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AMINO ACIDS PROTOCOLS

Measurement of unlabeled and stable isotope-labeled homoarginine, arginine and their metabolites in biological samples by GC–MS and GC–MS/MS

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Abstract Circulating and excretory L-homoarginine (hArg) and asymmetric dimethylarginine (ADMA) are cardiovascular risk factors. L-Arginine (Arg) is the common precursor of hArg and ADMA. This protocol describes gas chromatography–mass spectrometry (GC–MS) and gas chromatography–mass spectrometry–mass spectrometry (GC–MS/MS) methods for the quantitative determination of hArg, Arg and ADMA in biological samples, including human plasma, urine and sputum. Aliquots (10 µL) of native urine, plasma or serum ultrafiltrate (cutoff, 10 kDa), and acetone-deproteinized sputum samples are evaporated to dryness. Then, amino acids are derivatized to their methyl ester *N*-pentafluoropropionyl derivatives. In parallel, trideuteromethyl ester *N*-pentafluoropropionyl derivatives of hArg, Arg and ADMA are de novo synthesized from the unlabelled amino acids and used as internal standards. Alternatively, commercially available stable isotope-labeled analogs of hArg, Arg and ADMA are used as internal standards, and they are added to the native biological samples. Quantification is performed by selected ion monitoring in GC–MS and selected reaction monitoring in GC–MS/MS. By these protocols, unlabelled and stable isotope-labeled hArg, Arg and their metabolites including ADMA and ornithine can be measured equally accurately and precisely by GC–MS and GC–MS/MS in several different biological fluids in experimental and clinical settings.

Keywords GC–MS · GC–MS/MS · Guanidino compounds · Plasma · Protocols · Quantification · Saliva · Stable isotope · Urine

Abbreviations

ADMA	Asymmetric dimethylarginine (N^G,N^G-dimethyl-L-arginine)
AGAT	Arginine:glycine amidinotransferase
Arg	Arginine
CID	Collision-induced dissociation
EA	Ethyl acetate
ECNICI	Electron-capture negative-ion chemical ionization
GC–MS	Gas chromatography–mass spectrometry
GC–MS/MS	Gas chromatography–mass spectrometry–mass spectrometry
hArg	Homoarginine
ID	Internal diameter
IS	Internal standard
Me	Methyl
MeOH	Methanol
NO	Nitric oxide
NOS	Nitric oxide synthase
Orn	Ornithine
PFP	Pentafluoropropionyl
PFPA	Pentafluoropropionic anhydride
QC	Quality control
SDMA	Symmetric dimethylarginine (N^G,$N^{G'}$-dimethyl-L-arginine)
SIM	Selected-ion monitoring
SRM	Selected-reaction monitoring
TSQ	Triple-stage quadrupole

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Introduction

Figure 1 illustrates the chemical structures of the amino acids L-homoarginine (hArg), L-arginine (Arg) and asymmetric dimethylarginine (ADMA) (Fig. 1). hArg is a non-essential, non-proteinogenic, cationic amino acid, the structural homologue of Arg which is a semi-essential, proteinogenic, cationic amino acid. The common structural feature of hArg, Arg and ADMA is the N-terminal guanidine group. The structural difference of hArg and Arg is an additional methylene (CH_2, 14 Da) group in the amino acid backbone of Arg (Fig. 1).

Arg is a multifunctional amino acid and plays an important role in metabolism and nutrition in growth, health and disease (Wu et al. 2009). Arg is the substrate of nitric oxide synthase (NOS) isoforms which catalyze the oxidation of one of the N atoms of the guanidine group of Arg to nitric oxide (NO), with L-citrulline being the second reaction product (Moncada and Higgs 1993; Tsikas 2008a). hArg may serve both as a substrate and as an inhibitor of NOS activity (Moali et al. 1998, 2000). ADMA is an endogenous inhibitor of NOS activity (Tsikas 2008b). Although less than 0.1 % of Arg is converted to NO in healthy humans via NOS, the Arg/NO pathway has multiple important implications in health and disease. NO possesses various biological activities including vasodilation, inhibition of vascular inflammation and platelet aggregation, and prevention of adhesion of immune cells (Moncada and Higgs 1993; Leiper and Vallance 1999).

High ADMA concentration in plasma and serum (<0.4 μM) is a well-established cardiovascular risk factor in adult subjects, and the cardiovascular risk of ADMA is generally assigned to its inhibitory action on NOS activity (Leiper and Vallance 1999; Horowitz and Heresztyn 2007; Teerlink 2007; Tsikas 2008a). Recently, low circulating hArg concentrations were found to be associated with cardiovascular and all-cause mortality in humans (März et al. 2010; Pilz et al. 2011; Drechsler et al. 2011; Choe et al. 2013). Mean plasma and serum hArg concentrations are of the order of 2 μM in healthy humans. Higher hArg concentrations were measured in pregnancy, and it is assumed that hArg is involved in the pathology of preeclampsia (Valtonen et al. 2008; Khalil et al. 2013). Lower circulating hArg concentrations were measured in male smokers compared to male non-smokers, while ADMA plasma concentrations were higher in smokers compared to non-smokers (Sobczak et al. 2014). Because of this diametral effects of smoking, we hypothesized that the molar ratio of hArg to ADMA (hArg/ADMA) may provide more information about the cardiovascular risk than the concentrations of hArg and/or ADMA alone (Tsikas and Kayacelebi 2014; Kayacelebi et al. 2014a).

The biosynthesis of hArg (Ryan and Wells 1964; Ryan et al. 1969) and its biological activities in humans are incompletely understood. L-Arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) catalyzes the transamination of L-lysine by L-arginine to form hArg (Davids et al. 2012a; Choe et al. 2013). Increased AGAT expression was found to be associated with increased hArg synthesis in a cell model (Choe et al. 2013).

A variety of chromatographic and mass spectrometric methods are currently available for Arg and ADMA (Tsikas et al. 2003; Schwedhelm 2005; Horowitz and Heresztyn 2007; Schwedhelm et al. 2007; Teerlink 2007; Martens-Lobenhoffer and Bode-Böher 2014; Tsikas 2008b; Wu et al. 2009). The emerging importance of hArg as a novel biomarker of cardiovascular diseases resulted in the extension of previous methods to hArg for its quantitative determination in biological samples (Atzler et al. 2011; Davids et al. 2012b; Kayacelebi et al. 2014b).

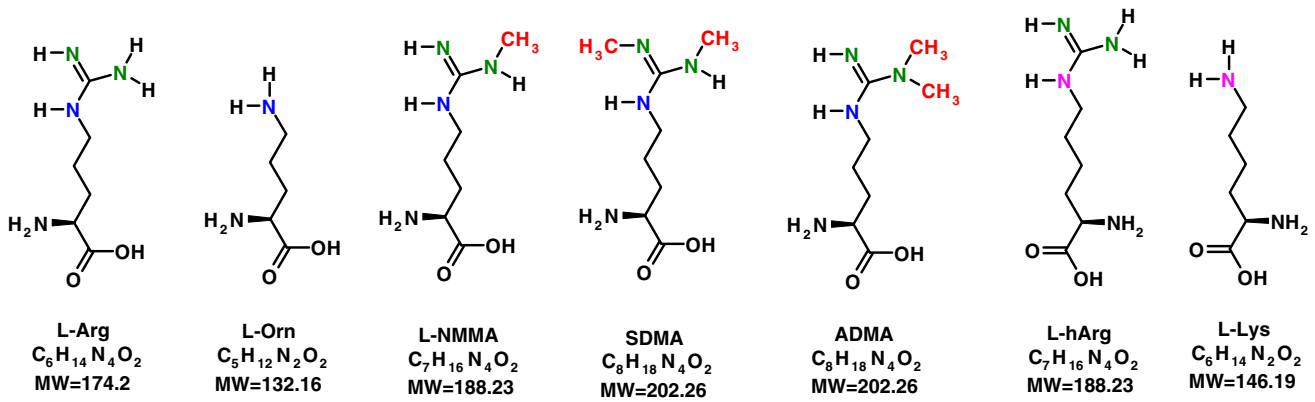

Fig. 1 Chemical structure, molecular formula and molecular weight (MW) of L-arginine (L-Arg), L-ornithine (L-Orn), L-N^G-monomethylarginine (L-NMMA), symmetric dimethylarginine (SDMA), asymmetric dimethylarginine (ADMA), L-homoarginine (hArg), and L-lysine (L-Lys)

Based on previously reported gas chromatography–mass spectrometry (GC–MS) and gas chromatography–mass spectrometry–mass spectrometry (GC–MS/MS) methods for ADMA and Arg (Tsikas et al. 2003), we extended these methods for the accurate and sensitive quantitative determination of hArg in plasma, serum, urine and saliva (Kayacelebi et al. 2014b). These GC–MS and GC–MS/MS methods also allow for the simultaneous analysis of hArg, Arg and ADMA in biological samples. In the present work, we describe detailed protocols which are routinely used in our group for the measurement of hArg, Arg and ADMA in plasma, serum, saliva and sputum in basic and clinical research.

Materials

Chemicals and reagents

L-Arginine hydrochloride (≥98 %)	SIGMA, #A-5131
Asymmetric dimethylarginine hydrochloride (≥98 %)	SIGMA, #D-4268
Boric acid	MERCK, #1.00165
Ethyl acetate (for analysis)	MERCK, #1.09623
GC columns, type Optima 17	MACHEREY–NAGEL
Dimensions: Length:	30 or 15 m
Internal diameter:	0.25 mm
Film thickness:	0.25 µm
L-Homoarginine hydrochloride (98 %)	SIGMA, #H-1007
Hydrochloric acid (37 %, ultrapure)	APPLICHEM, #A2427
Methanol (HPLC grade, >99.9 %)	SIGMA, #34860
$^2H_4$-Methanol (99.8 atom% ^2H)	SIGMA, #151947
Molecular sieve (0.4 nm)	MERCK, #1.05708
Pentafluoropropionic anhydride	Thermo, #TS-65193
Sodium hydroxide (pure)	MERCK, #1.06482
Toluene (anhydrous, 99.8 %)	SIGMA, #244511
Water (purified, Ampuwa)	FRESENIUS, #4801694

Equipment

GC–MS and GC–MS/MS apparatus and conditions

The protocols reported in this article are based on GC–MS and GC–MS/MS analyses performed on quadrupole mass spectrometers in the electron-capture negative-ion chemical ionization (ECNICI) mode. GC–MS analyses were performed on a single-stage quadrupole apparatus model DSQ from ThermoElectron (Austin, Texas, USA) directly interfaced with a gas chromatograph model Focus from ThermoElectron (Milano, Italy) equipped with an autosampler model AS 3000 from ThermoElectron (Milano, Italy). GC–MS and GC–MS/MS analyses were performed on the apparatus model TSQ 7000 from ThermoScientific (previously Finnigan MAT; San Jose, CA, USA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler model AS 2000 from CE Instruments (Austin, TX, USA).

The gas chromatograph of the GC–MS apparatus model was equipped with a 15-m-long fused-silica capillary column Optima 17 (0.25-mm I.D., 0.25 µm film thickness) from Macherey–Nagel (Düren, Germany). The following oven temperature program was used with helium (at a constant flow rate of 1 mL/min) as the carrier gas: 0.5 min at 90 °C, then increased to 210 and 320 °C at a rate of 15 and 35 °C/min, respectively. Interface, injector and ion-source were kept at 280, 280 and 250 °C, respectively. Electron energy was set to 70 eV and electron current to 100 µA. Methane (2.4 mL/min) was used as the reagent gas.

The gas chromatograph of the GC–MS/MS apparatus model TSQ7000 was equipped with a 30-m-long fused-silica capillary column Optima 17 (0.25-mm I.D., 0.25-µm film thickness) from Macherey–Nagel (Düren, Germany). The following oven temperature program was used with helium (at a constant flow rate of 1 mL/min) as the carrier gas: 1 min at 90 °C, then increased to 225 and 320 °C at a rate of 15 and 30 °C/min, respectively. Interface (280 °C), injector (280 °C) and ion-source (180 °C) were kept at constant temperatures. Electron energy was set to 200 eV and electron current to 300 µA. Methane (530 Pa) and argon (0.13 Pa collision pressure) were used as reagent and collision gases, respectively. Routinely, collision energy was set to 18 eV.

In both mass spectrometers, aliquots (1 µL) from toluene extracts were injected in the splitless mode by means of the respective autosampler. In quantitative analyses, the dwell time was 50 ms for each ion both in GC–MS (SIM mode) and in GC–MS/MS (SRM mode). The electron multiplier voltage ranged between 1.6 and 2.8 kV, in dependence upon the nature of biological samples and analytes.

Small laboratory equipment

1. Aluminum crimp cap, N 11, silver, center hole, natural rubber/butyl red–orange/TEF colorless, hardness 45° shore A, 1.0 mm, Macherey–Nagel (Düren, Germany), #70256.
2. Centrifuge, Sorval RC 6+, rotor Sorcvall SM24, ThermoFisher, USA.
3. Centrifuge, Multifuge 3 S-R, rotor 3332, Heraeus, USA.
4. Centrifuge, Multifuge 3 S-R, rotor 75006445, Heraeus, USA.

5. Crimp neck vials N 11 (wide opening), colorless, flat bottom, 1.5 mL, 11.6 × 32 mm, Macherey–Nagel (Düren, Germany), #70201HP.
6. Heating block with inserts for sample vials, flat, MBT 250 (Kleinfeld, Germany).
7. Magnetic stirrer, IKAMAG RCT, IKA (Staufen, Germany).
8. Micro-vials with crimp neck N 11, colorless, conical, 1.1 mL, 11.6 × 32 mm, Macherey–Nagel (Düren, Germany), # 702141.
9. Nitrogen evaporator, TurboVap LV evaporator; sample tray capacity for 50 vials; Zymark (Idstein/Taunus, Germany).
10. Pipette tips for piston-stroke Sarstedt (Nümbrecht, Germany).
11. Piston pipette, mLine, Sartorius (Göttingen, Germany).
12. Vivaspin 2 Hydrosart cartridges (cutoff, 10 kDa; volume 2 mL), Sartorius (Göttingen, Germany), #VS02H02.
13. Vivaspin 500 Hydrosart cartridges (cutoff, 10 kDa; volume 0.5 mL), Sartorius (Göttingen, Germany), #VS0102.
14. Vortex mixer model Reax 2000, Heidolph (Schwabach, Germany).

Stock and working solutions

Timing: about 2 h

A. 100 mM Arg:21.07 mg L-arginine HCl is dissolved in 1000 µL water.
B. 100 mM ADMA: the entire content of the original 10-mg ADMA-HCl containing flask is dissolved in 362 µL water.
C. 100 mM hArg: 22.47 mg L-homoarginine HCl is dissolved in 1000 µL water.
D. 2 M HCl in MeOH: 16 mL of 37 % hydrochloric acid is added slowly under stirring into 80 mL methanol. **!CAUTION**: heat develops.
E. 2 M HCl in d_4-MeOH: 11.2 mL of 37 % hydrochloric acid are added slowly under stirring into 56 mL d_4-methanol (volume of the original closed flask).
!CAUTION: heat develops. *Note*: old d_4-methanol flasks may contain less than 56 mL d_4-methanol. Measure the volume prior to use.
F. PFPA-EA (1:4, v/v): Add the content of a 1-mL pentafluoropropionyl (PFPA) containing ampoule to 3 mL ethyl acetate (EA). *Note*: prepare this solution freshly. *Note*: PFPA possesses an unpleasant smell.
G. Borate buffer: 6.183 g boric acid is dissolved in 240 mL water. The pH is (0.4 M pH 8.5) adjusted to 8.5 by 2 M NaOH. The final volume is adjusted to 250 mL by water.

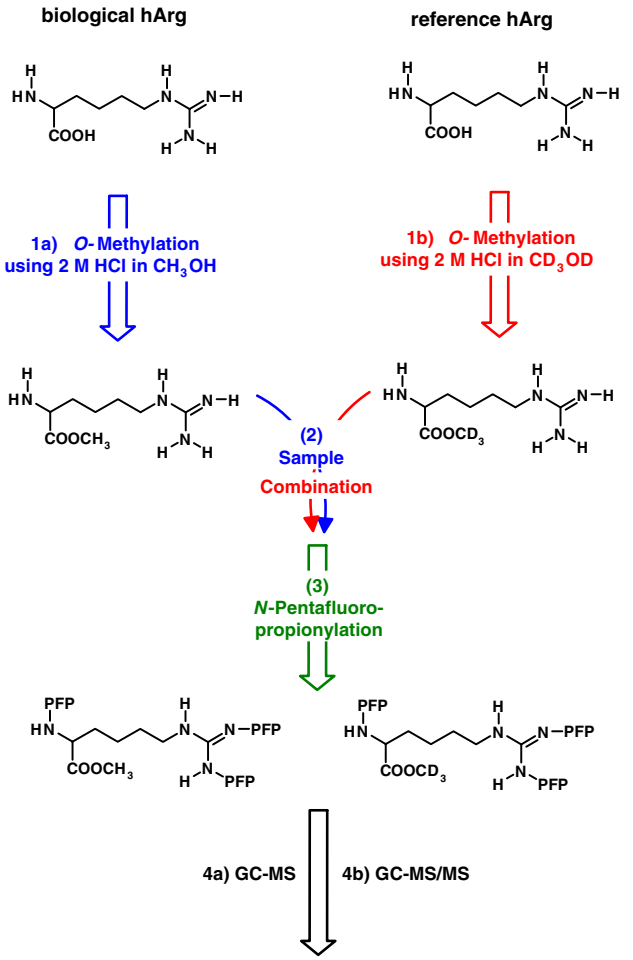

Fig. 2 Schematic of the main steps of the GC–MS and GC–MS/MS protocols for the analysis of hArg representing amino acids such as L-Arg and its derivatives, as well as L-Orn and L-Lys

Protocols

General aspects

The principle and the main steps of the methods described in the protocols that follow are illustrated in Fig. 2. Analysis of amino acids by GC–MS-based methods generally requires conversion of the amino acids into thermally stable and volatile derivatives by chemical reactions.

The carboxylic group is preferably converted to a methyl ester. Conventional esterification of amino acids is performed in an alcohol such as methanol (MeOH) in the presence of an inorganic acid such as hydrochloric acid (HCl) serving as the catalyst. The yield of this derivatization reaction is about 90 % and the esters such as methyl esters are sufficiently stable for storage for several weeks.

Primary and secondary amino groups are converted to their amides (*N*-acyl derivatives) by acylation reagents such

as carbonyl chlorides and anhydrides. For highest sensitive GC–MS-based analysis in the ECNICI mode use of fluorinated reagents such as pentafluoropropionyl anhydride (PFPA) is recommended, for instance as a solution in ethyl acetate (EA). The order of derivatization is first esterification and then *N*-acylation, because amides are susceptible to hydrolysis. *N*-Acylation with PFPA is impaired in the presence of proteins (Beckmann et al. 2008). Therefore, biological samples rich in proteins, such as plasma, serum and sputum, must be deproteinized prior to derivatization. Plasma and serum proteins are best removed by ultrafiltration. Removal of proteins from viscose samples such as saliva or sputum is best performed by volatile organic solvents such as acetone.

Unknown interferences from commercially available ultrafiltration cartridges may be present in the generated ultrafiltrate samples and impair the *N*-acylation of amino acids such as ADMA (Beckmann et al. 2008). Given the high sensitivity of GC–MS-based analysis of amino acids in the ECNICI mode, the above interference is minimized using small ultrafiltrate volumes such as 10 µL instead of 100 µL (Beckmann et al. 2008). Urine samples can be subjected to derivatization in their native form or after dilution with phosphate buffer.

In the present protocols, we use de novo synthesized deuterium-labeled analogs of hArg, Arg and ADMA (Tsikas 2009). This requires separate esterification of biological and synthetic amino acids. Thus, endogenous hArg, Arg and ADMA are *O*-methylated using HCl in unlabelled methanol (CH_3OH) to generate the unlabelled methyl esters (d_0Me) of the endogenous amino acids (e.g., d_0Me-d_0hArg). Synthetic amino acids are *O*-methylated using HCl in deuterium-labeled methanol (CD_3OD) to generate the [$^2H_3$]methyl esters (d_3Me) of the amino acids (e.g., d_3Me-d_0hArg). After methylation the samples are combined to a single sample for subsequent common *N*-pentafluoropropionylation with PFPA (Fig. 2).

Commercially available stable isotope-labeled analogs of hArg, Arg and ADMA can also be used. In that case, the internal standards are added to the original biological samples and the de novo synthesis step is entirely omitted.

Higher, unlabeled and stable isotope-labeled alcohols such as ethanol and propanol can also be used. Yet, this does not provide any analytical benefit and is moreover considerably more expensive.

The type of anticoagulation in blood sampling has different effects on the concentrations of hArg, ADMA and Arg independent of the analytical approach. ADMA concentrations in plasma and serum are almost identical. In contrast, Arg concentrations are considerably higher in serum compared to plasma. With respect to hArg, serum and plasma hArg concentrations are very similar, yet the difference seems to be greater and more variable when compared to ADMA (Kayacelebi et al. 2014b).

The concentrations of hArg, Arg and ADMA are of different order of magnitude in many biological samples, with hArg and ADMA being almost one order of magnitude smaller compared to Arg, for instance in plasma and serum. In quantitative GC–MS and/or GC–MS/MS analysis, selection of the detector voltage, e.g., the electron multiplier voltage must be optimized for the analytes. Otherwise, due to the high analytical sensitivity, the detector respond does linearly depend upon the analyte concentration in the upper range, despite the use of stable isotope-labeled analogs.

For most biological samples, GC–MS and GC–MS/MS provide very close concentrations and high correlation coefficients for hArg, Arg and ADMA (Kayacelebi et al. 2014b). Deviations are likely to occur for ADMA at very low analyte concentrations (Tsikas et al. 2003).

By these protocols and appropriate modifications, hArg, Arg and ADMA can be analyzed simultaneously or individually as desired. These protocols are extendable to symmetric dimethylarginine (SDMA) in many biological samples such as urine and microdialysate samples. For not yet known reasons, the analysis of SDMA in plasma and serum is less precise, accurate and reproducible, especially when compared to ADMA. Finally, these protocols are not useful for L-citrulline for not yet recognized reasons. In contrast, the protocols can easily be extended to L-ornithine (Becker et al. 2009).

Biological samples

Urine samples

Collect urine by spontaneous micturition (e.g., 10–50 mL), put the sample into an ice bath, transfer a 1-mL aliquot into a safely pre-labeled 4-mL polypropylene tube, and store the sample at −20 °C until further analysis.

Blood plasma samples

Draw blood (e.g., 3 mL) from the antecubital vein using monovettes containing EDTA, shake gently, and put the sample into an ice bath. Centrifuge the blood sample immediately (5 min, 800×*g*, 4 °C), transfer a 1-mL aliquot of the plasma into a safely pre-labeled 4-mL polypropylene tube, and store the sample at −80 °C until further analysis.

Sputum samples

Sputum is collected by a routine procedure. Put immediately the sample into an ice bath and store it at −80 °C

Table 1 Concentrations of hArg, Arg and ADMA added to the QC2 plasma, serum or urine samples

Amino acid	Added concentration (μM)	
	Plasma or serum	Urine
hArg	2	1
Arg	50	1
ADMA	0.5	20

until analysis. After thawing on ice, portions of the sputum sample are weighed (range 30–240 mg). Add under vortexing the fourfold volume of ice-cold acetone to inhibit enzymatic activity and precipitate proteins. After centrifugation (5 min, 800×*g*, 4 °C), 100-μL aliquots of the supernatants are evaporated to dryness under a stream of nitrogen, and the residues are subjected to derivatization as described below for plasma and serum.

Quality control samples

The reliability of the measurements is monitored and documented by implementing a quality control (QC) system. QC samples are prepared using pooled plasma (or serum) and urine samples from blood and urine donated by healthy volunteers, respectively. Study samples (for instance 20–30 samples per run), two unspiked QC samples (QC1) and two spiked QC samples (QC2) are worked up in parallel. The added concentrations of hArg, Arg and ADMA in the samples in QC2 are as listed in Table 1.

The accuracy (recovery %) and the imprecision (RSD %) by which study samples were analyzed are calculated using the QC samples. Recovery is determined using Formula (F1):

$$\text{Recovery } (\%) = ([\text{QC2}]-[\text{QC1}]/[\text{added}]) \times 100 \quad \text{(F1)}$$

whereas, [QC2] is the concentration (in μM) of the amino acids measured in the QC2 sample, [QC1] is the measured concentration (in μM) of the amino acids in the QC1 sample, and [added] is the concentration (in μM) of the amino acids added to the sample as indicated above.

Recovery and imprecision should be within generally acceptable ranges, i.e., 100 ± 20 % and <20 %, respectively (Fig. 3).

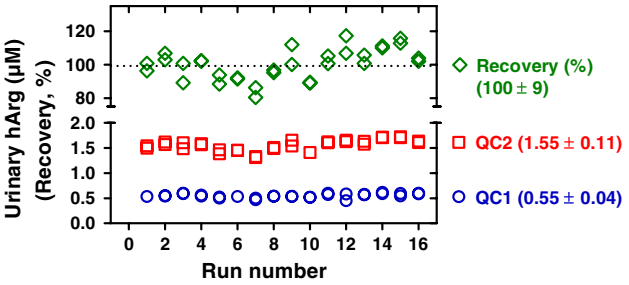

Fig. 3 Concentration of hArg in pooled urine sample of a healthy volunteer, serving as quality control (QC), without external addition of synthetic hArg (QC1) and with external addition of 1 μM synthetic hArg (QC2), and accuracy (recovery, %) by which the 1-μM hArg added was measured. About 1000 urine samples of a clinical study were analyzed in parallel with the QC samples in 16 runs. hArg was analyzed by GC–MS. QC1 and QC2 samples were analyzed in duplicate. QC1 and QC2 were measured with a mean imprecision (relative standard deviation) of 7.3 and 7.1 %, respectively

Ultrafiltration procedures for plasma and serum

Timing: 1–2 h

A. Procedure for sample volumes greater than 200 μL

1. Turn on the centrifuge Sorvall RC 6 (rotor Sorvall SM24).
2. Cool the centrifuge to 4 °C.
3. 200–2000 μL plasma (or serum) is pipetted into the Vivaspin 2 ultrafiltration cartridges.
4. Set the ultrafiltration cartridges in the rotor.
5. Centrifuge for 30 min at 8000×*g*.
6. Transfer the ultrafiltrates into 500-μL Eppendorf tubes and store them at −20 °C until further processing.

B. Procedure for sample volumes ranging between 30 and 200 μL

1. Turn on the centrifuge Multifuge 3 S-R (rotor 3332).
2. Cool the centrifuge to 4 °C.
3. 30–200 μL plasma (or serum) is pipetted into the Vivaspin 500 ultrafiltration cartridges.
4. Set the ultrafiltration cartridges in the rotor.
5. Centrifuge for 30 min at 21,000×*g*.
6. Transfer the ultrafiltrates into 500-μL Eppendorf tubes and store them at −20 °C until further processing.

C. Procedure for sample volumes less than 30 μL

1. Turn on the centrifuge Multifuge 3 S-R (rotor 3332).
2. Cool the centrifuge to 4 °C.
3. Dilute plasma (or serum) samples (<30 μL) with water to a final volume of 200 μL. Note the volumes of plasma, serum and water.
4. Pipette the 200-μL samples into the Vivaspin 500 ultrafiltration cartridges.
5. Set the ultrafiltration cartridges in the rotor.
6. Centrifuge for 30 min at 21,000×*g*.
7. Transfer the ultrafiltrates into 500-μL Eppendorf tubes and store them at −20 °C until further processing.

Derivatization of amino acids from plasma or serum samples

Timing: 3–4 h

1a. 10-µL aliquots of plasma or serum ultrafiltrate are transferred to a 1.5-mL glass vial.
1b. 10-µL aliquots of a standard solution of hArg, Arg and ADMA are placed in a 1.5-mL glass vial. *Note*: at this point precise pipetting is very important.
2. Samples 1a and 1b are evaporated to dryness by means of a nitrogen stream.
3a. The residue of sample 1a is reconstituted in 100 µL 2 M HCl in MeOH.
3b. The residue of sample 1b is reconstituted in 100 µL 2 M HCl in d_4-MeOH.
4. The glass vials are tightly closed with crimp caps, placed in a heating block, and heated for 1 h at 80 °C. *Note*: be sure that the crimp caps are tightly closed; otherwise there will be loss of sample.
5. After cooling to room temperature and opening the glass vials, the content of the sample 1b is quantitatively transferred into the glass vial of the sample 1a.
6. To the glass vial of the sample 1b, 100 µL of 2 M HCl in MeOH is added using a new pipette tip, gently shaken, and the whole sample is transferred into the glass vial of sample 1a using the same pipette tip. *Note*: avoid sample loss. Take care for a complete transfer of the samples into a common glass vial. (Time 1 h)
7. The solvent of the mixture is evaporated to dryness by means of a nitrogen stream.
8. The residue is reconstituted in 100 µL of PFPA in EA (1:4, v/v).

!CAUTION: PFPA is hazardous. It is corrosive. Avoid contact with skin and eyes.

9. The glass vial is tightly closed with crimp caps, placed in a heating block, and heated for 30 min at 65 °C. *Note*: be sure that the crimp caps are tightly closed.
10. After cooling to room temperature and opening the glass vial, the mixture is evaporated to dryness by means of a nitrogen stream.
11. The residue is reconstituted with 200 µL borate buffer, immediately thereafter 200 µL toluene is added, the glass vial is sealed, and the mixture is vortexed for 60 s.
12. The glass vial is centrifuged for 5 min at 4500×*g* and 4 °C.
13. About 100 µL of the upper toluene phase is transferred into a conical vial, which is sealed with a crimp cap and stored at 4 °C until GC–MS analysis.

Derivatization of amino acids from urine samples

Timing: 3–4 h, depending up on the number of samples

1a. 10-µL aliquots of native urine are transferred to a 1.5-mL glass vial.
1b. 10-µL aliquots of a standard solution of hArg, Arg and ADMA are placed in a 1.5-mL glass vial. *Note*: at this point precise pipetting is very important.
2. Samples 1a and 1b are evaporated to dryness by means of a nitrogen stream.
3a. The residue of sample 1a is reconstituted in 100 µL 2 M HCl in MeOH.
3b. The residue of sample 1b is reconstituted in 100 µL 2 M HCl in d_4-MeOH.
4. The glass vials are tightly closed with crimp caps, placed in a heating block, and heated for 1 h at 80 °C. *Note*: be sure that the crimp caps are tightly closed.
5. After cooling to room temperature and opening the glass vials, the content of the sample 1b is quantitatively transferred into the glass vial of the sample 1a.
6. To the glass vial of the sample 1b, 100 µL of 2 M HCl in MeOH is added, gently shaken, and the whole sample is transferred into the glass vial of sample 1a using the same pipette tip. *Note*: avoid sample loss. Take care for a complete transfer of the samples into a common glass vial. (Time 1 h)
7. The solvent of the mixture is evaporated to dryness by means of a nitrogen stream.
8. The residue is reconstituted in 100 µL of PFPA in EA (1:4, v/v).

!CAUTION: PFPA is hazardous. It is corrosive. Avoid contact with skin and eyes.

9. The glass vial is tightly closed with crimp caps, placed in a heating block, and heated for 60 min at 65 °C. *Note*: be sure that the crimp caps are tightly closed.
10. After cooling to room temperature and opening the glass vial, the mixture is evaporated to dryness by means of a nitrogen stream.
11. The residue is reconstituted with 200 µL borate buffer, immediately thereafter 1000 µL toluene is added, glass vial is sealed, and the mixture is vortexed vigorously for 60 s.
12. The glass vial is centrifuged for 5 min at 4500×*g* and 4 °C.
13. About 700 µL of the upper toluene phase is transferred into a 1.5-mL autosampler vial which is sealed with a crimp cap and stored at 4 °C until GC–MS analysis.

Table 2 Quantification of the indicated amino acids as methyl ester (Me) pentafluoropropionyl (PFP) derivatives by GC–MS in the SIM mode and by GC–MS/MS in the SRM mode using their trideuteromethyl (d_3Me) ester as internal standard

Amino acid	Derivative	GC–MS (SIM) (*m/z*)	GC–MS/MS (SRM) (*m/z* to *m/z*)
Orn	Me-$(PFP)_2$	418 $(M - 1 \times HF]^-)$	Not determined
d_3Me-Orn	d_3Me-$(PFP)_2$	421 $(M - 1 \times HF]^-)$	Not determined
Arg	Me-$(PFP)_3$	586 $(M - 2 \times HF]^-)$	586 → 295
d_3Me-Arg	d_3Me-$(PFP)_3$	589 $(M - 2 \times HF]^-)$	589 → 298
hArg	Me-$(PFP)_3$	600 $(M - 2 \times HF]^-)$	600 → 366
d_3Me-hArg	d_3Me-$(PFP)_3$	603 $(M - 2 \times HF]^-)$	603 → 366
ADMA	Me-$(PFP)_3$	634 $(M - 1 \times HF]^-)$	634 → 378
d_3Me-ADMA	d_3Me-$(PFP)_3$	637 $(M - 1 \times HF]^-)$	637 → 378
SDMA[a]	Me-$(PFP)_3$	634 $(M - 1 \times HF]^-)$	634 → 246
d_3Me-SDMA	d_3Me-$(PFP)_3$	637 $(M - 1 \times HF]^-)$	637 → 249

[a] The relative retention time of the Me-$(PFP)_3$ derivatives of SDMA to that of ADMA is 0.879 (Tsikas et al. 2011)

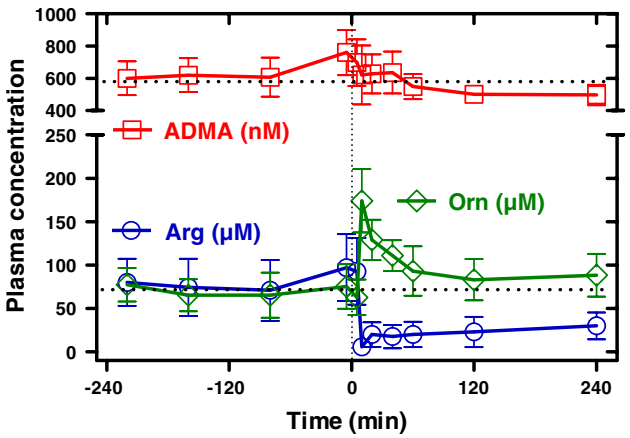

Fig. 4 Time course of the plasma concentration (mean ± SD) of ADMA, Arg and Orn before and after orthotopic liver transplantation in nine patients measured by the protocols described in the present article. This figure was constructed with the data of Table 2 of a previous study (Becker et al. 2009). The time point "0" signifies the time point of the start of the reperfusion of the transplanted liver

Quantification by GC–MS and GC–MS/MS

Timing: about 20 min per sample

Quantification is performed by ECNICI GC–MS in the SIM mode, or by ECNICI GC–MS/MS in the SRM mode. Table 2 summarizes the ions and mass transitions in quantitative analyses. In GC–MS/MS analyses, the same collision energy and collision gas pressure are used.

The peak area (PA) of the methyl ester pentafluoropropionyl derivatives of endogenous amino acids (A) and their IS are calculated either automatically by the software or in the manual mode and used for the determination of concentrations by the Formula (F2):

$$[A] = [IS] \times (PA_A/PA_{IS}) \quad \text{(F2)}$$

whereas [A] is the concentration of the amino acid, [IS] is the concentration of the respective internal standard, PA_A is the peak of the analyte, and PA_{IS} is the peak of the respective internal standard.

Biomedical applications

The L-arginine/NO pathway in orthotopic liver transplantation

The protocols reported in this work have been used in our group in several in vitro and in vivo studies in animals and in clinical studies. An example of a clinical application is shown in Fig. 4. Nine patients with end-stage liver disease underwent orthotopic liver transplantation. Plasma and urine samples of the patents taken before and after transplantation were analyzed for several biochemical parameters including the L-arginine/NO pathway (Becker et al. 2009). The plasma concentrations of ADMA, Arg and Orn were relatively constant before transplantation. Upon reperfusion (time point "0") of the transplanted liver, the plasma concentration of Arg decreased to about 5 μM, while concomitantly the plasma Orn concentration increased greatly to a maximum value of about 170 μM, indicating arginase activity most likely released by the reperfused liver. The ADMA plasma concentration increased slightly upon reperfusion as well. After reperfusion, Arg plasma concentration increased slowly, while the plasma concentration of Orn and ADMA decreased continuously with time.

Like other amino acids, Orn derivatization with 2 M HCl in MeOH yields the methyl ester of which the two primary amine groups are acylated with PFPA to form Me-$(PFP)_2$ derivatives. ECNICI of the Me-$(PFP)_2$ derivative of Orn produces intense ($[M - 1 \times HF]^-$ and $[M - 2 \times HF]^-$) ions (Fig. 5; Table 2) which are useful for GC–MS quantification in the SIM mode.

The L-arginine/NO pathway in a mouse aorta transplantation model

Recently, we showed that physical exercise reduces transplant arteriosclerosis in a mouse aorta transplantation model (Sommer et al. 2015). In blood samples of this study, we measured the synthesis of ^{15}NO and L-[*guanidine*-$^{15}N_2$]-homoarginine ($^{15}N_2$-hArg) from L-[*guanidine*-$^{15}N_2$]-arginine given in drinking water as described elsewhere (Tsikas 2004) with some modifications. The major modification

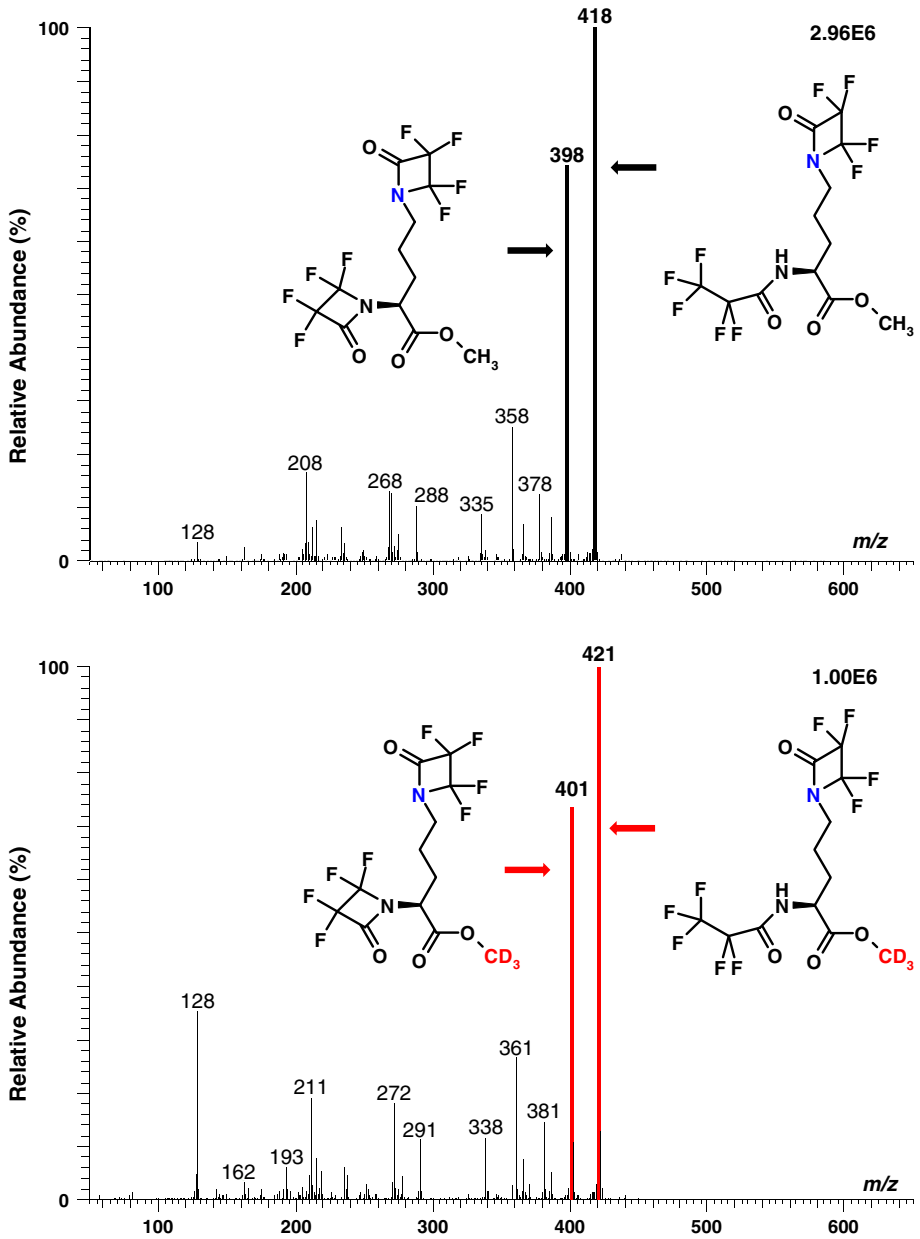

Fig. 5 GC–MS spectra of PFP derivatives of unlabeled Orn (*upper panel*) and trideuteromethyl ester (*lower panel*) in the ECNICI mode. *Insets* show the proposed structures for the ions *m/z* 418, *m/z* 398, *m/z* 421, and *m/z* 401

was that L-[*guanidine*-$^{15}N_2$]-arginine was orally administered for 4 weeks after transplantation of segments of the thoracic aorta from C57.Bl6 (H2b) or C3H.HeJ (H2k) mice into the abdominal aortas of CBA.Ca mice (H2k), resulting in two groups, i.e., BL6 → CBA and C3H → CBA, respectively. Each one group of mice underwent physical exercise (E), the second, non-exercising animal group (nE) did not and served as a control. Blood samples obtained after 2, 3 and 4 weeks of transplantation were analyzed for unlabeled and ^{15}N-labeled nitrite and nitrate by GC–MS (Tsikas 2000); L-arginine and L-[*guanidine*-$^{15}N_2$]-arginine were analyzed by GC–MS (Tsikas et al. 2003). $^{15}N_2$-hArg and unlabeled hArg ($^{14}N_2$-hArg) were analyzed in 1-µL blood samples by GC–MS/MS after derivatization. SRM of *m/z* 602 to *m/z* 368 for $^{15}N_2$-hArg and *m/z* 600 to *m/z* 366 for $^{14}N_2$-hArg was performed. The peak area values of the analytes were determined and the peak area ratio (PAR) values of ^{15}N to ^{14}N for each analyte were calculated. In these analyses, no internal standards were added to the samples for nitrite, nitrate, Arg and hArg.

In all blood samples analyzed the PAR values for Arg, nitrite and nitrate were above the natural abundance of the *N* isotopes, indicating that L-[*guanidine*-$^{15}N_2$]-arginine was absorbed and converted to ^{15}NO by all mice (data not shown). Interestingly, in the BL6 → CBA group but not in the C3H → CBA group the PAR of ^{15}N-nitrite to ^{14}N-nitrite as well as the PAR of ^{15}N-nitrate to ^{14}N-nitrate was at some time points higher in the exercising groups compared to

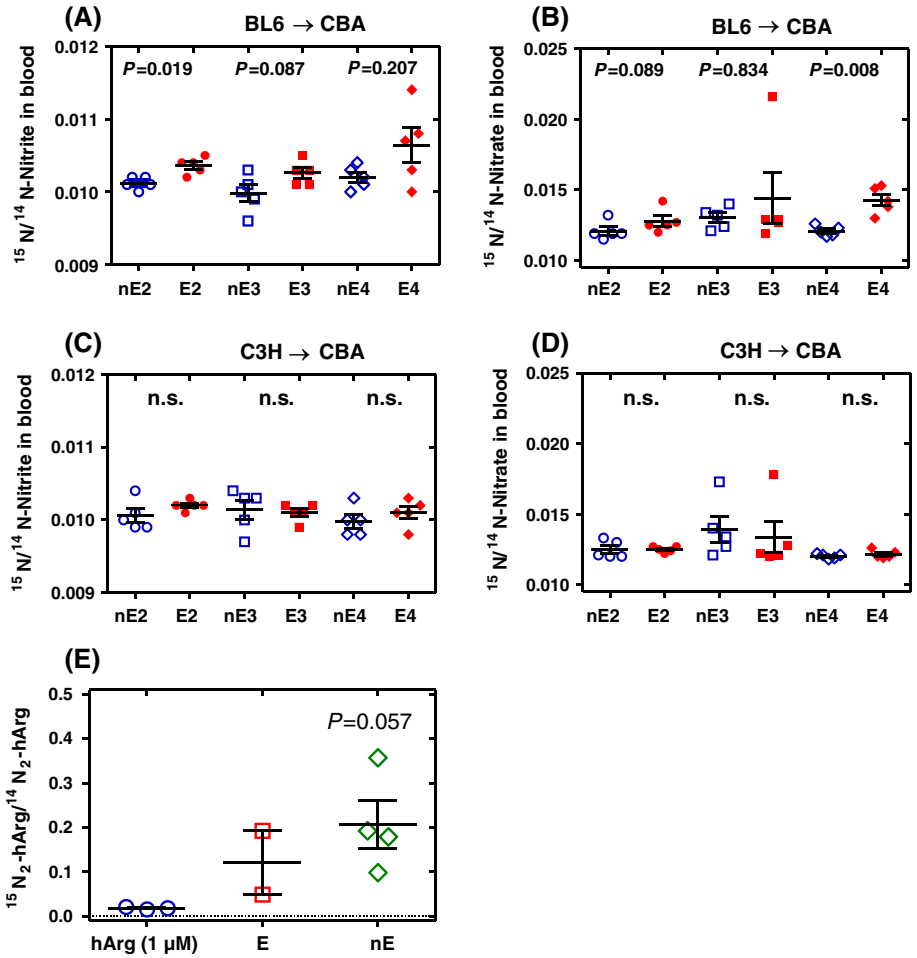

Fig. 6 Effects of physical exercise on the metabolism of L-[*guanidine*-$^{15}N_2$]-arginine to NO (**a**–**d**) and L-[*guanidine*-$^{14}N_2$]-homoarginine (E) in transplanted mice. Mice ($n = 5$ per group) drank L-[*guanidine*-$^{15}N_2$]-arginine-containing drinking water (100 µg/mL, 568 µM) for 4 weeks. Segments of the thoracic aorta from C57.Bl6 (H2b) mice were transplanted into the abdominal aortas of CBA.Ca mice (H2k), i.e., group BL6 → CBA. Similarly, segments of the thoracic aorta from C3H.HeJ (H2k) mice were transplanted into the abdominal aortas of CBA.Ca mice (H2k), i.e., group C3H → CBA. Each one group underwent physical exercise (E, *closed symbols*), the second non-exercising animal group (nE, *open symbols*) served as a control. Blood samples were obtained at weeks 2, 3 and 4 (*numbers behind the symbol E*) after transplantation and were analyzed for ^{15}N-nitrite, ^{14}N-nitrite, ^{14}N-nitrate and ^{15}N-nitrate by GC–MS (Tsikas 2000). L-[*guanidine*-$^{15}N_2$]-Homoarginine ($^{15}N_2$-hArg) and L-[*guanidine*-$^{14}N_2$]-homoarginine ($^{14}N_2$-hArg) were measured in some of these samples by GC–MS/MS after derivatization as described in the present protocol. SRM of *m/z* 602 to *m/z* 368 for $^{15}N_2$-hArg and *m/z* 600 to *m/z* 366 for $^{14}N_2$-hArg was performed. The peak area ratios of ^{15}N to ^{14}N were calculated for nitrite, nitrate and hArg, and compared by the Mann–Whitney test. This study was performed as described elsewhere (Sommer et al. 2015). *n.s.* not significant

non-exercising groups (Fig. 6a–d), suggesting that exercise increased the formation of ^{15}NO from L-[*guanidine*-$^{15}N_2$]-arginine. This finding is in agreement with functional and morphological observations which indicate that physical exercise reduces the extent of arteriosclerosis in this mouse aorta transplantation model (Sommer et al. 2015). With respect to L-[*guanidine*-$^{15}N_2$]-arginine there were no differences between the groups, indicating similar absorption rates of L-[*guanidine*-$^{15}N_2$]-arginine from the drinking water.

Of the 15 blood samples (each about 1 µL) available for hArg analysis, we found $^{15}N_2$-hArg and $^{14}N_2$-hArg only in two E and in four nE mice (Fig. 6e). A concomitantly analyzed standard sample of unlabeled hArg (i.e., 1 µL, 1 µM) corresponding to 1 pmol hArg yielded a PAR value of 0.0179 ± 0.0015 ($n = 3$). The PAR values $^{15}N_2$-hArg/$^{14}N_2$-hArg in the E and nE mice were 0.1206 ± 0.0718 ($n = 2$) and 0.2064 ± 0.0543 ($n = 4$). Statistical comparison of these PAR values is not possible. Mann–Whitney test between the hArg standard (which provides the ratio of the natural abundance of $^{15}N_2$-hArg and $^{14}N_2$-hArg) and the nE data indicates that the difference between these groups failed very nearly statistical significance ($P = 0.057$). These data suggest that use of L-[*guanidine*-$^{15}N_2$]-arginine

and GC–MS/MS analysis of $^{15}N_2$-hArg may be useful as an AGAT activity assay in vivo. Further studies and larger blood volumes are required to address the question whether hArg protects from arteriosclerosis and whether the beneficial effect of exercise is due to hArg.

Conclusions

Analysis of amino acids by GC–MS or GC–MS/MS requires preceding derivatization to form volatile and thermally stable derivatives. Here, we report protocols for the accurate, precise and sensitive quantitative determination of the guanidine amino acids Arg and hArg and their metabolites of guanidine methylation, namely ADMA and SDMA, and of Orn, the arginase metabolite of Arg in various biological samples including human plasma and urine. Quantification is performed using de novo synthesized trideuteromethyl esters of the respective synthetic amino acids serving as internal standards. This synthesis is performed in a solution of 2 M HCl in CD_3OD methanol and is generally applicable to amino acids. These protocols are equally useful in vitro and in vivo studies in animals and humans.

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

Ethical standard All animals received humane care in compliance with the German animal protection legislation, the Principles of Laboratory Animal Care, and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council. Principles of laboratory animal care, and the guide for the care and use of laboratory animals. Washington, DC: National Academies Press; 1996). The study was approved by the government board for animal welfare of Lower Saxony, Germany.

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