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Stable isotope dilution assay for liquid chromatography–tandem mass spectrometric determination of L-homoarginine in human plasma

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ABSTRACT

Nitric oxide (NO), the endogenous modulator of vascular tone and structure, originates from oxidation of L-arginine catalysed by NO synthase (NOS). The L-arginine derivative L-homoarginine serves as an alternative NOS substrate releasing NO, competing with L-arginine for NOS, arginase, and arginine transport. In the present article we report a liquid chromatography–tandem mass spectrometric (LC–tandem MS) method for the determination of L-homoarginine in human plasma by stable-isotope dilution. L-[$^{13}C_6$]-Homoarginine was used as internal standard. This method provides high sample throughput of 25-μl aliquots of plasma with an analysis time of 4 min using LC–tandem MS electrospray ionisation in the positive mode (ESI+). Specific transitions for L-homoarginine and L-[$^{13}C_6$]-homoarginine were m/z 245 → m/z 211 and m/z 251 → m/z 217, respectively. The mean intra- and interassay CVs were 7.4 ± 4.5% (±SD) for 0.1–50 μmol/L and 7.5 ± 2.0% for 2 and 5 μmol/L, respectively. Applying this method, a mean plasma concentration of L-homoarginine of 2.5 ± 1.0 μmol/L was determined in 136 healthy humans.

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1. Introduction

L-Homoarginine is a cationic amino acid of which the carbon chain is by one CH_2 group longer than in L-arginine. It is putatively formed by the transamination of L-lysine by the enzyme L-arginine:glycine-amidinotransferase (AGAT, EC 2.1.4.1) [1,2]. Given its structural similarity to L-arginine, L-homoarginine can interact and interfere with L-arginine metabolism and signalling. L-Homoarginine can serve as a substrate and competitive inhibitor of nitric oxide synthases (NOS) [3], the enzymes which generate nitric oxide (NO) from L-arginine [4–6]. NO plays an important role in the vascular system, it inhibits vascular inflammation, prevents adhesion of immune cells and aggregation of platelets and acts as vasodilator. Furthermore NO maintains smooth muscle cells in a nonproliferative quiescent state [4,6]. Thus, competition of L-homoarginine with L-arginine for binding to the NOS' active site may interfere with endothelial function. To date, reports of L-homoarginine plasma concentrations in health and disease and its relation to human pathophysiology are sparse. Valtonen et al. reported increased L-homoarginine plasma concentrations during the second and third trimester of pregnancy [7]. There are several metabolic diseases such as hyperornithinaemia, hypercitrullinaemia and hyperammoniaemia in which L-homoarginine and L-homocitrulline excretion into the urine is increased [8]. A recent study by März et al. suggested that low L-homoarginine plasma concentrations are associated with cardiovascular and all-cause mortality in patients subjected to coronary angiography or hemodialysis [9].

Analytical methods for the measurement of arginine and arginine derivatives using gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) have been reviewed elsewhere [10–14]. LC–tandem MS-based methods for the quantitative determination of asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and L-arginine have been widely applied in clinical studies and have elucidated the detrimental role of ADMA and SDMA in human pathophysiology [15]. The LC–tandem MS method described herein allows the quantification of L-homoarginine in human plasma. A stable isotope labeled homolog of L-homoarginine was used as internal standard for L-homoarginine. With the ability to avoid the need of solid phase extraction, this LC–tandem MS-based method is suitable to analyse L-homoarginine in large clinical trials and may be applicable for clinical laboratory routine.

2. Experimental

2.1. Chemicals and materials

L-Arginine hydrochloride reference standard was purchased from US Pharmacy (Rockville, MD, USA). L-Homoarginine

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hydrochloride, L-N-monomehtylarginine (L-NMMA), acetate, and N_ε-acetyllysine were obtained from Sigma–Aldrich (Steinheim, Germany). Aqueous stock solutions of L-homoarginine, L-NMMA and N_ε-acetyllysine were made by weighing authentic material supplied by the manufacturer. L-[$^{13}C_6$]-Lysine dihydrochloride (L-[1,2,3,4,5,6-$^{13}C_6$]-lysine, 97–99 atom% ^{13}C) was purchased from Euriso-top (Saint-Aubin, France). L-[$^{13}C_6$]-Homoarginine was synthesized in-house. 96-Well 0.20-μm Multiscreen HTS™ microfiltration plates were purchased from Millipore (Molsheim, France), u-shaped 96-well polypropylene plates were from Greiner bio-one (Frickenhausen, Germany), acetate foil, skirted multiply-PCR 96-well polypropylene plates were obtained from Sarstedt (Nümbrecht, Germany), and Slide-A-Lyzer™ dialysis cassettes were purchased from Pierce (Rockford, IL, US). All other chemicals were purchased from Sigma–Aldrich.

2.2. Synthesis of the stable isotope labeled internal standard

L-[$^{13}C_6$]-Homoarginine synthesis was performed according to the procedure of Kleinmaier and Gschwind [16]. Briefly, 250 mg thiourea were dissolved under stirring in 50 ml acetone at 50 °C. 0.22 ml methyliodide were added in one portion via syringe. The mixture was stirred at 55 °C under reflux for 3 h. After cooling to room temperature, the reaction mixture was poured into ice-cold diethylether and kept in the cooling bath to allow complete precipitation of the product methylthiourea, which was then filtered and rinsed with cold diethylether. Accordingly a copper–[$^{13}C_6$]-lysine-complex was prepared. Methylthiourea was dissolved in 25% ammonia solution and the copper–[$^{13}C_6$]-lysine-complex was added. The mixture was incubated at room temperature for 24 h. The mixture was then filtered, and the filtrate was dried under vacuum. The residue was reconstituted in methanol:water (50:50), filtered, and dried again under vacuum. The residue was dissolved in 0.1 M HCl and stored at −20 °C.

2.3. Plasma dialysis

A 12–30 ml Slide-A-Lyzer™ cassette was used for plasma dialysis as described by the manufacturer. In brief, after the cassette was hydrated in dialysis buffer (0.9% NaCl), the cassette was carefully filled with 20 ml of individual plasma of pooled specimen. The remaining air in the cassette was removed via syringe. The cassette was floated in 1.5 l dialysis solution (0.9% NaCl) for 72 h (4 °C) and the dialysis buffer was changed every 24 h. After three days, the dialysed plasma was removed carefully from the cassette. The dialysed plasma was utilised for the validation of the LC–tandem MS method in order to prevent interferences between endogenous and spiked compounds in the plasma.

2.4. Sample preparation

For protein precipitation, 96-well 0.20-μm microfiltration plates were used. Each well of the microfiltration plate was filled with 100 μl of internal standard solved in methanol. L-[$^{13}C_6$]-Homoarginine was used as internal standard at a final concentration of 10 μmol/L plasma sample. The 96-well filtration plate had to be placed on top of a 96-well polypropylene plate. Subsequently, each well was piled with a 25-μl sample aliquot. Proteins were precipitated quantitatively by shaking the 96-well plates for 15 min using an orbital shaker. To separate analytes from precipitated proteins, the microfiltration plates on top of the polypropylene plates were centrifuged for 10 min at 800 × *g* (Eppendorf centrifuge 5810R, 2000 rpm, Hamburg, Germany). After centrifugation, the eluates in the polypropylene plate were dried by heating at 85 °C on a 96-well aluminium block for 30 min. Compounds were derivatized to their butyl ester derivatives by adding 100 μl of 1 M HCl in 1-butanol. The plates were coated with acetate foil, and the 96-well polypropylene plates were heated at 65 °C for 30 min. The plates were centrifuged for 1 min at 800 g and the acetate foil was removed. The derivatization reagent was dried by heating at 85 °C for 30 min. The samples were redissolved in 100 μl of methanol:water (25:75) containing 0.1% ammonium formate, adjusted to pH 5 with formic acid. Samples were transferred in another 96-well 0.20-μm microfiltration plate on top of a polypropylene plate and centrifuged as described above. The calibrators and the quality controls were treated exactly the same as the plasma samples. Afterwards, polypropylene plates were placed in a CTC PAL autosampler (Varian, Palo Alto, CA, USA), and 20-μl aliquots were subjected to further analysis.

2.5. Liquid chromatography–tandem mass spectrometry

Analyses were performed on a Varian 1200L Triple Quadrupole MS equipped with two Varian ProStar model 210 HPLC pumps (Varian, Lake Forest, CA, USA). Separation of analytes from major matrix components was achieved with a Polaris C18-Ether column (Varian; 50 × 2.0 mm) using an elution gradient of (A) 0.1% formic acid in water and (B) acetonitrile–methanol (50/50, v/v) containing 0.1% aqueous formic acid (0:00 min 95/5, A/B (v/v) – 0:30 95/5–2:00 50/50–2:01 95/5–4:00 95/5) at 30 °C, with a flow rate of 0.3 ml/min. Nitrogen was used as the nebulizing and drying gas (250 °C) at 90 and 180 L/h, respectively. For positive electrospray ionisation (ESI+) the needle and shield voltage were set at 5000 and 600 V, respectively. For quantitative analyses the analytes were detected by means of characteristic product ions formed from protonated molecules by collision-induced dissociation (CID) using the multiple reaction monitoring (MRM) mode after fragmentation with argon (2 Pa): m/z 245→211 (collision energy 14 eV) for L-homoarginine, and m/z 251→217 (14 eV) for L-[$^{13}C_6$]-homoarginine. Dwell time was set to 0.5 s for L-homoarginine and L-[$^{13}C_6$]-homoarginine.

2.6. Calibration, validation, and ion suppression

Calibration and validation were performed in dialysed plasma. Calibration standards were prepared in the same manner as plasma samples. The calibration curve was constructed from integrated chromatograms using peak area ratios of L-homoarginine to the internal standard L-[$^{13}C_6$]-homoarginine. The lower limit of quantification (LLOQ), intraassay and interassay accuracy and precision of L-homoarginine were determined by calculating the recovery of added L-homoarginine to dialysed human plasma. For LLOQ and intraassay accuracy and precision nine different concentrations (n = 4, each) of L-homoarginine, i.e. 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 25, and 50 μmol/L were added to dialysed human plasma. Interassay accuracy and precision were performed by adding two different concentrations (2 and 5 μmol/L, n = 2, each) on seven separate 96-well plates. The limit of detection (LOD) was estimated according to FDA guidelines [Guidance for the industry – Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Centre for drug Evaluation and Research, May 2001]. To investigate differences between native and dialysed human plasma, we also determined intraassay accuracy and precision in native human plasma (eight concentrations, range 0.1–25 μmol/L, n = 5). In addition, we studied ion suppression by infusion experiments for native and dialysed plasma of three individuals as described elsewhere [14].

2.7. Human plasma samples

For the determination of the basal plasma concentrations of L-homoarginine, 136 apparently healthy volunteers provided

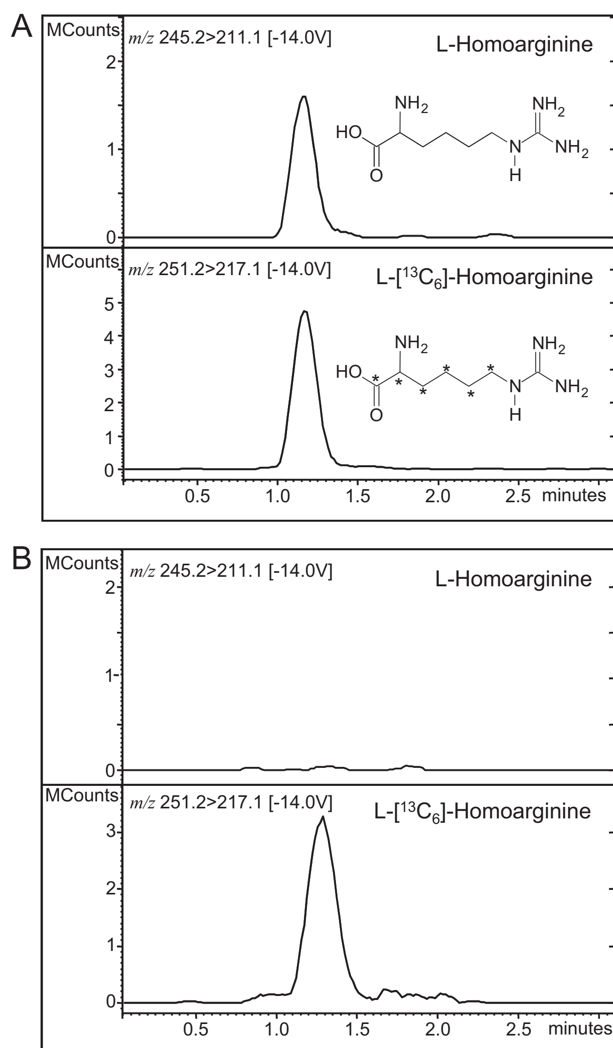

Fig. 1. (A) Typical LC–MS/MS chromatogram for L-homoarginine and L-[$^{13}C_6$]-homoarginine (IS). The following transitions were used for quantifying these compounds after fragmentation with argon: m/z 245.1 > 211.1 for L-homoarginine, and m/z 251.1 > 217.1 for [$^{13}C_6$]-homoarginine butyl ester derivatives. (B) Partial chromatograms from the LC–MS/MS analysis of L-homoarginine and it internal standards in human plasma matrix after plasma dialysis (3 × 24 h against 0.9% NaCl).

informed consent and plasma samples. The study was approved by local Ethics Committee of Hamburg board of Physicians. All samples were analysed batch-wise on 96-well plates. L-Homoarginine was measured by the LC–tandem MS method described above.

3. Results

3.1. Plasma dialysis

Method calibration and validation were performed in dialysed human plasma. The determination of L-homoarginine before and after the dialysis demonstrated efficient removal of >99% of the analytes. The chromatograms depicted in Fig. 1 show the concentrations of L-homoarginine prior (A) and after (B) plasma dialysis.

3.2. LC–tandem MS of the butyl ester derivatives of L-homoarginine and its congeners L-NMMA and N_ε-acetyllysine

The parent ions formed from ESI+ of the butyl esters of L-homoarginine, its congeners L-NMMA and N_ε-acetyllysine as well as the stable-isotope labeled L-homoarginine represent the protonated molecular cations, i.e. $[M+H]^+$ (Table 1), being isobaric for unlabeled compounds. All compounds show specific fragmentation patterns when analysed as their butyl ester derivatives. For L-homoarginine a characteristic neutral loss (NL) of 34 Da was observed to give the product ion m/z 211 (Fig. 2A). L-[$^{13}C_6$]-Homoarginine showed a NL of 34 Da to give the product ion m/z 217 (Fig. 2B). By using both differently isotope labeled L-homoarginine analogs and differently derivatized analogs, we were able to demonstrate fragmentation of the guanidino group of L-homoarginine. From the isobaric molecular ions of L-NMMA and N_ε-acetyllysine we identified the formation of the specific daughter ions m/z 70 for L-NMMA (Fig. 2C) and m/z 143 for N_ε-acetyllysine (Fig. 2D), respectively. Ion transitions specific for the isobaric ions are summarized in Table 1. A representative chromatogram from the analysis of L-homoarginine and L-[$^{13}C_6$]-homoarginine in human plasma in the MRM mode is depicted in Fig. 1A.

Table 1
m/z values of parent ($[M+H]^+$) and product ions, and collision energy (CE) values used in quantitative analyses of L-homoarginine, L-[$^{13}C_6$]-homoarginine, L-NMMA, and N_ε-acetyllysine in human plasma as butyl ester derivatives.

Analyte	Parent ion $[M+H]^+$	CE (eV)	Product ion
L-Homoarginine	245	−14	211
L-NMMA	245	−16	70
N_ε-Acetyllysine	245	−15	143
L-[$^{13}C_6$]-Homoarginine	251	−14	217

3.3. Validation of the L-homoarginine determination

The limit of detection (LOD) for L-homoarginine was determined as 5 nmol/L (S/N = 9 ± 5). Data from the intraassay validation experiments of L-homoarginine are given in Table 2. Average precision (R.S.D.) for a concentration range from 0.1 to 50 μmol/L was 7.4 ± 4.5% (mean ± SD). The observed bias for all added concentrations was +1.1 ± 7.4%. The LLOQ based on this analysis was 0.1 μmol/L (n = 4). Linear regression analysis between peak area ratios (y) and added (x) concentrations yielded slope and y-axis intercept of 0.15 and 0.01 (r^2 = 0.99). The data from the interassay accuracy and precision of the method are summarized in Table 3. The mean interassay variability for 2 and 5 μmol/L was 7.5 ± 2.0%. In addition, validation experiments performed with native plasma samples showed average precision and accuracy of 9.4 ± 4.7% and −5.5 ± 13%, respectively. Also we performed infusion experiments which resulted in similar ion suppression caused by native and dialysed plasma (Supplemental Fig. 2). Mean ion suppression observed

Table 2
Intraassay accuracy and precision of the method for L-homoarginine in human plasma (n = 4 for each concentration).

L-Homoarginine added[a]	L-Homoarginine measured minus basal level[b]	Accuracy (% bias)	Precision (R.S.D., %)
0.05	0.08	62.4	29.4
0.1	0.11	10.9	12.4
0.25	0.26	2.8	12.9
0.5	0.55	10.5	12.2
1	1.03	3.2	3.1
5	4.97	−0.5	6.0
10	9.30	−7.1	1.7
25	22.5	−10.0	5.1
50	49.5	−0.9	5.6
Mean ± S.D.		1.1 ± 7.4[c]	7.4 ± 4.5[c]

[a] All concentrations are given in μmol/L.
[b] Mean basal level was 0.011 μmol/L in dialysed human plasma.
[c] For mean intraassay accuracy and precision only values in the range of ±20% were included.

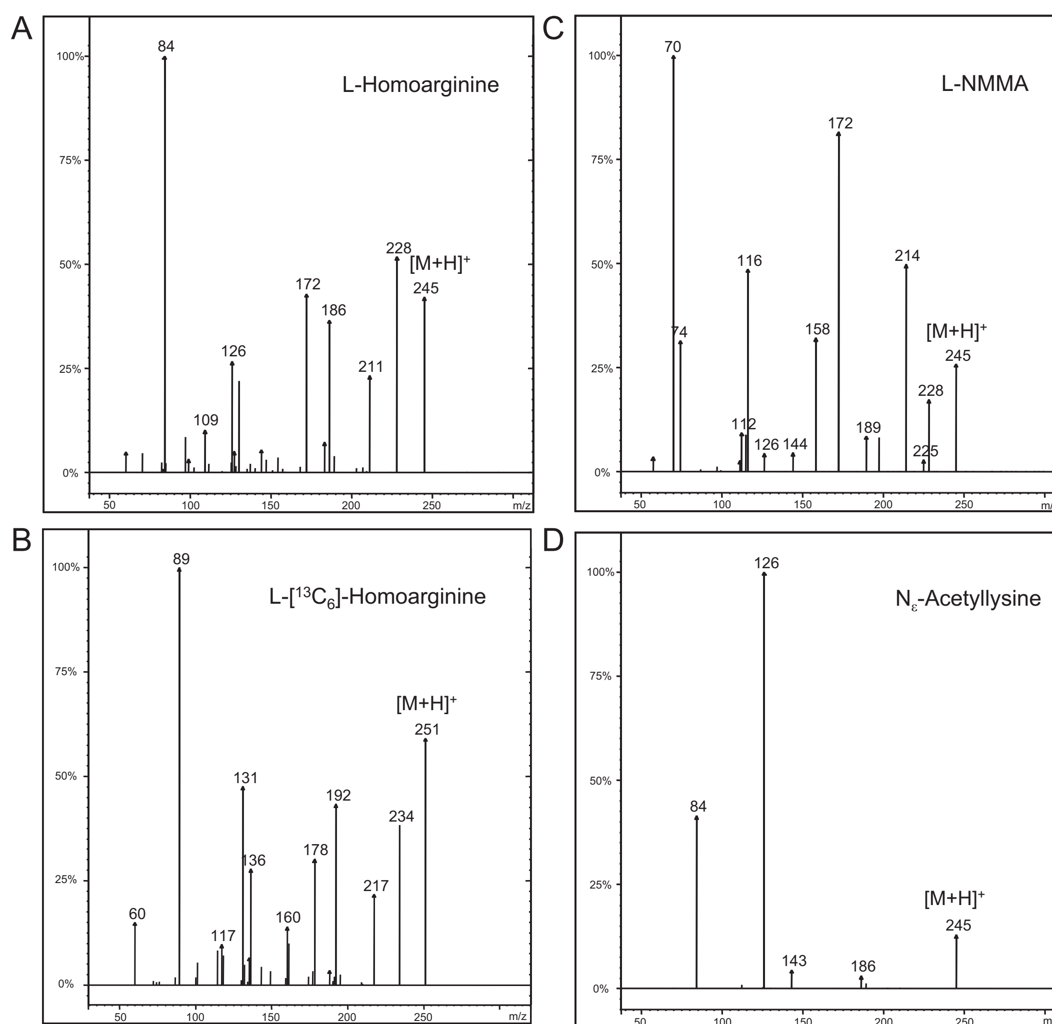

Fig. 2. ESI(+) tandem mass spectra of the butyl ester derivatives of L-homoarginine (A), L-[$^{13}C_6$]-homoarginine (B), L-NMMA (C), and N_ε-acetyllysine (D). The parent ions formed from ESI+ are indicated by $[M+H]^+$.

was 92 ± 3% and 89 ± 0.6%, for native and dialysed plasma, respectively (p = n.s.).

3.4. L-Homoarginine plasma concentration in human plasma

Fig. 1A shows a typical chromatogram from the LC–tandem MS analysis of L-homoarginine and the specific internal standard in 25 μl of human plasma. The mean plasma concentration of L-homoarginine in healthy humans was 2.5 ± 1.0 μmol/L (n = 136). Clinical characteristics of the participants were in the normal range (Supplemental Table 1). The distribution of L-homoarginine plasma concentration is depicted in Supplemental Fig. 1. In men, L-homoarginine plasma concentrations were significantly higher as compared with women (2.7 ± 1.0 vs. 2.3 ± 0.9 μmol/L; $p < 0.05$). There was no association between L-homoarginine plasma concentrations and age, in the individuals investigated.

Table 3
Interassay accuracy and precision of the method for L-homoarginine calculated in dialysed human plasma at two different levels (n = 13 for each concentration).

L-Homoarginine added[a]	L-Homoarginine measured minus basal level[b]	Accuracy (% bias)	Precision (R.S.D., %)
2.0	2.1	4.4	8.9
5.0	5.0	−0.2	6.1
Mean ± S.D.		2.1 ± 3.3	7.5 ± 2.0

[a] All concentrations are given in μmol/L.
[b] Mean basal level was 0.02 μmol/L in dialysed human plasma.

4. Discussion

The LC–MS/MS method described herein permits the determination of L-homoarginine in human plasma. Up to now, L-homoarginine was commonly used as internal standard for quantifying the methylated arginines ADMA and SDMA [17]. Knowledge about the endogenous occurrence of L-homoarginine complicated the use of L-homoarginine as internal standard for arginine derivatives in biological samples [18]. The presented method uses the in-house synthesized stable-isotope labeled internal standard L-[$^{13}C_6$]-homoarginine to determine L-homoarginine in human plasma. Advantageously, the stable-isotope ^{13}C contributes only by 1.1% to carbon isotopes and the physico-chemical properties of the applied internal standard are almost identical to the analyte. The derivatization to their butyl esters and collision-induced dissociation (CID) resulted in the formation of characteristic product ions (Table 1). Martens-Lobenhoffer et al. already addressed the potential interference of L-homoarginine with its congeners L-NMMA and N_ε-acetyllysine [19]. With m/z 245 the butyl esters of L-homoarginine, L-NMMA and N_ε-acetyllysine show similar product ion mass spectra. However, we were capable of identifying

specific daughter ions for each isobaric ion (Fig. 2). The observed MS/MS fragmentation of the butyl ester derivatives of the analyte and the internal standard allows simultaneous determination without chromatographic separation. Nevertheless an elution gradient was used to eliminate matrix components prior to detection of the analytes [14]. Thus, matrix constituents elute from the column first, while the butyl esters of the analytes are retained longer (Supplemental Fig. 2). This procedure enabled the abdication of solid-phase extraction (SPE) which has been used previously for the analysis of arginine and arginine analogs, resulting in disadvantageously large sample volumes (up to 1.5 ml) and long sample preparation times [11]. Hence, our method enabled fast sample preparation in a 96-well plate system. Jones et al. recently described a HPLC method for the analysis of L-homoarginine amongst other arginine derivatives using N-propyl L-arginine as internal standard [20]. The mean L-homoarginine plasma concentration in healthy volunteers measured by this HPLC method was 2.15 ± 0.75 μmol/L (mean ± SD, $n = 30$). The L-homoarginine plasma concentration Valtonen et al. reported was 5.07 ± 0.07 μmol/L (mean ± SD, $n = 3$) [18]. In the LUdwigshafen RIsk and Cardiovascular Health (LURIC) study, März et al. reported mean serum L-homoarginine concentrations in patients undergoing coronary angiography of 2.6 ± 1.1 μmol/L [9], determined by HPLC [21]. The differences between the mean L-homoarginine plasma concentrations determined with these different methods might be due to different internal standards or different populations investigated. A further advantage of our established method is the use of dialysed human plasma for calibration and LLOQ determination. With this approach, a matrix closely related to human plasma almost without an endogenous content of the analyte is available. The resulting calibration curve intercepted the *y*-axis close to zero (0.01 μmol/L), allowing calibration without the need of subtracting substantial amounts of endogenous compound. However, the dialysed plasma does not only lack the analyte, moreover it is likely to be short of polar major components. These components may interfere with the determination of the analyte of interest, i.e. by ion suppression. Being aware of this possible interference, we performed infusion experiments to account for differences in ion suppression observed for native and dialysed plasma. Continuous infusion of L-homoarginine and L-[$^{13}C_6$]-homoarginine show the same range of ion suppression sparked by native human plasma or dialysed human plasma, indicating similar characteristics of native and dialysed plasma (Supplemental Fig. 2).

In conclusion, this method allows the fast and reproducible quantitative determination of L-homoarginine in large clinical trials which is of crucial importance to elucidate the clinical consequence of disorders related to NO.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.06.016.

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