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Anisoin: A useful pre-chromatographic derivatization fluorogenic reagent for LC analysis of guanidino compounds

R. Gatti *, M.G. Gioia

Dipartimento di Scienze Farmaceutiche, Alma Mater Studiorum-Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy

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Abstract

The use of anisoin as pre-chromatographic reagent for LC analysis of guanidino compounds is proposed. The reagent reacts (5 min at 100 °C) with guanidino function and the resulting adducts can be chromatographed under reversed-phase conditions. A fluorescence detector (λ_{ex} = 325 nm; λ_{em} = 435 nm) was used to detect guanidino adducts. The derivatization and chromatographic conditions were optimised by a series of experiments. Application to the determination of arginine and creatine in pharmaceuticals and arginine, guanidine, methylguanidine, guanidinosuccinic acid, β-guanidinopropionic acid, γ-guanidinobutyric acid, guanidinoacetic acid and homoarginine in human urine is described. Quantitation limits ranged from 6 to 30 fmol, except for creatine (510 fmol).

Keywords: Reversed-phase liquid chromatography; Anisoin; Guanidino compounds; Fluorogenic pre-column derivatization; Pharmaceuticals; Human urine

1. Introduction

Several guanidino compounds occur in human physiological fluids and tissues. As example, among the biogenic guanidino compounds arginine (Arg) is a polar aliphatic amino acid that is best known as a growth hormone releaser. It is used as dietary supplement and in certain conditions accompanied by hyperammonaemia. Arg hydrochloride has also been used as an acidifying agent [1]. Creatine (CT) is an endogenous substance found mainly in skeletal muscle of vertebrates. CT phosphate has been tried in the treatment of cardiac disorders and has been added to cardioplegic solutions. CT monohydrate has been tried in metabolic disorders and used as a dietary supplement. It is also under investigation for the treatment of motor neurone disease [2]. Guanidinosuccinic acid (GSA) and methylguanidine (MG) have been considered to be uremic toxins in uremic syndrome since these compounds accumulate in the body fluids of uremic patients and give rise to a complex of symptoms similar to uremia [3–5]. Serum and urinary GSA levels are significantly decreased in cirrhotic patients [6], while extremely high plasmatic and urinary guanidinoacetic acid (GAA) concentration was the most specific abnormality in the guanidine acetate methyltransferase deficiency [7,8].

The analysis of these compounds is quite difficult due to their poor detectability owing to the absence of a strong chromophore and fluorophore. Pre- or post-column chemical derivatization in combination with fluorescence detection constitutes an effective approach to overcome the problem [5,9,10]. A great variety of reagents have been used for the derivatization of amines, thiols and carboxylic acids, while few probes have been described for the determination of guanidino compounds. Post-column derivatization and fluorescence detection have been reported for guanidino compounds employing an alkaline ninhydrin reagent [4–7]; the same compound has been investigated as pre-column reagent [11,12]. 9,10-Phenanthrenequinone can be used only for post-column derivatization, because the method gives a single fluorescent product, 2-amino-1H-phenantro[9,10-*d*]-imidazole, from all guanidino compounds [10,13,14]. Benzoin was found to react selectively and sensitively with guanidino compounds in alkaline medium in presence of β-mercaptoethanol (stabilizer for the fluorescent products) and sodium sulfite (suppresser of blank fluorescence) in an aqueous dimethylformamide or methylcellosolve solution to give fluorescent derivatives corresponding to the chemical structures of 2-substituted amino-4,5-diphenylimidazoles. The benzoin reaction has been largely applied in the fluorimetric and pre- or post-column deter-

* Corresponding author. Fax: +39 051 2099734.
E-mail address: rita.gatti2@unibo.it (R. Gatti).

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mination of guanidino compounds and Arg-containing peptides [3,8,9,14–21]. Besides, benzoin and various analogues were investigated as chemiluminogenic derivatization reagents [22].

On the basis of that, to develop additional useful fluorogenic reagents, our attention was directed to anisoin, the 4,4′-dimethoxy analogue of benzoin; the methoxy substituents of the aromatic structure make it potentially able to give highly fluorescent derivatives. This paper describes the application of anisoin as pre-column fluorogenic labelling reagent in the HPLC analysis of Arg and CT in pharmaceuticals and Arg, MG, GSA, GAA, homoarginine (H-Arg), guanidine (G), β-guanidinopropionic acid (β-GPA) and γ-guanidinobutyric acid (γ-GBA) in human urine. The HPLC method allows the quantitative determination at femtomole levels.

2. Experimental

2.1. Materials

Anisoin, guanidine hydrochloride, L-Arg hydrochloride, L-homoarginine hydrochloride, methylguanidine hydrochloride, guanidinosuccinic acid, β-guanidinopropionic acid, γ-guanidinobutyric acid, guanidinoacetic acid, creatine, creatinine (CTN), griseofulvin (used as internal standard, IS) and triethylamine (TEA) were obtained from Sigma–Aldrich (St. Louis, MO). Acetonitrile for chromatography were of HPLC grade from Romil (Delchimica Scientifica Glasswere, Neaples, Italy). Deionized, double distilled water was used for the mobile phase preparation. All the other chemicals were of analytical reagent grade.

2.2. Solutions

All the solutions were prepared freshly. The reagent anisoin standard solution (about 75 mM) was prepared in 5 ml of methoxyethanol (methylcellosolve). 3-Mercaptoproprionic acid (0.1 M) and sodium sulfite (0.2 M) were dissolved and diluted to a volume of 10 ml with distilled water to form a solution. Standard solutions of the described guanidino compounds were prepared in water (concentration under calibration graphs). The griseofulvin internal standard solution (13 μmol/ml) was prepared in acetonitrile. The GSA internal standard solution (5.6 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. Triethylammonium phosphate buffers (pH 4 and 9; 0.05 M) were 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) 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). 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 was treated with 75 μl of reagent solution (75 mM), 75 μl of 3-mercaptopropionic acid (0.1 M)–sodium sulfite (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 (3.0 ml) and cooled in ice-water. Then, 50 μl of the hydrochloric acid (4.8N) and 500 μl of IS solution under magnetic stirring were added and after filtration with 0.22 μm filter, 20 μl aliquots of the resulting homogeneous and clear solution were injected into the chromatograph directly or after dilution of 0.3 to 10 ml with the mixture triethylammonium phosphate buffer (pH 9; 0.05 M)–acetonitrile, in the ratio 80:20 (v/v). For the analysis of Arg and CT in pharmaceuticals, the derivatization procedure was also carried out by using a GSA solution as IS added before the derivatization reaction. At the end, to obtain a clear solution, 500 μl of acetonitrile was added.

2.5. Chromatographic conditions

The routine LC separations of guanidino compounds were performed at 33 ± 2 °C on a Phenomenex Gemini 5μ ODS (250 mm × 3.0 mm i.d.) stainless steel 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 9; 0.05 M) and B is acetonitrile at a flow-rate of 0.4 ml/min. A elution gradient with the following profile: $t = 0$ min, 20% B; $t = 20$ min, 20% B; $t = 30$ min, 40% B; $t = 40$ min, 20% B, for urine analysis was used. The analysis of Arg and CT in pharmaceuticals was carried out under isocratic conditions by using the mixture A:B in the ratio 80:20 (v/v). The LC separations of alone Arg from IS (griseofulvin) were carried out also at 33 ± 2 °C by a Phenomenex Prodigy 5μ ODS (250 mm × 3.2 mm i.d.) stainless steel column, with a guard column packed with the same stationary phase, under gradient elution. The mobile phase consisted of a mixture A:B, where A is triethylammonium phosphate buffer (pH 4; 0.05 M) and B is acetonitrile at a flow-rate of 0.4 ml/min and the gradient profile was: $t = 0$ min, 30% B; $t = 6$ min, 70% B; $t = 16$ min, 70% B; $t = 22$ min, 30% B. Fluorescence detection at $\lambda_{em} = 435$ nm with $\lambda_{ex} = 325$ 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 compounds standard solution was subjected to the described derivatization procedure. Triplicate

Table 1
Data for calibration graphs ($n = 5$), repeatability ($n = 6$), 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)	R.S.D. (%)	LOD (fmol)	LOQ (fmol)
Arg[b]	0.030306	−0.00292	0.9995	1.912–38.20	3.44	10	30
Arg[c]	0.030238	0.014455	0.9996	38.43–384.3	2.30	10	30
Arg[d]	0.005172	0.004178	0.9999	32.27–322.7	0.93	50	160
CT[b]	0.002987	0.004707	0.9998	52.4–1310.8	4.34	155	510
CT[c]	0.001012	0.019381	0.9996	51.12–511.2	3.25	155	510
GSA[b]	0.040036	0.024895	0.9998	2.18–43.75	2.97	8	25
H-Arg[b]	0.05957	−0.009039	0.9996	2.344–39.07	3.27	10	30
GAA[b]	0.038882	−0.00728	0.9998	8.51–425.5	5.08	8	25
β-GPA[b]	0.031322	0.017802	0.9997	2.135–42.70	4.41	8	25
γ-GBA[b]	0.0833	0.017527	0.9992	0.45–18.00	2.32	8	25
G[b]	0.015061	0.00754	0.9994	1.28–25.60	3.41	3	8
MG[b]	0.168224	−0.007039	0.9997	0.510–20.04	0.44	2	6

[a] According to $y = ax + b$, where x: analyte concentration expressed as nmol/ml and y: ratio of analyte peak-area to IS peak-area.
[b] Method under gradient elution conditions (pH 9) used for urine analysis (gain 100); IS: griseofulvin.
[c] Method under isocratic elution conditions (pH 9) used for pharmaceutical analysis (gain 100); IS: GSA.
[d] Method under gradient elution conditions (pH 4) used for pharmaceutical analysis (gain 10); IS: griseofulvin.

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 pharmaceuticals

2.7.1. Sample preparation

2.7.1.1. Effervescent tablets. Twenty tablets were powered and an amount of powder equivalent to about 0.21 mmol of Arg was treated with 10 ml of water in a 50 ml volumetric flask under ultrasonication till the disappearance of the effervescence and then diluted to volume with water. After filtration with 0.20 μm regenerated cellulose filters, an aliquot of 0.25 ml was directly diluted to 10 ml with water or after adding 100 μl aliquot of GSA solution.

2.7.1.2. Medicine in packets. An amount of powder equivalent to about 239.2 μmol of Arg and 635.5 μmol of CT was dissolved with 50 ml of water under ultrasonication for 10 min at ambient temperature. Then, an aliquot of 0.2 ml was directly diluted to 10 ml with water or after adding 100 μl aliquot of GSA solution.

2.7.1.3. Extemporaneous solution. The reservoir-plug power, equivalent to about 3.10 mmol of Arg was mixed, stirring till to homogeneity, with the appropriate solution of the bottle and was treated with 100 ml of water by ultrasonication for 3 min at ambient temperature; then, an aliquot of 0.3 ml was directly diluted to 100 ml with water or after adding 1 ml aliquot of GSA solution.

2.7.2. Assay procedure

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 human urine

Fasting morning urine was collected in plastic containers and the samples were freshly analysed or stored at −20 °C until analysis.

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

3. Results and discussion

3.1. Derivatization reaction

The potential of anisoin as derivatization reagent was studied with Arg whose detectability required enhancement. To achieve optimum conditions, the effects of the temperature, potassium hydroxide concentration and the reagent on the reaction were investigated. Reaction at 100 °C for 5 min using potassium hydroxide (1.6 M) in presence of 3-mercaptopropionic acid and sodium sulfite proved to be the most convenient conditions. Analogous results were obtained with ethanethiol or β-mercaptoethanol, which were rejected for their disgusting smell. The course of the reaction at different temperatures under magnetic stirring is reported in Fig. 1. A temperature of 100 °C was necessary to obtain a plateau after 4 min. At lower temperature (90 °C) reduced response was observed. The influence of the potassium hydroxide concentration on the adduct concentration is illustrated in Fig. 2. A 1.6–2.0 M potassium hydroxide concentration range gave the better response. Under the chosen conditions (5 min at 100 °C; 1.6 M potassium hydroxide) the response intensity increases to reach a plateau at a reagent to guanidino compound molar ratio of about 50 and further reagent excess does not interfere (Fig. 3). The stability of the adduct of Arg with anisoin in the mixture reaction

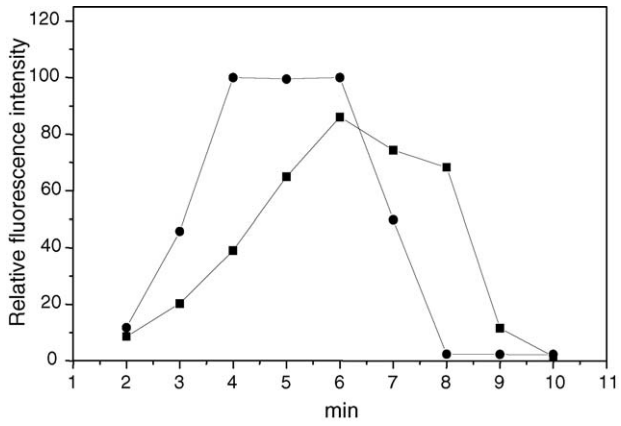

Fig. 1. Influence of the temperature on the derivatization reaction of Arg with anisoin: 90 °C (■) and 100 °C (●).

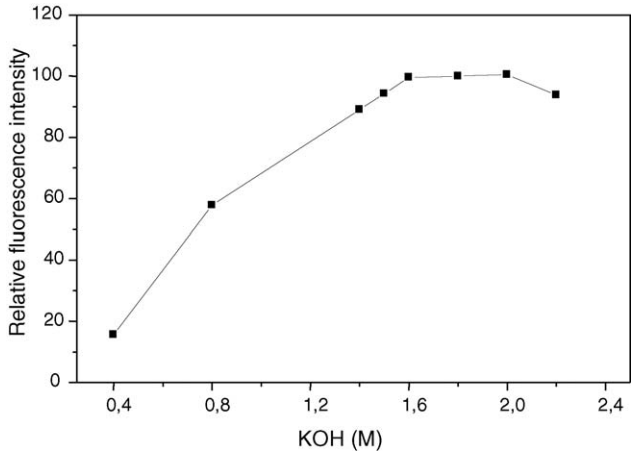

Fig. 2. Effect of the potassium hydroxide concentration on the derivatization reaction of Arg with anisoin.

was studied. In the presence of a thiol the derivative proved to be stable for at least 48 h. The developed reaction conditions were found in agreement with those described for benzoin [3].

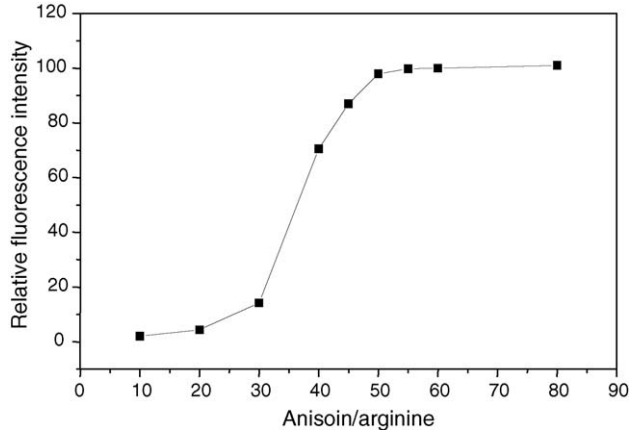

Fig. 3. Effect of the reagent to Arg molar ratio on the derivatization reaction.

3.2. Chromatography

The derivatization reaction yields different guanidino adducts which can be separated by LC and detected at $\lambda_{em} = 435$ nm with $\lambda_{ex} = 325$ nm. In the method development, columns (250 mm × 3.2 mm i.d. and 250 mm × 3.0 mm i.d.) packed with different RP materials (Prodigy 5μ and Gemini 5μ, respectively) were used. Prodigy™ columns, made with high purity HPLC silica phases and stable at pH range (2–9), represent a major advance in the analysis of basic, acidic and amphoteric compounds, at a cost savings. The ultra-low metal content silica (99.999% pure) and an inert bonded surface reduce the need for expensive and labor-intensive mobile phase modifiers or ion-pairing reagents. The Gemini column is a new column and it is described as stable silica column at a large pH range (1–12). It contains two-in-one technology™ (TWIN™ technology) which allows to combine the better of silica and polymer. The internal silica of the particle is unchanged during the production process, guaranteeing efficiency, while the silica-organic covering protects particle from chemical attacks.

Chromatographic separations were carried out under gradient elution conditions at 33 ± 2 °C on a reversed phase column ODS (Gemini 5μ at pH 9 and Prodigy 5μ at pH 4). The effects of composition and pH of the mobile phase on the resolution and fluorescence intensity of the guanidino derivatives were investigated. A triethylammonium phosphate buffer (pH 9; 0.05 M):acetonitrile mixture of varying composition (v/v) was found to be suitable for a guanidine compound separation and to obtain a significant fluorescence intensity (Fig. 4). As it can be seen, the reagent did not interfere with the analysis of the guanidino derivatives. The limit of detection, resulting in a peak height of three times to noise level (S/N = 3), was found to be in the range of 2–10 fmol of the injected derivatives, except for CT (155 fmol) (Table 1). The sensitivity obtained in the described

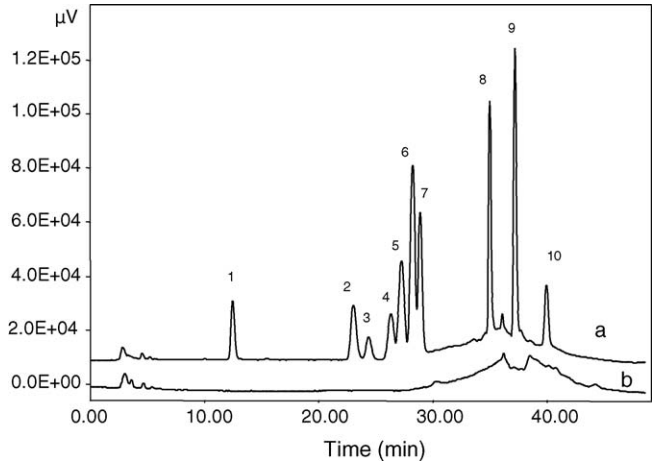

Fig. 4. Representative LC separation at 33 ± 2 °C of: (a) guanidino compounds derivatized with anisoin and (b) reagent under reaction conditions. Peaks: 1, GSA; 2, L-Arg; 3, CT + CTN; 4, H-Arg; 5, β-GPA; 6, GAA; 7, γ-GBA; 8, G; 9, MG; 10, griseofulvin (IS). LC conditions: Phenomenex Gemini 5μ ODS (250 mm × 3.0 mm i.d.) with a mixture of triethylammoniun phosphate buffer (pH 9.0; 0.05 M) and acetonitrile under gradient elution conditions as mobile phase; flow-rate: 0.4 ml/min. Fluorescence detection: $\lambda_{ex} = 325$ nm; $\lambda_{em} = 435$ nm. Gain = 100.

conditions was found about from 10- to 50-fold times higher than that described for the method which involves benzoin [3,17]. Besides, the Gemini column allowed also the simultaneous separation of Arg and CT under isocratic conditions (pH 9) useful for pharmaceutical analysis. However, the separation reproducibility using this column at pH 9, especially under gradient elution conditions, has not been completely satisfactory. On the other hand, using Prodigy column a minor number of guanidino compounds were separated. In particular, a mobile phase at pH 4 did not allow to detect creatine and creatinine and the detection limit of the other guanidino compounds was about 50–150 fmol.

3.3. Analysis of guanidino compounds

Both commercial formulations containing Arg and CT and urine were easily subjected to the derivatization and LC analysis (detection $\lambda_{em} = 435$ nm with $\lambda_{ex} = 325$ nm). Under the described chromatographic conditions a linear relationship between peak-area ratio (analyte to IS) and analyte concentration (nmol/ml) was observed for all guanidino compounds and the calibration parameters are shown in Table 1. The within-run precision (repeatability) of the methods (Table 1) was satisfactory as indicated by the R.S.D. value obtained from replicate ($n = 6$) analyses (derivatization and LC separation) of a standard solution of Arg (0.1 μmol/ml, pH 4) and guanidino compounds (0.17 μmol/ml for CT and about 0.02 μmol/ml for other guanidino compounds, respectively, pH 9).

3.3.1. Analysis of pharmaceutical formulations

Commercial pharmaceutical effervescent tablets, medicine in packets and extemporaneous solution, used as dietary supplements, were analysed by the proposed LC method (isocratic conditions, pH 9), based on pre-column derivatization with anisoin and fluorescence detection. The results obtained (Table 2) were found to be in agreement with the claimed content. The other ingredients of the formulations did not interfere with the analysis. The analysis of pharmaceuticals containing only Arg was carried out also by using the method at pH 4 and analogous results were obtained (Table 2). The accuracy of both methods (mobile phases pH 4 and 9) was verified by analysing commercial samples spiked with known amounts (20, 50 and 80%) of the claimed drug content; quantitative recoveries (99.9–100.0%, R.S.D. = 0.4–2.4% for method at pH 4 and 96.3–101.0%, R.S.D. = 1.3–5.1% for method at pH 9) were obtained. A sample chromatogram is reported in Fig. 5.

3.3.2. Analysis of urine

Arg, H-Arg, G, MG, GSA, β-GPA, γ-GBA, GAA, CT and CTN are present in urine [3–7,13,23] and were separated and quantified fluorometrically by the proposed method under gradient elution at pH 9, except CT and CTN co-eluting under the described conditions. A typical chromatogram obtained with urine from healthy subject is shown in Fig. 6. The identity of guanidino compounds was assigned by retention time coincidences, after analysing urine samples reinforced with authentic standards. The guanidino derivatives and the internal standard were separated in a reasonable time and the other biological components did not interfere with the analysis. The concentration values of individual guanidino compounds from five samples of healthy adult volunteers urine (Table 3) are in agreement with the data reported in literature [3,7,23]. The accuracy of the method was verified by analysing urine samples fortified with known amounts of guanidino compounds at three concen-

Table 2
Results for the LC determination of arginine and creatine in commercial pharmaceutical formulations

Formulation	Compound	%Found[a]	%R.S.D.
Effervescent tablets[b]	Arg hydrochloride	102.20[c]	2.90
		100.68[d]	1.39
Medicine in packets[e]	Arg	96.71[c]	3.33
		100.41[d]	1.11
	CT monohydrate	95.10[c]	3.74
Extemporaneous solution[f]	Arg glutamate	97.52[c]	2.68
		100.72[d]	2.01

[a] Mean of five determinations and expressed as a percentage of the claimed content.
[b] Inactive ingredients: citric acid, sodium bicarbonate, magnesium oxide, mannitol, sorbitol, ascorbic acid, lemon flavour, anhydrous sodium carbonate, aspartame, acesulfame-K, beet red (E162) and riboflavin 5′-(dihydrogen phosphate) monosodium salt.
[c] Method under isocratic elution conditions (pH 9).
[d] Method under gradient elution conditions (pH 4).
[e] Inactive ingredients: malt dextrin, 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, betacarotene, Vitamin E acetate, silicon dioxide, Vitamin B_6 hydrochloride, Vitamin B_1 hydrochloride, Vitamin B_{12} and folic acid.
[f] Inactive ingredients: saccharosium, citric acid, methyl *p*-hydroxybenzoate, sour black cherry oil, cobamamide, folinic acid calcium salt pentahydrate, mannitol and demineralized water.

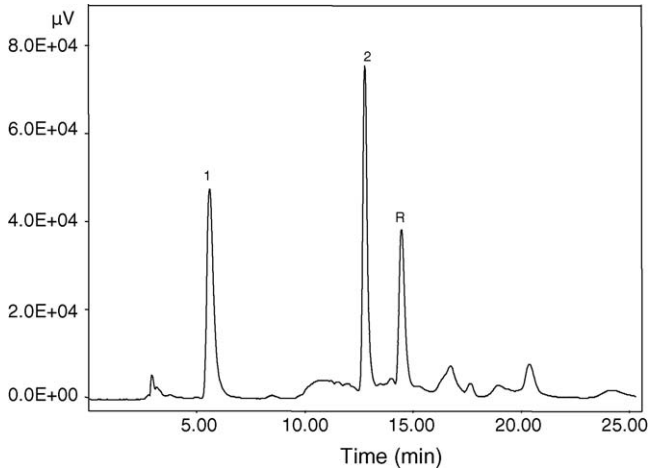

Fig. 5. LC chromatogram at 33 ± 2 °C of an Arg sample (effervescent tablets) derivatized with anisoin. Peaks: 1, Arg; 2, griseofulvin (IS); R, reagent. LC conditions: Phenomenex Prodigy 5μ ODS (250 mm × 3.2 mm i.d.) with a mixture of triethylammoniun phosphate buffer (pH 4.0; 0.05 M) and acetonitrile under gradient elution conditions as mobile phase; flow-rate: 0.4 ml/min. Fluorescence detection: $\lambda_{ex} = 325$ nm; $\lambda_{em} = 435$ nm. Gain = 10.

Table 3
Results of LC analysis of biogenic guanidino compounds in urine

Found[a] (%R.S.D.)							
GSA	Arg	H-Arg	β-GPA	GAA	γ-GBA	G	MG
2.8–33.1 (2.2–3.9)	3.28–18.1 (2.5–4.0)	<LOQ	<LOQ	52–350 (1.8–3.4)	0.48–9.1 (2.5–3.9)	3.45–19.8 (2.1–4.3)	<LOQ–4.1 (3.2–6.0)

[a] Range of found concentrations (nmol/ml) in five urine samples from different healthy adults volunteers. Each data was obtained as mean of five determinations.

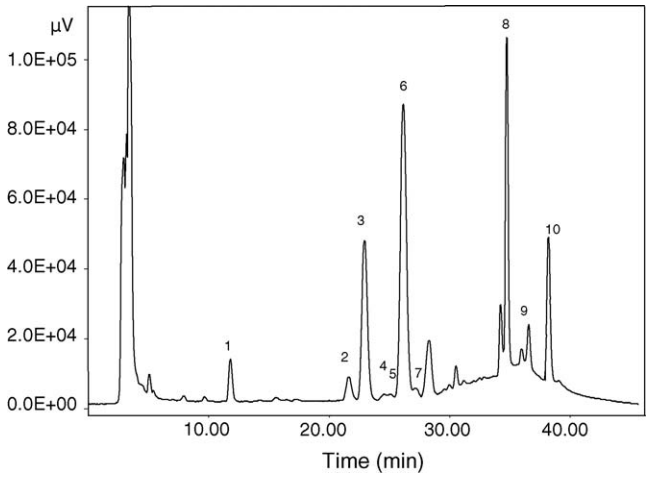

Fig. 6. LC chromatogram at 33 ± 2 °C of urine sample after derivatization with anisoin. Peaks: 1, GSA; 2, Arg; 3, CT + CTN; 4, H-Arg; 5, β-GPA; 6, GAA; 7, γ-GBA; 8, G; 9, MG; 10, griseofulvin (IS). LC conditions and detection as described in Fig. 4.

tration levels giving final solutions within the calibration range of the method. Essentially quantitative recoveries were obtained for three replicates of each concentration level (87.9–105.0%, R.S.D. = 1.2–5.4%).

4. Conclusions

Anisoin has been proven to be a useful reagent for the derivatization of guanidino compounds. The reagent exhibited good reactivity and selectivity towards the guanidino function. The derivatives showed high stability in the reaction mixture in presence of mercaptopropionic acid and were separated by reversed phase LC without interference from degradation products. Using basic mobile phase, Arg and creatine were determined in commercial pharmaceutical formulations under isocratic conditions, while Arg with related biogenic guanidino compounds were analysed in urine under gradient elution. The sensitivity of the method was about 2–10 fmol, except for creatine. The method proposed for urine analysis could be useful for diagnostic purpose to investigate the biological levels of guanidino compounds implicated as toxins, in patients with uremia, chronic renal failure and other diseases.

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