GC-MS quantification of urinary symmetric dimethylarginine (SDMA), a whole-body symmetric l-arginine methylation index

Analytical Biochemistry 556 (2018) 40–44

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

GC-MS quantification of urinary symmetric dimethylarginine (SDMA), a whole-body symmetric L-arginine methylation index

Alexander Bollenbach[a], Erik Hanff[a], Bibiana Beckmann[b], Ruan Kruger[c,d], Dimitrios Tsikas[a,*]

[a] Institute of Toxicology, Core Unit Proteomics, Hannover Medical School, 30623 Hannover, Germany
[b] Institute of Occupational Medicine, Hannover Medical School, 30623 Hannover, Germany
[c] Hypertension in Africa Research Team (HART), Department of Physiology, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa
[d] MRC Research Unit for Hypertension and Cardiovascular Disease, North-West University, Potchefstroom, South Africa

ARTICLE INFO

Keywords:
ADMA
Deuterium
Guanidine methylation
Pentafluoropropionic anhydride
SDMA

ABSTRACT

Circulating and excretory N^G,N'^G-dimethyl-L-arginine (symmetric dimethylarginine, SDMA) and N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) are cardiovascular risk factors. Despite close chemical structures, the gas chromatography-mass spectrometry (GC-MS) measurement of SDMA is remarkably more difficult than that of ADMA for as yet unknown reasons. Here, we describe an improved GC-MS method for the quantitative determination of SDMA in human urine using commercially available N^G,N'^G-di-[$^2H_3$]methyl-L-arginine (d_6-SDMA) as internal standard. The method is based on a single derivatization step with pentafluoropropionic anhydride (PFPA) in ethyl acetate (30 min, 65 °C) to *N,N,N,O-tetrakis*-pentafluoropropionyl derivatives, electron-capture negative-ion chemical ionization and selected-ion monitoring of the mass-to-charge (m/z) ions of m/z 456 for SDMA and m/z 462 for d_6-SDMA.

Free L-arginine is the substrate of nitric oxide synthases (NOS; EC 1.14.13.39)[1] [1]. Asymmetric dimethylarginine (ADMA, N^G,N^G-dimethyl-L-arginine) and symmetric dimethylarginine (SDMA, N^G,N'^G-dimethyl-L-arginine) are endogenously produced inhibitors of NOS [2]. Circulating and urinary ADMA and SDMA are considered cardiovascular risk factors mainly due to their inhibitory action on endothelial NOS (eNOS). However, the inhibitory potency of ADMA and SDMA towards eNOS is very weak (e.g., $IC_{50} \approx 12\,\mu M$ ADMA) and is unlikely to account to their cardiovascular risks (reviewed in Ref. [3]). ADMA and SDMA in proteins are produced by asymmetric and symmetric dimethylation of the guanidine (N^G) group of L-arginine residues, respectively, by the catalytic action of protein arginine methyltransferases (PRMT; EC 2.1.1.125) using *S*-adenosylmethionine (SAM) as the methyl donor [3]. The biological functions of ADMA- and/or SDMA-containing proteins are largely unknown. Asymmetric and symmetric dimethylated proteins are proteolyzed to free ADMA and SDMA which are differently eliminated by the body. Thus, SDMA is excreted almost unchanged in the urine, whereas ADMA is excreted in part unchanged (by about 10%) and in part as dimethylamine (DMA) upon hydrolysis by dimethylarginine dimethylaminohydrolase (DDAH; EC 3.5.3.18) [1]. Thus, the concentration of SDMA in urine may be a useful parameter to measure whole-body symmetric L-arginine dimethylation.

Nowadays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the most versatile technique to measure biological amino acids without derivatization, although esterification may improve the measurement of amino acid metabolites such as SDMA and ADMA [4]. Previously, we reported gas chromatography-mass spectrometry (GC-MS) based methods for the quantitative determination of SDMA and ADMA in human urine in experimental and clinical settings [1,5–7]. We often faced serious problems with the quantitative measurement of SDMA in plasma and urine for not fully recognized reasons. As these GC-MS methods rely on the esterification of the carboxylic groups of SDMA and ADMA in 2 M HCl in methanol (60 min, 85 °C), one explanation could be hydrolysis of the methylated guanidine group of SDMA. This phenomenon has been observed for L-citrulline in which the ureide group is hydrolyzed under the acidic conditions of the esterification to form L-ornithine [8]. We therefore searched for alternative derivatization procedures for the carboxylic group of SDMA.

Heptafluorobutyryl chloroformate has been reported to be useful for

* Corresponding author. Institute of Toxicology, Core Unit Proteomics, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

[1] Abbreviations used: ADMA, Asymmetric dimethylarginine; DDAH, Dimethylarginine dimethylaminohydrolase; ECNICI, Electron-capture negative-ion chemical ionization; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; Me, Methyl; NO, Nitric oxide; NOS, Nitric oxide synthase; PFP, Pentafluoropropionyl; PFPA, Pentafluoropropionic anhydride; PICI, Positive-ion chemical ionization; PRMT, Protein arginine methyltransferase; QC, Quality control; SAM, *S*-adenosylmethionine; SDMA, Symmetric dimethylarginine; SIM, Selected-ion monitoring.

https://doi.org/10.1016/j.ab.2018.06.021
Received 13 April 2018; Received in revised form 22 June 2018; Accepted 22 June 2018
Available online 23 June 2018

the derivatization of urinary amino-carboxylic compounds and their GC-MS analysis [9]. However, this multifunctional derivatization reagent was found not to be useful for L-arginine and L-citrulline [9] and is presumably not useful for SDMA as well. Because in our previous GC-MS methods for SDMA and ADMA, pentafluoropropionic anhydride (PFPA) emerged as a suitable derivatization reagent to convert their methyl esters to *N*-pentafluoropropionyl derivatives [5–7], we tested the utility of PFPA for the one-step derivatization of SDMA and its subsequent GC-MS analysis. Here, we report on the development, validation and successful application of a GC-MS method for the quantitative determination of SDMA in human urine using commercially available N^G,N'^G-di-[$^2H_3$]methyl-L-arginine (d_6-SDMA; Sigma-Aldrich, Germany; 99% isotopic purity of ^2H) as internal standard after a single derivatization step with PFPA.

GC-MS analyses were performed on a GC-MS apparatus consisting of a single-stage quadrupole mass spectrometer model ISQ, a Trace 1210 series gas chromatograph and an AS1310 autosampler from ThermoFisher (Dreieich, Germany). A fused-silica capillary column Optima 17 (15 m length, 0.25 mm I.D., 0.25 µm film thickness) from Macherey-Nagel (Düren, Germany) was used. Aliquots of 1 µL were injected in the splitless mode. The injector temperature was kept at 280 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was held at 90 °C for 0.5 min and ramped up to 210 °C at a rate of 15 °C/min and then to 320 °C at a rate of 35 °C/min. Interface and ion-source temperatures were set to 300 and 250 °C, respectively. Electron energy was 70 eV and electron current 50 µA. Methane was used as the reagent gas for both electron-capture negative-ion chemical ionization (ECNICI) and positive-ion chemical ionization (PICI), at a constant flow rate of 2.4 mL/min. Mass spectra of SDMA derivatives were generated by scanning in the mass-to-charge (*m/z*) range of 100–1000. In quantitative analyses, the dwell time was 100 m s for each ion in the selected-ion monitoring (SIM mode) and the electron multiplier voltage was set to 1900 V.

Each 5 nmol of synthetic d_6-SDMA and d_0-SDMA were derivatized with PFPA in ethyl acetate (100 µL, 1:4, v/v) by heating for 30 min at 65 °C as reported previously for amino acid methyl esters [5–7]. After cooling to room temperature, solvents and reagents were evaporated to dryness under a stream of nitrogen. Subsequently, residues were treated first with 200-µL aliquots of 400 mM borate buffer, pH 8.5, and immediately thereafter with 200-µL aliquots of toluene, followed by vortex-mixing for 60 s, and centrifugation (4000 × *g*, 5 min, 18 °C). Aliquots (150 µL) of the upper organic phase were transferred into autosampler glass vials equipped with micro inserts, the samples were sealed and subjected to GC-MS analysis.

GC-MS analysis of d_6-SDMA and d_0-SDMA revealed several GC peaks of which the mass spectra contained pairs of ions with differences of 6 Da or 3 Da due to the presence of 6 D atoms (two CD_3 groups) and 3 D atoms (presumably one CD_3 group), respectively. The retention times of the most abundant GC peaks of d_6-SDMA and the most intense ions in ECNICI mass spectra were 4.68 min (*m/z* 309), 6.02 min (*m/z* 366), 6.08 min (*m/z* 345), and 7.22 min (*m/z* 462) (Fig. S1, Supplement). The most intense ions in the ECNICI mass spectra of the d_6-SDMA and d_0-SDMA derivatives eluting at 7.22 and 7.28 min were *m/z* 462 and *m/z* 456 (Fig. 1), respectively, most likely produced by the neutral loss of a pentafluoroprionic acid molecule (164 Da) and a HF molecule (20 Da). The less intense ions at *m/z* 442 and *m/z* 436 are likely to have resulted from subsequent neutral loss each of a HF molecule. The most intense ions in the PICI mass spectra of the SDMA derivatives eluting at 7.22 and 7.28 min were at *m/z* 181 (100%) and *m/z* 483 (40%) for d_6-SDMA, and *m/z* 178 (100%) and *m/z* 477 (30%) for d_0-SDMA (Fig. S2). Clearly, the ions *m/z* 181 and *m/z* 178 (100%) carry one methyl group of the symmetric dimethyl guanidine groups of d_6-SDMA and d_0-SDMA, respectively. Based on the ECNICI and PICI mass spectra, the structures of the compounds eluting at the retention times of about 7.22 and 7.28 min were assigned to the *N,N,N,O-tetrakis*-pentafluoropropionyl derivatives of d_6-SDMA and d_0-SDMA, respectively (Fig. S3, Fig. S4). The most intense ions *m/z* 462 and *m/z* 456 were used in quantitative analyses of SDMA in human urine in the SIM mode.

For quantitative analyses of SDMA in human urine samples, 10-µL aliquots were spiked with d_6-SDMA and evaporated to dryness under a stream of nitrogen. Subsequently, derivatization with PFPA and solvent extraction were processed as described above for synthetic d_6-SDMA and d_0-SDMA. The method was validated for SDMA in 10-µL aliquots of urine samples from three healthy volunteers in the pathophysiologically relevant concentration range of 0–200 µM [1,5,7]. The concentration of d_6-SDMA in the validation experiments was 100 µM. The mean concentration of SDMA in the urine samples of these volunteers was determined to be 21.1 µM, 8.15 µM, and 68.6 µM. The accuracy (recovery, %) of the method was calculated by dividing the difference of measured and basal SDMA concentration by the added SDMA concentration and by multiplying the result by 100. The accuracy (mean recovery) of the method was 103% for 50 µM, 104% for 100 µM, and 113% for 200 µM spiked SDMA. These data indicate the analytical reliability of the GC-MS method for SDMA in human urine samples. Typical GC-MS chromatograms obtained from the quantitative measurement of SDMA in urine samples (10 µL aliquot) of two healthy children are shown in Fig. 2. Expectedly, the derivative of d_6-SDMA eluted constantly (about 3 s) in front of the d_0-SDMA derivative. The PFP derivative of SDMA in toluene is remarkably unstable, much less stable than the methyl ester PFP derivative. Reanalysis of a toluene sample that was stored for 8 days at room temperature indicated that the peak area of the internal standard decreased by a factor of about 5. Thus, urine samples should be analysed for SDMA immediately after PFPA derivatization for maximum sensitivity.

The method was used to investigate the reason for the difficult GC-MS measurement of SDMA as methyl ester pentafluoropropionyl derivative [7]. A mixture of synthetic d_6-SDMA and d_0-SDMA (each 5 nmol) was derivatized first with 2 M HCl in CD_3OD (60 min, 80 °C) and then with PFPA (30 min, 65 °C) and analysed in the ECNICI mode by SIM of *m/z* 462 and *m/z* 456 for the *N,N,O*-tripentafluoropropionyl derivatives, and of *m/z* 643, *m/z* 637 for the expected trideutero-methyl esters *N,N*-dipentafluoropropionyl derivatives of synthetic d_6-SDMA and d_0-SDMA [7]. The GC-MS analysis revealed two paired GC-peaks of comparable size/relative abundance. One pair of the GC peaks (*m/z* 462 and *m/z* 456) eluted at 7.22 and 7.27 min due to the *N,N,N,O-tetrakis*-pentafluoropropionyl derivatives of d_6-SDMA and d_0-SDMA (Fig. S5). This observation indicates incomplete esterification of d_6-SDMA and d_0-SDMA and/or hydrolysis of the trideutero-methyl esters during the subsequent PFPA derivatization. Previously, we found that the esterification of L-arginine with 2 M HCl in methanol is of the order of 85% [5]. The second pair of the GC-MS peaks (*m/z* 643, *m/z* 637) eluted at 6.73 and 6.77 min and is assigned to the trideutero-methyl esters *N,N,N-tris*-pentafluoropropionyl derivatives of synthetic d_6-SDMA and d_0-SDMA [7]. The ECNICI mass spectra of the trideutero-methyl esters *N,N,N-tris*-pentafluoropropionyl derivatives of d_6-SDMA and d_0-SDMA contained the most intense ion at *m/z* 236, and very weak ions (intensity, about 5%) at *m/z* 643 and *m/z* 637, respectively (Fig. S6). These observations suggest that the methyl ester pentafluoropropionyl (PFP) amide derivatives of SDMA and ADMA ionize very differently under ECNICI conditions. ECNICI of the methyl ester pentafluoropropionyl amide derivatives of ADMA yield abundant anions due to $[M–HF]^-$, whereas ECNICI of the methyl ester pentafluoropropionyl amide derivatives of SDMA yield very weak anions $[M–HF]^-$, and abundant anions at *m/z* 233, which is however common to many other amino acids. On the other hand, the anions at *m/z* 634 for the methyl ester of SDMA, *m/z* 637 for the trideutero-methyl ester and *m/z* 640 for d_6-SDMA are not intense enough for sensitive quantitative measurements of SDMA in biological samples.

We also applied the current method to the quantification of SDMA in spot urine samples of 80 healthy children (age, 6–8 years) collected in a previous study after approval by the local Ethics Committee [10]. Study samples were analysed within a single run together with two

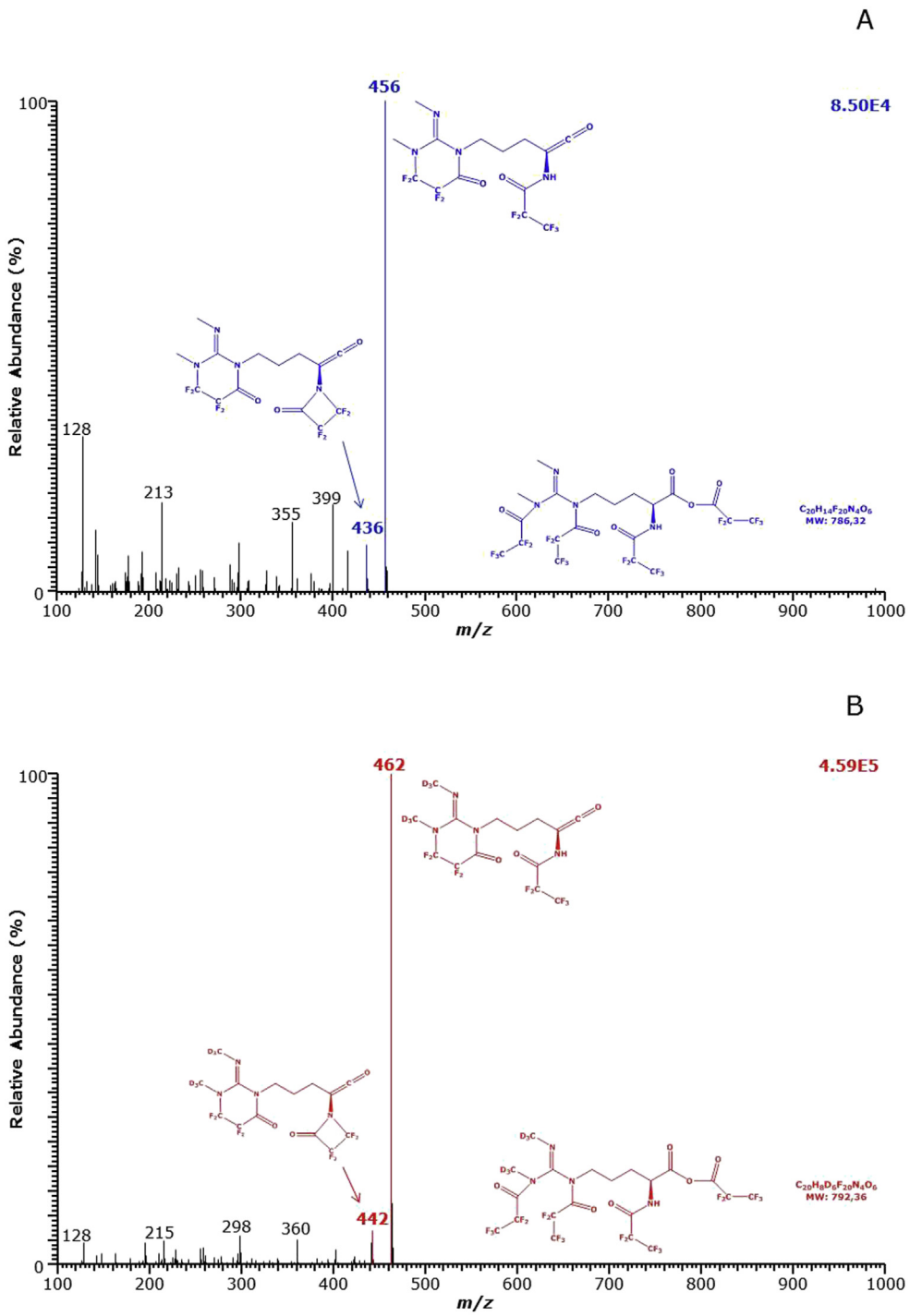

Fig. 1. ECNICI GC-MS spectra of the pentafluoropropionyl derivatives of synthetic unlabelled SDMA (A) and d_6-SDMA (B) eluted from the column at 7.28 and 7.22 min, respectively. Insets indicate the proposed structures for the ions *m/z* 462, *m/z* 442, *m/z* 456 and *m/z* 436.

quality control (QC) samples which were analysed in duplicate. The precision was 2.1% in the unspiked sample, and 2.5% in the urine sample spiked with 25 μM SDMA which was determined with an accuracy of 101%. In the QC samples, the peak area of the internal standard varied by 21%. Instrumental precision was determined to be 0.96 ± 0.38% for SDMA in a urine sample of a healthy child (n = 7). The retention times of the PFP derivatives of d_6-SDMA and d_0-SDMA from the analysis of 80 urine samples were 6.97 min (RSD, 0.50‰) and 7.02 min (RSD, 0.49‰). Creatinine-corrected excretion rate was determined to be 4.50 ± 0.80 μmol/mmol in the black boys (n = 39) and 4.89 ± 0.98 μmol/mmol in the white boys (n = 41). Thus, there was a small (8%), but statistically significant difference in the creatinine-corrected SDMA excretion between black and white boys (P = 0.037), suggesting a closely comparable yet weakly ethnicity-dependent whole-body symmetric dimethylation of L-arginine residues in proteins in the 6-8-year-old boys. This is in line with the small ethnic difference regarding arterial stiffness of these boys [10]. As creatinine is the most abundant methylated endogenous substance, the whole-body SDMA formation rate can be estimated in relation to that of creatinine and accounts to about 0.5%.

In summary, reliable GC-MS measurement of SDMA is possible in 10-μL aliquots of human urine after a single derivatization step with

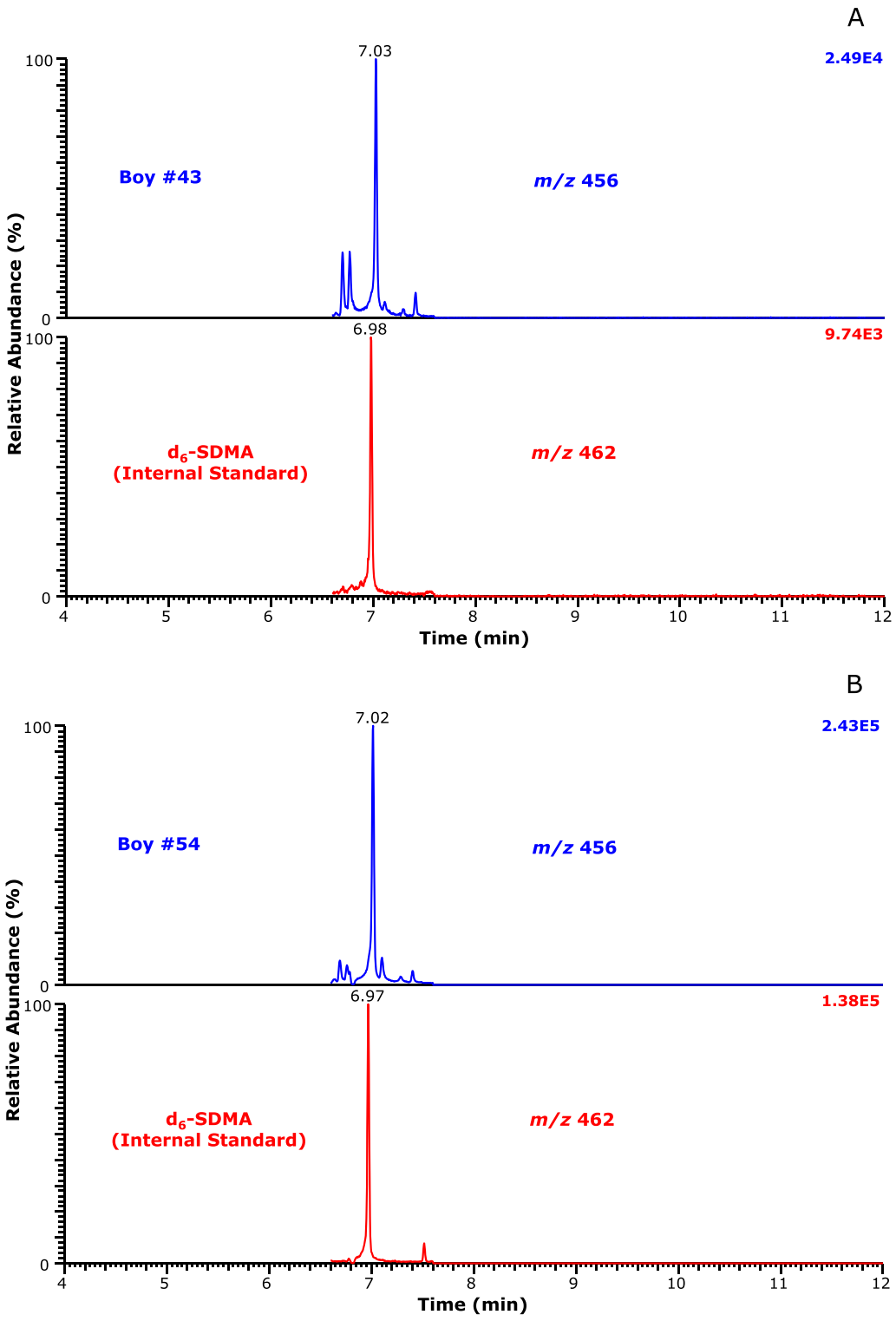

Fig. 2. Partial GC-MS chromatograms from the quantitative determination of SDMA in 10-μL aliquots of urine samples collected from two healthy children (A, black boy; B, white boy) in the SIM mode. The concentration of the internal standard d_6-SDMA was 20 μM with respect to the urine volume. The shorter retention times of the SDMA derivatives compared to those reported in legend to Fig. 1 are due to the column cut prior to start quantitative analyses.

PFPA in ethyl acetate. PFPA is not selective to amine groups, but also reacts with the carboxylic group of SDMA to form a mixed anhydride. Unlike *in situ* preparation of a trideutero-methyl ester of SDMA for use as the internal standard, one step derivatization of SDMA with PFPA requires the use of stable-isotope labelled SDMA analogs such as d_6-SDMA. SDMA undergoes only minor metabolism and is excreted unchanged in the urine. The creatinine-corrected excretion of SDMA urine may be a useful index for whole-body symmetric dimethylation of L-arginine residues in proteins.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This project was supported by grants from the South African National Research Foundation (NRF) (Grant ID: 99055) and the South Africa Sugar Association (SASA) (Grant ID: 240). Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2018.06.021.

References

[1] D. Tsikas, A critical review and discussion of analytical methods in the L-arginine/nitric oxide (NO) area of basic and clinical research, Anal. Biochem. 379 (2008) 139–163.
[2] D. Tsikas, J. Sandmann, A. Savva, P. Luessen, R.H. Böger, F.M. Gutzki, B. Mayer, J.C. Frölich, Assessment of nitric oxide synthase activity in vitro and in vivo by gas chromatography-mass spectrometry, J. Chromatogr. B 742 (2000) 143–153.
[3] D. Tsikas, A. Bollenbach, E. Hanff, A.A. Kayacelebi AA, Asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and homoarginine (hArg): the ADMA, SDMA and hArg paradoxes, Cardiovasc. Diabetol. 17 (2018) 1.
[4] J. Martens-Lobenhoffer, S.M. Bode-Böger, Mass spectrometric quantification of L-arginine and its pathway related substances in biofluids: the road to maturity, J. Chromatogr. B 964 (2014) 89–102.
[5] D. Tsikas, B. Schubert, F.-M. Gutzki, J. Sandmann, J.C. Frölich, Quantitative determination of circulating and urinary asymmetric dimethylarginine (ADMA) in human by gas chromatography-tandem mass spectrometry as methyl ester tri(*N*-pentafluoropropionyl) derivative, J. Chromatogr. B 798 (2003) 87–99.
[6] B. Beckmann, F.M. Gutzki, D. Tsikas, Sensitivity enhancement of a GC-MS/MS method for asymmetric dimethylarginine (ADMA) by plasma ultrafiltrate reduction, Anal. Biochem. 372 (2008) 264–266.
[7] D. Tsikas, B. Beckmann, F.M. Gutzki, J. Jordan, Simultaneous gas chromatography-tandem mass spectrometry quantification of symmetric and asymmetric dimethylarginine in human urine, Anal. Biochem. 413 (2011) 60–62.
[8] D. Tsikas, E. Hanff, T. Becker, Drastic decrease of global L-arginine bioavailability during orthotopic liver transplantation: a matter of ATP deficiency of the graft? Nitric Oxide 73 (2018) 96–97.
[9] P. Hušek, Z. Švagera, D. Hanzlíková, L. Řimnáčová, H. Zahradníčková, I. Opekarová, P. Šimek, Profiling of urinary amino-carboxylic metabolites by in-situ heptafluorobutyryl chloroformate mediated sample preparation and gas chromatography-mass spectrometry, J. Chromatogr. a 1443 (2016) 211–232.
[10] G.G. Mokwatsi, A.E. Schutte, R. Kruger, Ethnic differences regarding arterial stiffness of 6-8-year-old black and white boys, J. Hypertens. 35 (2017) 960–967.