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RAPID COMMUNICATION

A new selective pre-column ninhydrin-based derivatization for a RP-HPLC determination of plasma asymmetric dimethyl-L-arginine (ADMA) by fluorescence detection

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Abstract We report a new selective and direct pre-column ninhydrin-based derivatization reaction for determination of plasma ADMA levels. This original derivatization procedure matched to a validated and rapid RP-HPLC method can be a useful alternative to other assays in which time consuming and expensive extraction and/or purification steps are required.

Keywords ADMA · Homoarginine · Ninhydrin · Fluorescence

Introduction

Over the past two decades nitric oxide (NO) has evolved as a key bioactive molecule that plays a central role in many biological processes such as immune response, neurotransmission, and in the maintenance of vascular tone (Kerwin et al. 1995). NO is synthesized by three isoforms of NO synthases (NOS) (Griffith and Stuehr 1995) which catalyze the oxidation of the terminal guanidino group of L-arginine. All of the NOS isoforms exhibit varying degrees of susceptibility to the inhibition of several guanidine-substituted analogues of L-arginine (Rossiter et al. 2005). When this inhibition is framed in a pathological context the effect is a decrease of NO availability resulting in an endothelial vasodilator dysfunction which has been implicated as a key event in the pathogenesis of arteriosclerosis (Egashira 2002). Among the potential inhibitors of the NOS, some of the naturally occurring methylarginines can profoundly shift the balance of NO (Cardounel et al. 2005). Methylarginines derive from the methylated arginine residues released, as free molecules, after protein breakdown. They include N^G-monomethyl-L-arginine (L-NMMA), asymmetrical N^G,N^G-dimethyl-L-arginine (ADMA) and symmetrical N^G,N^G-dimethyl-L-arginine (SDMA). Only L-NMMA and ADMA show an inhibition activity toward NOS, but since ADMA concentration is tenfold higher than L-NMMA it is thought to represent a more important endogenous inhibitor of NOS. Therefore, there is a considerable demand for specific, sensitive, and rapid methods for ADMA measurement in biological fluids. However, this is difficult because the determination of ADMA revealed some problems. The structure of this methylarginine and its low circulating amount (0.33 ± 0.61 μmol/l), in fact, do not allow a sensitive UV absorbance detection. Besides, it is not easy to discriminate between ADMA and its symmetrical stereoisomer SDMA, whose levels are approximately equal to those of ADMA. Moreover, other amino acids may interfere with the method, which complicates ADMA detection. Therefore, the measurement of the naturally occurring methylarginines usually proceeds through a series of extraction and/or purification steps in order to concentrate them and to remove the other interfering amino acid. This phase is then followed by a pre- or post-column derivatization reaction in order to improve

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detection selectivity and to take advantage of the chromatographic properties of the resultant methylarginine-derivates. Despite this, the employment of a complex mobile phase or a gradient elution or special columns or equipment are often needed in order to reach a satisfactory separation and detection of the two enantiomers of dimethylarginine. Consequently, the currently available analytical methods are time consuming, expensive, and not very suitable to routine analysis. In this paper, we report the first use of pre-column derivatization using ninhydrin for a fast, selective and high sensitive fluorescence detection of plasma or serum ADMA concentration with isocratic reversed phase high-performance liquid chromatography separation.

Materials and methods

Chemicals

Acetonitrile (ACN), ethanol (EtOH), H_2SO_4 and tetrahydrofuran (THF) were obtained from Carlo Erba Reagenti (Milano, Italy). The guanidino compounds, homoarginine (HARG) and the methylarginines ADMA and SDMA as well KOH, ammonium bicarbonate (NH_4HCO_3) and ninhydrin were purchased from Sigma/Aldrich (St. Louis, MO, USA). High-purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA).

Solutions

Methylarginines and internal standard HARG (IS) were prepared respectively as 1 and 5 mmol/l stock solutions in ultrapure water and stored at −80°C until use. Fresh working solution (calibrators) were obtained by opportune serial diluition in ultrapure water of stock solutions. H_2SO_4 was prepared by diluting an opportune volume of concentrated reagent (17.96 mol/l) in ultrapure water to form a 5 mol/l solution. Ninhydrin and KOH were prepared by dissolving an opportune amount of powdered reagents, respectively in EtOH to form a 2% w/v and in H_2O to form a 5% w/v solutions.

Patients, sample collection and sample cleanup

Fifty apparently healthy volunteers (22 males, 28 females) aged from 19 to 84 years were enrolled for this study and carefully informed about the nature and purpose of the investigation before giving their voluntary consent to participate. After informed consent, whole blood was collected by venipuncture in 50 μl EDTA vacutainer tubes. Without delay, blood was centrifuged at $2{,}600 \times g$ for 10 min and the obtained plasma was immediately separated and stored in aliquots at −80°C until further processed. Prior to analyses, 200 μl of calibrators or plasma sample were mixed to 2.5 μl of internal standard (a 5 mmol/l HARG solution) then to 200 μl of ACN to precipitate the proteins. After vortex mixing, precipitated protein was removed by centrifugation at 15,000 rpm for 5 min.

Derivatization procedure

Twenty microliter of a 5% w/v KOH solution and 20 μl of a ethanolic 2% w/v ninhydrin solution were mixed to 300 μl of clear supernatant. After each adding step the samples were vortex mixing then were incubated at room temperature for 5 min in a light protected area. After this time, 20 μl of a 5 mol/l H_2SO_4 solution was added and after vortex-mixing the sample was incubated at 100°C for 10 min in a thermoblock heater.

Apparatus and chromatographic conditions

Chromatographic experiments were performed using a Waters (Mildford, MA, USA) HPLC system model Alliance 2695 equipped with a Waters 474 fluorescence detector. The separation was achieved by using a 4.6 mm × 15 cm Waters XBridge C18 5 μm column with a 4.6 × 20 mm guard column cartridge. The mobile phase consisted of an aqueous solution of 13 mmol/l NH_4HCO_3 containing 10% v/v THF. The eluent was isocratically delivered across the column at flow-rate of 1.3 ml min^{-1} and prior to use it was filtered through a disposable 0.22-μm filter (Millipore) to remove any particulate matter. Separation was carried out at room temperature (about 23–24°C) and amount of injection was 20 μl. Column eluates were detected with a fluorescent detector with the gain set at ×1,000 scale expansion and excitation and emission wavelengths set at 390 and 497 nm, respectively.

Results and discussion

Methylarginines are characterized from a mono- or dimethylated guanidino group that in the native state can reacts in strongly alkaline medium, typically pH ≥ 12, specifically with ninhydrin to yield a fluorescence adduct with a high quantum efficiency for fluorescence detection. The high-specific reaction between guanidine group and ninhydrin in alkaline medium was introduced by Conn and

Davis (Conn and Davis 1959) to detect and to measure guanidinium compounds such as creatine and arginine. To this regard, Buchberger and Ferdig (Buchberger and Ferdig 2004) have recently reported that for unknown reasons the reaction conditions cited in literature (Boppana and Rhodes 1990) do not lead to fluorescent adduct and have proposed an alternative explanation of the reaction mechanism and a new derivatization procedure. However by means of this scheme, and like others authors, only arginine and creatine were detected in a biological sample although the authors advanced the possibility that also others compounds with a guanidine moiety may be measured. In our experiments we followed the Buchberger and Ferdig's scheme (Buchberger and Ferdig 2004), which substantially differs from Conn and Davis's procedure (Conn and Davis 1959) for the addition of a heating step carried out in acidic conditions. In our approach, however, some aspects were profoundly changed because the original indications did not work or did not lead to satisfactory results. Essentially, to 200 µl of calibrators or plasma sample 20 µl of internal standard (a 0.625 mmol/l homoarginine solution) and 200 µl of acetonitrile to precipitate the proteins were mixed. Recently, we have examined the effects of the deproteinization by 100% ACN on sample recovering of arginine and dimethylarginines (Zinellu et al. 2007), discovering that for a ratio sample/ACN above 1:1, without addition of ammonia, the loss of these compounds was about 20 and 10%, respectively. Due to the selectivity of the reaction of ninhydrin towards the guanidino group, a thorough deproteinization was not required in this work; therefore, also in consideration of the cross-reaction of ninhydrin with ammonia, a ratio sample/ACN of 1:1 without addition of ammonia was employed. Once precipitated, protein was removed by centrifugation at 15,000 rpm for 5 min then 300 µl of clear supernatant were derivatized with ninhydrin as described above.

Although the mechanism of condensation between guanidine group and ninhydrin is not fully understood, starting from the data collected for arginine, which is among the best characterized reactions, it can be assumed also for ADMA that the reaction happens in two steps, an initial fast reaction followed by a slower reaction rate (Friedman 2004). In the first step, which occurs in strongly alkaline medium, a tertiary alcohol is generated while in the slower stage there is the elimination of the tertiary alcohol group (Buchberger and Ferdig 2004) to form a Schiff base. In order to accelerate the elimination of tertiary alcohol, both acidic condition and heating are useful. As depicted in Fig. 1, in order to reach the greatest sensitivity quickly, the best compromise between temperature and reaction time of the second stage of the derivatization procedure was 10 min to 100°C. In contrast, an increase of the reaction time of the first step of the derivatization

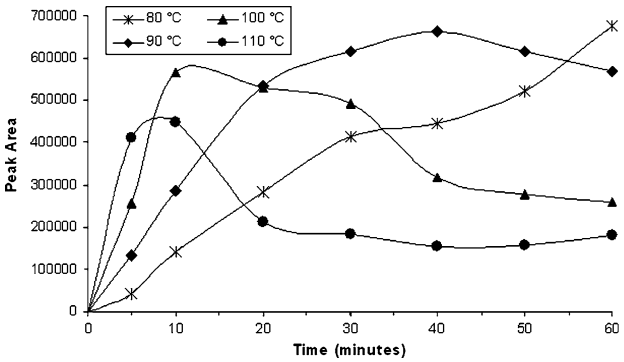

Fig. 1 Effect of temperature and reaction time of the second stage of the derivatization procedure on the ADMA peak area

procedure above 5 min, did not produce any improvement. The effects of concentration of ninhydrin, KOH and H_2SO_4 on the peak area are shown in Fig. 2a, b and c, respectively. Although an increased sensitivity occurred when a 10% w/v KOH solution was used, also a poor reproducibility and significant deviations from linearity were obtained; therefore, in order to alkalinize, a 5% w/v KOH solution was employed. By this way and contrary to common belief (Vanholder et al. 2001), for the first time we were able to carry out a pre-column derivatization of ADMA to the guanidine group by ninhydrin. Then we used the formed ninhydrin–ADMA adduct for a fast, specific and high sensitive fluorescence detection of the ADMA plasma levels by an isocratic reversed phase HPLC separation. Unexpectedly, under the same conditions the derivatization of SDMA did not succeed, but this was not a disadvantage considering its limited clinical relevance and its potential interference with ADMA. We explain the different behaviour of SDMA considering that both nitrogen atoms of its guanidine group are bound to a methyl group while only one nitrogen atom is dimethylated in ADMA, as depicted in Fig. 3. Therefore, it is likely that for the reaction with ninhydrin it is necessary that at least one of the nitrogen atoms of guanidine group is free. This is supported by the fact that in our experiments it was not difficult to derivatize a standard solution of L-NMMA, whose guanidine group has only one nitrogen atom bound to a methyl group, whereas the derivatization of creatinine, whose guanidine group is condensed into a cyclic structure was not feasible.

The optimum conditions for separation of the analytes, in terms of maximum resolution and short retention time, was achieved by employing as a mobile phase an aqueous solution of 10 mmol/l NH_4HCO_3 containing 10% v/v tetrahydrofuran. Under these conditions IS and ADMA were quickly baseline-separated with good resolution in less than 6.40 and 11.42 min, respectively, as depicted in Fig. 4a. Chromatographic peaks were identified both by

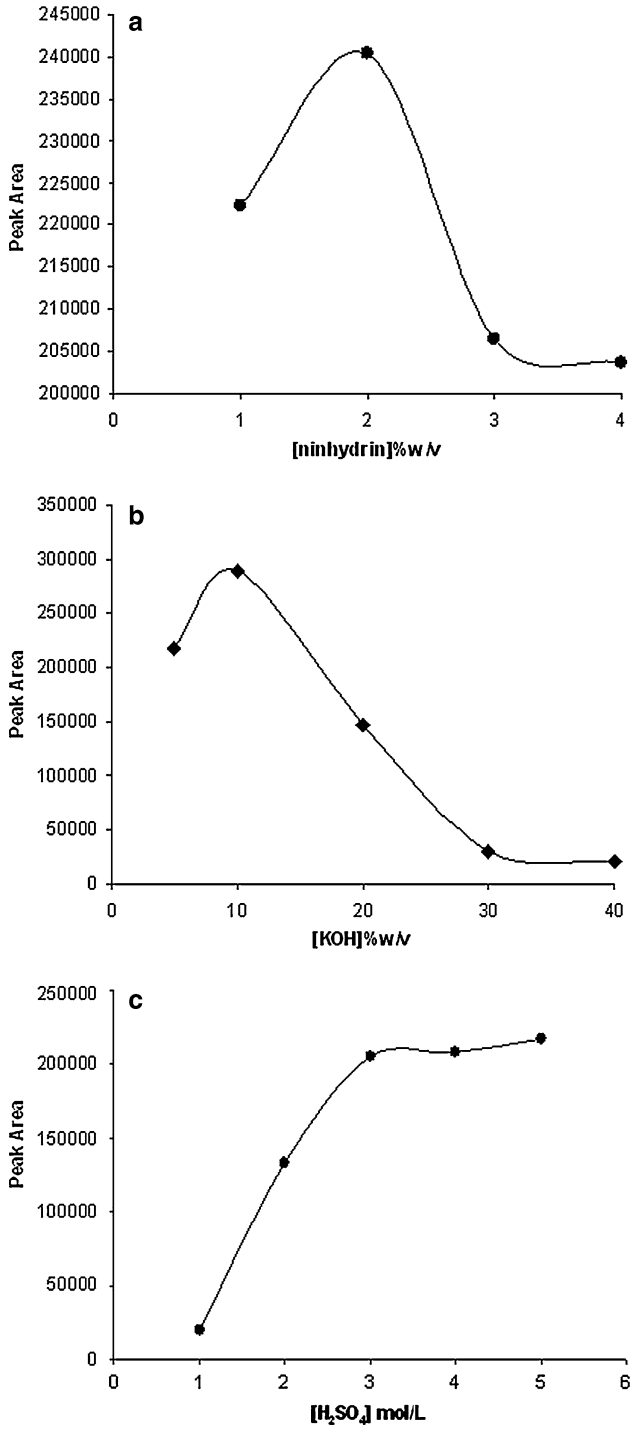

Fig. 2 **a** Effect of concentration of ninhydrin, KOH (**b**) and H_2SO_4 (**c**) on sensitivity expressed as peak area of ADMA

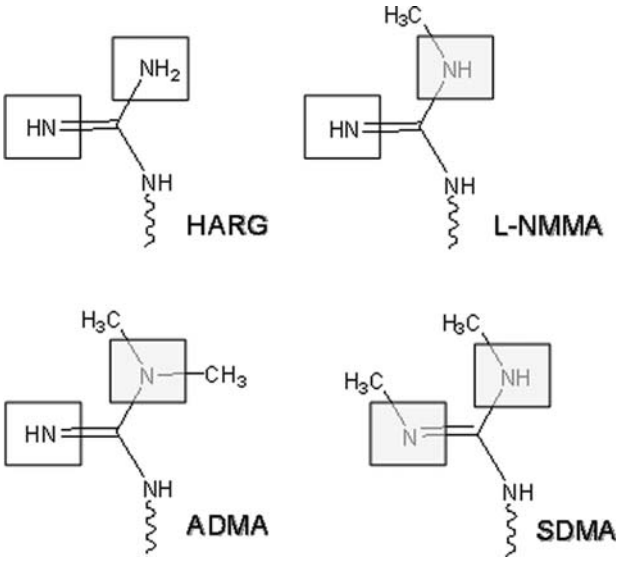

Fig. 3 Qualitative differences between guanidine group of HARG, L-NMMA, ADMA and SDMA. Unlike SDMA, HARG, L-NMMA and ADMA have at least a free guanidine nitrogen atom

spiking and by matching elution times of the plasma derivatives with those of authentic standards.

The calibration curve of a set of five non-zero calibration standards, ranging from 0.125 to 10 μmol/l, constructed by linear regression analysis of the ratio of ADMA and IS against concentrations of ADMA in the calibrators, showed a good coefficient of determination ($R \geq 0.99$) ensuring a linear response over the concentrations tested. Within-run precision of the method was evaluated by analyzing the same serum pool ten times consecutively while between-run precision was determined by analyzing the same sample in ten consecutive days. Intra- and inter-precision tests indicated a good repeatability of the method with an CV% for the intra-assay and inter-assay in the range of 8%. The average recovery, evaluated in five different experiments by adding 20 μl of 2, 4, 8 and 16 μmol/l of ADMA at sample level was between 98.03 and 105.15%. The limits of detection determined from signal-to-noise ratio of 3:1 was 4 nmol/l, while the limits of quantification evaluated from signal-to-noise ratio of 10:1 was 12 nmol/l. The average plasma level of ADMA in real samples obtained from fifty apparently healthy volunteers (22 males, 28 females) aged from 19 to 84 (mean 55.5) was 0.58 μmol/l and it was comparable to the data published by other authors for the age range considered (Teerlink 2005). Comparison of these results with the results obtained by analyzing the same samples with a validated CZE-UV method (Zinellu et al. 2007) showed good agreement and produced the Passing and Bablok regression HPLC Method = 0.0075 + CZE UV Method (see Fig. 4b). The 95% confidence intervals were −0.0615 to 0.0680 and 0.8844 to 1.1333 for intercept and slope, respectively. Cusum test showed no significant deviation of the regression line from linearity ($P > 0.10$).

The presented method allows a fast measurement of plasma ADMA levels by a new, direct and selective pre-column ninhydrin-based derivatization and fluorescence detection. Unlike other methods, extraction and/or

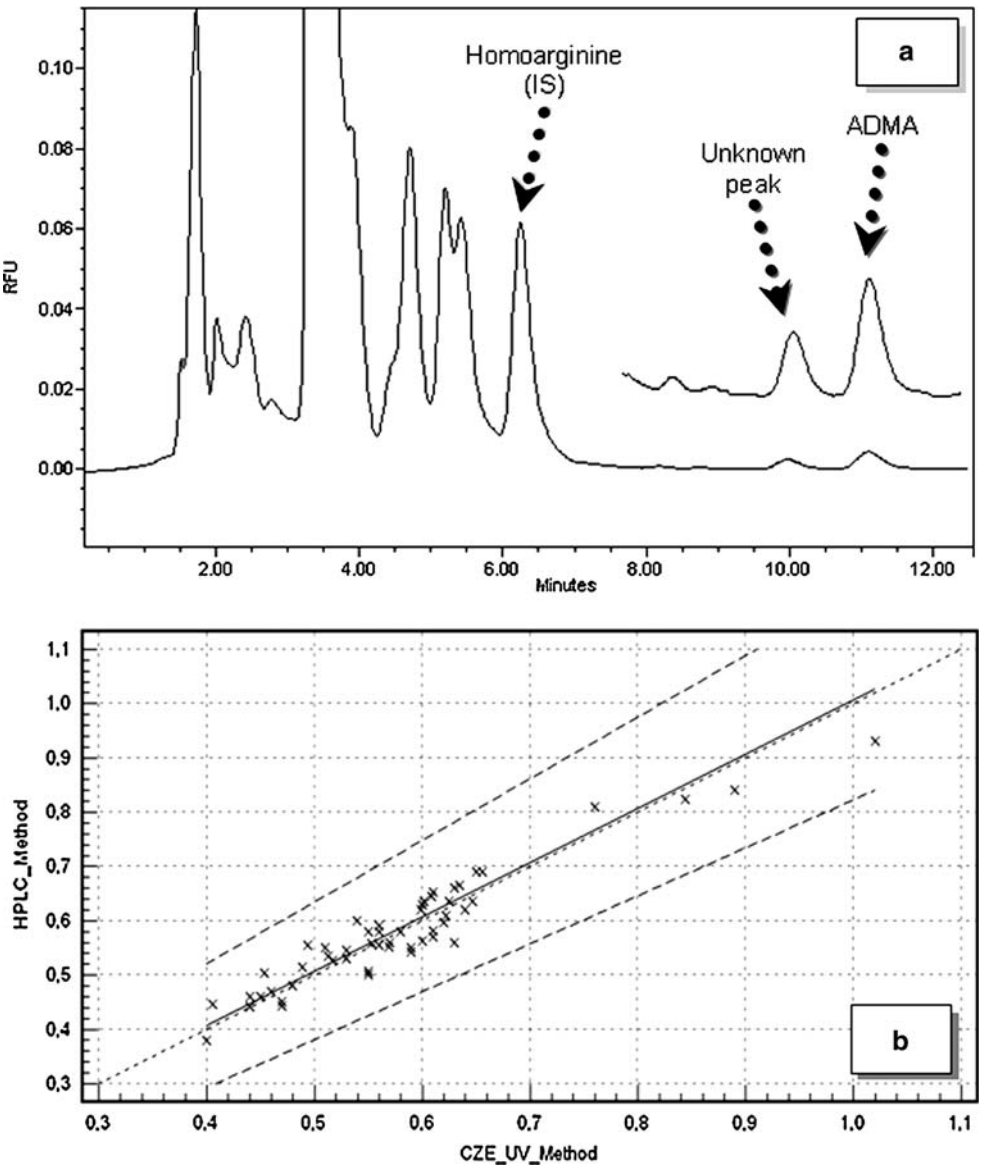

Fig. 4 **a** Typical chromatogram of a real sample. **b** Correlation (Passing Bablock) between 50 plasma ADMA measurements determined simultaneously by new HPLC method and by a validated CZE-UV method. Passing-Bablock regression was HPLC Method = 0.0075 + CZE UV Method. The 95% confidence intervals were −0.0615 to 0.0680 and 0.8844 to 1.1333 for intercept and slope, respectively. Cusum test showed no significant deviation of the regression line from linearity ($P > 0.10$)

purification steps to remove other interfering amino acids is not required. Also the problems of co-elution of its stereoisomer SDMA are overcome since its reaction with ninhydrin does not occur. On the whole, the new derivatization reaction permits to speed up the time of analysis of this clinically important amino acid. A comparison of the time required for the pre- and analytical phases of our previous method (Zinellu et al. 2007), shows that the total analysis time for ninhydrin-based derivatization (pre- and analytical phases) was drastically reduced, on average, from 5–6 h to only 32 min. Moreover, although this paper is focused only on the aspects of ADMA determination, from our preliminary experiments, this procedure can be extended to other compounds with a guanidine moiety such as arginine, creatine, guanidine acetic acid and related metabolites.

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