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ANALYSING GUANIDINO COMPOUNDS IN HUMAN SERUM AND URINE BY GAS CHROMATOGRAPHY

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ABSTRACT

Guanidino compounds are small polar molecules and are difficult to elute from gas chromatography (GC) column. The work examines derivatising reagents isovaleroylacetone (IVA) and isobutyl chloroformate (IBCF) for the GC determinations of 8 substances guanidine (G), creatinine (CTN), guanidinoacetic acid (GAA), methylguanidine (MG), guanidinopropanic acid (GPA), guanidinobutyric acid (GBA), guanidinosussinic acid (GSA) and arginine (Arg) from HP-5 (30 m × 0.32 mm id) column with thickness of film 0.25 µm. All the 8 compounds separated completely within 8 minutes, when eluted for 2 minutes at column temperature 90°C, programmed to 10°C/min up to 220°C with rate of flow of nitrogen 1.5 mL/min. The effect of variables on the derivatisation and elution were optimised and calibration curves were observed within 0.2 µg/mL–40.0 µg/mL and detection limits (LOD) and quantitation limits (LOQ) were within 4 ng/mL–110 ng/mL and 12 ng/mL–330 ng/mL. The identification and quantitation were repeatable (n = 4) with relative standard deviations (RSDs) within 2.1%–3.1%. The additions of pharmaceutical ingredients and amino acids did not interfere the analyses of the guanidino compounds. The procedure was studied for the analyses of guanidino substances from the biological fluids of uremic patients and with disease free volunteers. The quantity established in serum and urine of disease free volunteers were below limit of detection (BLOD) to 9.48 µg/mL and BLOD to 1,125 µg/mL with RSD 1.1%–3.1% and 1.5%–3.0%, respectively. Similarly, the amounts observed in serum and urine of patients suffering from uremic were 0.099 µg/mL–42.37 µg/mL and 0.014 µg/mL–1,338.0 µg/mL with RSD 1.3%–3.2% and 1.5%–3.2%,

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respectively. The recovery of guanidino compounds from spiked serum was calculated 93.3%–97.7% and from urine 95.1%–98.8% with RSD within 3.1% and 3.2%, respectively. The results obtained for disease free volunteers were compared with uremic patients and t-test was applied at 95% confidence level and a significant difference was recorded for number of guanidino compounds.

Keywords: Guanidino compounds, Gas chromatography, Serum and urine samples, Isovaleroylacetone (IVA), Isobutyl chloroformate (IBCF)

INTRODUCTION

The guanidino compounds are present at low concentrations within human physiological fluids and are excreted by healthy kidneys in urine. Their excretion and production are affected during renal failures and the condition may result into toxic effects of uremic (Kandro and Khuhawar 2010; Afzal *et al.* 2020). The guanidino blends are recognised from the existence of guanidinium radical in their structures (Marescau *et al.* 1992). The guanidino compounds have a number of functions in human body. Creatinine (CTN) is responsible for the function of chloride channel (De Deyn and Mac Donald 1990). Creatinine is reported in higher concentration in renal function of sickle cell disorder as compared to healthy volunteers (Airhomwanbor *et al.* 2018). Arginine (Arg) helps immune system and release of human growth hormones (Gatti and Gioio 2006). Hoffman (2019) has included CTN and guanidinoacetic acid (GAA) in dietary supplementation in sports and exercise. The methylguanidine (MG) and guanidinosussinic acid (GSA) are confined as uremic contaminations and agglomerate in the biological fluids of victims of uremic. They show indications similar to uremic (Hanai *et al.* 2000). The guanidino compounds are produced in human kidneys as the result of metabolism of proteins and amino acids (Taes *et al.* 2008). The clinical effects of uremic are because of the failure of excretory, metabolic, regulatory and endocrine functions of the kidneys. As a result, a number of guanidino compounds are found at higher concentration, together with other retained toxic metabolites to cause uremic syndrome (Majidano and Khuhawar 2012). These compounds are present at low concentrations in biological fluids of normal subjects (Vanholder *et al.* 2003), thus requiring sensitive analytical procedure for their detection and determination.

Different methods are described for the quantitation of guanidino compounds, but most of the sensitive and selective procedures are based on chromatography and capillary electrophoresis. The chromophoric methods mostly require derivatisation before their detection, because the guanidino composites are polar water soluble compounds. The methods comprised of high performance liquid chromatography (HPLC) (Kandro and Khuhawar 2010; Gatti and Gioio 2006; Carducci *et al.* 2001; Buchberger and Ferdinand 2004; Kandro and Khuhawar 2013), gas chromatography (GC) (Majidano and Khuhawar 2012; Valongo *et al.* 2004; Hunneman and Hanefeld 1997; Struys *et al.* 1998; Zounr *et al.* 2013; Zounr *et al.* 2016; Majidano and Khuhawar 2013) and capillary electrophoresis (Zinellu *et al.* 2006; Kandro *et al.* 2010; Kandro and Khuhawar 2014).

Among the chromatographic methods, HPLC procedures are more commonly used for the quantitation of guanidino composites, but GC procedures are simpler, involve less running cost and avoid the need of disposal of used solvents. However, GC may require derivatisation to convert the polar compounds to volatile derivatives. The guanidines are water soluble polar compounds and suitable derivatising reagents are used to convert guanidino compounds to volatile product. Hexafluoroacetylacetone (HFAA) together with trimethylsilyltrifluoroacetamide (Valongo *et al.* 2004; Hunneman and Hanefeld 1997),

trifluoroacetic anhydride (Patel and Cohn 1975), pentafluorobenzyl bromide or ethyl chloroformate (Zounr *et al.* 2013) are reported to convert guanidino compounds to volatile products. HFAA reacts with guanidino functional group to appear bis(trifluoromethyl) pyrimidine (Struys *et al.* 1998) and second reagent binds to imine or carboxylic acid group. HFAA comprises of two massive groups of trifluoromethyl and are creating hindrance during the synthesis of pyrimidine ring, as marked by the heating time of 2 hours at 80°C (Valongo *et al.* 2004; Hunneman and Hanefeld 1997). When HFAA was replaced with isovaleroylacetone (IVA) as derivatising reagent, the heating time was reduced to 40 minutes at 60°C (Zounr *et al.* 2016). The current work demonstrates the account of isobutyl chloroformate (IBCF) in place of ethyl chloroformate (ECF), together with IVA to further enhance the sensitivity with reduced analysis time.

MATERIALS AND METHODS

The chemicals guanidine (G), GAA, guanidinopropanic acid (GPA), Arg, CTN, MG (St Louis, Mo, Sigma-Aldrich), guanidinobutyric acid (GBA) (Switzerland, Sigma-Aldrich), GSA (Sigma-Aldrich G mbH, Germany) and IBCF (Fluka, Switzerland) were used. IVA was synthesised by Claisan reaction of 4-methylpentan-2-one (MIBK) with ethyl acetate as narrated (Lindoy *et al.* 1977). The ultraviolet (UV) and Fourier Transform Infrared Spectroscopy (FTIR) of IVA were correlated with valid specimen (Leghari *et al.* 2010). The solutions of standards of guanidino blends comprising 1 mg/mL were adapted with hydrochloric acid (0.05 N). Further solutions were diluted appropriately. Methanolic solutions (1%) were prepared for reagents IVA and IBCF. Solutions buffer (0.1 M) were correlated between pH 1–10 at 0.5 to 1.0 unit interval using potassium chloride and hydro chloric acid (HCl) (pH 1–2), sodium acetate and ethanoic acid (pH 3–6), ammonium acetate (pH 7), sodium tetraborate and boric acid (pH 7.5–8.5), sodium carbonate and sodium bicarbonate (pH 9) and ammonia solution and ammonium chloride (pH 10).

The solution pH was assessed with pH meter, Orion 420 (Boston, MA, Orion Research Inc.). GC system, Agilent 6890 connected with split injector and flame ionisation detection (FID) (Santa Clara, CA, Agilent Technologies) was employed. Generator hydrogen (Havorhill, Parker Baston, MA), nitrogen (99.9%) (Karachi, Pakistan, British Oxygen Company) and air compressor (Fountain Hills, AZ) attached with molecular sieve were connected to GC system. The Chemstation software with computer guarded the chromatograph. HP-5 (30 m × 0.32 mm id) capillary column with thickness of film 0.25 µm (Wilmington, NC, J & W Scientific GC column) was employed.

ANALYTICAL PLAN

Guanidino compounds solution (1.0 mL) containing (0.2 µg/mL–40 µg/mL each) were added IVA (1% v/v in methanol) (1 mL) and 0.5 mL buffer pH 8.5. The contents were heated at 60°C for 30 minutes and cooled at 30°C for 10 minutes. The solvent (0.5 mL) (water-acetonitrile-pyridine-methanol, 42, 42, 8, 8 v/v) and 0.5 mL IBCF (1% v/v) were mixed and sonicated for 15 minutes. Extracting solvent (0.5 mL) (Chloroform) was combined and contents were blended thoroughly. A portion of the chloroform layer was shifted to sample vial. Extract (1.0 µL) injected on HP-5 column at 90°C for 2 minutes and programmed at 10°C/min up to 220°C. The nitrogen flow rate was 1.5 mL/min with 10:1 split ratio. The injector was locked at 270°C and detector was at 280°C. The FID flow rates were established as follows: 45 mL/min nitrogen, 40 mL/min hydrogen and 450 mL/min air.

Analysis of Guanidines from Serum

Venous blood (2.0 mL) sampled from disease free volunteers or uremic subjects were centrifuged for 30 minutes at 3,000 rpm for 30 minutes. The methanol (2.0 mL) was added to collected supernatant layer (1 mL). The constituents were mixed thoroughly and centrifuged for thirty minutes at 3,000 rpm. The upper layer (1.0 mL) was shifted to a screw capped sample vial and processed as analytical plan. The freshly prepared regression equation linear y = ax + b achieved from calibration curve was used for quantitation.

Analysis of Guanidino Blends of Spiked Serum Specimens

Blood specimens (2.0 mL) were processed as analyses of G from serum, procured from disease-free volunteers and uremic victims. Two portions of deproteinised serum 1.0 mL each were taken and to one fraction was combined a mixture of 4.0 µg/mL each guanidino compound and both the solutions were then handled as analytical plan. The quantities were determined from increase in feedback of external calibration curves with standards added.

Analysis of Guanidino Compounds from Urine Specimens

Urine specimen (2.0 mL) was combined with methanol (2.0 mL), obtained from disease-free volunteers and uremic victims. The blend was centrifuged at 3,000 rpm for 30 minutes. Collected clear solution, and deproteinised urine sample (1.0 mL) was moderated to 5 mL with distilled water. The solution (1.0 mL) was handled as analytical plan. The freshly prepared calibration curves were used for quantitation.

Samples Collection

The uremic patients from the medical wards of Civil Hospital Dadu, and Jamshoro and Hyderabad Hospitals of Liaquat University of Medical and Health Sciences were requested for collection of their blood and urine samples, which they accepted by verbal/written permission. The metal free blood accumulating tubes, with > 1.5 mg dipotassium salt of ethylene diamine tetra acetic acid (K_2EDTA) were used to collect the blood samples. The sterilised plastic bottles were used to collect morning urine samples. The blood and urine samples of uremic patients recorded at hospitals were collected, but samples from patients with multiple diseases were not collected.

The research scholars and workers of the University of Sindh, Institute of Advanced Research Studies in Chemical Sciences, agreed to donate blood specimens. They had not used any medicine for last 7 days. The verbal/written permission was obtained before collecting their blood and urine samples. All the participants were briefed the purpose of work. Samples were immediately analysed as acquired. The approval of departmental ethical committee of Institute of Advanced Research Studies in Chemical Sciences, University of Sindh was obtained before starting the work.

Validation

The method was validated in terms of linearity of calibration curves, limits of detection (LOD), limits of quantitation (LOQ), precision (inter- and intra-day), accuracy and stability (effect of experimental variables and effect of diverse compounds). Statistical analyses

(mean, standard deviation, relative standard deviation and coefficient of determination) were calculated using Excel 2013 program.

Effects of Variables on Derivatisation

IVA and IBCF were used as derivatising reagents to optimise the GC separation. The effects were examined for pH, additions of derivatising reagents per analysis, warming time and temperature. IVA was examined first, followed by IBCF for optimisation. The pH for the reagent IVA was altered between 1–10 at 0.5–1.0 unit intervals. pH 8.5 indicated best return (mean peak height/ peak area, n = 4) using borate buffer. The warming temperature and time were altered 20°C–90°C at the interval of 10°C and 10 minutes–120 minutes with a gap of 10 minutes. The maximum responses were achieved at 60°C for 30 minutes. IVA (1%) was added 0.5 mL–2.0 mL at an interval of 0.5 mL. All the concentrations indicated a similar response and 1.0 mL was selected. For the optimisation of second derivatisation with IBCF, GC separation with both IVA and IBCF reagents was examined. The pH was varied 6–11 and better feedback was at pH 8.5 and 9.0. The pH 8.5 was selected to avoid the addition of second buffer. The sonication at indoor temperature (30°C) was changed for 5 minutes–20 minutes at a gap of 5 minutes and 15 minutes was selected. The addition of IBCF was changed from 0.5 mL–1.5 mL (1% in methanol) at a gap of 0.2 mL and IBCF addition was not effecting when enough was accessible in reaction mixture. Finally, the inclusion of 0.5 mL was preferred. The accession of solvent (water-acetonitrile-pyridine-methanol, 42:42:8:8 v/v) suggested (Husek 1991), was altered from 0.2 mL–1.0 mL at a gap of 0.2 mL and 0.5 mL was chosen. 1, 2-dichloroethane, tertiary butanol, ethyl acetate and chloroform were investigated for the separation of derivatives. Chloroform was preferred as reported (Husek 1991).

RESULTS AND DISCUSSION

G are small polar molecules and difficulties are generally encountered in elution from GC column but these could be eluted after appropriate derivatisation. The work examines two derivatising reagent IVA and IBCF for the determination of G, GPA, CTN, GAA, MG, GBA, Arg and GSA. The compounds could be eluted from GC after derivatisation with IVA (Zounr *et al.* 2016), but better sensitivity with a betterment in peak shape was observed using IVA and IBCF due to the binding of remaining polar groups. The GC of the compounds was examined from HP-5 column. Each of the derivative indicated a separate peak from derivatising reagents. Experiments were carried out to separate completely all the 8 clinically important guanidino compounds as well-formed peaks for the applicability of IVA and IBCF derivatisation method. Different temperature programs were tried to elute and separate the guanidino compounds with decreased time. Lastly, the elution program with column at 90°C for 2 minutes, then ramping at 10°C/min up to 220°C, with nitrogen flow rate 1.5 mL/min indicated better results with entire separation and was preferred. The factor resolution (R_s) among adjacent peaks was acquired > 1.5 (see Figure 1). The separation repeatability was investigated in terms of retention time (n = 4) at optimised experimental conditions and RSDs were attended in 2.5%.

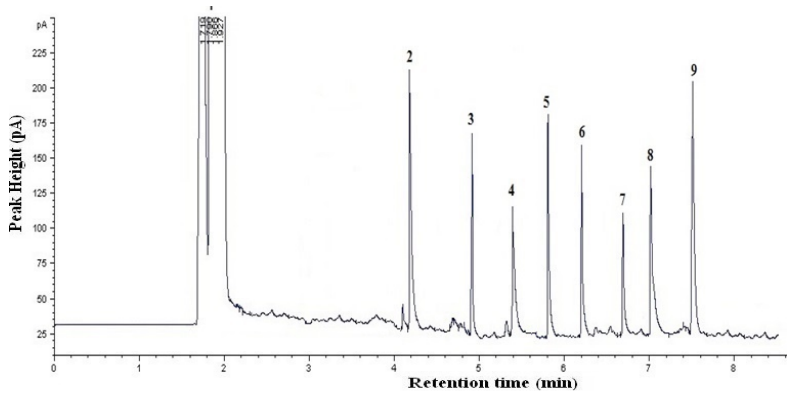

Figure 1: GC elution and separation of guanidino compounds after precolumn derivatisation with IVA and IBCF. Peak identification (1) derivatising reagents, (2) G, (3) MG, (4) CTN, (5) GAA, (6) GPA, (7) GBA, (8) Arg, (9) GSA.

Quantitation

Calibration curves linearity for all the eight G after derivatisation and extraction in organic phase was documented by plotting mean peak height/peak area (n = 4) versus concentrations (µg/mL) and were attained within 0.2 µg/mL–40 µg/mL with coefficient of determination (R^2) within 0.9931–0.9999. LODs and LOQs were evaluated as ratio of signal to noise (3:1) and (10:1) within 4 ng/mL–110 ng/mL and 12 ng/mL–330 ng/mL (see Table 1). Repeatability of derivatisation, quantitation and separation was observed inter (n = 4) and intra (n = 4) day variations with a 6 µg/mL each guanidino compound for retention time and peak height/peak area. The RSDs achieved were in 2.5%–3.1% and 2.1%–2.9% sequentially. The accuracy of the analysis was ascertained by analyses of four test solutions in calibration ranges for the mixtures of guanidino compounds and relative error was acquired in 3.6%.

Table 1: Quantitative data of GCs using IVA and IBCF as derivatising reagents.

Compound	Calibration Range (μgmL^{-1})	LOD (μgmL^{-1})	LOQ (μgmL^{-1})	Retention Time (min)	Coefficient of Determination (R^2)	Regression Equation
G	0.2–40	0.011	0.033	4.22	0.9931	y = 9.9532x + 1.1359
MG	0.2–40	0.030	0.090	4.93	0.9985	y = 11.912x + 0.875
CTN	0.2–40	0.004	0.012	5.41	0.9996	y = 12.816x + 0.9245
GAA	0.2–40	0.020	0.060	5.82	0.9999	y = 16.342x + 0.1777
GPA	0.2–40	0.012	0.072	6.22	0.9995	y = 20.795x – 0.455

(*continued on next page*)

Table 1: (*continued*)

Compound	Calibration Range (μgmL^{-1})	LOD (μgmL^{-1})	LOQ (μgmL^{-1})	Retention Time (min)	Coefficient of Determination (R^2)	Regression Equation
GBA	0.2–40	0.110	0.330	6.76	0.9999	y = 24.129x + 0.1982
Arg	0.2–40	0.004	0.012	7.10	0.9998	y = 25.519x + 0.5436
GSA	0.2–40	0.014	0.042	7.53	0.9995	y = 30.899x – 1.4532

Effect of Diverse Compounds

The obtrusive effects on the determinations of G with drugs additives and amino acids were investigated and galactose, fructose, lactose, starch, glucose, ascorbic acid, alanine, glycine, gum acacia and serine at two times the amount of G, GAA, CTN and Arg, and their accoutrements on separation, peak height and retention time (n = 4) were tested. The solution of standards of G derivatives were used to compare the responses. The effects of the amino acids and additives on the determinations were not observed and relative errors were not over 2.8%.

Serum Analyses

The post deproteinised serum was analysed for the specimens obtained from healthy volunteers and uremic victims for the load of G, GBA, GAA, GPA, GSA, MG, CTN and Arg. The chromatographic retention times (t_R) were used to identify the analytes by comparing with standard G's and spiking each of the compost in ordering. Ten disease-free volunteers in the age 20 years–29 years indicated average amounts (μg/mL)(n = 4) within G: 0.14–0.49, MG: below limit of detection (BLOD)–0.25, CTN: 6.45–9.67, GAA: 0.11–0.28, GPA: BLOD–0.03, GBA: 0.011–0.016, Arg: 3.99–6.45 and GSA: 0.033–0.053 with RSDs in 1.5%–3.1% (see Table 2). Likewise, the results of the analyses of serum of ten uremic victims in age of 25 years–62 years recorded amounts (μg/mL) for G: 0.23–0.62, MG: 0.19–0.32, CTN: 35.0–46.57, GAA: 0.19–0.35, GPA: 0.10–0.31, GBA: 0.019–0.24, Arg: 10.0–19.63 and GSA: 0.84–1.84 with RSDs in 1.2%–2.9% (see Table 3).

Serum specimen of disease-free volunteer and uremic victim were spiked with 4 μg/mL standard solution containing all the eight guanidino compounds. Hike in the feedback of each compound (peak area/peak height) was recorded with no alteration in the peak appearance (see Figure 2a and Figure 2b). The recovery percentage from serum of disease-free volunteer and uremic victim was measured G: 95.1%–96.5%, MG: 93.7%–96.0%, CTN: 95.6%–97.2%, GAA: 96.1%–96.2%, GPA: 93.9%–98.0%, GBA: 93.3%–95.8%, Arg: 94.2%–97.7% and GSA: 96.1%–97.2% with RSD within 3.1% (see Tables 2 and 3). The recovery percentage from serum samples was acceptable for the quantitation of the guanidino compounds.

Table 2: Concentration (μgmL^{-1}) of guanidino compounds in serum of healthy volunteers.

S. no	Age/ Sex	G	MG	CTN	GAA	GPA	GBA	Arg	GSA
1	27F	0.14(2.1)	0.10(2.1)	9.36(2.5)	0.10(1.8)	0.05(2.3)	0.012(1.1)	6.17(1.2)	0.041(2.5)
2	23M	0.19(3.0)	0.15(1.9)	8.11(1.4)	0.14(1.5)	0.03(1.7)	0.014(2.8)	3.99(1.9)	0.041(3.1)
3	29M	0.40(1.5)	0.17(2.0)	9.48(2.1)	0.20(2.3)	0.03(1.8)	0.011(2.5)	5.55(2.1)	0.053(1.9)
4	21M	0.27(1.4)	BLOD	7.12(1.2)	0.19(2.2)	BLOD	0.012(1.8)	4.99(1.5)	0.048(1.6)
5	22M	0.20(2.3)	0.09(3.0)	6.99(3.0)	0.11(1.2)	BLOD	0.015(1.5)	5.82(3.0)	0.036(2.5)
6	20F	0.22(1.4)	BLOD	6.45(2.9)	0.22(1.6)	BLOD	0.011(3.1)	5.11(1.2)	0.033(2.8)
7	28F	0.49(2.1)	0.25(1.3)	8.96(2.8)	0.28(2.9)	0.07(2.5)	0.016(1.5)	6.45(2.9)	0.049(1.3)
8	24M	0.14(1.5)	0.18(1.9)	7.33(1.5)	0.17(2.5)	0.09(2.9)	0.014(2.1)	5.00(1.4)	0.047(1.9)
9	25F	0.28(2.1)	0.14(2.4)	6.81(2.1)	0.20(3.1)	0.06(1.6)	0.015(2.9)	3.99(1.6)	0.042(1.6)
10	27M	0.29(2.5)	0.25(1.3)	9.67(2.9)	0.27(3.1)	0.051(1.5)	0.015(1.5)	6.22(2.9)	0.052(3.1)
*10		0.28(1.9)	0.24(2.2)	9.340(2.1)	0.26(1.7)	0.05(2.4)	0.014(2.7)	6.08(3.0)	0.050(2.5)

Note: *Spiked Sample (%RSD) = 1.2–3.1; BLOD = below limit of detection.

Table 3: Concentration (μgmL^{-1}) of guanidino compounds in serum of uremic patients.

S. no	Age/ Sex	G	MG	CTN	GAA	GPA	GBA	Arg	GSA
1	45M	0.45(1.5)	0.22(2.7)	38.12(1.5)	0.22(1.7)	0.12(3.1)	0.070(1.3)	15.11(3.0)	1.11(2.2)
2	49F	0.60(2.1)	0.19(2.5)	45.53(2.9)	0.31(2.6)	0.12(2.3)	0.087(2.7)	18.91(1.9)	1.13(3.0)
3	51F	0.41(2.1)	0.30(1.4)	40.15(1.5)	0.35(1.7)	0.21(2.9)	0.077(1.4)	16.52(3.0)	1.81(1.6)
4	43F	0.38(1.6)	0.19(2.3)	43.22(2.8)	0.23(2.9)	0.15(1.5)	0.019(1.8)	11.99(1.3)	1.32(2.2)
5	45M	0.39(3.2)	0.21(2.8)	39.68(2.5)	0.20(1.2)	0.13(2.1)	0.078(3.0)	114.27(1.3)	1.54(1.7)
6	40M	0.32(1.4)	0.19(2.9)	38.97(1.5)	0.20(1.5)	0.19(1.3)	0.055(1.5)	11.25(2.6)	0.99(1.3)
7	25M	0.23(2.9)	0.31(1.4)	38.0(2.6)	0.19(2.2)	0.11(2.0)	0.064(2.8)	10.50(2.8)	0.84(2.9)
8	35M	0.56(2.8)	0.20(1.9)	42.37(2.7)	0.24(2.9)	0.10(3.0)	0.210(1.9)	10.00(1.4)	1.11(2.1)
9	45M	0.39(2.2)	0.28(2.7)	39.99(1.8)	0.22(1.4)	0.20(2.6)	0.220(1.4)	12.74(1.7)	1.52(3.0)
10	62M	0.62(3.0)	0.32(2.9)	46.57(3.0)	0.27(1.8)	0.33(1.9)	0.240(2.7)	19.63(2.9)	1.84(2.2)
*10		0.59(1.9)	0.30(2.1)	44.55(1.5)	0.26(2.4)	0.31(2.5)	0.230(1.6)	18.50(1.3)	1.79(1.6)

Note: *Spiked Sample (%RSD) = 1.2–3.0.

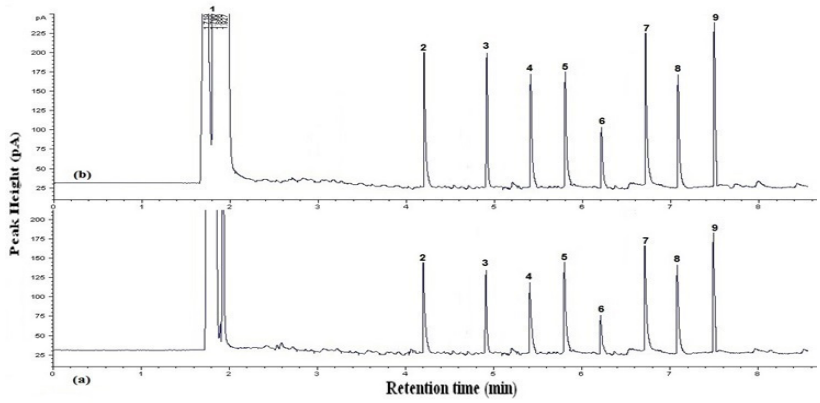

Figure 2: (a) GC separation and determination of guanidino compounds in serum samples of uremic patient. (b) GC response of guanidino compounds from uremic patient after spiking each compound with 4 µg/mL.

Urine Analyses

The urine specimens were also analysed. The mean magnitude (n = 4) observed from five disease-free volunteers in the age 21–25 years indicated (µg/mL) G: 2.45–3.99; MG: 0.53–6.61; CTN: 1,130–1,225; GAA: 50.29–60.11; GPA BLOD: 0.015; GBA: 0.074–0.20; Arg: 10.77–21.0 and GSA: 4.08–5.10 with RSDs in 1.5%–3.0% (see Table 4).

Table 4: Concentration (µgmL^{-1}) of guanidino compounds in urine of healthy volunteers

S. no	Age/ Sex	G	MG	CTN	GAA	GPA	GBA	Arg	GSA
1	31M	2.45(1.8)	0.53(2.2)	1,209(3.0)	50.29(2.1)	0.011(1.7)	0.074(2.1)	21.00(1.8)	4.58(2.7)
2	27M	2.88(2.3)	5.53(3.0)	1,130(1.8)	54.40(2.1)	0.013(2.1)	0.077(19)	13.93(2.5)	4.91(1.8)
3	28M	3.28(2.1)	4.99(2.3)	1,190(1.6)	59.32(1.9)	0.015(3.0)	0.200(1.5)	10.77(2.3)	4.21(3.0)
4	25M	3.99(2.9)	6.61(2.1)	1,225(1.9)	60.11(3.0)	BLOD	0.069(2.6)	15.45(2.6)	4.08(1.8)
5	26M	2.56(2.3)	6.11(1.9)	1,211(2.5)	51.12(2.8)	0.013(1.9)	0.075(2.1)	13.88(2.1)	5.10(2.3)
*5		2.53(3.0)	6.00(1.5)	1,180(2.3)	49.50(2.9)	0.0124(3.0)	0.074(1.6)	13.21(2.0)	4.90(1.9)

Note: *Spiked Sample (%RSD) = 1.5–3.0; BLOD = below limit of detection.

Likewise, the analyses of urine of six uremic victims within the age 49–61 years indicated the concentrations (µg/mL) of G: 2.99–4.43; MG: 4.13–6.44; CTN: 1,180–1,371; GAA: 5.01–6.48; GPA: 0.15–0.023; GBA: 0.29–0.37; Arg: 17.45–21.11 and GSA: 20.10–25.12 with RSDs in 1.6%–3.2% (see Table 5). A urine specimen of disease-free volunteer and uremic victim were spiked with 4 µg/mL each of the standard solution with all eight G. The return (peak area/peak height) was surged without variation in peak appearance (see Figure 3a and Figure 3b) and did not record the matrix effect of urine samples on the measurement of guanidino compounds. The feedback from urine of disease-free

volunteers and uremic victims was indicated G: 98.7%–98.8%; MG: 97.3%–98.1%; CTN: 95.3%–97.4%; GAA: 95.8%–96.8%; GPA: 95.3%–96.0%; GBA: 96.998%; Arg: 95.1%–97.8% and GSA: 96.0%–97.8% with RSDs within 3.2%.

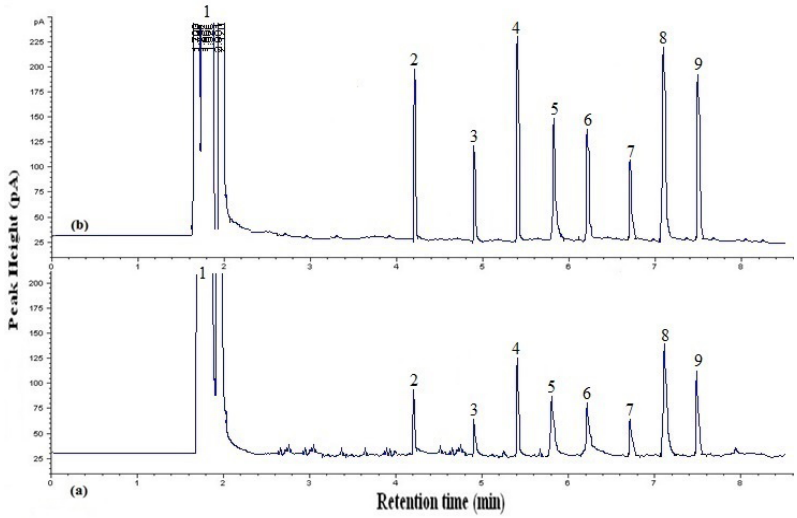

Figure 3: (a) GC determination of guanidino compounds in urine sample of uremic patient. (b) GC response of guanidino compounds from urine of uremic patient after spiking each compound with 4 µg/mL.

The mean values of serum samples of 10 disease-free volunteers serum samples were correlated with the mean value of ten uremic victims for every guanidino blend and *t*-test was exercised at 95% confidence levels and a significant change was found for G, CTN and GAA. Likewise, the mean concentration of urine samples of five disease free volunteers was correlated with mean concentration of five uremic victims for every G blend and *t*-test was also tested at 95% confidence level. An indicative change was also registered for G, GBA, CTN, GSA and Arg. The observations of higher concentrations of G in uremic victims than disease-free volunteers is supported by reported literature (De Deyn 2001).

The present procedure was related with symbolic recorded analytical procedures (see Table 6) using GC and HPLC procedures for ease of derivatisation, calibration range, time of separation, LOD and the number of G analysed. Gas chromatography-mass spectrometry (GC-MS) and HPLC-fluorimetric detection indicated better sensitivity, but the GC-MS procedure required higher reaction time and HPLC procedure depends upon gradient elution with more separation time. The present gas chromatography flame ionisation detector (GC-FID) method compares favourably with reported procedures, with shorter separation time (8 minutes) with acceptable sensitivity, applicable for analyses of biological samples for eight guanidino compounds.

Table 6: Comparison of present method with reported methods.

S.No	Technique	Derivatising reagent	Compound analysed	Calibration range	LOD	Optimal reaction time	Applications	Reference
5	HPLC-UV	Pyridoin	8	1.14–141 μmol L^{-1}	0.039–0.070 μmol L^{-1}	5 min	Human serum	Kandro & Khuhawa 2010
2	HPLC gradient elution	Anison	8	0.45–1310.8 nmol mL^{-1}	2–155 fM	5 min (Derivatisation time), speration time 40 min.	Pharmaceuticals and Human Urine	Buchberger & Ferdinand 2004
1	GC-MS	Hexafluroacetylacetone and Monotrimethylsilyltrifluoro acetamide	3	38–7325 μmol L^{-1}	1.22–1.54 μmol L^{-1}	2 hr (Derivatisation time), separation time 5 min for GAA and CTN.	Human Urine	Valongo *et al.* 2004
4	GC	Isovaleroylacetone and Ethylchloroformate	8	0.5–50 μgmL^{-1}	5–140 ngmL^{-1}	40 min (Derivatisation time), Separation Time 11 min.	Human Serum	Zounr *et al.* 2016
3	GC-MS	Hexafluroacetylacetone and Pentafluorobenzyl bromide	1	5–100 nmol	10 nmL^{-1}	2.25 hr (Derivatisation time), speration time 6.5 min for GAA	Human Urine and Plasma	Zinellu *et al.* 2006
6	GC-FID	IVA-IBCF	8	0.2–40 μgmL^{-1}	0.004–0.014 μgmL^{-1}	40 min	Human blood and urine samples	Present work

CONCLUSION

A capillary GC procedure was expended for the analysis and separation of eight G adapting IVA and IBCF as derivatising agents with reduced derivatisation time at lower temperature with an advancement in sensitivity with LODs at ng/mL levels using GC-FID. The approach provided a useful tool for quantitative analysis of G from human urine and serum of uremic victims and disease-free volunteers. The developed method compares well with reported GC and HPLC procedures for guanidino compounds. The analytical method is inexpensive and less time consuming using GC-FID system. The method can be used for clinical analyses.

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