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Liquid chromatographic analysis of guanidino compounds using furoin as a new fluorogenic reagent

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ABSTRACT

Furoin, a benzoin analogue, was examined as novel fluorogenic reagent for the selective and sensitive LC determination of various guanidines after pre-column derivatization. The derivatization reaction was carried out at 100 °C for 5 min to give adducts that were separated on a Phenomenex Synergi MAX-RP column and detected at λ_{em} = 410 nm with λ_{ex} = 325 nm. The reagent showed to be useful both for determining together arginine (Arg) and creatine (CT) in dietary supplements under elution isocratic conditions and for the simultaneous analysis of a variety of guanidines in biological samples (human plasma and urine) under elution gradient conditions. The detection limits ranged from 7 to 25 fmol. Recovery studies showed good results for all determined guanidino compounds (85.6–106.2%; R.S.D. = 1.1–6.2%).

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1. Introduction

A variety of currently used dietary supplements contains arginine (Arg) and creatine (CT). Arg is an amino acid essential for infant growth [1], while CT provides a necessary cellular reserve of high-energy phosphates and it has gained increasing attention as ergogenic aid in sports and as a possible therapeutic agent for various neuromuscular diseases [2,3]. On the other hand, also other guanidino compounds are involved in various biochemical processes including the urea and guanidine cycle. The guanidino compounds have been found in serum of nephritic patients, and some guanidines have been suspected to be uremic toxins. Methylguanidine (MG) was shown to be related to uremic polyneuropathy found in uremia. Guanidinosuccinic acid (GSA) was related to uremic bleeding diathesis, and was shown to inhibit excitatory neurotransmission. A variety of guanidino compounds increases in blood, urine and cerebrospinal fluid of nondialyzed as well as hemodialyzed uremic patients [4–6].

The sensitive and selective analysis of guanidino compounds in biological samples is important for diagnostic of diseases. The enzymatic analysis methods of all available guanidino compounds lack sensitivity for the measurement of these compounds in blood. Chromatographic methods are useful for the analysis of such mixtures with high sensitivity. However, some methods are time concerning and reproducibility was not satisfactory.

Guanidines are very polar compounds usually existing as ionic form and their structure does not allow sensitive and selective UV absorbance detection. One way to increase selectivity and sensitivity is the application of pre- or post-column derivatization using fluorogenic reagents such as ninhydrin, benzoin and 9,10-phenanthrenequinone, which allow the formation of products showing fluorescence properties. Ninhydrin and 9,10-phenanthrenequinone were used above all in post-column derivatization owing to forming instable products or a single derivative from all guanidino compounds [6–15], while benzoin was the most investigated compound both as pre- or post-column reagent and as chemiluminogenic reagent [15–26].

Recently [27], we have reported anisoin, 4,4′-dimethoxy analogue of benzoin, to be useful reagent for the derivatization of guanidino compounds giving stable and fluorescent adducts. However, the reproducibility of the chromatographic separation under gradient elution conditions was not completely satisfactory.

The aim of the present work was to increase the range of reagents available for guanidine analysis proposing furoin (I), the furanyl analogue of benzoin, as pre-column fluorogenic reagent (Fig. 1). Furoin, a compound commercially available, is devoid of significant intrinsic fluorescence, but is able to form highly fluorescent derivatives (II) at λ_{em} = 410 nm with λ_{ex} = 325 nm. However, in order to

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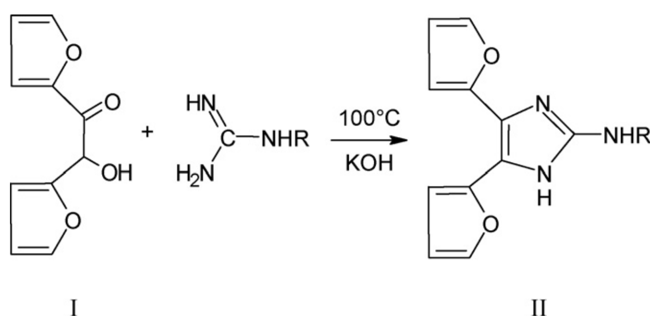

Fig. 1. Reaction derivatization scheme of guanidino compounds with furoin (I).

obtain reproducible and reliable analyses optimum derivatization reaction and chromatographic conditions were investigated. Arg and CT were determined together in dietary supplements by isocratic elution conditions (procedure A), while all tested guanidines, corresponding to GSA, CT, creatinine (CTN), Arg, guanidinoacetic acid (GAA), β-guanidinopropionic acid (β-GPA), homoarginine (H-Arg), γ-guanidinobutyric acid (γ-GBA), α-*N*-acetyl arginine (α-*N*-AA), guanidine (G) and MG were simultaneously analysed under gradient elution conditions (procedure B) in human plasma and urine.

2. Experimental

2.1. Materials

Furoin (1,2-di-2-furanyl-2-hydroxy ethanone), G hydrochloride, Arg hydrochloride, H-Arg hydrochloride, MG hydrochloride, GSA, β-GPA, γ-GBA, GAA, CT monohydrate, CTN, α-N-AA, 2,3-dimethylquinoxaline (used as internal standard, IS), triethylamine (TEA), 3-mercaptopropionic acid, methoxyethanol (methylcellosolve) and methanol for chromatography were obtained from Sigma–Aldrich (Milan, Italy). Acetonitrile for chromatography were of HPLC grade from Romil (Delchimica Scientific Glassware, Neaples, Italy). Sodium sulphite anhydrous and 5-sulfosalicylic acid (SSA), used as deproteinization agent, were purchased from Fluka (Milan, Italy). Purified water by a Milli-RX apparatus (Millipore, Milford, MA, USA) was used for the preparation of all solutions, buffers and mobile phase. All the other chemicals were of analytical reagent grade. Blood and urine samples were provided by healthy volunteers.

2.2. Solutions

All solutions were freshly prepared. The furoin reagent solution (about 75 μmol/mL) was prepared in 5 mL of methoxyethanol. A solution containing 3-mercaptopropionic acid at a concentration of 0.1 M and sodium sulfite at a concentration of 0.2 M was prepared by dissolving and diluting appropriate amounts to a volume of 10 mL with deionized water. Standard solutions of the described guanidino compounds were prepared in water (concentration under calibration graphs) diluting appropriately the stock solutions to 10 mL, after adding as internal standard (IS) 300 μL of GSA solution for the dietary supplement analysis (procedure A) and 1 mL of 2,3-dimethylquinoxaline solution for the biological sample analysis (procedure B). The 2,3-dimethylquinoxaline IS solution (about 100 μmol/mL) was prepared in water/methoxyethanol (60/40, v/v). The GSA IS solution (about 2 μmol/mL) was prepared in water. Potassium hydroxide (about 4.48 g) was dissolved in sufficient water to obtain a concentration of 1.6 M. Borate buffer (pH 9.5, 0.5 M) solution was prepared adjusting the pH of a boric acid solution to 9.5 with sodium hydroxide (1 M). Triethylammonium phosphate buffer (pH 8; 0.05 M) was prepared adding orthophosphoric acid to an aqueous TEA solution up to the desired pH value.

2.3. Equipment

The liquid chromatograph consisted of a Jasco Model LG-980-02S ternary gradient unit, a Jasco PU-1580 pump and a Jasco FP-920 fluorescence detector (Tokyo, Japan) connected to a personal computer AcerView 34TL. The integration program Borwin (Jasco, Tokyo, Japan) was used. Manual injections were carried out using a Rheodyne model 7125 injector with 20 μL sample loop. A column inlet filter (0.5 μm × 3 mm i.d.) model 7335 Rheodyne was used. The solvents were degassed on line with a degasser model Gastorr 153 S.A.S Corporation (Tokyo, Japan). The derivatization reaction was carried out on a heating and stirring apparatus Reacti-Therm™ (Pierce, Rockford, USA). The centrifugation was performed both by an ALC 4235 A and an eppendorf Centric 150 Tehtnica (Opto-Lab, Concordia, Modena, Italy) centrifuge. Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) equipment with thermostatically controlled heating (30–80 °C) was used for ultrasonication.

2.4. Derivatization procedure

A 150 μL aliquot of the guanidino compound solution containing IS was treated with 75 μL of reagent solution (75 μmol/mL), 75 μL of 3-mercaptopropionic acid (0.1 M)-sodium sulphite (0.2 M) solution and 150 μL of potassium hydroxide (1.6 M). The mixture was heated at 100 °C for 5 min under magnetic stirring in a micro reaction vessel (1.0 mL) and cooled in ice–water. Then, 50 μL of the hydrochloric acid (4.8 N) and 500 μL of acetonitrile under magnetic stirring were added. An aliquot of 0.6 mL of this solution was diluted to 5 mL with a mixture A/B, were A is triethylammonium phosphate buffer (pH 8; 0.05 M) and B is methanol/acetonitrile (50:50, v/v), in the ratio 73:27 (v/v) (procedure A) or methanol/acetonitrile (80:20, v/v), in the ratio 75:25, (v/v) (procedure B). Finally, after filtration with 0.22 μm filter, an 20 μL aliquot of the resulting homogeneous and clear solution was injected into the chromatograph.

2.5. Chromatographic conditions

In the development of the method a Phenomenex Gemini 5 μm ODS (250 × 3.0 mm i.d.) column and a Phenomenex Synergi 4 μm MAX-RP (250 × 3.0 mm i.d.) stainless steel column were used. The routine LC separations of guanidino compounds were performed at 33 ± 2 °C on a Phenomenex Synergi column, with a guard column packed with the same stationary phase, using a mobile phase consisting of a mixture A:B, where A is triethylammonium phosphate buffer (pH 8; 0.05 M) and B is methanol/acetonitrile with different composition at a flow-rate of 0.4 mL/min, under isocratic (procedure A) or gradient (procedure B) elution. The analysis under isocratic conditions, B is methanol/acetonitrile (50:50, v/v), in the ratio A:B 73:27 (v/v), while under elution gradient B is methanol/acetonitrile (80:20, v/v), with the following profile t = 0 min, 25% B; t = 30 min, 30% B; t = 50 min, 50% B; t = 60 min, 25% B. Fluorescence detection at λ_{em} = 410 nm with λ_{ex} = 325 nm was used.

2.6. Calibration graphs

Individual standard solutions of guanidino compounds were prepared in water (concentration ranges in Table 1). A 150 μL volume of guanidino compound standard solution containing IS (GSA for procedure A and 2,3-dimethylquinoxaline for procedure B) was subjected to the described derivatization procedure. Triplicate

Table 1
Data for calibration graphs ($n = 5$), limit of detection (S/N = 3) and limit of quantitation (S/N = 10)

Compounds	Slope[a]	y-intercept[a]	Correlation coefficient	Concentration range (nmol/mL)	LOD (fmol)	LOQ (fmol)
CT[b]	0.0125	−0.0050	0.9997	17.90–358.00	137.1	442.8
CT[c]	0.0531	−0.0020	1.0000	1.81–144.82	25.5	77.2
Arg[b]	0.0149	−0.0044	0.9997	11.13–222.63	37.9	109.6
Arg[c]	0.0974	0.0011	1.0000	0.22–79.36	16.8	41.5
GSA[c]	0.0800	−0.0005	0.9999	0.13–43.18	7.5	22.1
GAA[c]	0.0864	0.0003	0.9999	0.21–83.68	13.6	49.7
β-GPA[c]	0.1178	−0.0008	0.9997	0.25–39.60	11.0	33.4
H-Arg[c]	0.0982	−0.0016	0.9999	0.25–39.96	12.1	35.8
γ-GBA[c]	0.0846	0.0001	0.9998	0.15–38.58	9.6	28.5
α-N-AA[c]	0.1179	0.0006	0.9999	0.26–84.51	9.2	27.9
G[c]	0.1384	−0.0020	0.9999	0.10–50.56	6.9	21.2
MG[c]	0.0815	0.0008	1.0000	0.22–44.40	8.3	24.3

[a] According to $y = ax + b$, where x = analyte concentration expressed as nmol/mL, y = ratio of analyte peak-area to IS peak-area.
[b] Procedure A; IS: GSA.
[c] Procedure B; IS: 2,3-dimethylquinoxaline.

injections for each standard solution were made and the peak-area ratio of analyte to IS was plotted against the corresponding guanidino compounds concentration to obtain the calibration graphs.

2.7. Analysis of dietary supplements

2.7.1. Soluble granules (type I, Neovis® plus; type II, Anasten® plus; type III, Argivit)

The content of twenty sachets for each formulation was finely ground and an amount of powder equivalent to about 118.6, 129.4 μmol of Arg and 277.1, 302.3 μmol of CT, for types I and II, respectively, was dissolved with 50 mL, while an amount equivalent to 474.6 μmol of Arg and 277.4 of CT for type III was dissolved with 200 mL of water, under ultrasonication for 10 min at ambient temperature. Then, an aliquot of 0.2 mL was diluted to 10 mL with water after adding 300 μL aliquot of GSA solution (IS).

A 150 μL aliquot of the sample solution was subjected to the described derivatization procedure and the guanidino compound content in each sample was determined by comparison with an appropriate standard solution.

2.8. Analysis of biological samples

2.8.1. Sample preparation

The blood and urine samples were obtained from healthy volunteers between 7:00 and 9:00 after they had fasted overnight.

2.8.1.1. Plasma. The venous blood sample (4 mL) was collected in siliconated sterile tubes containing potassium EDTA as anticoagulant. Plasma was obtained by centrifugation at 2900 *g* for 20 min at ambient temperature. Before the analysis plasma samples (400 μL) were added of 80 μL of IS solution (dimethylquinoxaline) and immediately deproteinized with 80 μL of 25% (w/v) 5-sulfosalicylic acid solution [28]. After vortex-mixing and subsequently icing for 10 min, the samples were centrifugated for 12 min at 6000 × g. Then, to 100 μL of the supernatant (deproteinized plasma) was added 60 μL of borate buffer (0.5 M; pH 9.5) and 40 μL of a mixture water/methoxyethanol (60/40, v/v). Finally, the sample was filtered through a 0.22 μm membrane filter. The residual deproteinized plasma was stored at −80 °C until analysis.

2.8.1.2. Urine. Fasting morning urine was collected in plastic containers. The samples were filtered and 2.5 mL aliquots were diluted to 10 mL with water, after adding 1 mL of IS solution (dimethylquinoxaline). The final sample solutions were filtered through a 0.22 μm membrane filter. The samples were freshly analysed or stored at −80 °C until analysis.

2.8.2. Assay procedure

A 150 μL aliquot of sample or spiked sample with guanidino compounds was subjected to the derivatization procedure as described above. The guanidino compound levels in each sample were calculated by comparison with an appropriate standard solution.

3. Results and discussion

The good results obtained with anisoin [27] and the fairly promising approaches in chemiluminescence using furoin [26], a further analogue of benzoin, have been a spur to take in consideration furoin as potential fluorogenic reagent of guanidines. The derivatization studies were carried out using Arg chosen as typical example of guanidino compound with poor intrinsic detectability characteristic.

3.1. Derivatization reaction

The optimum derivatization conditions were investigated considering the effect of temperature and reagent to Arg molar ratio (Fig. 2). Analogously to anisoin the most suitable conditions were obtained at 100 °C in the range of 4–7 min using potassium hydroxide (1.6 M) in presence of 3-mercaptopropionic acid to stabilize the fluorescent products. On the other hand, the response intensity increases to reach a plateau at a reagent to guanidine compound molar ratio of about 200 and the further reagent excess did not interfere. Furoin showed to be selective for guanidine function: other functional groups, such as carboxylic and aminic groups, did not react. The adducts with furoin were enough stable to guarantee a sufficient time for handling and manual injection of the samples.

3.2. Chromatography

The guanidine derivatives obtained using furoin reagent were separated by LC and detected at $\lambda_{em} = 410$ nm with $\lambda_{ex} = 325$ nm. Among the tested columns was chosen Synergi MAX-RP, which is a new C12 column with endcapping TMS. Its use is suggested for moderately polar or non polar, acidic and basic compounds in a wide pH range (1.5–10). It has a hydrophobic retention like C18 column, owing to a covering 25% higher than that of most C18 columns. The column stability at different pH allows to use acidic or basic mobile phases without damaging the column. The influence of composition and pH of the mobile phase on the resolution and fluorescence intensity of guanidine adducts was studied. Various solvent mixtures containing acetonitrile, methanol and tetrahydrofuran as organic modifiers were examined. Optimum separation,

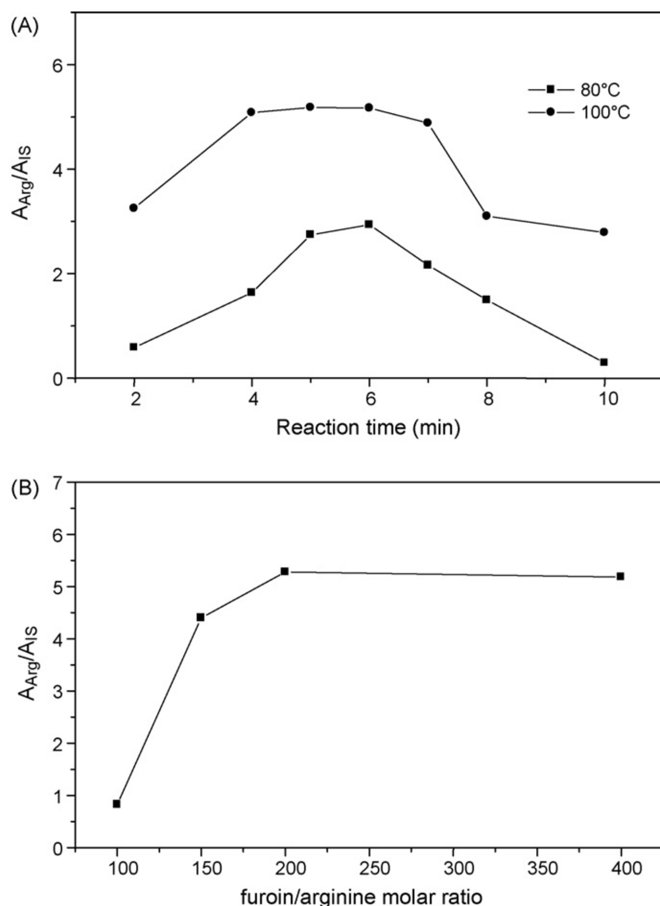

Fig. 2. Effect of the temperature (A) and reagent to Arg molar ratio (B) on the derivatization reaction between furoin and Arg.

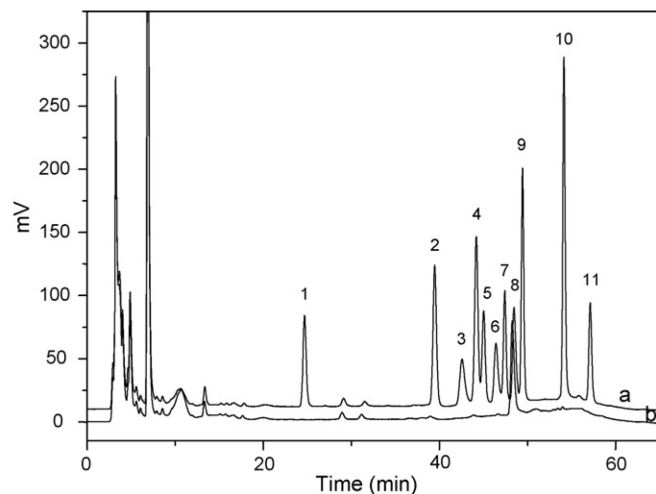

Fig. 3. Representative LC separation at 33 ± 2 °C of: (a) guanidino compounds derivatized with furoin; (b) reagent under reaction conditions. Peaks: 1 = GSA; 2 = CT + CTN; 3 = Arg; 4 = GAA; 5 = β-GPA; 6 = H-Arg; 7 = γ-GBA; 8 = 2,3-dimethylquinoxaline (IS); 9 = α-*N*-AA; 10 = G; 11 = MG. LC conditions: Phenomenex Synergi 4 μm MAX-RP (250 × 3.0 mm i.d.) with a mixture of triethylammoniun phosphate buffer (pH 8.0; 0.05 M), methanol and acetonitrile under gradient elution conditions as mobile phase; flow-rate: 0.4 mL/min. Fluorescence detection: λ_{ex} = 325 nm; λ_{em} = 410 nm. Gain = 100.

peak symmetry and reproducibility were obtained using Synergi column at pH 8 with a mixture of acetonitrile and methanol, while the addition of small volumes of tetrahydrofuran gave no significant improvement. As it can be seen in Fig. 3, under developed gradient elution conditions (procedure B) all examined guanidino compounds were separated, except CT and CTN. That is probably due to the transformation of the cyclic CTN into CT during the derivatization process allowing to identical chemical structure which cannot be separated [17]. However, it does not exclude the possibility that the problem can be overcome applying the post-column derivatization. As it can be seen the reagent did not interfere with the analysis of guanidines both for the absence of intrinsic fluorescence and for the elution of its degradation products close to the solvent front (Fig. 3b). The fluorescence intensity of guanidine derivatives at pH 8 showed to be similar to that at pH 9, while at lower pH values it decreases such as for benzoin adducts [29,30]. In general, at pH 9 a poor chromatographic reproducibility was observed. Gradient elution conditions were found suitable for the analysis of biological samples, while isocratic elution conditions (procedure A) were developed to analyze commercial dietary supplements in short time (Fig. 4).

3.3. Analysis of guanidines

Under the described chromatographic conditions (procedure A and B) the method was validated to analyze commercial dietary supplements and for its high sensitivity also plasma and urine. Linearity, repeatability, limit of detection (LOD), limit of quantitation (LOQ) and accuracy were verified. A linear relationship between peak-area ratio (analyte to IS) and analyte concentration was observed for each guanidine. The calibration parameters are shown in Table 1. The within-run precision (repeatability), covering derivatization and chromatographic separation, was satisfactory. The R.S.D. values, obtained from replicate (n = 6) analyses of a standard solution of Arg and CT (0.04 μmol/mL, respectively) were 0.91% for Arg and 1.34% for CT by the procedure A, while for all examined guanidino compounds (1–4 nmol/mL) the R.S.D. value range was 0.93–5.42% by the procedure B.

The detection limits were in the range of 7–25 fmol (Table 1) in accordance with guanidine derivatives obtained using anisoin reagent, except for CT, where the fluorescence intensity with furoin was found to be six-fold higher.

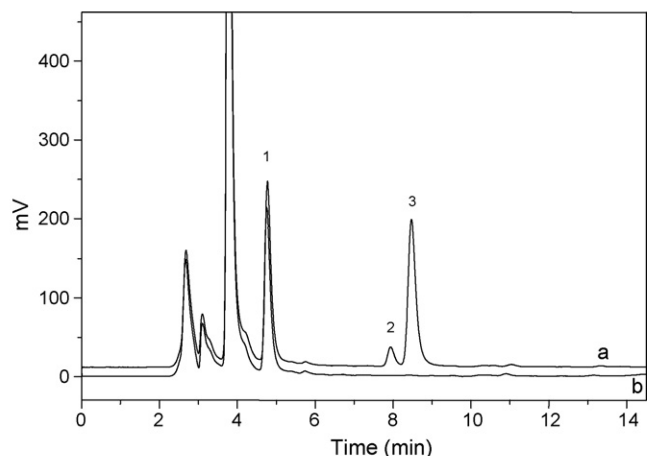

Fig. 4. LC chromatogram at 33 ± 2 °C under isocratic conditions of: (a) dietary supplement sample (soluble granules) after derivatization with furoin; (b) reagent under reaction conditions. Peaks: 1 = GSA (IS); 2 = CT; 3 = Arg. LC conditions: Synergi 4 μm MAX-RP (250 × 3.0 mm i.d.) column with a mixture A/B, where A is triethylammoniun phosphate buffer (pH 8.0; 0.05 M) and B is methanol/acetonitrile (50:50, v/v), in the ratio 73:27, v/v, as mobile phase; flow-rate: 0.4 mL/min. Fluorescence detection: λ_{ex} = 325 nm; λ_{em} = 410 nm. Gain = 100.

Table 2
Results for the LC determination of Arg and CT in commercial dietary supplements

Soluble granules	Compound	Claimed content (g/100 g)	%Found[a]	%R.S.D.
Type I[b]	Arg	8.333	97.95	2.85
	CT	16.667	115.42	3.37
Type II[c]	Arg	9.091	96.73	3.08
	CT	18.182	109.06	1.85
Type III[d]	Arg	33.33	102.32	1.88
	CT	16.67	106.77	2.08

[a] Mean of five determinations and expressed as a percentage of the claimed content.
Inactive ingredients: [b]Maltodextrin, citric acid, flavour, potassium phosphate, magnesium-L-aspartate, potassium-D-L-aspartate, L-carnitine tartrate, magnesium oxide, vitamin C, aspartame, saccharin sodium, L-glutathione, ferric phosphate, yeast with a guaranteed silicon content, β-carotene, vitamin E acetate, silicon dioxide, vitamin B_6 hydrochloride, vitamin B_1 hydrochloride, vitamin B_{12}, folic acid.
[c] Saccharosium, magnesium citrate, potassium aspartate, citric acid, flavour, vitamin C, L-carnitine fumarate, lecithin, magnesium oxide, acesulfame-K, zinc oxide, coenzyme Q10, silicon dioxide, selenium aspartate, coloring agent E102.
[d] Potassium aspartate, magnesium aspartate, maltodextrin, citric acid, silicon dioxide, flavour, β-carotene, aspartame, saccharin sodium, ferric phosphate, magnesium oxide, copper citrate, sodium selenite, vitamin C, vitamin E acetate.

3.3.1. Analysis of dietary supplements

Commercial dietary supplements are complex formulations owing to the presence of a variety of other ingredients (vitamins, carnitines, etc.) in addition to Arg and CT (Table 2). The procedure A allowed the selective determination of guanidines (Fig. 4) without interference from other compounds, due above all to the application of the derivatization reaction and the fluorescence detection (λ_{ex} = 325 nm; λ_{em} = 410 nm). Besides, the pre-column derivatization selectivity has simplified the sample treatment phase because it was sufficient to dissolve the soluble granules in water under ultrasonication without the need of laborious extractive operations procedures. The obtained results (Table 2) were found to be substantially in agreement with the claimed content. The method accuracy was verified by analysing commercial samples spiked with known amount (20%) of the claimed guanidine content; quantitative recoveries (98.04–104.05%, R.S.D.% = 1.10–3.51) were obtained for both compounds.

3.3.2. Analysis of biological samples (plasma and urine)

The urine samples were analysed after simple dilution and the plasma samples after deproteinization with 5-sulfosalicylic acid. The analytes were identified by comparison of chromatographic retention times (t_r) with those of standard guanidines and spiked amount of individual guanidino compounds. Representative LC separations, obtained in the analysis of plasma and urine, are illustrated in Fig. 5 (A and B). As it can be seen, in both samples the biological matrix did not interfere with the compound analysis. The assay results are reported in Table 3. The data agree substantially with literature data related to controls [8–10,22,25,27,31–34]. The accuracy of the method was verified by analysing biological samples (plasma and urine) fortified with known amounts of guanidino compounds at concentration level giving final solutions within the calibration graphs range. The recovery obtained for the replicates of each concentration level were in the range 85.61–106.25%; R.S.D. = 1.14–6.23%. The repeatability of the complete method (sample treatment, derivatization and chromatographic separation) obtained from replicate (n = 3) analyses of a biological sample (blood and urine) fortified with known amounts of guanidino compounds, at concentration level of about quantitation limits, was satisfactory. The R.S.D. values were in the range of 1.07–13.03% for blood and 1.64–12.12% for urine.

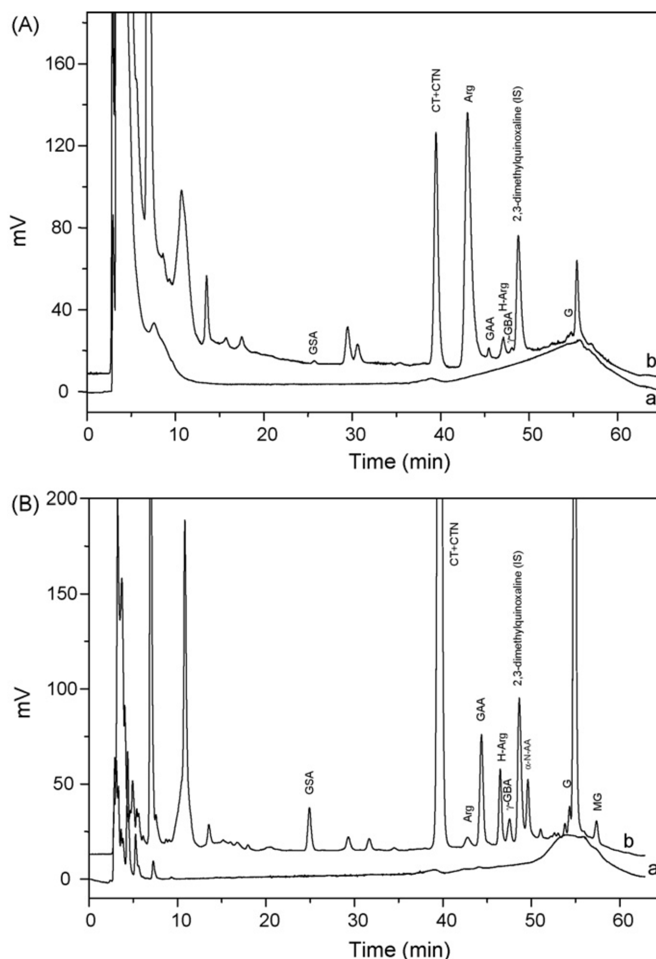

Fig. 5. LC chromatogram at 33 ± 2 °C of plasma (A) and urine (B) samples before (a) and after (b) derivatization with furoin. LC conditions and detection as described in Fig. 3.

Table 3
Results of LC analysis of biogenic guanidino compounds in urine and plasma samples (n = 5) from healthy volunteers

Guanidino compounds	Plasma concentration range[a] (μmol/L)	Urine concentration range[b] (μmol/L)
GSA	0.37–1.13	16.14–28.99
Arg	82.29–140.99	10.43–21.79
GAA	0.81–32.97	79.16–327.45
β-GPA	<LOD–0.45	<LOD
HArg	0.52–12.32	<LOD–30.22
γ-GBA	<LOD–0.43	<LOD–5.68
α-N-AA	<LOD–14.98	9.82–32.34
G	0.20–0.78	<LOD–4.46
MG	<LOD–0.52	4.54–13.29

[a] Mean of three determinations (plasma). R.S. D.: 1.7–7.5%.
[b] Mean of three determinations (diluted urine). R.S.D.: 2.2–8.2%.

4. Conclusions

Furoin has been proved to be a pre-column fluorogenic reagent useful for the analysis of guanidine in dietary supplements and in biological samples such as plasma and urine. The compound has not significant native fluorescence, but forms highly fluorescent adducts. The high sensitivity resulting in detection limits of about 7–25 fmol, selectivity and reproducibility of the proposed method can allow to determine target guanidino compounds for diagnostic purpose in metabolic diseases.

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