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ORIGINAL ARTICLE

Measurement of homoarginine in human and mouse plasma by LC–MS/MS and ELISA: a comparison and a biological application

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Abstract Homoarginine (hArg) is a non-essential amino acid that was identified as a risk marker for cardiovascular disease. Several analytical methods have been described for the quantification of hArg in biological samples. The aim of this study was to compare a liquid chromatography–tandem mass spectrometric (LC–MS/MS) approach with a commercially available enzyme-linked immunosorbent assay (ELISA). Determination of hArg concentrations in ELISA calibration standards measured by both methods revealed a correlation coefficient r^2 of 0.99, for LC–MS/MS calibrators r^2 was 0.997. However, linear regression analysis between the two assays for hArg concentrations in human plasma samples revealed a correlation coefficient r^2 of 0.78. Plasma concentrations obtained from LC–MS/MS are on average 29 % higher than those by ELISA. We investigated the hArg-isobaric N^{ε}-trimethyllysine as potential source for the higher observed values, but evaluation of mass spectra indicated that N^{ε}-trimethyllysine did not interfere with hArg quantification in our LC–MS/MS method. Both quantification methods were applied to measure hArg in (1) a case–control study of acute coronary syndrome and (2) L-arginine:glycine amidinotransferase-deficient mice. Our LC–MS/MS and the commercially available ELISA assay are suitable for hArg measurement in human and mouse plasma, but different reference values for each method need to be considered.

Keywords L-Arginine:glycine amidinotransferase · ELISA · Homoarginine · LC–MS/MS

Abbreviations

ACS	Acute coronary syndrome
AGAT	L-Arginine:glycine amidinotransferase
ANOVA	Analysis of variance
CID	Collision-induced dissociation
CV	Cardiovascular
ELISA	Enzyme-linked immunosorbent assay
ESI(+)	Positive electrospray ionization
GC–MS/MS	Gas chromatography–tandem mass spectrometry
hArg	Homoarginine
$^{13}C_6$-hArg	[$^{13}C_6$]-Homoarginine
HPLC	High-performance liquid chromatography
IQR	Interquartile range
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
m/z	Mass-to-charge ratio
NO	Nitric oxide
QC	Quality control
SAP	Stable angina pectoris

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SD	Standard deviation
SPE	Solid-phase extraction
UPLC-MS/MS	Ultrahigh-performance liquid chromatography–tandem mass spectrometry
Wt	Wild-type

Introduction

Homoarginine (hArg) is a naturally occurring amino acid that differs from arginine by an additional methylene group. Its formation is catalyzed by L-arginine:glycine amidinotransferase (AGAT) (Choe et al. 2013), the first enzyme in the biosynthesis of creatine. hArg competes with arginine as a weak substrate for endothelial nitric oxide (NO) synthase with fivefold to 15-fold decreased catalytic efficiency compared to arginine (Moali et al. 1998). Furthermore, hArg weakly inhibits the arginine-degrading enzyme, arginase (Hrabák et al. 1994), an interaction which may potentially enhance NO formation by increasing arginine concentrations. Clinical data indicate that low circulating hArg concentrations are associated with cardiovascular (CV) disease, CV mortality, and all-cause mortality (März et al. 2010; Pilz et al. 2011a, b, 2014, 2015; Atzler et al. 2013; Choe et al. 2013).

For the quantification of hArg in biological samples, several methods have been developed (Meinitzer et al. 2007; Di Gangi et al. 2010; Jones et al. 2010; Atzler et al. 2011; Davids et al. 2012; Midttun et al. 2013; Kayacelebi et al. 2014). A gas chromatography–mass spectrometry (GC–MS)-based approach to quantify hArg in plasma, serum, urine, and sputum involves preceding derivatization steps to yield the methyl ester tri(*N*-pentafluoropropionyl) derivative of hArg (Kayacelebi et al. 2014). A high-performance liquid chromatography (HPLC) method is based on solid-phase extraction (SPE) and pre-column derivatization of hArg with o-phenylendiamine followed by fluorescent detection, and its applicability is shown for human plasma and serum samples (Meinitzer et al. 2007). A further HPLC method for the quantification of hArg in human plasma samples with fluorescent detection utilizes separation of hArg with SPE and derivatization with the AccQ-Fluor™ reagent (Waters, Milford, MA, USA), containing 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Jones et al. 2010). An ultrahigh-performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) assay involves hArg derivatization to its butyl ester to determine hArg in plasma, serum and urine (Di Gangi et al. 2010). Two liquid chromatography–tandem mass spectrometric (LC–MS/MS) methods for the quantification of hArg after protein precipitation or SPE, respectively, and derivatization to its butyl ester were reported for the determination of hArg in human plasma (Atzler et al. 2011), and in plasma, cells, and tissues (Davids et al. 2012). Further, an LC–MS/MS assay was used by Midttun and colleagues with previous separation of hArg and other amino acids in plasma samples on phenyl reversed-phase material (Midttun et al. 2013).

The aim of the present study was to compare a previously published LC–MS/MS method (Atzler et al. 2011) with a commercially available enzyme-linked immunosorbent assay (ELISA; DLD Diagnostika GmbH, Hamburg, Germany). In addition, we modified these methods for hArg quantification in mouse plasma samples.

Materials and methods

Chemicals and materials

L-Homoarginine hydrochloride and N^{ε}-trimethyllysine were obtained from Sigma-Aldrich (Steinheim, Germany). hArg stock solutions were prepared in dialyzed plasma. [$^{13}C_6$]-Homoarginine ($^{13}C_6$-hArg) was synthesized in-house as previously described (Atzler et al. 2011) and a stock solution was prepared in methanol (Merck, Darmstadt, Germany). 96-Well 0.20 µm Multiscreen HTS™ microfiltration plates were purchased from Millipore (Molsheim, France), u-shaped 96-well polypropylene plates from Greiner bio-one (Frickenhausen, Germany), acetate foil, and sealing mats for 96-well plates were obtained from Sarstedt (Nümbrecht, Germany). hArg ELISA kits were purchased from DLD Diagnostika GmbH (Hamburg, Germany). Other chemicals were obtained from Merck.

LC–MS/MS method

Measurements were performed using a previously described LC–MS/MS assay (Atzler et al. 2011). Briefly, after addition of the stable-isotope labeled internal standard $^{13}C_6$-hArg (10 µM for human samples and 0.5 µM for mouse samples) and protein precipitation with methanol, analytes were converted to their butyl ester derivatives, and solvents were subsequently evaporated. Analytes were then dissolved in methanol/water (25/75, v/v) containing 0.1 % ammonium formate before subjection to a Varian 1200L Triple Quadrupole MS equipped with two Varian ProStar model 210 HPLC pumps (Agilent Technologies, Santa Clara, CA, USA). The analytes were separated on a Polaris C18-Ether column (Agilent, 50 × 2.0 mm) and quantified by multiple reaction monitoring of the transitions of mass-to-charge ratios (*m/z*) 245 → 211 for hArg and *m/z* 251 → 217 for $^{13}C_6$-hArg. hArg concentrations used for calibration were 0, 2, 5, and 10 µM for human plasma and 0, 0.1, 0.25, and 0.5 µM for mouse plasma samples. As quality controls (QC) hArg concentrations of 2 and

5 µM, or 0.1 and 0.25 µM were used for human and mouse samples, respectively. Mass spectra were recorded after injection of 10 µl aliquots of *O*-butylated hArg and N^{ε}-trimethyllysine (10 µM in methanol/water, 25/75, v/v) into the mass spectrometer using full scan monitoring of the product ion mass spectrum of *m/z* 245. For positive electrospray ionization (ESI+), needle and shield voltage were adjusted at 5000 and 600 V, respectively. Collision-induced dissociation (CID) with argon was performed at a collision energy of 14 eV.

ELISA method

For the competitive hArg ELISA (DLD Diagnostika GmbH, Hamburg, Germany), pretreated plasma samples containing acylated hArg were transferred into ELISA microtiter plates with solid-phase-bound hArg. After incubation with rabbit anti-hArg antiserum, free antigen and free antigen–antiserum complexes were removed by washing, and a secondary antibody (anti-rabbit) conjugated with the enzyme peroxidase was added. Free anti-rabbit antibody was removed by washing, and oxidation of the substrate 3,3′-5,5′-tetramethylbenzidine was monitored at 450 nm (reference wavelength 620 nm) using a Sunrise™ microtiter plate reader (Tecan, Crailsheim, Germany). For human samples, concentrations of calibrators were 0, 0.3, 0.8, 1.6, 3.2, and 7 µM hArg; concentrations of QC were 1 and 2.2 µM (DLD Diagnostika GmbH, homoarginine ELISA package insert, 2014). To increase ELISA assay sensitivity for the quantification of hArg in mouse plasma, a higher volume of pretreated samples was used for the ELISA assay and incubation time with anti-hArg antiserum was prolonged. For mouse plasma 0, 0.1, 0.15, 0.3, 0.8, and 1.6 µM hArg were used for calibration; 0.15 and 0.8 µM hArg were used as QC. Newly prepared standard and QC samples were prepared by diluting calibration standards with standard 1 (0 µM hArg). Detailed protocols for human and mouse plasma samples are given in Online Resource 1.

Human plasma samples

A total of 144 human plasma samples were analyzed. They included 13 healthy controls (54 % male, mean age ± standard deviation (SD) 59.5 ± 10.5 years), 36 patients diagnosed with stable angina pectoris (SAP; 83 % male, 64.6 ± 9.8 years), and 95 patients who had symptoms of acute coronary syndrome (ACS; including unstable angina pectoris, non-ST-elevation myocardial infarction, and ST-elevation myocardial infarction; 70 % male, 69.3 ± 12.2 years). The study complied with the standards set forth in the Declaration of Helsinki and was approved by the Ethics Committee of the University Medical Center Hamburg-Eppendorf, Germany. All patients gave their written informed consent prior to inclusion into the study.

Mouse plasma samples

Plasma samples from 20 mice in total were analyzed, comprising eight female AGAT wild-type (wt) and 12 female AGAT-deficient ($^{-/-}$) mice. The German animal welfare laws for the care and use of animals were followed.

Statistical analyses

Data are reported as median and interquartile range (IQR) or mean and SD. Statistical differences were tested by Wilcoxon matched pairs test, Mann–Whitney test, both two-tailed, or analysis of variance (ANOVA). Differences were considered significant for $P < 0.05$. For method comparison, linear regression analyses, Bland–Altman plot, and linear function analysis using F test (Araujo 2009) were applied. PRISM version 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical evaluation of data.

Results

Analyses of calibrators and controls with LC–MS/MS and ELISA

hArg concentrations of ELISA calibration standards and controls, and LC–MS/MS calibrators and controls were quantified applying LC–MS/MS (Table 1). On average, ELISA calibration standard and control hArg concentrations measured by LC–MS/MS were 0.2 ± 14.9 % (mean ± SD) lower than specified values. In addition, hArg concentrations of ELISA calibration standards and controls, and LC–MS/MS calibrators and controls were also quantified by ELISA (Table 2). On average, LC–MS/MS calibrators and controls we found 0.1 ± 13.0 % lower than specified. Linear regression analysis between LC–MS/MS and ELISA revealed correlation coefficients r^2 of 0.99 for ELISA calibration standards (Fig. 1), and of 0.997 for LC–MS/MS calibrators. Linear function analysis (Araujo 2009) revealed non-linearity of determined hArg concentrations of ELISA calibration standards measured by LC–MS/MS compared to ELISA. Calculated Fisher variance ratio was 5.4, and thus larger than tabulated value for the 0.05 significance level with 3.05 (3 and 22 degrees of freedom).

Homoarginine analyses in human plasma samples

To compare hArg plasma concentrations measured either by LC–MS/MS or ELISA, a total of 144 human plasma samples were analyzed in parallel by both methods. Linear regression

Table 1 Analysis of calibrators and controls with LC–MS/MS

	Added (µM hArg)	Measured minus basal level[a] (µM hArg)		Recovery (%)		*n*
		Mean ± SD	RSD	Mean ± SD	RSD	
ELISA calibration standards (Std) and controls (Ctrl) measured by LC–MS/MS						
Std 2	0.3	0.24 ± 0.03	14.6	79.2 ± 11.6	13.8	6
Std 3	0.8	0.89 ± 0.13	14.5	111.0 ± 16.1	12.4	6
Std 4	1.6	1.52 ± 0.18	11.8	95.1 ± 11.2	14.2	6
Std 5	3.2	3.40 ± 0.15	4.3	106.3 ± 4.5	4.2	6
Std 6	7	7.01 ± 0.34	4.8	100.1 ± 4.8	4.6	6
Ctrl 1	1	1.02 ± 0.18	17.2	102.4 ± 17.7	14.5	6
Ctrl 2	2.2	2.29 ± 0.29	12.8	104.1 ± 13.4	15.0	6
LC–MS/MS calibrators (Cal) and controls (Con) measured by LC–MS/MS						
Cal 1	2	1.96 ± 0.07	3.6	97.9 ± 3.5	3.5	6
Cal 2	5	4.69 ± 0.38	8.1	93.8 ± 7.6	9.2	6
Cal 3	10	8.74 ± 0.38	4.4	87.4 ± 3.8	4.1	6
Con 1	2	1.75 ± 0.13	7.6	87.5 ± 6.6	7.5	6
Con 2	5	4.65 ± 0.21	4.5	93.0 ± 4.2	4.6	6

[a] 0.02 µM hArg

Table 2 Analysis of calibrators and controls with ELISA

	Added (µM hArg)	Measured minus basal level[a] (µM hArg)		Recovery (%)		*n*
		Mean ± SD	RSD	Mean ± SD	RSD	
ELISA calibration standards (Std) and controls (Ctrl) measured by ELISA						
Std 2	0.3	0.39 ± 0.06	15.1	130.3 ± 19.6	15.1	6
Std 3	0.8	0.85 ± 0.08	9.3	106.0 ± 9.9	9.3	6
Std 4	1.6	1.51 ± 0.12	7.8	94.4 ± 7.3	7.8	6
Std 5	3.2	2.97 ± 0.28	9.3	92.9 ± 8.6	9.3	6
Std 6	7	6.90 ± 0.71	10.3	98.6 ± 10.2	10.3	6[b]
Ctrl 1	1	0.94 ± 0.07	7.9	93.6 ± 7.3	7.9	6
Ctrl 2	2.2	1.83 ± 0.12	6.6	83.1 ± 5.5	6.6	6
LC–MS/MS calibrators (Cal) and controls (Con) measured by ELISA						
Cal 1	2	1.87 ± 0.16	8.4	93.3 ± 7.8	8.4	8
Cal 2	5	5.25 ± 0.75	14.2	105.1 ± 14.9	14.2	8
Cal 3	10	9.52 ± 1.30	13.7	95.2 ± 13.0	13.7	8
Con 1	2	2.09 ± 0.23	11.0	104.4 ± 11.4	11.0	14
Con 2	5	4.95 ± 0.73	14.7	98.9 ± 14.5	14.7	13

[a] 0.01 µM hArg

[b] Two values outside calibration range, outliers were not included in the calculation

[c] 0.05 µM hArg

analysis revealed a correlation of $r^2 = 0.78$ between the two assays (Fig. 2). hArg concentrations obtained from LC–MS/MS measurements were consistently higher than those determined by ELISA (i.e., median [IQR] 2.11 [1.15] vs. 1.56 [0.93] µM; LC–MS/MS vs. ELISA; $P < 0.001$, Wilcoxon matched pairs test). To analyze comparability between the two assays, a Bland–Altman plot was created. This analysis showed increasing bias with increasing hArg plasma concentrations (Fig. 3). The mean difference ± SD between the two methods was 0.50 ± 0.39 µM. On average, values measured by LC–MS/MS were 29 % higher than those obtained by ELISA.

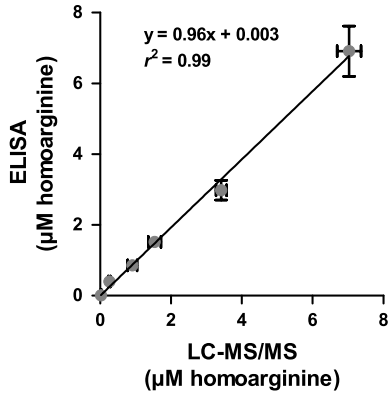

Fig. 1 Linear regression analysis of homoarginine concentrations in ELISA calibration standards measured by LC–MS/MS and ELISA. Mean ± SD; $n = 4$–6

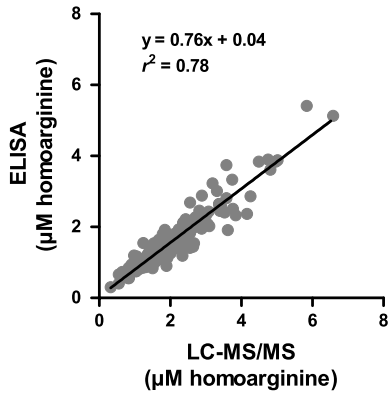

Fig. 2 Linear regression analysis of homoarginine plasma concentrations in humans measured by LC–MS/MS and ELISA

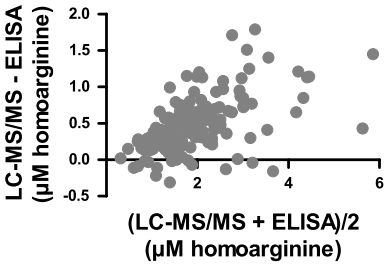

Fig. 3 Bland–Altman plot of human plasma samples measured by LC–MS/MS and ELISA

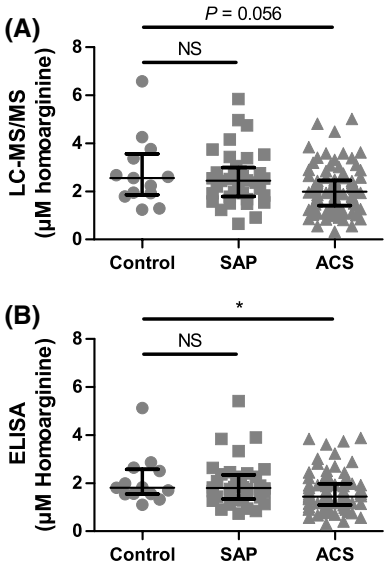

Fig. 4 Homoarginine plasma concentrations of the clinical cohorts stratified by disease status measured by LC–MS/MS (**a**) and ELISA (**b**). Median, IQR. *$P < 0.05$; *NS* not statistically significant (Mann–Whitney test, two-tailed)

The applicability of both methods was tested in plasma samples from patients with CV disease. Also after stratifying hArg measurements by disease status into different groups, both, LC–MS/MS and ELISA, showed lower hArg plasma concentrations for ACS patients compared with healthy subjects (Fig. 4; $P = 0.056$ and 0.03, respectively, Mann–Whitney test). Median hArg concentrations determined were (LC–MS/MS vs. ELISA) 2.56 [1.71] vs. 1.80 [1.03] µM in the control group, 2.45 [1.20] vs. 1.80 [1.01] µM in patients with SAP, and 1.99 µM [1.05] vs. 1.44 µM [0.89] in patients with ACS.

Homoarginine analyses in mouse plasma samples

The applicability of both methods was also tested in plasma samples obtained from genetically modified mice and wild-type littermates. Plasma samples from 20 mice in total were analyzed (8 AGAT wt and 12 AGAT$^{-/-}$ mice). Linear regression analysis revealed a correlation coefficient r^2 of 0.76 between LC–MS/MS and the modified ELISA assay. Both LC–MS/MS and the modified ELISA showed lower hArg plasma concentrations in AGAT$^{-/-}$ mice compared with AGAT wt mice (Fig. 5; $P < 0.001$ and $P < 0.05$, ANOVA). Median plasma concentrations were 0.54 [0.19] vs. 0.43 [0.11] µM (LC–MS/MS vs. ELISA) in AGAT wt mice and 0 [0.06] vs. 0.06 [0.02] µM in AGAT$^{-/-}$ mice.

ESI(+) tandem mass spectra of the butyl ester derivatives of homoarginine and N^{ϵ}-trimethyllysine

O-Butylated N^{ϵ}-trimethyllysine and hArg showed some common product ions (*m/z* 84, 130, and 186) from CID of the isobaric parent ion with *m/z* 245 (Fig. 6). In contrast to N^{ϵ}-trimethyllysine, the product ion mass spectrum of hArg contained the ion *m/z* 211.

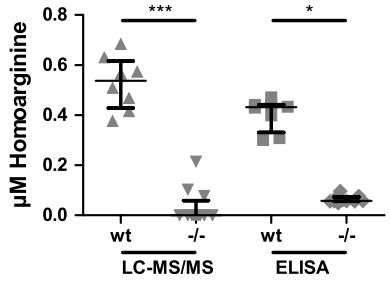

Fig. 5 Homoarginine plasma concentrations in AGAT wt vs. AGAT$^{-/-}$ mice, measured by LC–MS/MS and ELISA. Median, IQR. ***$P < 0.001$; *$P < 0.05$ (ANOVA)

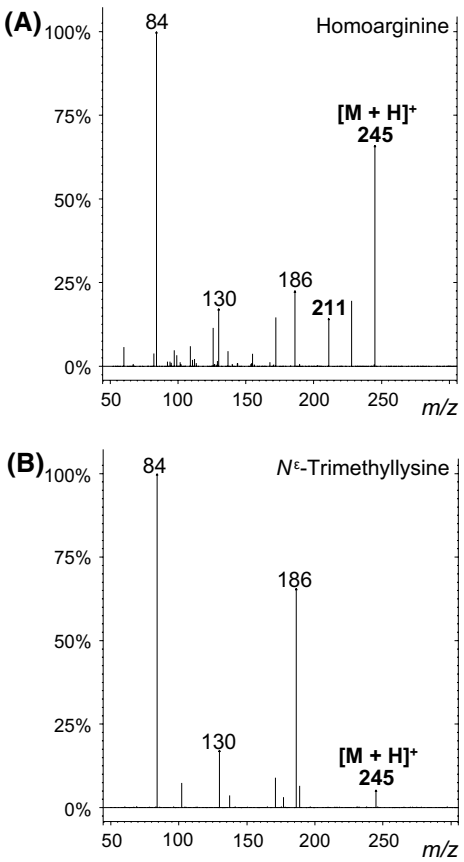

Fig. 6 ESI(+) tandem mass spectra of homoarginine (**a**) and N^{ε}-trimethyllysine (**b**). The parent ions ([M + H]$^+$) underwent CID with argon at a collision energy of 14 eV

Discussion

By comparing the results of the quantification of calibrators and controls with both methods, i.e., LC–MS/MS and ELISA, we did not observe differences from the expected to the measured concentrations. Linear function analysis (Araujo 2009) revealed non-linearity of ELISA calibration standards measured by LC-MS/MS compared to ELISA due to some lack of fit of values, despite a strong correlation ($r^2 = 0.99$) found by linear regression analysis. Furthermore, we compared the applicability of both methods quantifying hArg in (1) a case–control study of ACS and (2) AGAT$^{-/-}$ mice. Linear regression analysis of hArg concentrations in human plasma samples revealed a correlation of $r^2 = 0.78$. Overall, values obtained by LC–MS/MS were consistently higher than those measured by ELISA. In theory, the latter may be due to impaired selectivity of the LC–MS/MS method or due to lower sensitivity of the ELISA. Acylated hArg in the sample competes with solid-phase-bound hArg for a settled number of anti-hArg antiserum-binding sites. After establishing the equilibrium, free antigen and free antigen–antiserum complexes are removed by washing. Solid-phase-bound antigen–antiserum complexes get coupled with the enzyme peroxidase, and for quantification its catalytic reaction with 3,3′-5,5′-tetramethylbenzidine is monitored. An imbalance of hArg and anti-hArg antibody concentrations or an inadequate sensitivity of the antiserum may possibly lead to lower sensitivity of the ELISA. Another possible explanation for the higher observed values obtained by our LC–MS/MS method might be the measurement of different hArg fractions in plasma with ELISA and LC–MS/MS. Plasma matrix components, e.g., proteins, might disguise hArg. Recently, it was shown for asymmetric dimethylarginine that 30 % of this amino acid is bound to human serum albumin (Sitar et al. 2015), which is close to the 29 % higher hArg concentrations measured by LC–MS/MS, compared with ELISA. Sample preparation of our LC–MS/MS method includes protein precipitation which might release hArg from binding sites of plasma proteins. Another difference between the two approaches used in this study is that different matrices were used for calibration. In our LC–MS/MS method for calibration, we use dialyzed plasma with added hArg to calibrate in a matrix similar to the samples. For ELISA, hArg in aqueous protein-free buffer matrix is used for calibration (DLD Diagnostika GmbH, personal communication, 2015). However, we obtained similar results by cross-evaluating calibrators. A further explanation for the higher values given by the LC–MS/MS assay might be a lower selectivity of this method. The parent ions generated by ESI(+) with *m/z* ratios of 245 for hArg and 251 for $^{13}C_6$-hArg undergo fragmentation by CID with argon, and one specific fragment is used as quantifier. There might be isobaric substances with similar physico-chemical properties, yielding a fragment with the same *m/z* of 211 used for the quantification of hArg. We investigated the fragmentation pattern of N^{ε}-trimethyllysine, which is isobaric to hArg and is also present in human plasma (Lehman et al. 1987). *O*-Butylated N^{ε}-trimethyllysine and hArg show some common fragments, i.e., *m/z* 84, 130, and 186, but the specific transition that we use in our LC–MS/MS assay (*m/z*

Table 3 Overview of hArg values in healthy subjects measured by different methods

References	Matrix	HArg (μM)	*N*	Age (years)	Male (%)	Method
Meinitzer et al. (2007)	Plasma	2.48 (1.42–4.62) [median (2.5–97.5th percentile)]	78	26.7 ± 4.0 (mean ± SD)	100	HPLC
		2.58 (1.38–5.29) [median (2.5–97.5th percentile)]	93	41.6 ± 4.0 (mean ± SD)		
		2.37 (1.43–4.69) [median (2.5–97.5th percentile)]	82	57.5 ± 4.2 (mean ± SD)		
		2.49 (1.11–4.68) [median (2.5–97.5th percentile)]	39	69.6 ± 3.3 (mean ± SD)		
Di Gangi et al. (2010)	Plasma	0.99 ± 0.56 (mean ± SD)	20	42.5 ± 5.0 (mean ± SD)	NR	UPLC–MS/MS
Jones et al. (2010)	Plasma	2.15 ± 0.75 (mean ± SD)	30	16–61 (range)	40	HPLC
Atzler et al. (2011)	Plasma	2.5 ± 1.0 (mean ± SD)	136	48.5 ± 11.0 (mean ± SD)	51	LC–MS/MS
Davids et al. (2012)	Plasma	2.01 ± 0.647 (mean ± SD)	27	NR	NR	LC–MS/MS
Midttun et al. (2013)	Plasma	1.93 (1.17–3.31) [median (5–95th percentile)]	171	21–68 (range)	60.2	LC–MS/MS
DLD (2014)	Plasma, serum	2.0 ± 0.7 (mean ± SD)	252[a]	NR	NR	ELISA
Kayacelebi et al. (2014)	Plasma, serum	1.87 ± 0.67 (mean ± SD)	10	NR	NR	GC–MS/MS

DLD 2014, DLD Diagnostika GmbH, homoarginine ELISA package insert, 2014; *NR* not reported

[a] DLD Diagnostika GmbH, personal communication, 2015

245 → 211) is not present in the product ion mass spectrum of N^{ε}-trimethyllysine. Previously, other endogenous compounds isobaric to hArg, i.e., N^{G}-monomethylarginine and N^{ε}-acetyllysine, were also shown not to interfere with the ion transition *m/z* 245 → 211 in hArg analysis by LC–MS/MS (Atzler et al. 2011). Thus, these endogenous metabolites do not interfere with the quantification of hArg by the LC–MS/MS method investigated.

In our healthy control group ($n = 13$), we found median plasma concentrations of 2.56 [1.71] μM by LC–MS/MS and 1.80 [1.03] μM by ELISA. Many different methods for the determination of hArg in biological samples have already been described (Meinitzer et al. 2007; Di Gangi et al. 2010; Jones et al. 2010; Atzler et al. 2011; Davids et al. 2012; Midttun et al. 2013; Kayacelebi et al. 2014). Our study is the first that systematically compares two different methods, i.e., a previously reported LC–MS/MS method (Atzler et al. 2011) with a commercially available ELISA method. Reported hArg concentrations in plasma and serum of healthy subjects vary between 0.99 μM (mean; Di Gangi et al. 2010) and 2.58 μM (median; Meinitzer et al. 2007) (Table 3). This range for circulating hArg may be due to the different anthropometric characteristics of the individuals (e.g., age, gender) analyzed, or by differences between the quantification methods used. Further studies are required to investigate the cause of these discrepancies between the different studies, e.g., by analyzing varying healthy study populations, or by analyzing replicate samples by several methods for comparison. Consistently with the results from the Bland–Altman plot analysis of the present study, indicating a systematic bias, both methods showed a comparable relative difference of hArg plasma concentrations observed for ACS patients and healthy controls. We adapted both methods to measure hArg in mouse plasma samples which have lower hArg levels than human plasma samples. In line with the observation that AGAT catalyzes hArg formation in vivo, the application of both methods confirmed previous findings that $AGAT^{-/-}$ mice have significantly lower hArg plasma concentrations compared with their wt littermates (Choe et al. 2013).

Our study suggests that the investigated LC–MS/MS method and the commercially available ELISA are both suitable for the determination of hArg in human and mouse plasma samples. Our LC–MS/MS method yields constantly higher hArg values than the commercially available ELISA method tested. When comparing hArg concentrations reported from experimental and clinical studies, the analytical method used to quantify hArg needs to be explicitly mentioned. Therefore, methods have to be harmonized or different reference values and intervals have to be formulated for different analytical methods for hArg.

In our study, we only applied two methods, LC–MS/MS (Atzler et al. 2011) and a commercial available ELISA assay, to quantify hArg in human and mouse plasma. A multi-method comparison could clarify the cause of the considerable but consistent deviations found for LC–MS/MS and ELISA in our study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical statement The study complied with the standards set forth in the Declaration of Helsinki and was approved by the Ethics Committee of the University Medical Center Hamburg-Eppendorf. All patients gave their written informed consent prior to inclusion into the study. The German animal welfare laws for the care and use of animals were followed.

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