Improved Gas Chromatographic Determination of Guanidino Compounds Using Isovaleroylacetone and Ethyl Chloroformate as Derivatizing Reagents

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An improved GC method in terms of sensitivity and decrease in the analysis time has been developed for the analysis of eight guanidino compounds: guanidine (G), methylguanidine (MG), creatinine (CTN), guanidinoacetic acid (GAA), guanidinobutyric acid (GBA), guanidinopropionic acid (GPA), arginine (Arg), and guanidinosuccinic acid (GSA), using isovaleroylacetone (IVA) and ethyl chloroformate (ECF) as derivatizing reagents. The separation was obtained from column HP-5 (30 m × 0.32 mm i.d.) with film thickness of 0.25 µm within 11 min. The linear calibrations were obtained with 0.5 to 50 µg/mL with coefficient of determination (R^2) within 0.9969 – 0.9998. Limits of detections (LODs) were within 5 – 140 ng/mL. The derivatization, separation and determination was repeatable ($n = 6$) with relative standard deviation (RSD) within 1.2 – 3.1%. The guanidino compounds were determined in deproteinized serum of healthy volunteers and uremic patients within below LOD to 8.8 µg/mL and below LOD to 43.99 µg/mL with RSD within 1.4 – 3.6%. The recovery of guanidino compounds calculated by standard addition from serum was within 96.1 – 98.9%, with RSD 1.4 – 3.6%.

Keywords Gas chromatography, guanidino compounds, derivatization, isovaleroylacetone, ethyl chloroformate, serum

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Introduction

The guanidino compounds are characterized by the presence of basic guanidinium radical. They differ considerably in their metabolism, time distribution, biological properties and physiological functions.¹ They are generated *in vivo* as the result of protein and amino acid metabolism in the mammalian kidneys.² A number of them are labeled as uremic toxins because they have an ability to interact with reactive species. The guanidino compounds found in mammals include creatinine (CTN), guanidinosuccinic acid (GSA), guanidinoacetic acid (GAA), arginine (Arg), and guanidine (G).³ GSA and methylguanidine (MG) are considered as uremic toxins and accumulate in the body fluids of uremic patients and indicate symptoms similar to uremia.⁴ A high concentration of GAA is reported in the plasma and urine of those with guanidine acetate methyl transferase deficiency.^{5,6} Similarly, the levels of guanidino compounds are disturbed in the serum of patients with various metabolic disorders including hyper argininemia and cirrhosis.^{7,8} Therefore, the determination of guanidino compounds in serum has clinical importance.

A number of analytical procedures have been developed for the determination of guanidino compounds, but they involve small molecules and derivatization is carried out before their detection. The analytical procedures are mainly based on high performance liquid chromatography (HPLC),^{5,9–16} gas

chromatography (GC)^{6,17–20} and capillary electrophoresis.^{21–22}

HPLC is more frequently used for the analysis of guanidino compounds,⁴ but capillary GC has high potential for the separation and analysis of volatile organic compounds with short analysis time. The guanidino compounds have been converted to volatile species by derivatization with hexafluoroacetylacetone (HFAA) and trimethylsilyltrifluoroacetamide,^{6,19} HFAA and trifluoroacetic anhydride,²³ HFAA and pentafluorobenzyl bromide²⁰ or HFAA and ethylchloroformate.¹⁸ HFAA reacted with guanidine compounds to form bis (trifluoromethyl) pyrimidine²⁰ and another reagent reacted with imino or carboxylic acid group. HFAA contains bulky trifluoromethyl groups, which causes steric hinderance in the formation of a pyrimidine ring and is indicated by warming time of 2 h at 80°C.^{6,20} It was considered that if bulky trifluoromethyl groups are replaced with methyl and isobutyl groups in HFAA, the formation of a pyrimidine ring with guanidino compounds could take place at lower temperatures with shorter reaction time. The present work therefore examines the use of isovaleroylacetone (IVA) instead of HFAA as a derivatizing reagent for shorter reaction time at lower temperatures. The work also looks in to increasing the number of guanidino compounds analyzed by GC from biological fluids.

Experimental

The compounds G, MG, GAA, guanidinopropanic acid (GPA) (Sigma-Aldrich, St. Louis, MO), guanidino butyric acid (GBA) (Sigma-Aldrich, Switzerland), GSA (Sigma-Aldrich, GmbH,

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Germany), Arg and CTN (Sigma-Aldrich, St. Louis, MO) were used. IVA was prepared by Claisen condensation of methyl isobutyl ketone (MIBK) with ethyl acetate²⁴ as reported by Sparge *et al.*²⁵ and Adams and Hauser.²⁶ FT-IR and UV of IVA was compared with an authenticated sample.²⁷ Ethylchloroformate (ECF) (Fluka, Switzerland) and methanol (RDH, Germany) were used. Guaranteed reagent grade hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, sodium tetraborate, boric acid, sodium bicarbonate, sodium carbonate, ammonium chloride and ammonia solutions were obtained from E. Merck, Darmstadt, Germany.

The stock solutions containing 1 mg/mL were prepared with 0.05 N hydrochloric acid in water. Further solutions were prepared by appropriate dilution. The derivatizing reagent IVA (1% v/v) was prepared in methanol. ECF was used without purification for derivatization. Buffer solutions (0.1 M) between pH 1 – 10 were prepared from the following: potassium chloride adjusted with hydrochloric acid (pH 1 – 2), acetic acid-sodium acetate (pH 3 – 6), ammonium acetate (pH 7), boric acid-sodium tetraborate (pH 7.5 – 8.5), sodium bicarbonate-sodium carbonate (pH 9) and ammonium chloride ammonia solution (pH 10).

Equipment

The pH measurements were made with an Orion 420 A pH meter (Orion Research Inc., Boston, MA) with combined glass electrode and reference internal electrode. A spectrophotometric study was carried out with Perkin Elmer 35 (Perkin Elmer, Singapore) spectrophotometer with dual 1 cm path length quartz Cuvettes. GC-flame ionization detection (FID) studies were carried out on an Agilent Model 6890 network GC system interfaced with FID and split injector (Agilent Technologies, Santa Clara, CA), hydrogen generator (Parker Boston, Analytical Gas System, H2-90, Parker Hannifin, Haverhill, MA), and using pure nitrogen (99.9%) (British Oxygen Co., Karachi, Pakistan). The air for FID was supplied from a Fountain Air Compressor (Fountain Hills, AZ) connected with a molecular sieve. A computer with Chemstation software controlled the gas chromatograph. A HP-5 capillary column (30 m × 0.32 mm i.d.) with film thickness of 0.25 µm (J & W Scientific GC column, Wilmington, NC) was used throughout the study.

Analytical procedure

Boric acid-sodium tetraborate buffer pH 8.5 (0.5 mL) and IVA (1% v/v in methanol) (1 mL) were added to the solution (0.5 – 1.0 mL) containing guanidino compounds (0.5 – 50 µg/mL each). The contents were warmed on water bath at 60°C for 40 min and allowed to cool at room temperature for 10 min. To this solution was added 0.5 mL of solvent system (acetonitrile-water-methanol-pyridine, 42:42:8:8 v/v/v/v) and ECF (0.5 mL) and the contents were sonicated for 15 min. Chloroform (0.5 mL) was added and the contents were mixed well. The layers were allowed to separate and an aliquot of organic layer was transferred to a screw capped sample vial. The extract (1 µL) was injected on GC with DB-5 column with a temperature program of 90°C for 2 min, followed by an increase in the temperature at a rate of 10°C/min up to 220°C. The nitrogen flow rate was 1 mL/min with split ratio of 10:1. The injector and detector temperatures were fixed at 270 and 280°C, respectively. The flow rates for FID were fixed as follows: nitrogen 45 mL/min, hydrogen 40 mL/min and air 450 mL/min.

Determination of guanidino compounds from serum

The blood samples (5 mL) collected from healthy volunteers or uremic patients were kept at room temperature (30°C) for 1 h and centrifuged at 3000 rpm for 30 min. The supernatant layer of the serum (2.5 mL) was separated and methanol (5.0 mL) was then added. The contents were mixed well and again centrifuged at 3000 rpm for 30 min. The supernatant layer was collected and 1.0 mL of the solution was transferred to a screw capped sample vial and processed as analytical procedure. The quantitation was carried out from linear regression equation $y = ax + b$ derived from the external calibration curve.

Analysis of guanidino compounds from spiked serum samples

Blood samples (5 mL) collected from uremic patients or healthy volunteers were processed as described above. Two portions of 1.0 mL were taken from each after deproteinization with methanol and to one portion was added a mixture of guanidino compounds 4 µg/mL each, then both the solutions were processed as analytical procedure. The quantitation was made from the external calibration curve and from the increase in the response with added standards.

The blood samples of uremic patients were collected with verbal consent from the medical ward of the Liaquat University of Medical and Health Sciences Hospital in Jamshoro and Hyderabad, Pakistan. The blood samples were collected in sterilized EDTA tubes by vein puncture. The blood samples of healthy volunteers who had not taken any medicine for at least one preceding week were collected from the students and employs of the Institute of Advanced Research Studies in Chemical Sciences, University of Sindh, Jamshoro, Pakistan. The samples were collected with verbal consent. The uremic patients and healthy volunteers were informed about the objectives of the project. The collected samples were quickly analyzed as received.

Results and Discussion

The guanidino compounds did not elute from the GC column, but eluted after derivatization with IVA and the GC response in terms of peak height/peak area increased 20 – 40% after the second derivatization with ECF. It was therefore the separation of guanidino compounds as derivatives of IVA and ECF that were examined for simultaneous optimization of the derivatization conditions in terms of the effect of the pH, warming time and temperature, solvent system for derivatization and ease of solvent extraction of the derivatives.

The GC of guanidino compounds from the column HP-5 first as derivative of IVA and then as derivatives of IVA and ECF were examined. Each derivative eluted from the GC as a single peak and separated from the derivatizing reagent. An attempt was made to completely separate all the eight guanidino compounds as symmetrical peaks. Different temperature elution programs and nitrogen flow rates were examined. The initial temperature was varied from 80 – 100°C for 1 to 4 min, followed by heating rate of 10 to 20°C/min up to 200 to 250°C with nitrogen flow rate of 1 – 2 mL/min. The elution program with initial temperature of 90°C for 2 min, followed by heating rate of 10°C/min up to 220°C, with the nitrogen flow rate of 1 mL/min gave better sensitivity with complete base line separation and was selected. The resolution factor (R_s) between adjacent peaks was observed >1.5 (Figs. 1a and 1b). The order of the elution of the compounds was (1) derivatizing reagents (2) G, (3) MG, (4) CR, (5) GAA, (6) GPA, (7) GBA, (8) AR, and (9) GSA.

Fig. 1 GC elution and separation of guanidino compounds after using (a) IVA, (b) IVA and ECF as derivatizing reagents. Peak identifications of (1) Derivatizing reagents, (2) G, (3) MG, (4) CRT, (5) GAA, (6) GPA, (7) GBA (8) Arg (9) GSA from column HP-5 (30 m $\times$ 0.32 mm i.d.) with film thickness of 0.25 μ m at an initial column temperature of 90°C for 2 min, followed by heating rate of 10°C/min up to 220°C with nitrogen flow rate of 1 mL/min. The split ratio was 10:1.

Table 1 Quantitative data of guanidino compounds by GC using isovaleroylacetone and ethyl chloroformate as derivatizing reagents

Compound	Calibration range/ μ g mL ⁻¹	LOD/ μ g mL ⁻¹	LOQ/ μ g mL ⁻¹	Retention time/min	Coefficient of determination (r^2)	Regression equation ($n = 4$)
G	0.5 – 50	0.012	0.036	3.5	0.9994	$y = 8.8659x + 0.4777$
MG	0.5 – 50	0.02	0.06	4.42	0.9991	$y = 10.016x + 1.0495$
CTN	0.5 – 50	0.005	0.015	4.90	0.9997	$y = 22.077x + 0.432$
GAA	0.5 – 50	0.03	0.09	5.85	0.9989	$y = 9.9839x + 1.0186$
GPA	0.5 – 50	0.009	0.027	6.49	0.9969	$y = 12.153x + 1.9949$
GBA	0.5 – 50	0.14	0.42	7.00	0.9994	$y = 10.827x + 0.7486$
Arg	0.5 – 50	0.005	0.015	8.82	0.9997	$y = 14.913x + 0.7623$
GSA	0.5 – 50	0.015	0.045	10.5	0.9998	$y = 13.902x + 0.0636$

Abbreviations are described in the text.

Repeatability of the separation was examined in terms of retention time ($n = 5$) and relative standard deviations were observed within 2.8%. GC separation obtained using IVA was used to optimize the effect of pH, warming time and temperature and addition of derivatizing reagent per analysis. The pH was varied between 1 – 10 at an interval of 0.5 unit and maximum response (average peak height/peak area ($n = 4$)) was observed at pH 8.5 using boric acid-sodium tetraborate buffer; as such these parameters were used. The warming temperature was varied from 30 – 90°C at the interval of 10°C and maximum response was obtained at 60°C, so this was the temperature used for further studies. Warming time was varied from 30 – 140 min at an interval of 10 min and maximum response was recorded at 40 min so warming time was set at 40 min. Now comparing the warming time and temperature with commonly used HFAA as derivatizing reagent, which requires warming time of 2 h at 80°C,^{6,20} a decrease in warming time and temperature in the present method has resulted due to a decrease in steric hindrance in the formation of the pyrimidine ring. The addition of the derivatizing reagent IVA (1% in methanol) was varied from 0.5 – 2.0 mL at an interval of 0.5 mL. The addition of derivatizing reagent was not critical as long as an excess of the reagent was available in the reaction mixture. However, to

complete the reaction in a shorter time, the addition of 1.0 mL was selected.

In order to optimize the reaction conditions with the second derivatizing reagent ECF, the GC separation obtained using both derivatizing reagents IVA and ECF was used. The effect of pH was examined within 6 – 10 and maximum response was again obtained at pH 8.5 and 9.0. For the ease of not adding buffer a second time, pH 8.5 was selected. The variation in the sonication of the reaction mixture at room temperature (30°C) was examined for 5 – 20 min at an interval of 5 min and the maximum response was obtained at 15 min and longer, and was used. The solvent system is reported to have an influence on derivatization with ECF,²⁸ therefore the addition of methanol, acetonitrile and the solvent system (acetonitrile–water–methanol–pyridine 42:42:8:8 v/v/v/v) to the reaction mixture was checked after the first derivatization with IVA. The addition of (acetonitrile–water–methanol–pyridine 42:42:8:8) solvent was selected as reported for similar reactions.²⁸ The effect of order of addition of solvent and ECF for second derivatization was examined, but no significant effect was observed. Chloroform, tertiary butanol and ethyl acetate were examined as solvents for the extraction of the derivatives but chloroform indicated better extraction as reported by other researchers.²⁸

Table 2 Concentration ($\mu\text{g/mL}$) of guanidino compounds in serum

Serum No.	Age/Sex	G	MG	CTN	GAA	GPA	GBA	Arg	GSA
1	22M	0.19 (3.0)	0.13 (2.6)	8.49 (1.8)	0.12 (2.7)	0.08 (3.0)	0.015 (1.6)	4.99 (2.3)	0.046 (2.9)
2	19M	0.21 (2.8)	0.12 (2.4)	7.99 (2.3)	0.16 (3.0)	0.07 (2.9)	0.016 (2.2)	4.82 (1.4)	0.049 (3.2)
3	25M	0.2 (1.7)	0.12 (1.8)	8.88 (1.9)	0.23 (1.3)	0.07 (2.5)	0.014 (2.4)	5.11 (2.4)	0.045 (3.4)
4	23M	0.21 (1.4)	0.13 (2.0)	7.87 (1.8)	0.21 (1.6)	0.09 (2.0)	0.014 (2.9)	4.67 (1.8)	0.051 (2.3)
5	21M	0.2 (2.8)	0.12 (3.2)	7.58 (2.4)	0.19 (1.8)	—	0.016 (2.7)	5.0 (3.1)	0.048 (2.6)
6	19F	0.19 (2.8)	0.14 (3.0)	7.45 (3.1)	0.41 (1.6)	—	0.015 (2.0)	4.81 (2.7)	0.053 (1.9)
7	26M	0.22 (3.1)	0.14 (2.2)	8.78 (3.0)	0.33 (2.7)	0.07 (2.2)	0.015 (1.9)	5.99 (2.1)	0.051 (2.0)
8	22M	0.19 (2.5)	0.21 (3.1)	7.77 (2.2)	0.19 (3.1)	—	0.12 (2.3)	4.87 (2.0)	0.053 (3.3)
9	23M	—	0.16 (1.6)	7.64 (1.9)	0.15 (2.5)	—	0.016 (2.0)	4.79 (1.9)	0.046 (1.8)
10	26M	0.22 (2.9)	0.14 (2.5)	8.56 (2.3)	0.16 (1.8)	0.09 (1.7)	0.017 (1.7)	5.12 (3.1)	0.0465 (3.0)
10*		0.19 (2.8)	0.137 (3.0)	8.4 (2.4)	0.15 (1.5)	0.08 (2.4)	0.016 (2.1)	4.99 (3.0)	0.043 (2.1)
11	48M	0.26 (2.7)	0.24 (2.3)	41.89 (1.9)	0.19 (2.0)	0.12 (3.6)	0.09 (1.6)	12.89 (3.3)	1.25 (1.4)
12	51F	0.42 (2.3)	0.22 (2.1)	43.33 (2.8)	0.21 (2.5)	—	0.095 (1.7)	14.65 (1.9)	1.22 (1.7)
13	55M	0.37 (2.0)	0.28 (1.9)	43.99 (2.5)	0.22 (1.8)	0.14 (3.1)	0.089 (2.4)	15.11 (3.1)	0.99 (1.7)
14	40M	0.44 (2.3)	0.14 (2.3)	41.65 (1.8)	0.2 (1.6)	—	0.023 (1.9)	13.21 (2.2)	0.89 (1.5)
15	46F	0.46 (1.5)	0.18 (3.4)	41.0 (3.1)	0.18 (2.0)	0.13 (2.2)	0.089 (2.2)	13.0 (2.5)	1.24 (1.5)
16	38M	0.39 (1.4)	0.18 (2.4)	41.0 (1.8)	0.18 (2.7)	—	0.099 (3.1)	12.87 (1.9)	1.24 (1.8)
17	28M	0.28 (1.7)	0.27 (2.0)	40.84 (2.1)	0.21 (2.6)	0.14 (3.0)	0.26 (2.4)	12.54 (3.1)	1.26 (1.6)
18	32F	0.45 (3.2)	0.21 (1.8)	40.22 (1.7)	0.22 (2.7)	0.14 (2.9)	0.24 (3.2)	12.55 (1.8)	0.98 (2.2)
19	41M	0.41 (1.9)	0.22 (2.5)	41.37 (1.9)	0.2 (2.3)	0.16 (1.7)	0.26 (3.2)	13.51 (2.7)	0.99 (2.9)
20	60M	0.55 (2.1)	0.26 (3.2)	43.99 (2.1)	0.22 (1.9)	0.34 (2.9)	0.026 (2.0)	15.55 (1.8)	0.89 (2.2)
20**		0.53 (1.4)	0.25 (1.9)	43.55 (1.8)	0.217 (1.4)	0.33 (2.2)	0.025 (2.8)	15.24 (1.9)	0.87 (1.8)

Healthy volunteers (1 to 10) and uremic patients (11 to 20). *Spiked sample; %RSD, 1.3 – 3.4. **Spiked sample; %RSD, 1.4 – 3.6.

Repeatability for derivatization, solvent extraction and GC separation was examined ($n = 5$) in terms of retention time and peak height/peak area and RSDs were observed within 1.3 – 2.7%.

Quantitation

Linear calibration curves were recorded by plotting average peak height/peak area ($n = 4$) against concentration within 0.5 – 50 $\mu\text{g/mL}$ with coefficient of determination (R^2) within 0.9969 – 0.9998 with 19 calibrators at selected operating conditions. The limits of detection (LODs) and limits of quantitation (LOQs) calculated as signal to noise (S/N) ratio 3:1 and 10:1 were within 0.01 – 0.15 $\mu\text{g/mL}$ and 0.03 – 0.45 $\mu\text{g/mL}$ respectively (Table 1). An improvement in LOD and LOQ was observed by the use of IVA instead of HFAA¹⁸ due to the increase in the sensitivity in GC-FID response owing to the increase in carbon number in IVA. The reproducibility of quantitation measured in terms of peak height and retention time was examined at inter ($n = 5$) and intra ($n = 5$) days for all the eight guanidino compounds at a concentration of 10 $\mu\text{g/mL}$. The changes in peak height inter and intra day were observed with RSDs within 2.1 and 2.9%, respectively. The corresponding RSDs for retention times were 1.8 and 2.5%, respectively. The analysis of test mixtures ($n = 5$) of guanidino compounds within calibration range was conducted with relative error within $\pm 3.2\%$.

The interfering effect of pharmaceutical additives and amino acids were examined on the determination of the guanidino compounds. The compounds glucose, galactose, starch, lactose, fructose, glycine, serine, alanine, ascorbic acid, and gum acacia were added twice to the concentration of guanidino compounds and their effect on separation, retention time and peak height were examined. The average responses ($n = 4$) were compared to the standard solution of guanidino compounds. Their presence did not affect the determination with relative error within $\pm 3.0\%$.

Sample analysis

The method was applied for the analysis of guanidino compounds from the serum of healthy volunteers and uremic patients. Pre-deproteinized serum was examined for the contents of G, MG, CTN, GAA, GPA, GBA, Arg and GSA. A blank was also prepared from serum without addition of derivatizing reagents, but the elution of the guanidino compounds was not indicated. The identification of each analyte was based on the comparison of retention times with that of standards and by spiking of each compound in sequence. The amounts of guanidino compounds from the serum of 10 healthy volunteers ranging in age from 19 to 26 years were as follows: G, below the limit of detection (BLOD) – 0.22 $\mu\text{g/mL}$; MG, 0.12 – 0.16 $\mu\text{g/mL}$; CTN, 7.45 – 8.88 $\mu\text{g/mL}$; GAA, 0.12 – 0.41 $\mu\text{g/mL}$; GPA, BLOD – 0.09 $\mu\text{g/mL}$; GBA, 0.014 – 0.12 $\mu\text{g/mL}$; Arg, 4.67 – 5.99 $\mu\text{g/mL}$ and GSA, 0.043 – 0.053 $\mu\text{g/mL}$ with RSDs within 1.3 – 3.4%. Similarly, the analysis of the serum of 10 uremic patients in the age range of 28 – 60 years indicated concentrations in $\mu\text{g/mL}$ as follows: G, 0.26 – 0.53; MG, 0.14 – 0.28; CTN, 40.22 – 43.99; GAA, 0.18 – 0.22; GPA, BLOD – 0.34; GBA, 0.023 – 1.266; Arg, 12.54 – 15.55 and GSA, 0.87 – 1.26 with RSDs within 1.4 – 3.6% (Table 2). A sample from healthy volunteers and a sample from uremic patients were spiked with standards of all eight guanidino compounds and a corresponding increase in peak height was indicated without change in peak shape (Fig. 2a). It indicated that the deproteinized serum did not interfere with the derivatization, solvent extraction and GC separation of the guanidino compounds. The recovery from the serum of healthy volunteers and uremic patients was observed within G, 96.36 – 97.7%; MG, 96.1 – 97.8%; CTN, 98.1 – 98.9%; GAA, 98.1 – 98.6%; GPA, 97.0 – 97.7%; GBA, 96.1 – 97.0%; Arg, 97.4 – 98.0% and GSA, 97.7 – 98.7% with RSDs within 3% (Fig. 2b).

The average value of 10 healthy volunteers was compared with the average value of 10 uremic patients for each guanidino compound and a t -test was applied at 95% confidence limits and

Fig. 2 (a) GC determination of guanidino compounds in serum samples of uremic patients. Peaks and conditions as in Fig. 1. (b) GC response of guanidino compounds from serum of uremic patients after spiking each compound with 4 µg/mL. Derivatization was with IVA and ECF peak positions and conditions as in Fig. 1.

Table 3 Comparison of present method with reported methods

Serum No.	Technique	Derivatizing reagent	Compound analyzed	Calibration range	LOD	Optimal reaction time	Applications	Ref.
1	GC-MS	Hexafluoroacetylacetone and monotrimsilyltrifluoroacetamide	3	38 – 7325 µmol/L	1.22 – 1.54 µmol/L	2 h (derivatization time), separation time 5 min for GAA and CTN	Human urine	6
2	HPLC gradient elution	Anison	8	0.45 – 1310.8 nmol/mL	2 – 155 fM	5 min (derivatization time), separation time 40 min	Pharmaceuticals and human urine	13
3	GC-MS	Hexafluoroacetylacetone and pentafluorobenzyl bromide	1	5 – 100 nmol	10 nM/L	2.25 h (derivatization time), separation time 6.5 min for GAA	Human urine and plasma	21
4	GC	Isovaleroylacetone and ethylchloroformate	8	0.5 – 50 µg/mL	5 – 140 ng/mL	40 min (derivatization time), separation time 11 min	Human serum	Present method

a significant difference was indicated for G, MG, CTN, GBA, GSA and Arg. The results of analysis obtained agreed with reported values for GAA from healthy volunteers^{21,29} and GSA, GBA and MG for both uremic patients and healthy volunteers.

The present method was also compared with representative reported analytical procedures (Table 3) in terms of ease of derivatization, calibration range, LODs, separation time and guanidino compounds analyzed. The present method compares favorably with reported procedures.

Conclusions

An analytical procedure has been developed for the separation and determination of eight guanidino compounds using IVA and ECF as derivatizing reagents with shorter derivatization time and temperature, as well as an improvement in GC-FID sensitivity. The method is applicable for the determination of guanidino compounds from deproteinized serum from healthy volunteers and uremic patients.

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