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ORIGINAL ARTICLE

Analysis of L-homoarginine in biological samples by HPLC involving precolumn derivatization with *o*-phthalaldehyde and *N*-acetyl-L-cysteine

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Abstract L-Homoarginine (hArg) may play a role in regulating the metabolism of its structural homologue L-arginine via multiple pathways (including nitric oxide synthase) in animals. Accurate measurement of hArg is essential for studying its synthesis and utilization by cells and the whole body. Here, we describe a simple, sensitive and automated method for analysis of hArg in biological samples by high-performance liquid chromatography involving precolumn derivatization with *o*-phthalaldehyde (OPA) and *N*-acetyl-L-cysteine (NAC) as the thiol. The hArg–OPA–NAC derivative was separated at 25 °C on a reversed-phase C_{18} material and detected by fluorescence at excitation and emission wavelengths of 340 and 450 nm, respectively. The total running time for one sample (including the time for column regeneration) was 20 min, with the retention time for hArg being 10.03 min. The limit of detection was 188 fmol hArg, which was equivalent to 12 nM hArg in the 160-µl assay mixture. The assay was linear between 1.0 and 80 pmol hArg injected into the HPLC column (equivalent to 0.0625 and 5 µM hArg in the 160-µl assay mixture, respectively). The precision (relative deviation, %) and bias (relative error, %) of the HPLC method were 0.52–1.16 and 0.42–1.12, respectively, for aqueous solutions of hArg and for various biological samples (e.g., plasma, liver, brain and kidney). This is a highly sensitive, accurate, efficient and easily automated method for analysis of hArg in biological samples and provides a useful tool for studying the biochemistry, nutrition, physiology and pharmacology of hArg and arginine in animals and humans.

Keywords L-Homoarginine · Derivatization · *o*-Phthalaldehyde · *N*-Acetyl-L-cysteine · HPLC

Abbreviations

AA	Amino acids
AGAT	L-Arginine:glycine amidinotransferase
hArg	L-Homoarginine
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LOD	Limit of detection
MS	Mass spectrometry
NAC	*N*-Acetyl-L-cysteine
NOS	Nitric oxide synthase
OPA	*o*-Phthaldialdehyde
SPE	Solid-phase extraction

Introduction

L-Homoarginine (hArg), a structural homologue of L-arginine, was first synthesized chemically 90 years ago (Steib 1926) and subsequently identified as a naturally occurring substance in plants (Bell 1962). Compared with L-arginine, hArg has an additional $-CH_2$ group on its main carbon chain. Synthesis of hArg by rats and humans was discovered by Ryan and Wells (1964), with the major sites of synthesis including liver and kidneys (Ryan et al. 1968, 1969). In animals, L-arginine:glycine amidinotransferase (AGAT) may play a role in synthesizing hArg from L-arginine and L-lysine (Davids et al. 2012a, b; Ryan et al.

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1969). Concentrations of hArg in plasma are relatively low (approximately 2 μM) in healthy humans and may increase up to 20 μM in hyperargininemic patients (Marescau et al. 1985).

Research over the past half century led to new knowledge of hArg biochemistry and physiology in animals and humans (Davids et al. 2012a, b; Kayacelebi et al. 2015; Lustig and Kellen 1971; May et al. 2015; Michel 2013; Nunn et al. 2010). Of interest, hArg has been reported to inhibit L-arginine transport by cells (Greene et al. 1993), arginase (Hrabák et al. 1994), as well as liver and bone alkaline phosphohydrolases (Lin and Fishman 1972), while serving as a substrate for nitric oxide (NO) synthase (NOS) (Moali et al. 1998). Whether hArg has a beneficial or an adverse effect on NO production likely depends on cell type, extracellular and intracellular concentrations of L-arginine, and activities of competing pathways for hArg and L-arginine metabolism (Atzler et al. 2015; Khalil et al. 2013; Michel 2013; Tsikas and Kayacelebi 2014). Clearly, extensive investigation is warranted to address this important issue. Nonetheless, emerging evidence shows that low concentrations of hArg in plasma are associated with a high risk of cardiovascular disease in humans and animals (Atzler et al. 2015).

An accurate and sensitive method for the measurement of hArg is essential for studying its synthesis and utilization by cells and the whole body. This is a challenging task for the following reasons: First, concentrations of hArg in animal or human tissues are relatively low (May et al. 2015; Ryan and Wells 1964). Second, hArg itself is neither a chromophore nor a fluorophore and, therefore, cannot be directly analyzed by spectrophotometric or fluorescent detection methods. To date, many techniques have been developed to determine hArg in biological samples (Martens-Lobenhoffer and Bode-Böger 2014). These methods include: (1) gas chromatography (GC)–mass spectrometry (MS) and GC–tandem MS (Kayacelebi et al. 2014); (2) liquid chromatography (LC)–electrospray ionization tandem MS (Atzler et al. 2011; Davids et al. 2012a, b; Di Gangi et al. 2010, 2012; Martens-Lobenhoffer et al. 2013; Midttun et al. 2013; Servillo et al. 2013); (3) ninhydrin-based automated amino acid analyzer with UV absorbance detection (Marescau et al. 1985); (4) capillary zone electrophoresis with UV absorbance detection (Zhao et al. 1999); and (5) high-performance liquid chromatography (HPLC) involving precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor) (Jones et al. 2010), with phenyl isothiocyanate derivatization (Khan et al. 1994), or with *o*-phthalaldehyde (OPA) plus 3-mercaptopropionic acid (Teerlink et al. 2002).

The MS-based approaches require expensive instruments and are limited to only specialized laboratories (Martens-Lobenhoffer and Bode-Böger 2014), whereas UV absorbance detection in capillary zone electrophoresis, amino acid (AA) analyzers and HPLC analysis affords low sensitivity of detection (8 μM) (Khan et al. 1994; Marescau et al. 1985; Zhao et al. 1999). While HPLC equipment is more widely available in laboratories than MS, all of the current HPLC-based methods require preparation of biological samples using solid-phase extraction (SPE) cartridges and subsequent solvent evaporation. Also, the total running time (including the time for column regeneration) for each sample is rather long (e.g., 1 h or longer) (Jones et al. 2010; Khan et al. 1994). Other significant drawbacks of these HPLC methods are tedious and time-consuming procedures, additional workload, increased risk of analytical errors and variations due to multiple steps of sample preparation, and high costs of the single use of SPE cartridges. In this study, we describe a simple, sensitive and automated HPLC method for the analysis of hArg in biological samples, which involves precolumn derivatization with OPA in the presence of *N*-acetyl-cysteine (NAC) (Fig. 1) and the fluorescence detection of the hArg–OPA–NAC derivative.

Materials and methods

The sources of HPLC-grade water, HPLC-grade methanol, hArg, OPA, NAC and other chemicals, as well as HPLC columns, were the same as previously described (Dai et al. 2014a, b). Plastic tubes (Fisher Scientific) were used to prepare hArg standard and store extracts of biological samples.

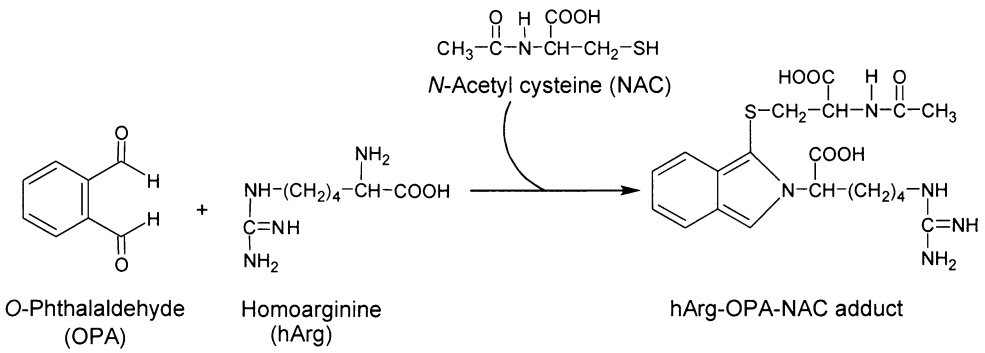

Fig. 1 Reaction of L-homoarginine (hArg) with OPA in the presence of NAC to yield a highly fluorescent hArg–OPA–NAC derivative under alkaline conditions

Equipment

The Waters HPLC apparatus used for this study consisted of the following: (1) a Model 600E Powerline multisolvent delivery system with 100-µl heads; (2) a Model 600E system controller; (3) a Model 717plus autosampler (or a Model 712 WISP autosampler); (4) an analytical column (Supelco 3-µm C_{18} column, 150 × 4.6 mm I.D.) protected by a guard column (Supelco 40-µm C_{18} column, 50 × 4.6 mm I.D.); (5) a Model 2475 Multi λ fluorescence detector (or a Model 420-AC fluorescence detector); and (6) a Millenium-32 Workstation or a Model 810 Baseline Workstation (Waters Inc., Milford, MA). All mobile phase solutions were degassed with helium for 30 min before use. To prevent accumulation of salts in the HPLC system, both columns and pumps were thoroughly washed with HPLC-grade water for at least 20 min at 1.1 ml/min and then with methanol at 1.1 ml/min for at least 20 min before and after running a sample set. Additionally, the HPLC columns were equilibrated with 86 % solvent A and 14 % solvent B (see below) for 15 min at the flow rate of 1.1 ml/min, and the autosampler was purged with HPLC-grade water twice before starting the run of a sample set.

Preparation of reagents and HPLC solvents

The OPA–NAC reagent solution (30 mM OPA, 23.7 mM NAC, 40 mM sodium borate and 3.1 % Brij-35; pH 9.5) was prepared by mixing 50 mg OPA (stored at 4 °C), 50 mg NAC and 1.25 ml methanol in a brown bottle, to which was added 11.2 ml of 40 mM sodium borate buffer (pH 9.5) and 0.4 ml Brij-35. The solution was mixed thoroughly, stored at 4 °C and used within 36 h after preparation. Benzoic acid (1.2 %) was prepared by adding 8.4 g benzoic acid (an antibiotic) to 525 ml HPLC-grade water, followed by addition of 175 ml of saturated $K_2B_4O_7$ (prepared in H_2O). Solvent A (0.1 M sodium acetate; pH 7.2) was prepared by adding 27.3 g sodium acetate (trihydrate) and 96 µl of 6 M HCl to 1.6 L HPLC-grade water in a glass bottle, followed by addition of 180 ml methanol, 10 ml tetrahydrofuran and 210 ml HPLC-grade water. Solvent B was 100 % HPLC-grade methanol.

Animals

Male Sprague–Dawley rats (Harlan Laboratories, Houston, Texas, USA) were used to provide plasma and tissues for the present study. Between 5 and 9 weeks of age, the animals were housed individually at 25 °C and had free access to drinking water and a semi-purified diet, as we described previously (Jobgen et al. 2009). At 9 weeks of age (300 to 320 g body weight), rats were anesthetized with CO_2 and killed by cervical dislocation. Cardiac blood samples (0.5 ml/tube) were withdrawn into heparinized tubes and transferred into 1.5-ml microcentrifuge tubes, which were immediately centrifuged at 10,000×*g* for 1 min for collection of the supernatant fluid (plasma). In addition, liver, kidney and brain were rapidly dissected from the rats. Tissue samples (approximately 1 g each) were immediately placed in liquid nitrogen. Both plasma and tissues were stored at −80 °C until analysis within one week.

Preparation of plasma samples for hArg analysis

An aliquot of plasma sample (60 µl) was mixed with 60 µl of 1.5 M $HClO_4$, followed by addition of 30 µl of ice-cold 2 M K_2CO_3. The tube was centrifuged at 10,000×*g* and 25 °C for 1 min. The supernatant fluid was directly used for hArg analysis.

Preparation of tissue samples for hArg analysis

A portion of frozen tissue (e.g., 50 mg liver, brain or kidney) was homogenized at 25 °C with a battery-powered plastic pestle in 100 µl of 1.5 M $HClO_4$, followed by addition of 50 µl of 2 M K_2CO_3. The solution was transferred to a 1.5-ml microcentrifuge tube, which was centrifuged at 10,000×*g* for 1 min. The supernatant fluid was directly used for hArg analysis.

Procedure for tissue AGAT enzyme assays to generate hArg

hArg was generated from arginine and lysine by AGAT in tissue homogenate, as described previously (Davids et al. 2012a, b; Ryan et al. 1969), with modifications. Briefly, a portion of frozen tissue (e.g., 50 mg liver, brain or kidney) was homogenized at 25 °C with a battery-powered plastic pestle in 250 µl of 80 mM sodium phosphate buffer (pH 7.5). The whole homogenate was used for AGAT enzyme assays. The sample tube consisted of 50 µl of 30 mM L-arginine plus 30 mM L-lysine, and the reaction was initiated by addition of 50 µl tissue homogenate. After a period of 15-min incubation at 37 °C, 50 µl of 1.5 M $HClO_4$ was added to the sample tube, followed by neutralization with 25 µl of 2 M K_2CO_3. The blank was run in parallel except that 50 µl of 1.5 M $HClO_4$ was added to the blank tube 2 min before the addition of tissue homogenate. All tubes were centrifuged at 10,000×*g* for 1 min, and the supernatant fluid was used directly for hArg analysis. The data on hArg production are used to determine tissue AGAT activity.

HPLC analysis of hArg

All procedures were performed at room temperature (25 °C). For hArg standard, 10 µl of 0–50 µM hArg, 140 µl HPLC-grade water and 10 µl of 1.2 % benzoic acid (a total volume of 160 µl) were added to a 250-µl microinsert

placed within a 4-ml glass vial. For the analysis of hArg in plasma or brain extracts, 50 µl sample, 100 µl HPLC-grade water and 10 µl of 1.2 % benzoic acid (a total volume of 160 µl) were added to a 250-µl microinsert placed within a 4-ml glass vial. For the analysis of hArg in tissue (e.g., liver and kidney) extracts or in AGAT assay preparations, 10 µl sample, 140 µl HPLC-grade water and 10 µl of 1.2 % benzoic acid (a total volume of 160 µl) were added to a 250-µl microinsert placed within a 4-ml glass vial. The vials were vortexed for 30 s and then placed onto the autosampler. An aliquot (15 µl) of the 160 µl of a sample or hArg standard assay solution was programmed to mix automatically with 15 µl OPA in the derivatization loop. Exactly after 1-min mixing, the hArg–OPA–NAC solution (30 µl) was injected into the HPLC column. The 20-min solvent gradient for hArg separation (including the time for column regeneration after the run of each sample), expressed as % of solvent B, was as follows: 0–8 min, 14; 10–12 min, 100; 12.1–20 min, 14. Fluorescence of the hArg–OPA–NAC derivative was monitored at excitation and emission wavelengths of 340 and 450 nm, respectively.

Determination of the recovery of hArg from biological samples as well as the precision and accuracy of hArg analysis

Rates of the recovery of hArg from water, plasma, as well as brain, kidney or liver homogenates, were determined by adding either 100 µl of water or hArg standard (5 or 20 µM) to 100 µl of water, plasma or tissue (e.g., brain, kidney and liver) homogenates. The samples were then processed for hArg analysis as described previously. Recovery (%) of hArg from the sample was calculated as (Concentration of total hArg in the sample with added hArg standard − Concentration of hArg in the unspiked sample)/Concentration of hArg added to the sample × 100. For determining the precision and accuracy of hArg analysis in water, plasma or tissue (e.g., brain, kidney and liver) homogenates, 10 pairs of replicates (2 tubes/replicate) were run by adding 100 µl of hArg standard (5 or 20 µM) to 100 µl of water, plasma or tissue (e.g., brain, kidney and liver) homogenates (Stenesh 1984). The samples were then processed for hArg analysis, as described previously.

Statistical analyses

Results are expressed as the mean ± SEM. Statistical analyses of data were performed by one-way analysis of variance using the general linear model procedures (Assaad et al. 2014). Differences among treatment means were determined using the Student–Newman–Keuls multiple comparison method (Assaad et al. 2014). A probability value was ≤ 0.05, which was taken to indicate statistical significance.

Results and discussion

HPLC separation of hArg

Representative HPLC chromatograms for the hArg standard alone and a mixture of hArg plus other AA standards are illustrated in Fig. 2. hArg was satisfactorily separated from other AA using a 20-min gradient program (including the time for column regeneration for the next sample on the automated system). Our analysis time per sample (a total of 20 min) is much shorter than that (approximately 1 h or longer) for the previous HPLC- or ninhydrin-based methods (Jones et al. 2010; Khan et al. 1994; Marescau et al. 1985). No peak for hArg appeared in the water blank or a mixture of all AA standards without hArg. The retention time of hArg was 10.03 ± 0.024 min (mean ± SEM, $n = 10$). OPA itself does not yield fluorescence, and substances without a primary amino group cannot react with OPA to form a fluorescent derivative (Campíns-Falcó et al. 2001). Thus, only those molecules with a primary amino group (e.g., hArg and proteinogenous AA) were detected in our analysis, and very stable baselines were obtained for HPLC chromatograms.

Using the method described herein, we successfully determined hArg in samples from rats, including the plasma, liver and kidney (Fig. 3). This HPLC technique was also very useful to determine AGAT activity in animal tissues. Only a negligible amount of hArg was detected in blank samples (e.g., when AGAT in tissue extracts was denatured by 1.5 M $HClO_4$ before addition of enzyme substrates), as shown for the blank in AGAT assays involving acidified extracts of the rat liver (Fig. 4). In contrast, we detected relatively large amounts of hArg generated by AGAT during 15-min enzyme assays involving buffer-prepared extracts from the rat liver and kidney (Fig. 4). The present HPLC method for hArg analysis is free of interference by other AA or molecules (including ammonia, 3-methylhistidine, carnosine, glutathione and γ-aminobutyrate) in animal tissues and in extracts of AGAT assay preparations.

One guard column and one analytical column can be used to analyze at least 500 biological samples. Of particular note, our HPLC method did not require any use of SPE cartridges to extract hArg from plasma or tissues, as necessitated in all the previous MS- and LC-based techniques (Martens-Lobenhoffer and Bode-Böger 2014). Thus, the derivatization of hArg with OPA in the presence of NAC, followed by HPLC separation and fluorescence detection, allows for the determination of hArg in complex mixtures of proteins, peptides and other substances in biological samples.

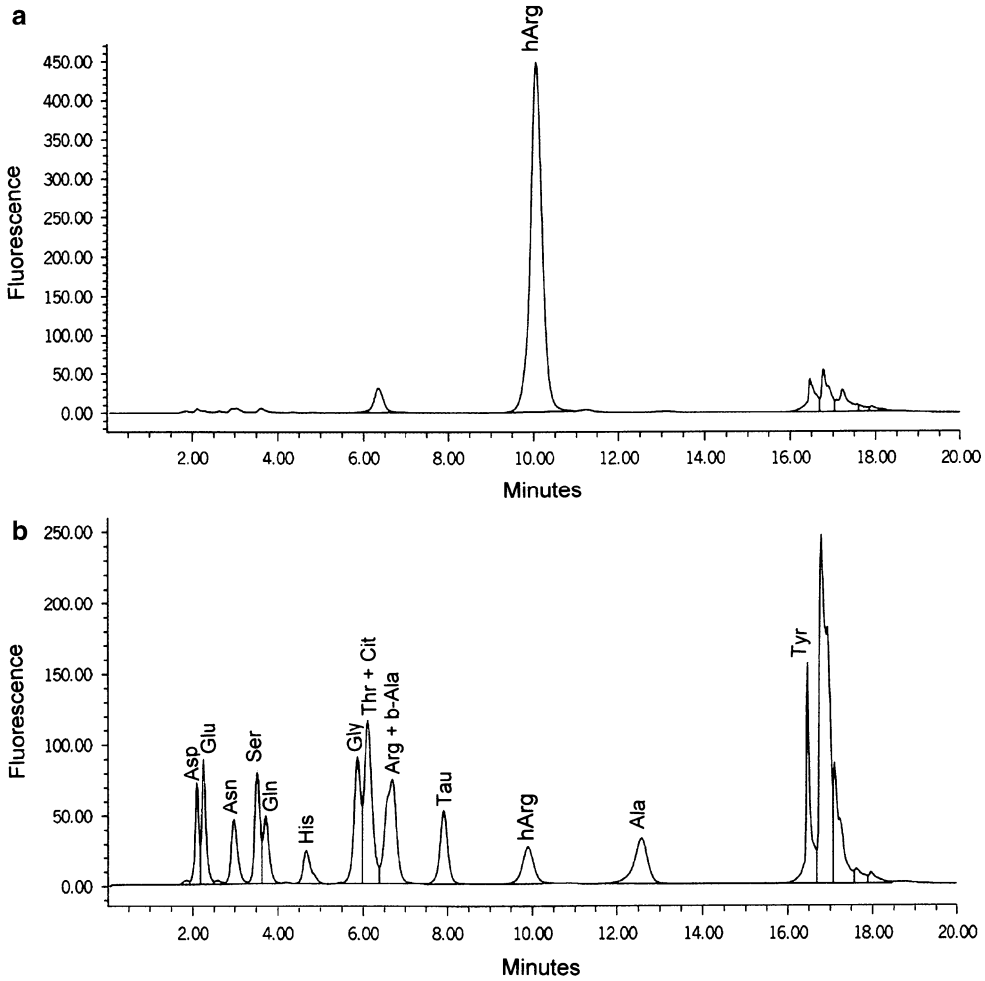

Fig. 2 Representative HPLC chromatograms for L-homoarginine (hArg) standard in aqueous solutions in the absence (**a**) or presence (**b**) of other AA standards. In a 160-µl assay mixture, hArg standard (2 µM; **a**) or hArg plus other AA (0.12 µM each; **b**) found in physiological fluids and protein reacted with OPA in the presence of NAC to yield highly fluorescent derivatives. Non-hArg AA standards were alanine (Ala), β-alanine (b-Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), citrulline (Cit), cysteine, glutamate (Glu), glutamine (Gln), glycine (Gly), histidine (His), hydroxyproline, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine (Ser), taurine (Tau), threonine (Thr), tryptophan, tyrosine (Tyr) and valine. The hArg–OPA–NAC derivative was well separated on a reversed-phase C_{18} column and detected by fluorescence. Peak areas for hArg in **a** and **b** were 8.53×10^6 and 0.49×10^6, respectively. Cysteine, hydroxyproline or proline did not react with OPA under the conditions employed in the present study. OPA–NAC derivatives for isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, tryptophan and valine were eluted from the HPLC column between 17 and 19 min of the automated program

Stability, linearity and detection limit of the hArg–OPA–NAC derivative

In separate solutions, hArg, OPA and NAC are stable at 25 °C for 12.5 h (Dai et al. 2014a, b). However, hArg–OPA–NAC derivative, like other AA-OPA-NAC derivatives, is not known to be stable (Campíns-Falcó et al. 2001). Thus, an autosampler is essential for obtaining high reproducibility and accuracy for the OPA-based analysis of AA (Wu and Meininger 2008). In our automated method, hArg was programmed to react with OPA in the presence of NAC for exactly 1 min, and the hArg–OPA–NAC derivative was then immediately injected into the HPLC column without any delay. In one set of 32 hArg standard vials (each having the same concentration), hArg peak areas did not differ between the first and last vials. Similar results were obtained for rat samples (e.g., plasma, liver and kidney). Thus, a sample set consisting of two hArg standard vials (one at the beginning and one at the end), and 30 biological sample vials can be run without affecting the precision and accuracy of our HPLC method.

The limit of detection (LOD, defined as a signal-to-noise ratio of 3:1) was 188 fmol hArg, which was the equivalent of 12 nM hArg in the 160-µl assay mixture contained in

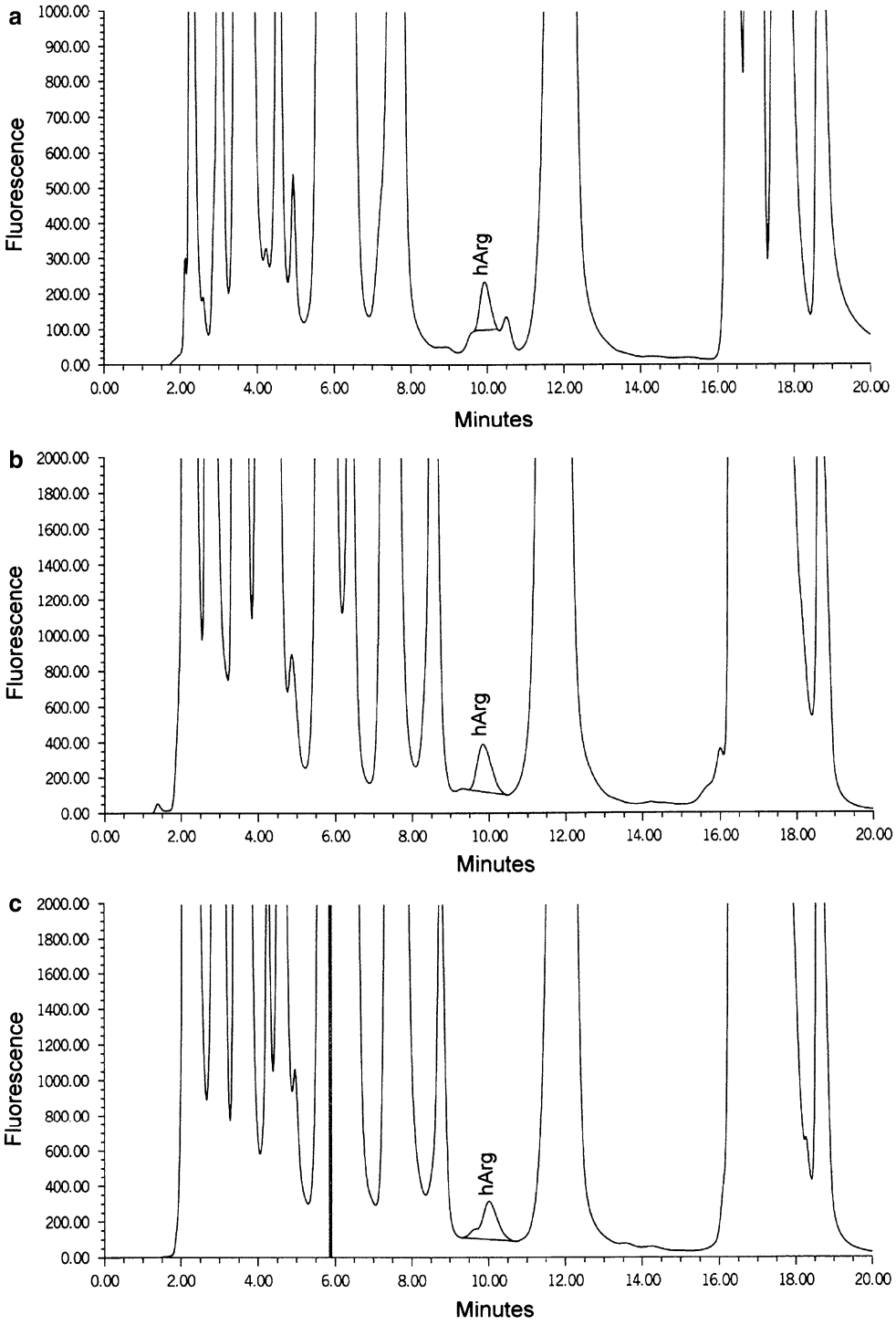

Fig. 3 Representative HPLC chromatograms for the analysis of L-homoarginine (hArg) in rat plasma (**a**), liver (**b**) and kidney (**c**). Plasma (60 µl) was deproteinized with an equal volume of 1.5 M $HClO_4$, while a liver or kidney sample (50 mg) was homogenized in 100 µl of 1.5 M $HClO_4$. Neutralized extracts were used directly for hArg analysis, as described in text. hArg reacted with OPA in the presence of NAC to yield the hArg–OPA–NAC derivative, which was separated on a reversed-phase C_{18} column and detected by fluorescence. Peak areas for hArg in **a**, **b** and **c** were 2.43×10^6, 6.86×10^6 and 6.27×10^6, respectively

the microinsert of the HPLC vial. The assay was linear between 1.0 and 80 pmol hArg injected into the HPLC column when known amounts of hArg standard were added to water (HPLC-grade), plasma, liver, brain and kidney, with correlation coefficiencies (r^2) of 0.998, 0.993, 0.995, 0.997 and 0.996, respectively. The amounts of 1.0 and 80 pmol hArg injected into the HPLC column were equivalent to 0.0625 and 5 µM in the 160-µl assay mixture contained in the microinsert of the HPLC vial. Thus, our HPLC method is more sensitive than the UV absorbance detection-based capillary zone electrophoresis (LOD, 8 µM; Zhao et al. 1999), the AccQ-Fluor method (LOD, 0.3 µM) and the UV absorbance detection-based phenyl isothiocyanate method (LOD, 8 µM) (Khan et al. 1994).

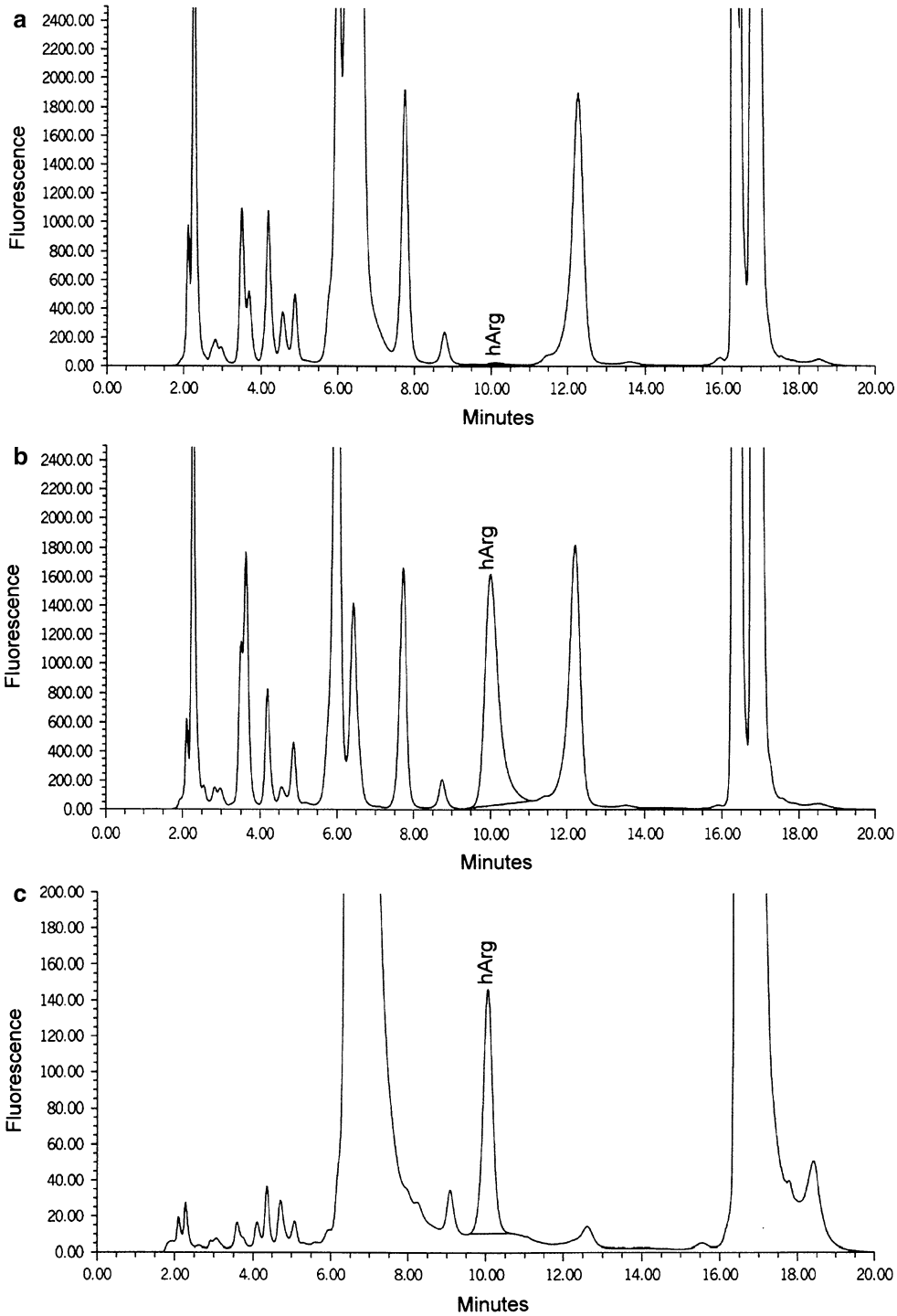

Fig. 4 Representative HPLC chromatograms for L-homoarginine (hArg) generated by AGAT in rat tissues. AGAT assays were performed with extracts from the acid-treated liver (**a**) and from buffer-prepared liver (**b**) and kidney (**c**) homogenate. In the acid-treated liver homogenate, AGAT was inhibited by 1.5 M $HClO_4$, followed by addition of enzyme substrates. In the buffer-prepared tissue homogenate, AGAT assays were performed as described in text. Neutralized extracts were directly used for hArg analysis, as described in text. hArg reacted with OPA in the presence of NAC to yield the hArg–OPA–NAC derivative, which was separated on a reversed-phase C_{18} column and detected by fluorescence. A negligible amount of hArg appeared in the AGAT assay mixture containing an acid-treated liver sample (the blank), as compared with the buffer-prepared liver homogenate. Peak areas for hArg in **a**, **b** and **c** were 0.32×10^6, 4.08×10^6 and 2.40×10^6, respectively

Recovery of hArg from biological samples as well as the precision and accuracy of hArg analysis

The rates of recovery of hArg from plasma or tissue (e.g., kidney, brain and liver) homogenate were approximately 97 % (Table 1), which were greater than the values of 82–88 % reported for the use of SPE cartridges (Davids et al. 2012a, b; Martens-Lobenhoffer and Bode-Böger 2014; Teerlink et al. 2002). The precision (agreement between replicate measurements) of the HPLC method, as evaluated by the relative deviation (the mean of absolute deviation/the mean of replicate measurements × 100 %) (Stenesh 1984) for hArg analysis, was 0.52–1.16 % in water, plasma or tissue (kidney, brain and liver) homogenate (Table 1). The accuracy (the closeness of an experimental value to the true value) of the HPLC method, as determined with

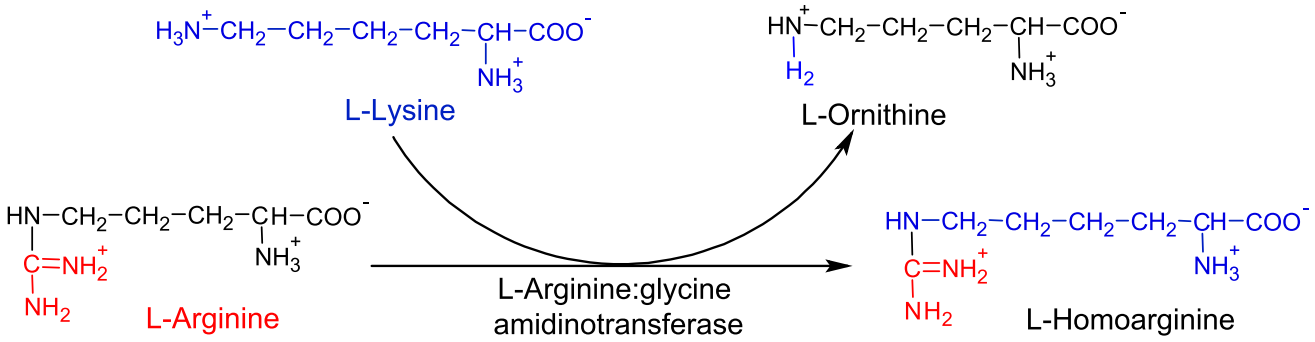

Fig. 5 Synthesis of L-homoarginine from L-arginine and L-lysine in animal tissues. L-Arginine:glycine amidinotransferase is known to transfer the amidino group from L-arginine to glycine to generate guanidinoacetate in the mitochondria of kidneys, pancreas, and liver. This enzyme can also catalyze the transfer of the amidino group from L-arginine to the ε-amino group of L-lysine to form L-homoarginine in animal tissues (e.g., kidney and liver). L-Ornithine is a co-product of the reaction

known amounts of hArg standards and expressed as the relative error (bias) [(measurement value − true value)/true value × 100 %)] (Stenesh 1984), was 0.42–1.12 % in water and the above biological samples (Table 1). The precision and accuracy of our new HPLC method are comparable to those reported for MS-based techniques for hArg determination (Martens-Lobenhoffer and Bode-Böger 2014). Intra-day and inter-day variations for hArg analysis, which were measured with 2.5 and 10 μM hArg standard ($n = 10$) and calculated as variation coefficients [(mean/standard deviation) × 100 %], were 0.47 and 0.64 %, respectively. These results indicate the high reproducibility and reliability of our method for analysis of hArg not only in water, but also in biological samples.

Application of the HPLC method to quantitative analysis of hArg in biological samples

A limitation of hArg analysis using UV absorbance detection-based methods is their low sensitivity. Thus, large amounts of biological samples are generally required to obtain sufficient amounts of hArg for its quantification. With the improvement of LOD by >100 times over the UV absorbance detection-based techniques, our highly sensitive HPLC method allows for the analysis of hArg in 60 μl plasma or 50 mg animal tissue. The amount of a biological sample can be further reduced when the volumes of the acid and base employed for sample processing are proportionately decreased.

Using the external standard calibration (Dai et al. 2014b), we could readily quantify hArg in various biological samples. Data on hArg concentrations in the rat plasma, brain, kidney and liver, as well as AGAT activity in these tissues, are summarized in Table 2. This mitochondrial enzyme catalyzes the transfer of the amidino group from L-arginine to L-lysine to form hArg, with L-ornithine being a product (Fig. 5). Assuming that water content is 70 % in brain, kidney and liver, concentrations of hArg are estimated to be approximately 1.5, 100 and 115 μM in these tissues, respectively. Although AGAT activity in the liver (measured at maximal substrate concentrations) was 20 times greater than that in the kidney, concentrations of hArg in the liver were only 15 % greater than those in the kidney (Table 2). This may be explained by: (1) a relatively low concentration of arginine in the liver (~50 μM) as compared with a much higher concentration (~1.5 mM) in the kidney (Wu and Morris 1998; Wu et al. 2009) and (2) the possibility of a higher rate of hArg catabolism in the liver than in the kidney. This new analytical technique is expected to provide a useful and low-cost tool for studying hArg synthesis and catabolism in cells and within the whole body. As there is growing interest in hArg metabolism and its possible role in cardiovascular function (Atzler et al. 2015; Khalil et al. 2013; Michel 2013; Tsikas and Kayacelebi 2014), our current HPLC method for analysis of hArg in body fluids and tissues will greatly facilitate research in this new field of AA biochemistry and nutrition.

In conclusion, hArg readily reacts with OPA in the presence of NAC under alkaline conditions to form the highly fluorescent hArg–OPA–NAC derivative, which is efficiently separated from other AA–OPA–NAC derivatives on a reversed-phase C_{18} material at 25 °C using a 20-min gradient program. This HPLC method is applicable to the analysis of hArg in aqueous solutions and various biological samples at satisfactory precision and accuracy. Our simple, rapid and fluorescence detection-based HPLC technique offers the advantages of high sensitivity, high specificity and easy automation for determining nM concentrations of hArg in small amounts of plasma and animal tissues. Collectively, the results of this study indicate that our HPLC method provides a useful research tool for

Table 2 Concentrations of hArg and AGAT activity in plasma and tissues from rats

	Plasma	Brain	Kidney	Liver
HA[e]	2.12 ± 0.05^{c}	1.05 ± 0.06^{d}	69.3 ± 2.4^{b}	79.8 ± 2.8^{a}
AGAT[f]	ND	0.69 ± 0.03^{c}	11.8 ± 0.40^{b}	231 ± 7.5^{a}

Values are the mean ± SEM, $n = 8$

ND not determined

[a–d] Within a row, means with different superscript letters differ ($P < 0.05$)

[e] Concentration and content of hArg are expressed as μM for plasma and nmol/g wet weight for tissue (brain, kidney and liver), respectively

[f] Enzymatic activity is expressed as nmol hArg/g wet tissue weight per min

Table 1 Recovery of hArg from biological samples as well as the precision and accuracy of hArg analysis

Sample	Recovery	Relative deviation from the mean[c]	Relative error[d]
Water	100 ± 0.22^{a}	0.52 ± 0.039^{b}	0.42 ± 0.045^{b}
Plasma	97.2 ± 0.48^{b}	1.03 ± 0.060^{a}	1.08 ± 0.053^{a}
Kidney	97.8 ± 0.40^{b}	1.16 ± 0.064^{a}	1.12 ± 0.047^{a}
Brain	97.0 ± 0.32^{b}	1.12 ± 0.086^{a}	1.02 ± 0.051^{a}
Liver	96.6 ± 0.35^{b}	1.01 ± 0.081^{a}	1.08 ± 0.130^{a}

Values, expressed as % and the mean ± SEM, $n = 10$, were determined with 2.5 and 10 μM hArg standard

[a,b] Within a column, means with different superscript letters differ ($P < 0.05$)

[c] A measure of precision (i.e., the agreement between replicate measurements) of analysis as the mean of absolute deviation/the mean of replicate measurements × 100 %

[d] A measure of accuracy (the closeness of a measured value to the true value) of analysis as the relative error [(measurement value − true value)/true value × 100 %)]

studying the biochemistry, nutrition, physiology and pharmacology of hArg and arginine in animals and humans.

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

Ethics statement The use of animals for this research was approved by the Institutional Animal Care and Use Committee of Texas A&M University.

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