several purposes [41]. First, it increases the detectability of the amino acids by converting them into adducts that can be detected with high sensitivity using UV absorbance, fluorescence, or electrochemical detection. Second, derivatization usually increases the hydrophobicity of the very polar amino acids, making them amenable to separation by HPLC in reversed-phase mode. Third, for amino acids that contain reactive groups in their side chain, e.g. the sulfhydryl group in cysteine and homocysteine, selectivity can be increased dramatically by selective labeling of that functional group with a proper derivatization reagent. Unfortunately, selective reagents for the derivatization of methylated arginine species have not been described, and therefore selectivity is most often increased by solid-phase extraction (SPE). Most published HPLC methods are suitable for the simultaneous determination of arginine, ADMA, and SDMA. Plasma concentrations of MMA are usually very low, and determination of this analyte in plasma is usually not performed. However, intracellular concentrations of MMA may be much higher and have been shown to be of similar magnitude as ADMA concentrations in endothelial cells [42], and even higher than ADMA concentrations in neuronal cells [43]. Measurement of MMA may thus provide additional information if intracellular metabolism is studied.

2.2. Sample cleanup

Almost all published methods were specifically developed for the analysis of plasma or serum samples and require sample volumes of 0.02–1.5 ml. In a small number of methods, sample cleanup is limited to precipitation of proteins with 5-sulfosalicylic acid, followed by centrifugation and filtration [44–46], or precipitation with ethanol, followed by lyophilization of the supernatant [47]. Although simple to perform, this procedure leads to complex chromatograms with long run times. Most methods rely on a more elaborate cleanup by SPE on cation-exchange columns, exploiting the basic character of both arginine and its methylated analogs. After conditioning of the SPE columns by sequential washing steps with methanol and

water or buffer, diluted plasma is applied to the columns, although in some methods a protein precipitation step is included in the protocol [48,49]. Tissue homogenates prepared in perchloric acid should be neutralized before application to the columns [50]. Tissue or protein hydrolysates, that are usually prepared by heating in 6 M hydrochloric acid, can be neutralized by evaporation of the hydrochloric acid and dissolving the residue in a suitable buffer [51]. After sample loading, the bulk of the matrix components, including neutral and acidic amino acids, is removed from the SPE column by a series of washing steps. In the final step, basic amino acids, including the analytes of interest, are eluted from the column. This is achieved by lowering the pH in the case of weak cation-exchange columns or increasing the pH in the case of strong cation-exchange columns. In the latter case, an ammonia/methanol mixture is often used, although triethylamine has been used as well [49,52]. To allow subsequent removal of the solvent by evaporation, only volatile buffers should be used. Furthermore, the eluting solvent should not only effectively break the interaction between the analytes and the stationary phase, but also be a good solvent for the analytes. We have for instance noted that a mixture of ammonia with pure methanol is effective in eluting ADMA and SDMA, but leads to a low recovery of arginine, because of limited solubility [53]. Although this can be overcome by increasing the polarity of the solvent by adding some water, it should be carefully checked that all analytes, including the internal standard, are recovered to the same extent. Most SPE columns are silica based, but polymeric columns (e.g. Oasis MCX from Waters or Strata-X-C from Phenomenex) are equally effective and have the advantage that preconditioning is not essential and their performance is not adversely affected if the columns run dry between the sample application, washing, and elution steps [53,54]. However, blank chromatograms should be checked for the presence of spurious peaks, because some batches of columns may contain impurities, necessitating a conditioning step before use [55]. Sample cleanup by SPE is labor intensive, but the procedure can be fully automated [55]. The use of small SPE columns in microtiter plate format also holds promise for increasing throughput.

2.3. Derivatization reagents

Although underivatized arginine, ADMA, and SDMA have been quantified in plasma by UV detection at 200 nm [56], most HPLC methods employ pre-column derivatization. Reported derivatization reagents include *o*-phthaldialdehyde (OPA) [44,45,47,52,53,57,58], naphthalene-2,3-dicarboxaldehyde (NDA) [59], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [46,49], 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [54], and phenyl isothiocyanate (PITC) [60]. OPA is by far the most widely used fluorogenic reagent. Major advantages of this reagent are the almost instantaneous reaction at room temperature and the fact that OPA itself is non-fluorescent, leading to relatively clean chromatograms. It is of note that the reaction between OPA and amino groups depends on the presence of a thiol. For this purpose both 2-mercaptoethanol (ME) and 3-mercaptopropionic acid (MPA) can be used. OPA-ME adducts are rather unstable, necessitating fully automated derivatization in order to obtain reliable results. OPA-MPA adducts are more stable, allowing off-line derivatization. It is very important to realize that both ME and MPA do not merely allow the reaction between OPA and the amino acid to proceed, but are also incorporated in the final adduct (Fig. 2). Therefore, these adducts not only differ in stability, but also with respect to their chromatographic properties. In general, at neutral pH, OPA-MPA derivatives are slightly more polar then their OPA-ME counterparts due to the presence of the additional carboxylate group.

2.4. Choice of internal standard

Both sample cleanup by SPE and the derivatization reaction are sources of random errors, necessitating the use of an internal standard that should be added at the very beginning of the analytical procedure, i.e. before sample cleanup. A suitable internal standard should meet a number of requirements. First, it should be a basic compound to ensure recovery by cation-exchange SPE. Second, it should contain a primary amino group to allow its derivatization together with the analytes of interest. Third, the polarity of the derivatized internal standard should be similar to that of the derivatized analytes, but differ enough to allow full chromatographic resolution. Finally, the compound chosen should preferably not be present in biological samples. If the latter requirement cannot be met, it should be ensured that concentrations of endogenous substances are at least two orders of magnitude lower than the concentration used as internal standard. In most published methods for the quantification of arginine, ADMA, and SDMA, either homoarginine or MMA is used as internal standard. MMA is an obvious choice, because endogenous plasma levels of this compound are very low. However, because intracellular MMA concentrations are much higher [42,43], measurement of this compound in cells and tissues may be of interest. In that case, homoarginine can be used as internal standard. N^{ω}-Propyl-L-arginine (N-PLA), a selective inhibitor of neuronal nitric oxide synthase (NOS) that does not occur endogenously, has also been successfully used as internal standard [59].

2.5. Chromatography

Chromatographic separation of the derivatives of arginine and its methylated analogs is usually performed by reversed-phase chromatography using either isocratic or gradient elution. Because ADMA and SDMA are structurally very similar, separation of this pair of compounds is most difficult to accomplish. Using OPA as derivatization reagent, the ADMA adduct usually elutes before the SDMA adduct on a C18 column. If separation is performed on a phenyl column, this elution order is reversed [57,61]. This reversal of elution order on phenyl columns has also been observed for NDA derivatives of ADMA and SDMA [59]. Total run time of the separation is usually around 30 min or even longer, which severely limits the throughput of these methods. We have recently succeeded in decreasing the analysis time of the HPLC separation by using a silica-based monolithic column (Chromolith Performance RP18-e from Merck) [55]. These

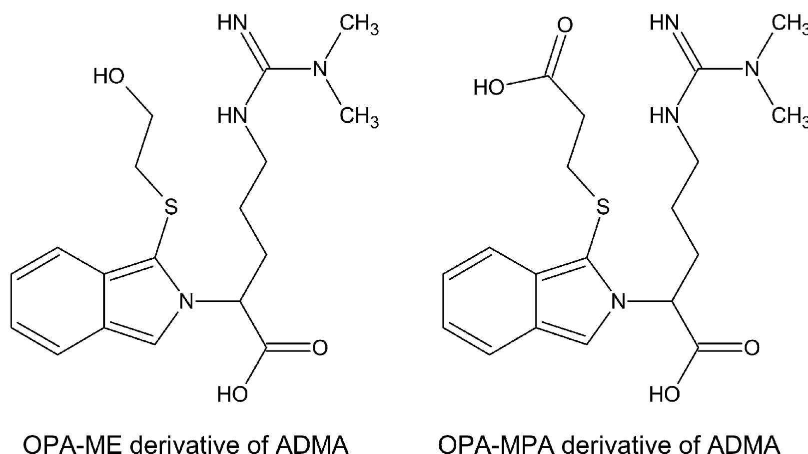

Fig. 2. Reaction products of ADMA with *o*-phthaldialdehyde (OPA) reagent, containing either 2-mercaptoethanol (ME) or 3-mercaptopropionic acid (MPA).

columns, consisting of a silica rod with a bimodal pore structure, are highly permeable and can be operated at high flow rates. Most important, monolithic columns have a high separation efficiency and the backpressure is much lower in comparison to conventional columns packed with silica particles [62,63]. Comparison of a separation of OPA derivatives on a monolithic column with separation on a conventional C18 column [51,53], shows that the retention times are reduced 2-fold without loss of resolution (Fig. 3). Likewise, the total run time, including a strong solvent wash to elute strongly retained compounds and column re-equilibration, was reduced from 30 to 15 min. The high flow rate that can be used with monolithic columns may lead to a loss of resolution if the separation is performed at an elevated temperature. The reason for this decreased separation efficiency is not a decrease in plate count, but the development of a temperature gradient across the column, caused by insufficient temperature equilibration of the mobile phase. The magnitude of this effect depends not only on flow rate and temperature, but also on the design of the column oven.

Sufficient separation of ADMA and SDMA in standard solutions does not guarantee that these compounds can be measured free from interference in biological samples. Analyte concentrations, but also interference by endogenous compounds may differ between matrices, patient groups, and even between species. In healthy subjects, plasma concentrations of ADMA and SDMA are almost equal, but in patients with moderate renal failure the increase of SDMA is more pronounced than the increase of ADMA (Fig. 4). This reflects the fact that SDMA is exclusively eliminated by renal excretion, whereas degradation by DDAH is the major route of disposal for ADMA. Conversely, plasma concentrations of ADMA in healthy dogs are approximately 3- to 5-fold higher than in humans, whereas SDMA concentrations are comparable [64] (Fig. 4). In human urine, ADMA and SDMA concentrations are approximately equal, but 10- to 100-fold higher than concentrations in plasma, whereas the concentration of arginine is lower than in plasma (Fig. 5). Apparently, tubular reabsorption, which is highly efficient for arginine, is less efficient for its methylated analogs. It is of interest that concentrations of ADMA in rat urine are almost negligible [19], again showing an inter-species difference in the metabolism of methylated arginines (see Section 1.1). In culture medium of human umbilical vein endothelial cells both ADMA and SDMA can be detected, but ADMA reaches much higher concentrations than SDMA (Fig. 6).

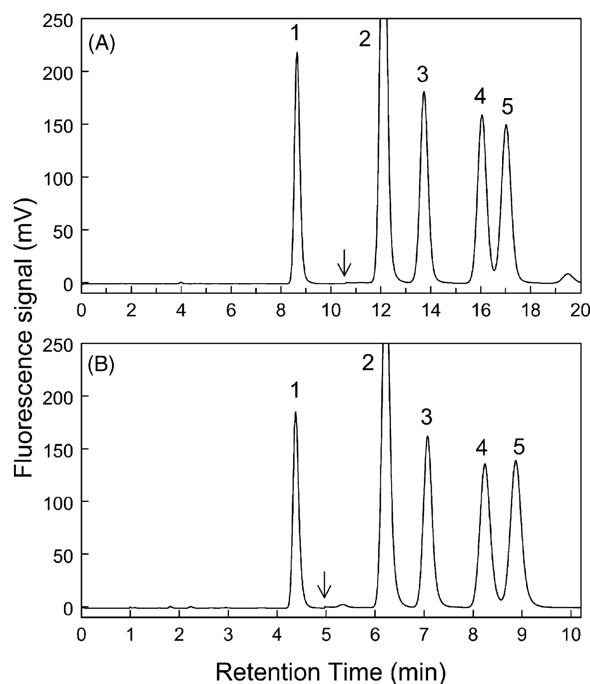

Fig. 3. Chromatograms of a combined standard, containing 100 μM arginine, 10.5 μM homoarginine, 10.1 μM ADMA, and 10.8 μM SDMA, separated by reversed-phase chromatography on a conventional column (Symmetry C18; 3.9 mm × 150 mm; 5 μm particle size from Waters) with a mobile phase of 50 mM potassium phosphate buffer (pH 6.5), containing 8.7% acetonitrile at a flow rate of 1.1 ml/min and a column temperature of 30 °C [53] (A) or on a monolithic column (Chromolith Performance RP-18e; 100 mm × 4.6 mm from Merck) with a mobile phase of 25 mM potassium phosphate buffer (pH 6.5), containing 7.0% acetonitrile at a flow rate of 2.0 ml/min and a column temperature of 30 °C according to [55] (B). The arrow indicates a gain switch of the fluorescence detector. Peak identification: 1, arginine; 2, internal standard (MMA; final concentration 20 μM); 3, homoarginine; 4, ADMA; 5, SDMA.

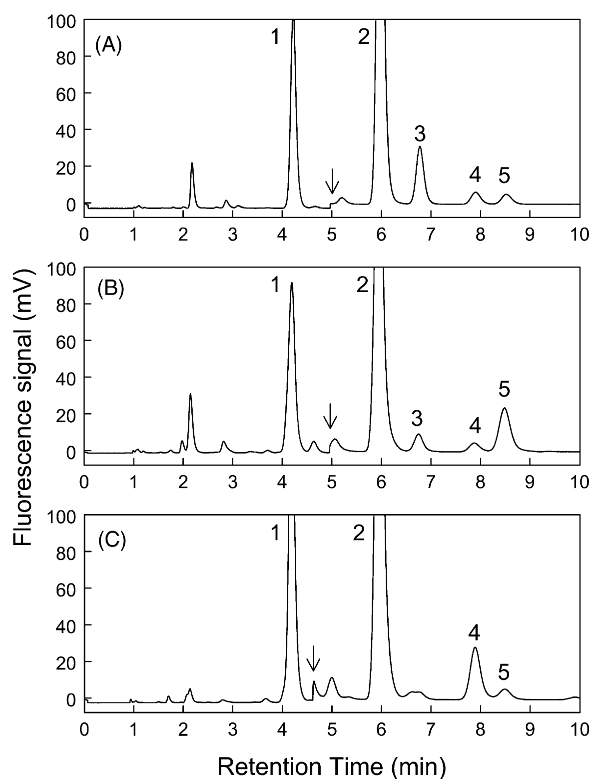

Fig. 4. Chromatograms of plasma from a healthy volunteer, containing 77 μM arginine, 0.65 μM ADMA, and 0.55 μM SDMA (A), of plasma from a patient with moderate renal failure, containing 89 μM arginine, 0.57 μM ADMA, and 2.97 μM SDMA (B), and of plasma from a healthy dog, containing 110 μM arginine, 2.41 μM ADMA, and 0.50 μM SDMA (C). Sample cleanup and chromatography were performed according to [55]. See legend to Fig. 3B for chromatographic conditions. The arrow indicates a gain switch of the fluorescence detector. Peak identification: 1, arginine; 2, internal standard (MMA; final concentration 20 μM); 3, homoarginine; 4, ADMA; 5, SDMA.

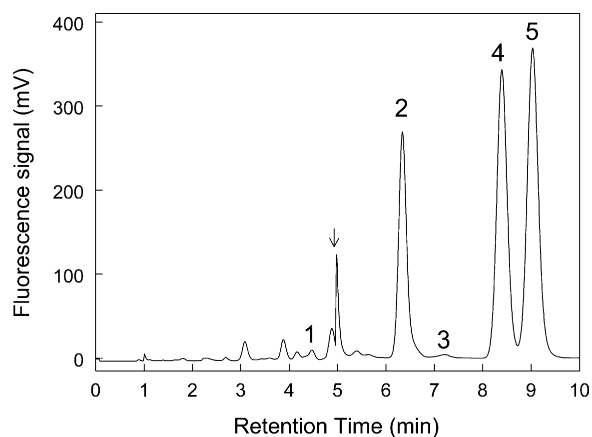

Fig. 5. Chromatogram of urine from a healthy volunteer, containing 66 μM ADMA, and 73 μM SDMA. Note the almost complete absence of arginine and homoarginine. Sample cleanup and chromatography were performed according to [55]. See legend to Fig. 3B for chromatographic conditions. The arrow indicates a gain switch of the fluorescence detector. Peak identification: 1, arginine; 2, internal standard (MMA; final concentration 20 μM); 3, homoarginine; 4, ADMA; 5, SDMA.

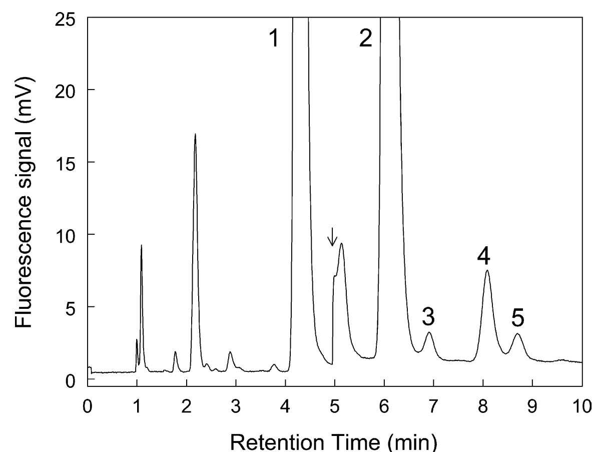

Fig. 6. Chromatogram of medium of a human umbilical vein endothelial cell culture, containing 250 μM arginine, 0.53 μM ADMA, and 0.16 μM SDMA. The medium was harvested 96 h after the start of the culture. Sample cleanup and chromatography were performed according to [55]. See legend to Fig. 3B for chromatographic conditions. The arrow indicates a gain switch of the fluorescence detector. Peak identification: 1, arginine; 2, internal standard (MMA; final concentration 20 μM); 3, homoarginine; 4, ADMA; 5, SDMA.

3. Method validation

3.1. Linearity and lower limit of quantification

HPLC methods using fluorescence detection usually show a linear detector response over several orders of magnitude, with the upper limit of the linear range far above the concentrations present in biological samples. However, at the high end, linearity may be limited by the concentration of the derivatization reagent, which can be recognized by a decreased area of the internal standard. The lower limit of quantification of most published methods ranges from 0.01 to 0.3 μM, which is sufficient to allow measurement in plasma and urine samples. However, in other matrices, such as cerebrospinal fluid [53,65], ADMA and SDMA concentrations are much lower and can only be measured by methods with high sensitivity. The lower limit of quantification is often defined at a signal/noise ratio of 10. It is important to note that at this signal/noise ratio, assay imprecision may be as high as 10%, which is far from optimal (see Section 3.2.).

3.2. Accuracy and precision

As a first step in ascertaining accuracy of the method, recovery of exogenously added analytes should be investigated. Both recovery after SPE and recovery of the entire analytical procedure should be investigated. But even full recovery of added analytes from biological samples is no proof for accuracy. Coelution of matrix components with the analytes of interest leads to measurement of falsely high concentrations in samples but has no effect on the percentage recovery from spiked samples. Accuracy is often expressed in terms of bias from the expected concentration, using a certified reference material. For the time being such reference material for the analysis of ADMA and

SDMA is not available, which may in part explain the wide range of reported values in healthy subjects (see Section 5). On the other hand, a recent inter-laboratory comparison showed a fair agreement between reported values, although further improvement is certainly desirable [61].

Both intra-assay and inter-assay imprecision are commonly expressed as a coefficient of variation (CV). Although intra-assay CV may be important when related samples are to be compared (e.g. samples from a patient before and after treatment), inter-assay CV is often more relevant, because in most clinical studies samples are analyzed in multiple series. Reported inter-assay imprecision for ADMA and SDMA HPLC assays range from approximately 2% to values exceeding 10%. It should be noted that imprecision is not solely determined by the design of the particular HPLC method, but to a large extent by other factors, such as equipment and skills of the analytical personnel. Inter-laboratory transfer of a particular method may therefore result in a change of assay performance in terms of precision. A widely held view is that desirable imprecision should be <0.50 times the within-subject variability (CV_I), and optimal imprecision <0.25 CV_I [66]. For ADMA and SDMA no solid data on CV_I is available, but it is reasonable to assume that CV_I is smaller than the between-subject variability (CV_G). In a large cohort study in the general population ($n = 2311$; see also Section 5) we measured mean concentrations of 0.497 ± 0.063 μM for ADMA ($CV_G = 12.7\%$), and 0.526 ± 0.101 μM for SDMA ($CV_G = 19.2\%$). From these data, it can be estimated that optimal imprecision is <3% and <5% for ADMA and SDMA, respectively. It is clear that not all published HPLC methods meet these criteria. Although methods with higher imprecision can certainly yield relevant results, their use may lead to low statistical power in clinical trials and to a severe underestimation of associations in epidemiological studies [67].

4. Pre-analytical factors

Analysis of arginine, ADMA, and SDMA is most often performed in serum or plasma. If plasma is used, the choice of anticoagulant may affect the measured concentrations to some extent. We have observed no significant differences in concentrations of arginine, ADMA, and SDMA in a small study in which 15 paired EDTA– and heparin–plasma samples were compared [53]. On the other hand, citrate–plasma should not be used, because the rather large volume of citrate solution in the blood collection tubes leads to a considerable dilution. Theoretically, allowance for this dilution can be made, but accurate correction is difficult because dilution of the plasma depends on both the level of filling of the tubes and the hematocrit. The additional error introduced by this correction may be detrimental, considering the very small biological variation of plasma concentrations of ADMA and SDMA (see also Sections 3.2 and 5). A comparison of paired serum and heparin–plasma samples ($n = 10$) revealed no significant difference for ADMA concentrations (0.389 ± 0.044 μM versus 0.387 ± 0.045 μM; $P = 0.54$), approximately 2% higher SDMA concentrations in serum (0.405 ± 0.084 μM versus 0.397 ± 0.085 μM; $P = 0.010$), and 58% higher concentrations of arginine in serum (115.5 ± 16.1 μM versus 73.3 ± 14.3 μM; $P < 0.001$) [53]. Regarding the quantification of arginine it is important to avoid hemolysis, because falsely low arginine levels may be obtained in hemolytic plasma due to degradation by arginase released from erythrocytes.

Storage conditions for plasma are not very critical. Plasma can be stored indefinitely at −70 °C or below, and at least several years at −20 °C without alteration in concentration of (methylated) arginine. In addition, it has been shown that repeated freeze/thaw cycles do not influence ADMA and SDMA concentrations [68]. We have observed that even at room temperature plasma concentrations did not change significantly during 24 h.

For the reliable analysis of arginine, ADMA and SDMA in tissues it is important to consider that after tissue homogenization, proteolysis may lead to a very rapid increase in free ADMA and SDMA [69]. In addition, ADMA and MMA may be degraded by DDAH, which is present in many tissues. Furthermore, several tissues contain arginase and other enzymes that either consume or generate arginine. Therefore, in order to avoid artefactual increases or decreases of arginine and its methylated analogs, it is important to use low temperatures and stop enzymatic reactions by the addition of acid as soon as possible after tissue homogenization. Homogenization of frozen tissue in 0.6 M perchloric acid is a convenient procedure to preclude these problems [50].

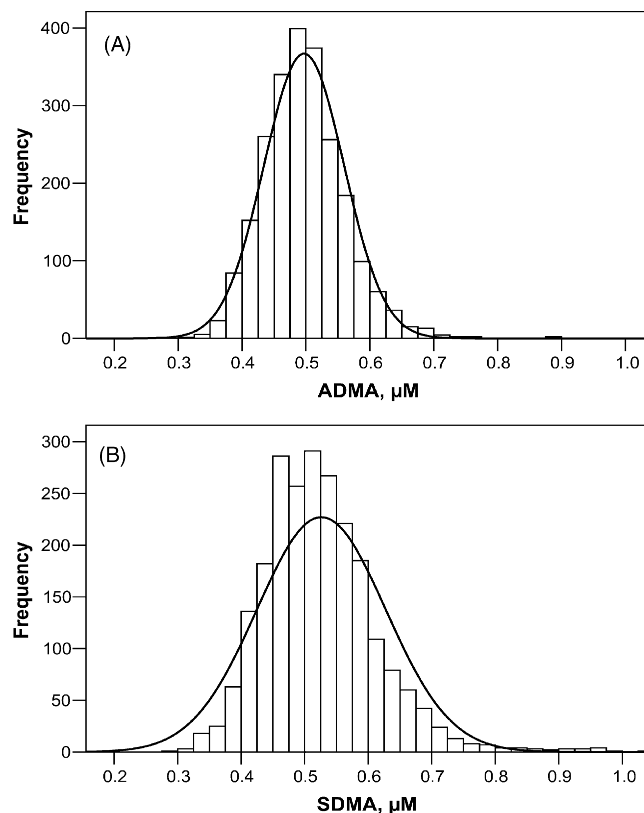

Fig. 7. Distribution of plasma concentrations of ADMA (A) and SDMA (B) in the general population. Samples ($n = 2311$) were from participants (aged between 50 and 75 years) of the Hoorn Study, a population-based cohort study.

5. Reference values

In most publications on HPLC methods, basal plasma levels of ADMA in small numbers of healthy individuals are reported. In the past, these concentrations tended to differ widely, from values as low as 0.2 μM to values exceeding 1 μM. Values reported in more recent publications are more consistent and tend to converge around 0.5 μM (reviewed in [61]). Recently, Schulze et al. reported reference values for ADMA, that were derived from measurement of ADMA in plasma of 500 healthy non-smoking subjects by a commercially available ELISA [70]. A mean value of 0.69 ± 0.20 μM was found in the whole group, but values were age- and sex-dependent. In the Hoorn Study, a large cohort study in the general population ($n = 2311$; aged 50–75 years), we have recently measured ADMA and SDMA by HPLC. Mean concentrations were 0.497 ± 0.063 μM for ADMA, and 0.526 ± 0.101 μM for SDMA, and the 95% reference intervals were 0.39–0.63 and 0.38–0.73 μM for ADMA and SDMA, respectively. Histograms of the distributions are shown in Fig. 7. ADMA concentrations were higher in females compared to males and showed a positive association with age ($r = 0.224$; $P < 0.001$), in accordance with the data of Schulze et al. [70].

6. Concluding remarks

Since the recognition of ADMA as an important endogenous inhibitor of nitric oxide synthase, described in a landmark paper by Vallance and colleagues in 1992 [16], many groups of investigators have studied the role of ADMA and other methylated arginine analogs in health and disease. A large number of analytical approaches has been described, most of which are based on HPLC with fluorescence detection. Reliable quantification of low plasma concentrations of ADMA and SDMA in the presence of many other amino acids that are present in far higher concentrations constitutes an analytical challenge. Considering the very narrow distribution of ADMA concentrations in healthy subjects, the importance of low imprecision of the analytical procedure cannot be overemphasized. Although ADMA holds promise as a biomarker of cardiovascular disease, it should be noted that at the moment ADMA does not fulfil all requirements for clinical use as a biomarker (reviewed in [71]). However, as the role of ADMA as a risk marker or even risk factor in cardiovascular disease is becoming ever more clear, it is conceivable that in the future ADMA will be used in conjunction with traditional risk factors to improve cardiovascular risk assessment. By that time, standardized, rapid, and cost-effective ADMA assays will be required that can be run in clinical chemistry laboratories, preferably on the same fully automated laboratory platforms that are used for routine clinical chemistry assays. For the time being, a commercially available ELISA system is the only way to perform ADMA assays outside specialized research laboratories [68,72,73]. On the other side of the analytical spectrum, specialized laboratories will increasingly use GC–MS/MS and LC–MS/MS methods for the analysis of methylated arginines. The superior selectivity of these techniques allows reliable and rapid quantification of these compounds, without elaborate sample cleanup. This approach is described in detail elsewhere in this issue (see [74,75] for recent examples). However, for many research laboratories, HPLC with fluorescence detection will remain the method of choice for the quantification of methylated arginine analogs. Irrespective of the analytical technique that is used, quality control aspects, determination of age- and sex-specific reference values, and both short- and long-term intra-individual variability remain important issues to be addressed.

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References

[1] J.D. Gary, S. Clarke, Prog. Nucleic Acid Res. Mol. Biol. 61 (1998) 65.
[2] J.R. Cook, J.H. Lee, Z.H. Yang, C.D. Krause, N. Herth, R. Hoffmann, S. Pestka, Biochem. Biophys. Res. Commun. 342 (2006) 472.
[3] A.E. McBride, P.A. Silver, Cell 106 (2001) 5.
[4] F.M. Boisvert, J. Côté, M.C. Boulanger, S. Richard, Mol. Cell. Proteomics 2 (2003) 1319.
[5] F. Bachand, P.A. Silver, EMBO J. 23 (2004) 2641.
[6] T.B. Miranda, P. Khusial, J.R. Cook, J.H. Lee, S.I. Gunderson, S. Pestka, G.W. Zieve, S. Clarke, Biochem. Biophys. Res. Commun. 323 (2004) 382.
[7] W.A. Smith, B.T. Schurter, F. Wong-Staal, M. David, J. Biol. Chem. 279 (2004) 22795.
[8] G.L. Cuthbert, S. Daujat, A.W. Snowden, H. Erdjument-Bromage, T. Hagiwara, M. Yamada, R. Schneider, P.D. Gregory, P. Tempst, A.J. Bannister, T. Kouzarides, Cell 118 (2004) 545.
[9] Y. Wang, J. Wysocka, J. Sayegh, Y.H. Lee, J.R. Perlin, L. Leonelli, L.S. Sonbuchner, C.H. McDonald, R.G. Cook, Y. Dou, R.G. Roeder, S. Clarke, M.R. Stallcup, C.D. Allis, S.A. Coonrod, Science 306 (2004) 279.
[10] A.J. Bannister, T. Kouzarides, Nature 436 (2005) 1103.
[11] T. Ogawa, M. Kimoto, K. Sasaoka, Biochem. Biophys. Res. Commun. 148 (1987) 671.
[12] M. Kimoto, H. Tsuji, T. Ogawa, K. Sasaoka, Arch. Biochem. Biophys. 300 (1993) 657.
[13] C.T. Tran, M.F. Fox, P. Vallance, J.M. Leiper, Genomics 68 (2000) 101.
[14] J.M. Leiper, J. Santa Maria, A. Chubb, R.J. MacAllister, I.G. Charles, G.S. Whitley, P. Vallance, Biochem. J. 343 (1999) 209.
[15] V. Achan, M. Broadhead, M. Malaki, G.S. Whitley, J. Leiper, R. MacAllister, P. Vallance, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1455.
[16] P. Vallance, A. Leone, A. Calver, J. Collier, S. Moncada, Lancet 339 (1992) 572.
[17] B. Marescau, G. Nagels, I. Possemiers, M.E. De Broe, I. Becaus, J.M. Billiouw, W. Lornoy, P.P. De Deyn, Metabolism 46 (1997) 1024.
[18] R.J. Nijveldt, P.A. van Leeuwen, C. van Guldener, C.D. Stehouwer, J.A. Rauwerda, T. Teerlink, Nephrol. Dial. Transplant. 17 (2002) 1999.
[19] R.J. Nijveldt, T. Teerlink, C. van Guldener, H.A. Prins, A.A. van Lambalgen, C.D. Stehouwer, J.A. Rauwerda, P.A. van Leeuwen, Nephrol. Dial. Transplant. 18 (2003) 2542.
[20] K.A. Carello, S.E. Whitesall, M.C. Lloyd, S.S. Billecke, L.G. D'Alecy, Am. J. Physiol. Heart Circ. Physiol. 290 (2006) H209.
[21] R.J. Nijveldt, T. Teerlink, M.P. Siroen, A.A. van Lambalgen, J.A. Rauwerda, P.A. van Leeuwen, Clin. Nutr. 22 (2003) 17.
[22] R.J. Nijveldt, M.P. Siroen, T. Teerlink, A.A. van Lambalgen, J.A. Rauwerda, P.A. van Leeuwen, Liver Int. 24 (2004) 510.
[23] M.P. Siroen, J.R. van der Sijp, T. Teerlink, C. van Schaik, R.J. Nijveldt, P.A. van Leeuwen, Hepatology 41 (2005) 559.
[24] D. Tsikas, I. Rode, T. Becker, B. Nashan, J. Klempnauer, J.C. Frölich, Hepatology 38 (2003) 1063.
[25] M.P. Siroen, M.C. Warlé, T. Teerlink, R.J. Nijveldt, E.J. Kuipers, H.J. Metselaar, H.W. Tilanus, D.J. Kuik, J.R. van der Sijp, S. Meijer, B. van der Hoven, P.A. van Leeuwen, Liver Transpl. 10 (2004) 1524.

[26] M.P. Siroen, T. Teerlink, R.J. Nijveldt, H.A. Prins, M.C. Richir, P.A. van Leeuwen, Annu. Rev. Nutr. 26 (2006) 203.
[27] J.P. Cooke, V.J. Dzau, Annu. Rev. Med. 48 (1997) 489.
[28] J.P. Cooke, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2032.
[29] R.H. Böger, Clin. Chem. Lab. Med. 41 (2003) 1467.
[30] J.P. Cooke, Circulation 109 (2004) 1813.
[31] H. Miyazaki, H. Matsuoka, J.P. Cooke, M. Usui, S. Ueda, S. Okuda, T. Imaizumi, Circulation 99 (1999) 1141.
[32] C. Zoccali, F.A. Benedetto, R. Maas, F. Mallamaci, G. Tripepi, L.S. Malatino, R. Böger, J. Am. Soc. Nephrol. 13 (2002) 490.
[33] P.W. Nanayakkara, T. Teerlink, C.D. Stehouwer, D. Allajar, A. Spijkerman, C. Schalkwijk, P.M. ter Wee, C. van Guldener, Kidney Int. 68 (2005) 2230.
[34] R.J. Nijveldt, T. Teerlink, B. van der Hoven, M.P. Siroen, D.J. Kuik, J.A. Rauwerda, P.A. van Leeuwen, Clin. Nutr. 22 (2003) 23.
[35] C. Zoccali, S.M. Bode-Böger, F. Mallamaci, F.A. Benedetto, G. Tripepi, L.S. Malatino, A. Cataliotti, I. Bellanuova, I. Fermo, J.C. Frölich, R.H. Böger, Lancet 358 (2001) 2113.
[36] T.M. Lu, Y.A. Ding, S.J. Lin, W.S. Lee, H.C. Tai, Eur. Heart J. 24 (2003) 1912.
[37] R. Schnabel, S. Blankenberg, E. Lubos, K.J. Lackner, H.J. Rupprecht, C. Espinola-Klein, N. Jachmann, F. Post, D. Peetz, C. Bickel, F. Cambien, L. Tiret, T. Münzel, Circ. Res. 97 (2005) e53.
[38] J.T. Kielstein, S.M. Bode-Böger, G. Hesse, J. Martens-Lobenhoffer, A. Takacs, D. Fliser, M.M. Hoeper, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1414.
[39] I.E. Bultink, T. Teerlink, J.A. Heijst, B.A. Dijkmans, A.E. Voskuyl, Ann. Rheum. Dis. 64 (2005) 1362.
[40] J.B. Schnog, T. Teerlink, F.P. van der Dijs, A.J. Duits, F.A. Muskiet, Curama Study Group, Ann. Hematol. 84 (2005) 282.
[41] T. Teerlink, J. Chromatogr. B 659 (1994) 185.
[42] M. Beppu, S. Obayashi, T. Aso, M. Goto, H. Azuma, J. Cardiovasc. Pharmacol. 39 (2002) 192.
[43] A.J. Cardounel, J.L. Zweier, J. Biol. Chem. 277 (2002) 33995.
[44] B.M. Chen, L.W. Xia, R.Q. Zhao, J. Chromatogr. B 692 (1997) 467.
[45] B.M. Chen, L.W. Xia, S.X. Liang, G.H. Chen, F.L. Deng, W.R. Zhang, L. Tao, Anal. Chim. Acta 444 (2001) 223.
[46] B. Anderstam, K. Katzarski, J. Bergström, J. Am. Soc. Nephrol. 8 (1997) 1437.
[47] W.Z. Zhang, D.M. Kaye, Anal. Biochem. 326 (2004) 87.
[48] K.S. Park, H.W. Lee, S.Y. Hong, S. Shin, S. Kim, W.K. Paik, J. Chromatogr. 440 (1988) 225.
[49] T. Heresztyn, M.I. Worthley, J.D. Horowitz, J. Chromatogr. B 805 (2004) 325.
[50] M.P. Siroen, T. Teerlink, A.C. Bolte, R.M. van Elburg, M.C. Richir, R.J. Nijveldt, B. van der Hoven, P.A. van Leeuwen, Gynecol. Obstet. Invest. 62 (2006) 7.
[51] T. Teerlink, Methods Mol. Med. 108 (2005) 263.
[52] A. Pettersson, L. Uggla, V. Backman, J. Chromatogr. B 692 (1997) 257.
[53] T. Teerlink, R.J. Nijveldt, S. de Jong, P.A. van Leeuwen, Anal. Biochem. 303 (2002) 131.
[54] S. Nonaka, M. Tsunoda, K. Imai, T. Funatsu, J. Chromatogr. A 1066 (2005) 41.
[55] S. de Jong, T. Teerlink, Anal. Biochem. 353 (2006) 287.
[56] R.J. MacAllister, M.H. Rambausek, P. Vallance, D. Williams, K.H. Hoffmann, E. Ritz, Nephrol. Dial. Transplant. 11 (1996) 2449.
[57] D. Tsikas, W. Junker, J.C. Frölich, J. Chromatogr. B 705 (1998) 174.
[58] J. Pi, Y. Kumagai, G. Sun, N. Shimojo, J. Chromatogr. B 742 (2000) 199.
[59] M. Marra, A.R. Bonfigli, R. Testa, I. Testa, A. Gambini, G. Coppa, Anal. Biochem. 318 (2003) 13.
[60] S. Ueno, A. Sano, K. Kotani, K. Kondoh, Y. Kakimoto, J. Neurochem. 59 (1992) 2012.
[61] E. Schwedhelm, Vasc. Med. 10 (Suppl. 1) (2005) S89.
[62] K. Cabrera, J. Sep. Sci. 27 (2004) 843.
[63] T. Ikegami, N. Tanaka, Curr. Opin. Chem. Biol. 8 (2004) 527.
[64] L.G. Pedersen, I. Tarnow, L.H. Olsen, T. Teerlink, H.D. Pedersen, Res. Vet. Sci. 80 (2006) 336.
[65] C. Mulder, L.O. Wahlund, M. Blomberg, S. de Jong, G.J. van Kamp, P. Scheltens, T. Teerlink, J. Neural Transm. 109 (2002) 1203.
[66] C.G. Fraser, P.H. Petersen, Clin. Chem. 45 (1999) 321.
[67] T. Teerlink, Clin. Chem. Lab. Med. 43 (2005) 1130.
[68] P. Valtonen, J. Karppi, K. Nyyssönen, V.P. Valkonen, T. Halonen, K. Punnonen, J. Chromatogr. B 828 (2005) 97.
[69] T. Teerlink, Vasc. Med. 10 (Suppl. 1) (2005) S73.
[70] F. Schulze, R. Maas, R. Freese, E. Schwedhelm, E. Silberhorn, R.H. Böger, Eur. J. Clin. Invest. 35 (2005) 622.
[71] R.S. Vasan, Circulation 113 (2006) 2335.
[72] F. Schulze, R. Wesemann, E. Schwedhelm, K. Sydow, J. Albsmeier, J.P. Cooke, R.H. Böger, Clin. Chem. Lab. Med. 42 (2004) 1377.
[73] J. Martens-Lobenhoffer, S. Westphal, F. Awiszus, S.M. Bode-Böger, C. Luley, Clin. Chem. 51 (2005) 2188.
[74] D. Tsikas, B. Schubert, F.M. Gutzki, J. Sandmann, J.C. Frölich, J. Chromatogr. B 798 (2003) 87.
[75] E. Schwedhelm, J. Tan-Andresen, R. Maas, U. Riederer, F. Schulze, R.H. Böger, Clin. Chem. 51 (2005) 1268.