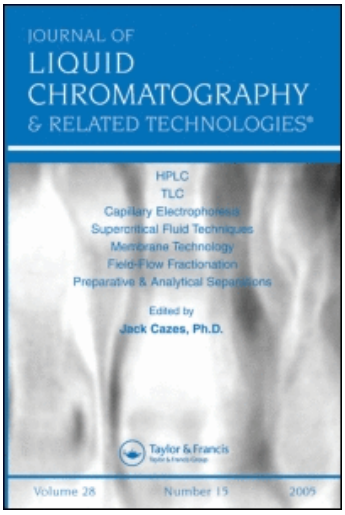

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A. J. Kandhro[a]; M. Y. Khuhawar[a]
[a] Institute of Advanced Research Studies & Chemical Sciences, University of Sindh, Jamshoro, Pakistan

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HPLC DETERMINATION OF GUANIDINO COMPOUNDS IN SERUM OF UREMIC PATIENTS USING PYRIDOIN AS DERIVATIZING REAGENT

A. J. Kandhro and M. Y. Khuhawar

Institute of Advanced Research Studies & Chemical Sciences, University of Sindh, Jamshoro, Pakistan

□ *Guanidino compounds: guanidine (GD), methylguanidine (MG), guanidinoacetic acid (GAA), guanidinopropionic acid (GPA), guanidinobutyric acid (GBA), arginine (Arg), guanidinosuccenic acid (GSA), and creatinine (CRN) as derivatives of pyridoin were separated from Kromasil C-18, 5 μm column, when eluted with methanol-water-sodium tetraborate buffer (0.1 M, pH 8.8) (57:28:15 v/v/v) with a flow rate of 1 mL/min. The UV detection was at 228 nm, linear calibrations were observed with 1.14–141 μmol/L and limits of detection (LOD) within 0.039–0.070 μmol/L. The method was used for the determination of guanidino compounds from serum of uremic patients, and the results have been interpreted as compared to that of healthy volunteers. The results obtained were reproducible (n = 4) with RSD within 4.2% and were higher in uremic patients as compared to healthy volunteers.*

Keywords guanidino compounds, healthy volunteers, HPLC, pyridoin, uremic patients

INTRODUCTION

Guanidino compounds are small water soluble solutes to which neurotoxic effects have been attributed.[1] These are excreted from healthy human bodies by healthy kidneys. However, the concentration of guanidino compounds, especially guanidine (GD), guanidinosuccenic acid (GSA), creatinine (CRN), and methylguanidine (MG) are highly increased in uremic biological fluids and tissues.[2] A number of these are uremic toxins. The MG is shown to be related to the uremic polyneuropathy,[3] and GSA is related to uremic bleeding diathesis and uremic encephalopathy.[4] The compounds, GSA, MG, GD, and CRN are suggested to cause chronic and generalized seizures after systemic and intracerebroventricular administration in mice.[5,6] Guanidino compounds do not show a similar

Address correspondence to M. Y. Khuhawar, Institute of Advanced Research Studies & Chemical Sciences, University of Sindh, Jamshoro, Pakistan. E-mail: jkandhro04@yahoo.com

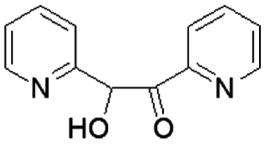

FIGURE 1 Structural diagram of Pyridoin.

kinetic behavior as urea, with easy removal by dialysis strategy. Eloot et al.[7] observed that GD, MG, CRN, GSA, and guanidinoacetic acid (GAA) indicated markedly larger distribution volumes as compared to urea. This resulted in the decrease in the effective removal on the dialysis, requiring an increase in the dialysis duration and or frequency. Thus, determination of guanidino compounds in the biological fluids of uremic patients has clinical importance.

The analytical procedures reported for the determination of the guanidino compounds are based on high performance liquid chromatography (HPLC),[8–12] gas chromatography (GC),[13,14] and capillary electrophoresis (CE).[15] The derivatization is generally carried out before the detection to enhance the absorbance, fluorescence, or the volatility of the compounds. The derivatizing reagents used for HPLC determination are ninhydrin,[16] benzoin,[12–19] anisoin,[9] furoin,[8] 9,10-phenanthrenequinone,[20] and 9,10-phenanthrenequinone-3-sulfonate.[21] The detection was usually repor-ted by spectrophotometric or spectrofluorimetric.[8,9,16–26] The chromatography of guanidino compounds has been reviewed.[22] Yonekura et al. examined benzoin and its analogues including pyridoin as chemiluminogenic reagents for arginine (Arg) containing peptides.[26]

The work examined pyridoin [1,2-di-2-pyridyl-2-hydroxyethanone] (Figure 1) as a precolumn derivatizing reagent to develop a simple HPLC procedure using isocratic elution for the separation and determination of guanidino compounds: GD, MG, GAA, GPA, GBA, Arg, GSA, and CRN using UV detection. The conditions for the derivatization and separation of guanidino compounds are optimized and examined in the terms of linearity, limit of detection (LOD), limit of quantitation (LOQ), repeatability (inter- and intra-day), and accuracy.

EXPERIMENTAL

Chemicals and Solutions

2-Pyridinecarboxaldehyde (Fluka, Switzerland), GD, MG, GAA, Arg, CRN (Sigma Louis, USA), GBA (Sigma, Switzerland), GSA (Sigma GmbH, Germany), methanol (RDH, Germany), guaranteed reagent grade

acetonitrile, β-mercaptoethanol, sodium sulphite, potassium hydroxide, hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, ammonium acetate, sodium tetraborate, boric acid, sodium bicarbonate, ammonium chloride, and ammonia solution were used from E. Merck (Germany).

The stock solutions of guanidino compounds containing 1.14–141 μmol/L were prepared in (0.05 M) hydrochloric acid. Further solutions were prepared by appropriate dilutions. The derivatizing reagent pyridoin was prepared as reported[28] from 2-pyridinecarboxaldehyde, following benzoin type condensation in pyridine-water (30:70 v/v) as the solvent. The derivatizing reagent pyridoin solution (4 mmol) was prepared in formamide-methanol; whereas, pyridoin (85 mg) was dissolved in 20 ml of formamide and the volume adjusted to 100 mL with methanol. The solutions of β-mercaptoethanol (0.1 M), sodium sulfite (0.2 M) and potassium hydroxide (2 M) were prepared in distilled water.

Buffer solutions (0.1 M) between pH 1–10 at unit intervals were prepared from the following: hydrochloric acid and potassium chloride (pH 1–2), acetic acid and sodium acetate (pH 3–6), ammonium acetate (pH 7), boric acid and sodium tetraborate (pH 8–9), sodium bicarbonate and sodium carbonate (pH 9), and ammonium chloride and ammonia (pH 10).

Equipment

The pH measurements were made with an Orian 420A pH meter with combined glass electrode and reference internal electrode. The spectrophotometric study was carried out with Perkin Elmer Lambda 35 UV-vis spectrophotometer (Perkin Elmer Life and Analytical Sciences, Ayer Rajah Crescent, Singapore) with dual 1 cm silica cuvettes. IR spectrum of pyridoin was recorded on an Avatar 330 FT-IR (Thermo Nicolet, Thermo Electron Corporation, USA) with attenuated total reflectance (ATR) (accessory smart partner) within a range of 4000–660 cm^{-1}.

HPLC studies were carried out with Hitachi L-7100 liquid chromatograph (Hitachi (Pvt) Ltd, Tokyo, Japan) connected with variable wavelength UV-Vis Varian 9050 detector (Varian Instrument Group Walnut Creek, Ca, USA), Rheodyne 7125 injector with 20 μl sample loop and Hitachi Chromato-integretor 2500. Column Kromasil 100, C-18, 5 μm (150 × 4.6 mm id) (Teknokroma S. Corp. Ltd, Barcelona, Spain) was used throughout the study.

Spectrophotometric Procedure

An aqueous solution (0.5 mL) containing GD (4.2–37.5 μmole/L), MG (3.65–36.7 μmole/L), GAA (3.4–40.6 μmole/L), GPA (3.1–35.4 μmole/L),

GBA (2.72–38.2 μmole/L), GSA (2.28–45.8 μmole/L), Arg (2.3–92.3 μmole/L), and CRN (3.52–140.5 μmole/L) was placed in a 5 ml volumetric flask separately, and each was treated with 0.5 mL (4 mmole) 2,2-pyridoin, 0.3 ml β-mercaptoethanol (0.1 M), 0.3 ml sodium sulfite (0.2 M), and 0.5 ml potassium hydroxide (2 M) with cooling in ice water. The mixture was heated in a boiling water bath for 5 min., cooled in ice water for 2 min., and then added to a 0.5 mL sodium tetraborate buffer (0.1 M, pH 8.8). The contents were mixed well. A slight turbid solution formed and was then added to a solvent system of acetonitrile:methanol: water (40:40:20 v/v/v) to clear the solution, and the volume was adjusted to 5 ml. The absorption spectra were recorded against a reagent blank within 600–200 nm and absorbance was measured at 358 nm.

Analytical Procedure

An aqueous solution 0.5 ml containing GD, MG, GAA, GPA, GBA, GSA, Arg, and CRN within the concentration range as indicated is shown in Table 1 was placed in 5 mL volumetric flask and was treated as mentioned previously in the Spectrophotometric Procedure section. The solution 20 μl was injected on column Kromasil 100, C-18, 5 μm (150 × 4.6 mm id) and eluted with methanol-water-sodium tetraborate buffer (0.1 M, pH 8.8) (57:28:15 v/v/v) with a flow rate of 1 mL/min. UV detection was at 228 nm.

Analysis of Guanidino Compounds from Serum

The blood sample (5 mL) collected from healthy volunteers and uremic patients was kept at room temperature (30°C) for 1 hr and centrifuged at 3000× g for 30 min. The supernatant layer of serum (2.5 mL)

TABLE 1 Quantitative Data of Guanidino Compounds by HPLC as Derivatizing Reagent 2,2-pyridoin

S.No.	Guanidino Compounds	Calibration Range μmol/L	Limit of Quantitation (LOQ) μmol/L	Limit of Detection (LOD) μmol/L	Coefficient of Determination (r^2)	Linear Regression Equations
1	GSA	1.14–44.2	0.11	0.039	0.9988	$1.6932 \times +2.7865$
2	GAA	1.72–39.5	0.17	0.061	0.9979	$1.259 \times +2.0916$
3	GPA	1.58–34.7	0.16	0.052	0.9984	1.0732×-1.7365
4	CRN	1.77–141	0.18	0.062	0.9981	$1.858 \times +3.2366$
5	GBA	1.39–37.5	0.15	0.050	0.9971	1.1663×-2.2349
6	Arg	1.15–92.5	0.12	0.038	0.9969	$1.4345 \times +3.1198$
7	GD	2.1–36.4	0.21	0.070	0.9981	1.1263×-1.9848
8	MG	1.84–36.0	0.18	0.062	0.9982	$0.9982 \times +1.3041$

was separated and it was then added 2.5 mL of methanol. The contents were mixed well and again centrifuged at 3000× g for 20 min. The supernatant layer was collected and 2.5 ml solution was transferred to a 5 mL volumetric flask and the procedure described previously in the Analytical Procedure section was followed. The quantification was carried out from the linear regression equation $y = a\times + b$ derived from the external calibration curve.

Analysis of Guanidino Compounds from Serum Using Linear Calibration with Spiked Samples

A blood sample (10 mL) from a uremic patient (No 10, Table 2) was treated as previously described in the Analytical Procedure section. Deproteinized serum with methanol (2.5 mL) was taken in duplicate and one part was added GPA (0.5 mL) (5 μmol/L). Both the solutions were processed as previously described in the Analytical Procedure section. The quantitation was from the external linear calibration curves and from an increase in the response with an added standard.

TABLE 2 Concentration of Guanidino Compounds in Serum of Uremic Patients by HPLC (μmol/L) as Derivatizing Reagent 2,2-pyriodoin

S.No.	Age/ Sex	GSA μmol/L RSD (%)	GAA μmol/L RSD (%)	GPA μmol/L RSD (%)	CRN μmol/L RSD (%)	GBA μmol/L RSD (%)	Arg μmol/L RSD (%)	GD μmol/L RSD (%)	MG μmol/L RSD (%)
1	54 M	7.97	3.89	1.13	455.63	0.91	72.87	2.23	2.39
		(2.1)	(1.9)	(2.7)	(1.6)	(3.1)	(1.9)	(1.2)	(1.6)
2	61 M	8.15	4.27	0.98	462.74	1.13	79.26	3.12	2.53
		(1.7)	(1.1)	(3.9)	(2.8)	(2.9)	(2.3)	(2.8)	(1.3)
3	46 M	8.65	5.57	1.41	425.42	1.21	80.14	3.17	2.68
		(3.6)	(1.4)	(2.3)	(1.3)	(2.4)	(3.6)	(1.9)	(2.2)
4	59 M	8.86	5.81	1.03	483.51	1.34	86.36	2.46	2.07
		(2.6)	(1.7)	(1.5)	(3.2)	(1.6)	(1.3)	(1.7)	(3.1)
5	52 F	5.28	3.67	1.17	517.37	0.95	87.46	2.92	2.73
		(2.3)	(3.4)	(3.3)	(1.9)	(2.5)	(1.4)	(3.6)	(3.4)
6	55 M	7.91	3.38	1.32	519.65	1.04	96.52	3.19	2.26
		(2.1)	(1.8)	(2.9)	(3.4)	(2.8)	(3.1)	(3.5)	(4.2)
7	48 F	5.26	4.29	1.11	494.80	1.10	90.48	3.78	2.17
		(3.8)	(3.2)	(2.8)	(2.5)	(2.2)	(4.2)	(2.7)	(1.1)
8	46 M	6.12	4.13	1.07	481.32	1.15	94.61	3.65	2.75
		(3.1)	(2.7)	(2.4)	(3.3)	(2.6)	(2.1)	(3.7)	(2.8)
9	57 M	7.64	4.27	1.20	513.45	1.25	85.74	2.29	2.16
		(2.9)	(1.5)	(1.6)	(1.7)	(1.7)	(3.2)	(2.5)	(4.3)
10* Direct	50 F	7.69	2.65	**0.93**	464	1.43	84.7	2.8	1.17
		(3.5)	(2.2)	**(1.7)**	(2.4)	(2.6)	(1.6)	(2.7)	(1.9)
Spiked				**0.98**					
				(2.3)					

RESULT AND DISCUSSION

Optimization of Derivatization

2,2-pyridoin an analogue of benzoin was examined for its reactions towards guanidino compounds. The reactions were initially monitored spectrophotometrically, following a similar reaction conditions as reported by Kai et al.[12] using benzoin as the derivatizing reagent. The reactions of pyridoin with guanidino compounds were examined in an alkaline media in the presence of β-mercaptoethanol and sodium sulfite. The reaction was monitored at 358 nm. The effect of a heating temperature between 70°C and 100°C at intervals of 10°C was examined, and the maximum response was observed at 100°C as reported with related compounds;[8,9] and, heating in boiling water was selected. Heating time was varied from 2 to 10 min with a variation of 1 min. A maximum response was observed at 4 to 7 min and a heating time of 5 min was selected. The addition of derivatization reagent pyridoin (4 mmole) was varied from 0.2–1.0 mL at intervals of 0.1 mL. The addition of derivatization reagent was not critical as long as the excess of the derivatizing reagent was available, and a similar response was observed with 0.4 to 0.7 mL; therefore, an addition of 0.5 mL was selected. An addition of buffer solution after the derivatization within pH 7–10 was examined with an interval of 0.25 units. Maximum absorbance was observed at pH 8.8 using sodium tetraborate buffer and, therefore, pH 8.8 was selected. At the end of the reaction, a slight turbid solution was generally observed, but when the solution was made up to volume (5 mL) with acetonitrile-methanol-water 40:40:20 v/v/v), a clear solution was observed. At the optimized conditions, the guanidino compounds indicated the values of molar absorptivity within 2.8 to 4.8×10^3 L.mole^{-1} cm^{-1} at 358 nm Table 3.

TABLE 3 Quantitative Data of Guanidino Compounds by Spectrophotometry using 2,2-pyridoin as Derivatizing Reagent

S.No.	Guanidino Compounds	Calibration Range µmol/L	Limit of Quantitation (LOQ) µmol/L	Limit of Detection (LOD) µmol/L	Coefficient of Determination (r^2)	λ-max 358 nm	ξ-Absorptivity mole^{-1} cm^{-1}
1	GSA	2.28–45.8	0.23	0.08	0.9971	358	3.9×10^3
2	GAA	3.4–40.6	0.35	0.11	0.9964	358	3.4×10^3
3	GPA	3.1–35.4	0.32	0.10	0.9959	358	2.8×10^3
4	CRN	3.52–140.6	0.36	0.12	0.9965	358	4.8×10^3
5	GBA	2.72–38.2	0.28	0.10	0.9949	358	2.9×10^3
6	Arg	2.3–92.3	0.24	0.09	0.9952	358	4.0×10^3
7	GD	4.2–37.5	0.43	0.13	0.9968	358	3.0×10^3
8	MG	3.65–36.7	0.36	0.12	0.70	358	2.9×10^3

CHROMATOGRAPHY

The guanidino compounds react with pyridoin to form different derivatives, which could be separated by HPLC and detected by UV detector. The separation was examined from column Kromasil 100, C-18, 5 μm (150 × 4.6 id) using isocratic elution. Solvent system consisting of methanol-water-sodium tetraborate buffer (0.1 M) was examined and the addition of acetonitrile or tetrahydrofuran did not improve the separation. Complete separation between the derivatizing reagent and eight guanidino compounds GSA, GAA, GPA, CRN, GBA, Arg, GD, and MG was obtained within 26 min using methanol-water-sodium tetraborate buffer (0.1 M, pH 8.8) (57:28:15 v/v/v) with a flow rate of 1 mL/min. The detection was possible at 356 nm; however, for better sensitivity, a UV region within 200 to 300 nm was examined and UV detection at 228 nm was selected. Using the conditions the resolution factor (Rs) between the adjacent peaks was obtained >1.7. The capacity factor (K′) for derivatizing reagent 2,2-pyridoin and guanidino compounds GSA, GAA, GPA, CRN, GBA, Arg, GD, and MG was calculated as 1.52, 4.56, 7.51, 10.17, 13.13, 15.74, 18.24, 21.23, and 24.12, respectively (Figure 2). Peak identification was based on comparison of retention times to the standards, followed by spiking each guanidino compound in sequence. The separation was repeatable (n = 5) with relative standard deviation (RSD) in retention times within 2.4%.

Quantitation and Validation

Linear calibration curves were obtained by plotting average peak height/peak area (n = 4) of each guanidino compound against concentration within the range 1.14–141 μmole/L with a coefficient of determination (r^2) within 0.9969–0.9988. The limits of detection (LODs) and limits of quantitation (LOQs) measured as signal to noise ratio (3:1) and (10:1) were calculated within 0.039–0.070 μmole/L and 0.11–0.21 μmole/L, respectively (Table 1); inter-day (n = 5) and intra-day (n = 5) variations, in the terms of retention time and peak height, were observed with RSD within 2.1% and 3.6%, respectively. The accuracy of the method was checked by the analysis of the test mixtures (n = 4) of guanidino compounds within the calibration range and relative error was observed within ±2.3%. The pharmaceutical additives and some amino-acids were examined for their possible interfering effects on the determination of the guanidino compounds. The compounds lactose, glucose monohydrate, starch, magnesium stearate, methylparabin, talc, gum acacia, *β*-alanine, and L-cystine were added in amounts twice the concentration of MG, and their effect was examined on separation, migration, and average peak height (n = 4). The responses were compared with standard solutions of

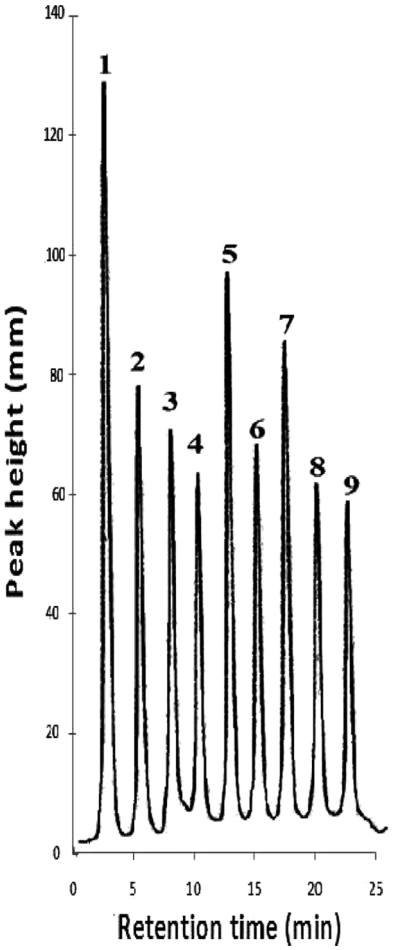

FIGURE 2 HPLC separation of guanidino compounds (1) Reagent (2) GSA (3) GAA (4) GPA (5) CRN (6) GBA (7) Arg (8) GD (9) MG as derivatives of pyridoin, Conditions: Column Kromasil 100, c-18 µm (150 × 4.6 mm id) eluted with methanol-water–sodium tetraborate buffer (0.1 M, pH 8.8) (57:28:15 v/v/v) with a flow rate of 1 mL/min. UV detection was at 228 nm.

the guanidino compound derivatives. They did not affect the determination of guanidino compounds with relative error within ±2.7%.

Analysis of Serum

Human serum after deproteinization with methanol was analyzed for the contents of the guanidino compounds. The analytes were identified by comparison of chromatographic retention time (t_r) with those of the standards. The serum samples of ten uremic patients and ten healthy volunteers were analyzed and responses corresponding to GSA, GAA, GPA, CRN, GBA, Arg, GD, and MG were examined. A representative

chromatograph obtained in the analysis of serum is illustrated in Figure 3a. The chromatograph obtained from the serum resembled Figure 2 recorded from standard guanidino compounds, and it seems that the biological matrix did not interfere with compound analysis. The results of the analysis are reported in Tables 2 and 4. The uremic patients within the age 46–61 years indicated concentrations μmol/L of GSA 5.26–8.86, GAA 2.65–5.81, GPA 0.93–1.41, CRN 425–519, GBA 0.91–1.43, Arg 72.87–96.52, GD 2.23–3.78, and MG 1.17–2.75 with RSD within 1.1–4.3% as compared to healthy volunteers within the age 22–34 years indicated the concentrations

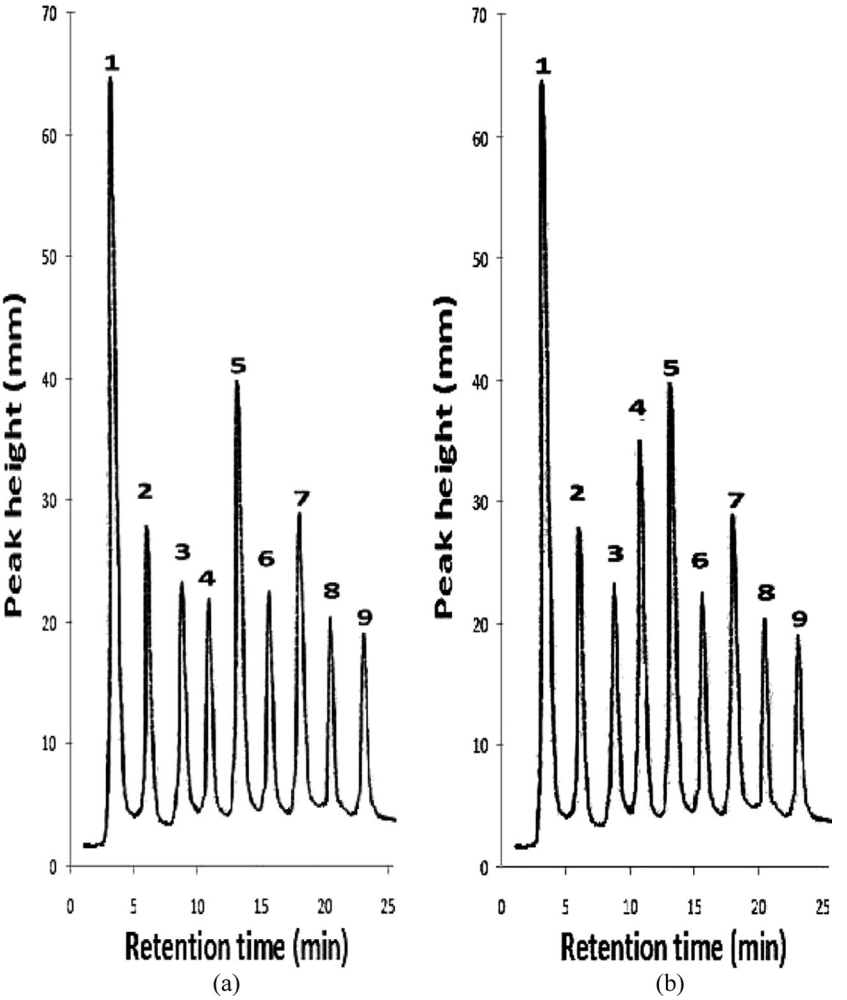

FIGURE 3 (a) HPLC separation of guanidino compounds from serum of uremic patient: (1) Reagent (2) GSA (3) GAA (4) GPA (5) CRN (6) GBA (7) Arg (8) GD (9) MG as derivatives of pyridoin, (b) after spiking the serum of uremic patient with GPA (4), conditions as Figure 2.

TABLE 4 Concentration of Guanidino Compounds in Serum of Healthy Volunteers by HPLC (μmol/L) as Derivatizing Reagent 2,2-pyriodoin

S.No.	Age/ Sex	GSA (μmol/L) RSD (%)	GAA (μmol/L) RSD (%)	GPA (μmol/L) RSD (%)	CRN (μmol/L) RSD (%)	GBA (μmol/L) RSD (%)	Arg (μmol/L) RSD (%)	GD (μmol/L) RSD (%)	MG (μmol/L) RSD (%)
1	24 F	1.23	1.16	0.18	82.36	0.25	29.95	0.30	0.29
		(2.3)	(3.5)	(3.1)	(1.4)	(2.4)	(3.6)	(2.7)	(2.5)
2	27 M	1.25	1.21	0.25	78.96	0.33	32.98	0.26	0.46
		(2.1)	(1.2)	(1.6)	(2.8)	(2.6)	(2.9)	(1.5)	(1.6)
3	22 F	0.98	0.93	0.32	66.46	0.37	31.12	0.63	0.39
		(1.4)	(2.8)	(3.9)	(1.9)	(1.2)	(3.3)	(3.5)	(3.3)
4	34 M	1.36	1.31	0.27	64.92	0.21	33.07	0.51	0.43
		(2.9)	(1.7)	(2.4)	(3.6)	(2.7)	(1.7)	(3.2)	(2.2)
5	26 M	1.29	1.24	0.42	79.76	0.41	30.16	0.42	0.41
		(3.5)	(1.9)	(2.3)	(1.5)	(3.1)	(3.9)	(1.9)	(2.4)
6	29 M	1.14	1.10	0.36	70.81	0.40	36.14	0.36	0.27
		(3.7)	(3.1)	(2.8)	(2.3.)	(2.9)	(2.8)	(4.1)	(1.2)
7	20 F	1.42	1.37	0.40	73.26	0.19	39.04	0.29	0.23
		(3.3)	(2.5)	(3.7)	(2.6)	(1.3)	(1.5)	(1.6)	(3.6)
8	28 M	1.18	1.15	0.30	97.73	0.17	42.06	0.34	0.35
		(2.4)	(3.8)	(1.9)	(3.1)	(3.6)	(2.2)	(3.8)	(2.3)
9	22 M	0.96	0.82	0.27	69.61	0.23	37.16	0.23	0.19
		(1.7)	(1.6)	(2.5)	(2.1)	(3.2)	(2.9)	(1.4)	(2.4)
10	25 F	1.15	1.13	0.31	71.13	0.31	38.19	0.32	0.25
		(1.3)	(1.4)	(3.4)	(3.2)	(2.5)	(3.1)	(2.1)	(2.8)

μmol/L of GSA 0.96–1.42, GAA 0.82–1.37, GPA 0.18–0.42, CRN 64.92–97.73, GBA 0.17–0.41, Arg 29.95–42.06, GD 0.26–0.63 and MG 0.19–0.46 with RSDs within 1.2–4.1%. The serum of a patient was also spiked with GPA and results of analysis agreed with that of linear calibration (Figure 3b). The repeatability of the whole procedure (sample treatment, derivatization, and chromatography) for 10 uremic patients and 10 healthy volunteers for the biological sample (blood serum), together with a spiked sample with a known amount of guanidino compound at the concentration within the calibration range, indicated an RSD within 4.3%. The results of analyses for guanidino compounds are within the range reported for uremic patients.[8,9,11,12,16,25,27] The reported HPLC procedures using benzoin,[12] anisoin,[9] and furoin[8] as derivatizing reagents are based on gradient elution, coupled with fluorimetric detection. The present work reports isocratic elution with UV detection.

CONCLUSION

An analytical procedure has been developed for separation and determination of eight guanidino compounds by HPLC using simple

isocratic elution. The detection was by UV at 228 nm. The derivatizing reagent used was pyridoin. The method indicated sensitivity and selectivity required for the analysis of guanidino compounds from biological samples (serum).

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