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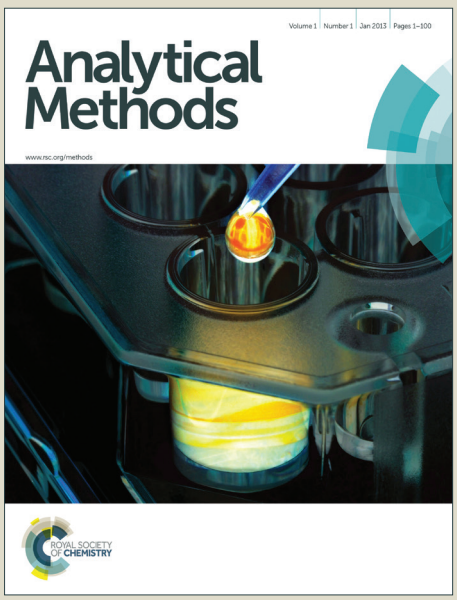

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GC Analysis of Guanidino Compounds in Serum and Urine of Healthy Volunteers and Uremic Patients Using Methylglyoxal and Ethyl Chloroformate as Derivatizing Reagent

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Abstract

Gas chromatographic (GC) determination of 9 guanidino compounds: guanidine, methylguanidine, guanidinoacetic acid, guanidinopropionic acid, guanidinobutyric acid, guanidinosuccinic acid, arginine, creatinine and creatine have been examined from the serum and urine of healthy volunteers and uremic patients using precolumn derivatization with methylglyoxal and ethyl chloroformate. GC elution and separation was from the column HP-5 (30m × 0.32mm id) with film thickness 0.25 µm within 10.5 min. The detection was carried out using FID. The linear calibration curves for guanidino compounds were within 0.1 – 20.0 µmol L^{-1} and lower limits of detection were 0.014 - 0.027 µmol L^{-1}. The amounts of guanidino compounds observed in the serum of healthy volunteers were 0.2 - 88.0 µmol L^{-1} as compared to uremic patients 0.8 - 521 µmol L^{-1} with relative standard deviations (RSD) within 1.5 - 3.7% and 1.0 - 3.8% respectively. The guanidino compounds in the urine of healthy volunteers and uremic patients were

observed in the range below the limit of detection to (BLOD) 7304 μmol L^{-1} and below LOD - 7541 μmol L^{-1} with RSD 1.1 - 3.7% and 1.0 - 3.8% respectively. The derivatization, separation and quantitation were repeatable with RSD < 4 %. Recovery of the guanidino compounds calculated by standard addition from serum and urine was obtained within 93 - 105% and 89 – 99% with RSD 1.4 - 2.9% and 1.8 – 4.3% respectively.

Key words: GC, Guanidino compounds, methylglyoxal, ethyl chloroformate, uremic patients, healthy volunteers, serum, urine.

Introduction

Guanidino compounds are present in the biological fluids and provide information about renal function. Their concentration is reported to increase in the uremic patients and some of these are considered as uremic toxins[1,2]. Methylguanidine (MG) is related to uremic polyneuropathy[3]. Guanidinosuccinic acid (GSA) is reported for uremic bleeding diathesis[4]. GSA, MG, guanidine (G) and creatinine (CTN) are reported to cause chronic and generalized seizures after systematic and intracerebroventricular administration in mice[5,6]. Several guanidino compounds are shown to affect leukocyte activity and homocysteine protein binding, possibly resulting in cardiovascular disease, which is one of the major cause of mortality in patients with chronic renal disease[7,8]. The concentration of GSA is reported to decrease in serum and urine of cirraotic patients[9] and concentration of GAA is increased in the abnormality in the guanidinoacetate methyltransferase deficiency[10,11].

The analyses of guanidino compounds have clinical importance. They are present in biological fluids at low concentration and they do not contain strong chromophore and

fluorophore groups. Pre or post HPLC column derivatization is generally carried for the sensitive spectrophotometric and fluorometric detection. A number of derivatizing reagents have been proposed for HPLC determination of guanidino compounds mainly benzoin[12-14], ninhydrin[15,16], 9,10-phenanthrenequinone[17], anisoin[18], furoin[19] and pyridoin[20].

Gas chromatography has high potential for the separation of organic compounds with low running cost and analysis could be completed with short running time. The GC of guanidino compounds has been carried out after precolumn derivatization with trifluoroacetic anhydride[21], hexafluoroacetylacetone[22], hexafluoracetylacetone-mono (trimethylsilyl) trifluoroacetamide[23,24], hexafluoroacetylacetone pentafluorobenzyl bromide[25], hexafluoroacetylacetone and ethyl chloroformate[26]. High sensitivity has been reported by using GC-MS, but requires reaction time (> 2h at 80 ^{0}C) and a limited number of guanidino compounds (2-6 compounds) have been reported and determined[23-25]. Recently glyoxal and glyoxal-ethyl chloroformate have been used for the separation and determination of 6 guanidino compounds[27-28].

Methylglyoxal (MGo) is an endogenous metabolite[29] and is known as precursor of advanced glycation end products (AGEs)[30,31], which accumulates in the plasma of diabetic and uremic patients[32,33]. Methylglyoxal is identified to react with guanidino group in proteins[29, 34] and the reaction have been shown to occur at physiological concentrations of methylglyoxal[35,36].

MGo is reported to react with guanidino compounds to form imidazolone adducts[27,36] and binds two imino nitrogens of guanidine groups, but the molecules still contain free carboxylic acid or imine groups. Ethyl chloroformate (ECF) is reported to

react with carboxylic acid and imine groups quickly in aqueous-organic phase to form derivatives amiable to GC[37-39]. The present work examines the use of MGo and ECF as precolumn derivatizing reagents for GC elution, separation and determination of the guanidino compounds from biological fluids (serum and urine). Experimental conditions for derivatization, GC elution and separation are optimized before determinations.

Experimental

Chemicals and Solutions

Methylglyoxal (MGo) (40% solution), ECF (Fluka, Buchs, Switzerland), guanidine (G), methylguanidine (MG), guanidinoacetic acid (GAA), guanidinopropionic acid (GPA), arginine (Arg), creatinine (CTN), creatine (CT) (Sigma Aldrich, St. Louis, MO, USA), guanidinobutyric acid (GBA) (Sigma Aldrich, Buchs, Switzerland), guanidinosuccinic acid (GSA) (Sigma Aldrich Chemie, GmbH, Mannheim , Germany), methanol (RDH Chemicals Co. Spring Valley CA, USA), chloroform (LAB-SCAN, Bangkok, Thailand), guaranteed reagent grade potassium hydroxide, hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, ammonium acetate, sodium tetraborate, boric acid, sodium bicarbonate, ammonium chloride and ammonia solution from E-Merck, Darmstadt, Germany were used.

The stock solutions of guanidino compounds containing 1000 μmol/L were prepared in water containing few drops of hydrochloric acid (1 M). Other solutions were prepared by appropriate dilution. 2,3-dimethyl-5,6-diphenyl-5,6-dihydropyrazine was used as internal standard and was prepared as reported[27] by refluxing together equimolar dimethylglyoxal with meso-stilbenediamine in methanol for 30 min.

Buffer solutions (0.1M) between pH 1 - 10 at unit interval were prepared from the following: potassium chloride adjusted with hydrochloric acid (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 – sodium carbonate (pH 9) and ammonium chloride – ammonia (pH 10).

Equipment

The pH measurements were made with an Orian 420A pH meter with combined glass electrode and reference internal electrode. IR spectrum of internal standard was recorded on an Avatar 330 FT-IR (Thermo Nicolet, Thermo Electron Coroporation, USA) with attenuated total reflectance (ATR) (accessory Smart Partner) within a range of 4000 - 600 cm^{-1}. Gas chromatographic studies were carried out on Agilent model 6890 network GC system (Agilent Technology Inc. USA), split injector, flame ionization detection (FID), hydrogen generator (Parker Balston Analytical Gas System, Parker Hannifin Havorhill, MA, USA) and pure nitrogen (British Oxygen Company, Karachi). The computer with Chemstation Software controlled the gas chromatograph. A capillary column HP-5 (30 m × 0.32 mm id) with layer thickness of 0.25 μm (J & W Scientific Corporation, USA) was used throughout the study.

Synthesis of Imidazolone Derivative of GBA and CTN

The solution of GBA or CTN (0.001M) in methanol – water (1:1 v/v) was added MGo (0.001M) in methanol. The contents were refluxed for 1 h. The reaction mixture turned from colourless to red during heating. The product was allowed to cool at room temperature and solvent was removed under reduced pressure. The oily mass obtained was washed several times with ether and n-hexane. Semi solid product obtained was

dried at 80 ^{0}C. The mass spectra of the derivatives were recorded at HEJ Research Institute of Chemistry, University of Karachi, on Jeol MS Route using direct probe intel and E1+ ionization mode. GBA derivative indicated main peaks at m/z (rel. intensity %) 196 (8.4), 180 (11.9), 152 (15), 138 (18.7), 124 (84.1), 110 (11.5) and 44 (100), FT-IR cm^{-1} at 1667, 1652, 1453, 1387, 1330, 1298, 1217, 1147, 1098, 1003 and 926. CTN derivative indicated main peaks at m/z (rel. intensity %) 166 (12.2), 139 (31.2), 111 (15.5), 83 (11.1), 69 (19.5) and 44 (100). FT-IR cm^{-1} at 1672, 1589, 1122, 1095, 1088, 1076, 1067, 822, 795, 785 and 776.

Analytical Procedure

To the guanidino solution 0.3 mL adjusted to 1 mL with distilled water (0.67 - 66.67 μmol L^{-1}) in 5 mL screw capped vial was added sodium tetraborate buffer pH 7.5 (0.3 mL), MGo (3% in methanol v/v) (0.3 mL) and 2,3-dimethyl-5,6-diphennyl-5,6-dihydropyrazine (33.33 μmol/L) (0.3 mL) as internal standard. The contents were warmed on water bath at 80 ^{0}C for 15 min and allowed to cool at room temperature (5 min). To this solution was added carbonate buffer (pH 9) (0.3 mL) and ECF (0.3 mL) and the contents were sonicated for 10 min. Chloroform (1 mL) was added and contents were mixed well. The layers were allowed to separate and an aliquot of the organic layer was transferred to a septum caped sample vial. The solution (1 μL) was analyzed on column HP-5 using a temperature program 80 ^{0}C for 3 min, followed by heating rate 20 ^{0}C min^{-1} up to 270 ^{0}C; total run time was as 13 min. Nitrogen flow rate was 2.5 mL min^{-1} with split ratio 10:1. The injector and detector temperatures were fixed at 270 ^{0}C and 280 ^{0}C respectively. The flow rates for FID were fixed 45 mL min^{-1} for nitrogen as make up gas 40 mL min^{-1} hydrogen and 450 mL min^{-1} for air.

Sample Collection and Pretreatment

The blood and urine samples of uremic patients with verbal / written consent were obtained from Liaquat University of Medicine and Health Sciences Hospital, Jamshoro. The blood samples were collected by vein puncture with hypodermic syringe and were transferred to EDTA tubes. Urine samples were collected in the morning in clean plastic bottle. The blood and urine samples of healthy volunteers were collected with verbal / written consent from the students and employees of Institute of Advanced Research Studies in Chemical Sciences, who had not taken any medicine at least during preceding week. All the donors of the blood and urine samples were informed about the objects of the research project and they agreed to participate in the project. The experimental work permed with the approval of ethical committee of University of Sindh.

Analysis of Guanidino Compounds from Serum

The blood sample (2 mL) collected from healthy volunteers and uremic patients was kept at room temperature (30 ^{0}C) for 1h and centrifuged at 3000 g for 30 min. The supernatant layer of the serum was separated and was added methanol (2 mL). The contents were mixed well and again centrifuged at 3000 g for 20 min. The supernatant layer was collected[37]. The deproteinized serum 1 mL was transferred to screw capped vial and the derivatization and GC analysis was carried out using analytical procedure. The quantitation was carried out using external linear calibration curves using linear regression equation $Y = ax + b$.

Analysis of Serum Using Linear Calibration with Spiked Sample

The blood sample (5 mL) collected from healthy volunteers and uremic patients was treated as above procedure. Four aliquots of (1 mL) of serum from the supernatant

layer after deproteinization with methanol was collected. Three serum samples (1 mL) was added to a mixture of G, MG, GAA, GPA, GBA, GSA, Arg, CTN and CT (0.3 mL) containing 5.0, 15.0 or 33.33 μmol L^{-1} each and all the four solutions were processed by analytical procedure. The quantitation was carried out by recording the responses (average peak height / peak area (n = 3) against the standard added and measuring the concentration at y = 0. The quantitation was also carried out from the increase in the response from the calibration curve with the added standards.

Analysis of Urine Samples

Urine sample (5 mL) collected from healthy volunteers and uremic patients was added methanol (5 mL) and was centrifuged for 30 min at 3000 g. The supernatant layer was collected. 1 mL of deproteinized urine was diluted to 5 mL with distilled water and 1 mL of diluted sample was taken and processed by analytical procedure. The quantitation was made by external calibration and from the ratio of the peaks using internal standard.

Analysis of Urine Using Linear Calibration with Spiked Sample

The urine sample (5 mL) collected from uremic patient was treated as above procedure. Four aliquots (1 mL) of urine from the supernatant layer after dilution and deproteinization with methanol were collected. Three urine samples (1 mL each) were added to a mixture of G, MG, GAA, GPA, GBA, GSA, Arg, CTN and CT (0.3 mL) containing 5.0, 15.0 or 33.33 μmol L^{-1} each and the solutions were processed by analytical procedure. The quantitation was carried out from the increase in the response from the calibration curve with the added standards and recording the average responses (n = 3) against standard added and measuring the concentration at y = 0.

Results and Discussion

The guanidino compounds are reported to react with methylglyoxal to form imidazolone adducts[29,38]. Expected chemical reaction of guanidino compounds with derivatizing reagents (MGo and ECF) is given in **Fig1**. An attempt was made to prepare pure imidazolone adducts by reaction of equimolar amounts of guanidino compounds and MGo in methanol – water. The reaction mixture quickly changed to red solution. The coloured adduct of GBA and CTN were examined for MS. The MS of GBA derivative indicated peaks at m/z 197 and 180 corresponding to $(M\text{-}OH)^+$ and $(M\text{-}COOH)^+$, respectively. This was followed by fragment peaks at m/z 152, 138 and 124 corresponding to the successive loss of $–CH_2$ groups. The signals obtained at m/z 124 corresponding to $C_5H_6N_3O$ with relative intensity of 84.1% supports the formation of imidazolone ring. Base peak (100%) was obtained at m/z 44 corresponding to CO_2 contributed from carboxylic group (**Fig** 2). FT-IR indicated bands at 1667 cm^{-1} and 1652 cm^{-1} for ν C=O and C=N respectively and supported the earlier investigators and the structures assigned[29,36,38](**Fig 3**). Similarly MS of CTN derivative indicated peaks at m/z 166 corresponding to $(M\text{-}OH)^+$ following by peak at m/z 139.1 due to opening of a ring and loss of fragment corresponding to C_2H_3. A fragment corresponds to C=O is further lost with observation of peak at m/z 111 corresponding to CTN molecule with loss of two hydrogen $[CTN\text{-}2H]^+$. FT-IR also indicated peaks at 1672 and 1589 cm^{-1} indicating the presence of C=O and C=N groups within the derivatized molecule.

It was initially observed that guanidino compounds without derivatization did not elute from GC column, but when excess of MGo was added to the guanidino compounds at pH 7.5 and the product was allowed at room temperature (30 ^{0}C) for 30 min, GC elution corresponding to guanidino compounds was observed, which separated from the

elution of the derivatizing reagent MGo. It was therefore the effect of reaction conditions were examined in terms of pH, amount of reagent (MGo) to be added per determination and warming time and temperature for the quantitation of guanidino compounds. The reaction was monitored by measuring average peak height or peak area (n = 3) and the condition which gave maximum response was considered optimum. The effect of pH was examined within 1 - 10 at unit interval and optimal response was obtained at pH 7 to 8 and pH 7.5 was selected. The addition of the derivatizing reagent MGo (3% v/v) was varied from 0.1 - 1.0 ml at an interval of 0.1 mL and it was observed that same response was obtained by the addition of 0.2 mL and above and 0.3 mL was selected per determination. The reaction required longer time for completion at room temperature, thus the warming of the reaction within 50 - 100 ^{0}C at an interval of 10 ^{0}C and warming time within 5 - 30 min at an interval of 5 min was examined. The warming at 80 ^{0}C for 15 min was selected.

At the optimized conditions the guanidino compounds G, MG, GAA, GPA, GBA, GSA, Arg, CTN and CT eluted and separated from the GC column, but to enhance the sensitivity and selectivity of the analytical procedure, the second derivatization with ECF was examined. An increase in the sensitivity in terms of increase in peak height / peak area for 10 – 20% was observed with an improvement in peak shape. It was therefore the conditions for second derivatization were examined. The effect of pH on second derivatization with ECF was examined within 6 – 10 at unit interval and maximum response was at pH 9 using carbonate buffer as has been reported for reactions with ECF[37]. The volume of buffer added per analysis was changed from 0.1 to 1.0 mL at an interval of 0.1 mL and change in the volume of buffer did not have effect on the average

response (n = 4) and volume of buffer was fixed to 0.3 mL. The addition of derivatizing reagent ECF was varied from 0.1 to 0.6 mL at an interval of 0.1 mL, but no change in peak height was observed by changing its volume and addition of 0.3 mL ECF per analysis was considered optimum. Chloroform, tertiary butanol, 1,2-dichloroethane and ethyl acetate were examined as solvents for the extraction of the derivatives. Chloroform indicated better extraction and was selected for the study. The conditions for GC elution and separation of the guanidino compounds from column HP-5 were examined. Each of the compounds gave a single peak and separated from the derivatizing reagents. Different temperature elution programs and nitrogen flow rates were examined for the separation of the guanidino compounds within short time. Finally initial column temperature of 80^0C for 3 min was fixed, followed by heating rate of 20^0C / min up to 270^0C with nitrogen flow rate of 2.5 mL / min. Total run time was 11.0 min with split ratio 10:1. All the nine guanidino compounds plus derivatizing reagent, and internal standard separated completely (**Fig 4 b**). Peak identification was based on retention time and by spiking of each of the guanidino compound in the sequence. The derivatives formed were examined after different intervals of the time and it was observed that the derivatives once formed were highly stable and did not show any change in peak height / peak area or retention time up to 12 h, with RSD within 5% (n = 6). The order of elution for derivatives of the compounds was (1) derivatizing reagents (2) G, (3) MG, (4) CTN, (5) GAA, (6) GPA, (7) CT, (8) GBA, (9) Arg, (10) GSA and (11) internal standard (2,3-dimethyl-5,6-diphenyl-5,6-dihydro pyrazine).

Quantitation

Linear calibration curves were observed by recording average peak height or peak area (n = 4) versus concentration within 0.1 - 20.0 μmol L^{-1} with coefficient of determination (r^2) within 0.9973 - 0.9997 for each of the guanidino compound using 11 calibrators (n = 11). Similarly linear calibrations were also obtained by recording ratio of average peak height or peak area (n = 4) to the internal standard within the same concentration range (0.1 - 20.0 μmol L^{-1}). The limit of detection (LOD) and limit of quantitation (LOQ) measured as signal to noise ratio (3:1) and (10:1) were obtained within 0.014 - 0.027 μmol L^{-1} and 0.042 - 0.081 μmol L^{-1} respectively (**Table 1**). The analysis of test mixtures of guanidino compounds within the calibration range indicated relative error within ±2.5 - 5.0%.

Precision

Repeatability of GC separation (n = 6) was examined in the terms of retention time and peak height and relative standard deviation (RSD) was obtained within 2.5 – 3.9 % and 1.7 – 4.3 % respectively. The derivatization, GC separation and quantitation was repeatable in terms of retention time and peak height or peak area for each of the guanidino compound inter (n = 5) and intra day (n = 5) by the same operator on the same day and different days at a concentration 10 μmol/L and RSD did not increase more than 4.0%.

The pharmaceutical additives methylparaben, propylparaben, gum acacia, manitol, lactose, fructose, glucose, galactose, sodium chloride, sodium laucryl sulphate and methyl hydroxypropyl cellulose were added twice the concentration of guanidino compound and analysis was carried out following analytical procedure. The results obtained were compared with guanidino compounds standards. The additives did not

affect the determination of guanidino compounds with relative error within 4.3 - 5.0%. The pharmaceutical preparations metformin, ranitidine, cimetidine and famotidine reported to elute from GC column after derivatization with MGo[40], were examined for possible interence of the guanidino compounds. The GC elution of the pharmaceutical preparations were examined at the optimized GC conditions for the guanidino compounds and it was observed that the pharmaceutical preparations eluted after guanidino compounds and did not interfere the determination of guanidino compounds.

Sample Analysis

MGo is an endogenous product and is reported to be present in serum and urine of healthy and uremic patients at the concentration of 0.04 – 0.29 μg mL^{-1} [29]. It was therefore the serum and urine sample was processed without addition of the derivatizing reagent MGo. No significant response for the guanidino compounds was observed above LOD and it was considered as blank (**Fig. 4 a**). Blood and urine samples of healthy volunteers and uremic patients were analyzed for the contents of guanidino compounds. Sixteen blood samples of healthy volunteers with ages 24 to 28 years were analyzed (**Fig. 4 c**). The amounts in μmol L^{-1} found were in the range GSA 1.1 - 1.4, GAA 0.9 - 1.5, GPA 0.17 - 0.3, CTN 82 - 86, GBA 0.1 - 0.29, Arg 30 - 35, G 0.1 - 0.28, MG 0.3 - 0.41 and CT 33 – 48 with RSDs within 1.5 – 3.7%. Similarly blood samples of 12 uremic patients within the age of 52 - 60 years were analyzed and amounts of guanidino compounds μmol L^{-1} were found within GSA 7.5 - 8.1, GAA 4.5 - 5.0, GPA 0.8 - 1.1, CTN 433 - 502, GBA 0.8 - 1.4, Arg 85 - 90, G 2.7 - 4.0, MG 1.8 - 2.1 and CT 38 - 48 with RSDs within 1.0 – 3.8% (**Table 2**).

Sixteen urine samples of healthy volunteers within the age of 24 - 28 years were also analyzed. The amounts found were μmol/L for GSA 22.9 - 25.3, GAA 323 - 346, GPA BDL - 0.08, CTN 4942 - 6518, GBA 0.38 - 0.43, Arg 39 - 65, G 13.8 - 16, MG BDL - 3.0 and CT 2297 - 2512 with RSDs within 1.1 – 3.7%.

Urine samples of 12 uremic patients within the age of 49-62 years were collected and analyzed (**Fig. 5 a**) and the results are summarized in **Table 2**. The amounts of guanidino compounds in urine samples were observed to contain μmol L^{-1} within the range GSA 126 - 139, GAA 31.0 - 36.2, GPA below the detection limit (BDL) to 0.08, G 20.4 - 23.9, CTN 6425 - 6903, GBA 1.34 - 1.81, Arg 114.0 - 120.8, MG 34.8 - 39.2 and CT 2697 - 2897 with RSDs within 1.5 – 3.7%. The urine sample was also spiked with standard solution of guanidino compounds and the recovery was observed; 96.5, 90.4, 96.5, 89.4, 89.3, 88.9, 98.9 and 96.1% with RSD 1.8 – 4.3% for GSA, GAA, CTN, GBA, Arg, G, MG and CTN, respectively (**Fig 5 b**).

The blood serum of healthy volunteers and uremic patients after deproteinization with methanol was spiked with the mixture of guanidino compounds and corresponding increase in the peak of compound was observed, without change in peak shape. Thus deproteinized serum matrix did not interfere with the determination of guanidino compounds. The results of analysis also agreed with calibration and average % recovery (n = 4) calculated were GSA 93.3 %, GAA 112 %, GPA 100 %, CTN 98.8 %, GBA 100 %, Arg 97.3 %, G 100 %, MG 75 % and CT 77.6 % with RSD 2.4 %, 1.7 %, 2.9 %, 3.5 %, 2.6 %, 3.1 %, 2.0 %, 3.2 % and 3.0 % respectively.

The mean values obtained for the guanidino compounds for serum of 12 uremic patients were compared with mean values of 16 healthy volunteers and t-test was applied.

A significant difference was obtained at 95% confidence level with higher concentrations for GSA, GBA, Arg. MG, GAA, GPA, CTN and G for uremic patients. However no significant difference was observed for CT. Similarly the average values for the urine of 12 uremic patients were compared with average values of 16 healthy volunteers and t-test was again examined. A significant difference was indicated at 95% confidence level between the averages with higher values for urine of uremic patients for GSA, GBA, Arg, MG, CTN and G. A significant difference was also observed for GAA with the observation of average lower concentrations of GAA in uremic patients. However no significant difference was indicated for GPA and CT in urine samples of uremic patients and healthy volunteers at 95% confidence level.

Comparing the results with reported values, it was observed that the values of GSA, GAA, GPA, GBA and MG for urine of healthy volunteers agreed with reported values of Gatti and Gioia[18]. The values for GAA, CTN, GBA and CT in the serum of healthy volunteers and GSA and CT of uremic patients agreed with reported values of Marescau et al[39]. The concentration of CTN, Arg, G and MG in the serum of uremic patients and GAA and CTN in the serum of healthy volunteers agreed with the reported values of Kikuchi et al[41].

The present analytical procedure was compared with reported analytical data for guanidino compounds using pre column derivatization methods involving HPLC, MEKC and GC. HPLC procedures combined with fluorimetric detection are sensitive, but require longer analysis time and are based on gradient elution. MEKC has been reported with sensitivity with shorter analysis time, but the equipment is not available with many laboratories for routine analysis of the compounds. GC procedures are reported for 2 to 6

compounds (**Table 3**). The present method compared favorably with the reported procedures in terms of sensitivity and selectivity. Use of simple reagent (MGo), complete separation of nine guanidino compounds with shorter GC elution time of 10 .5 min are the advantages of the present method.

Conclusion

An analytical procedure has been developed for GC determination of 9 guanidino compounds from deproteinized serum and urine of healthy volunteers and uremic patients using MGo and ECF as derivatizing reagents. The method indicated required sensitivity, selectivity and stability for the analysis of guanidino compounds from biological samples. The method could be used for clinical analysis of uremic patients.

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Description of Diagrams

Fig. 1. Reaction of guanidino compounds with MGo and ECF.

Fig. 2. Mass spectrum of GBA-MGo derivative.

Fig. 3. FT-IR spectrum of GBA-MGo derivative.

Fig. 4.

a = A GC response for the guanidino compounds in blood sample without derivatization.

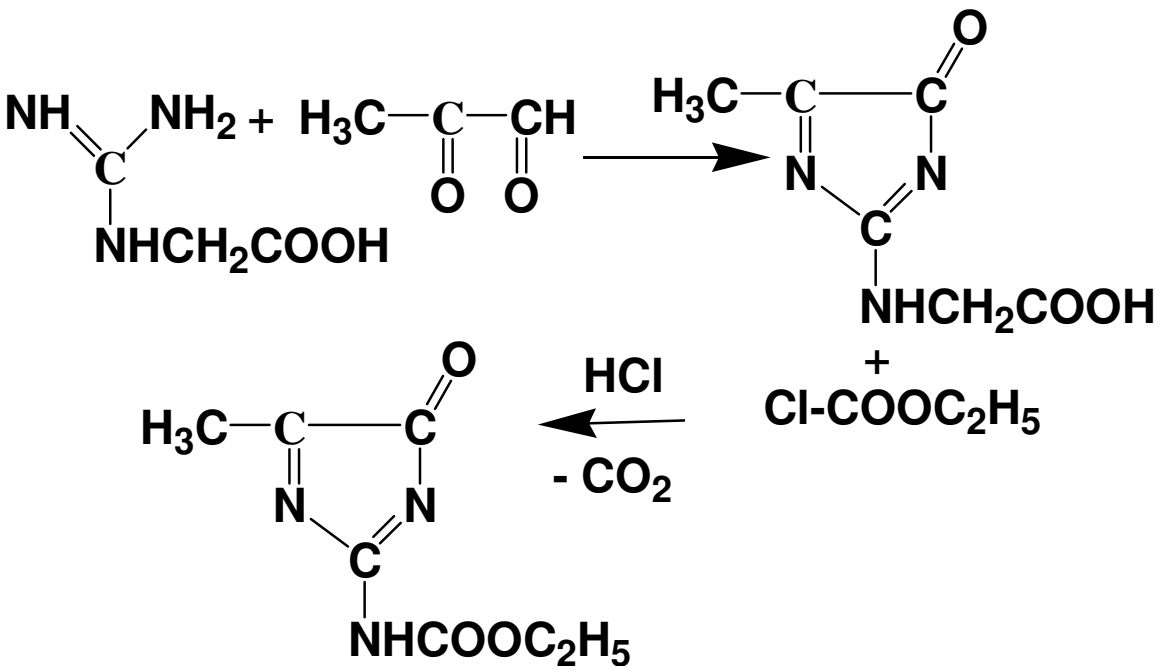
b = A separation of guanidino compounds using MGo as derivatizing reagents. (1) Reagent, (2) G, (3) MG, (4) CTN, (5) GAA, (6) GPA (7) CT, (8) GBA, (9) Arg, (10) GSA, (11) Internal Standard (2,3-dimethyl-5,6-diphenyl-5,6-dihydropyrazine).

c = GC response for derivatized guanidino compounds from serum sample of healthy volunteer. GC Conditions: as described in section “Analytical Procedure”.

Fig. 5.

a = GC response of derivatized guanidino compounds in urine sample of uremic patient.

b = GC response for derivatized guanidino compounds from urine sample of uremic patient after spiking with standards of guanidino compounds. Peak No as Fig 2 and GC Conditions: as described in section “Analytical Procedure”.



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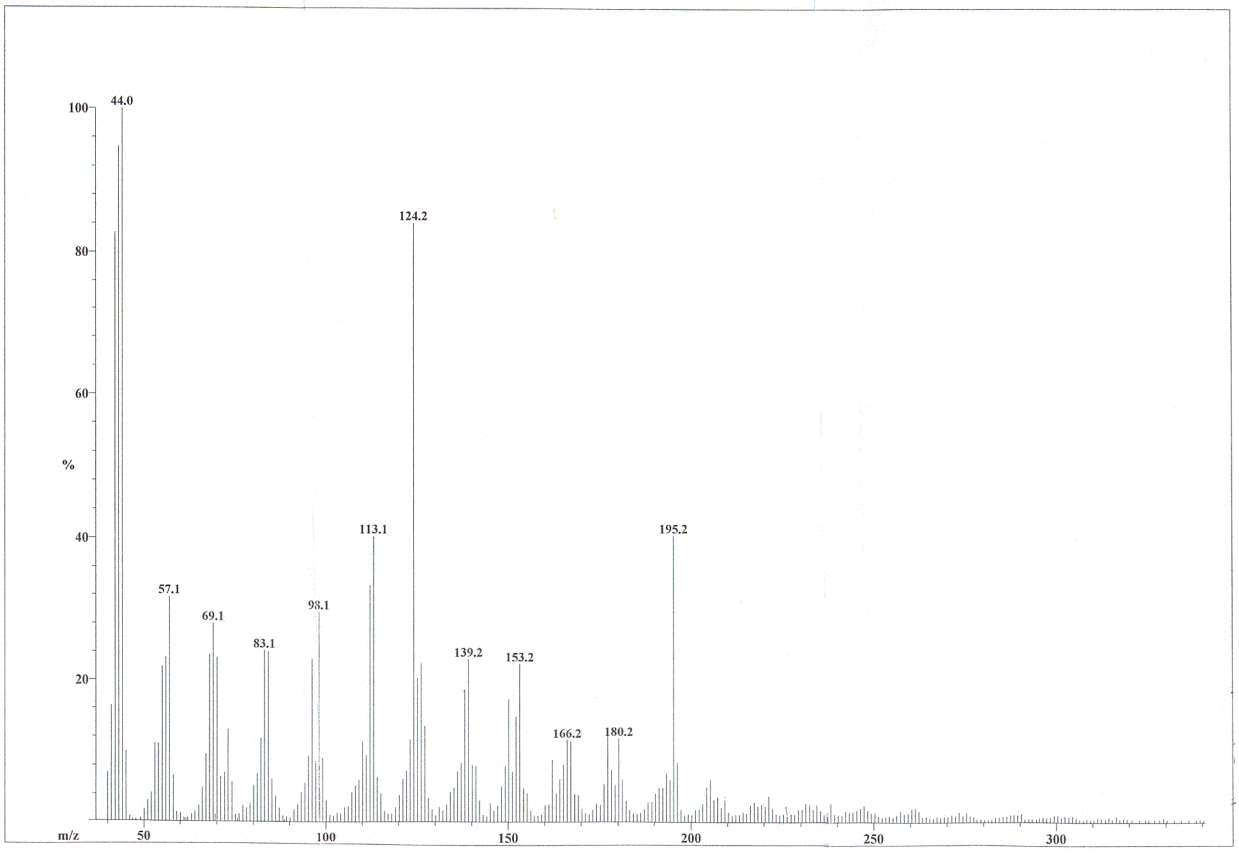

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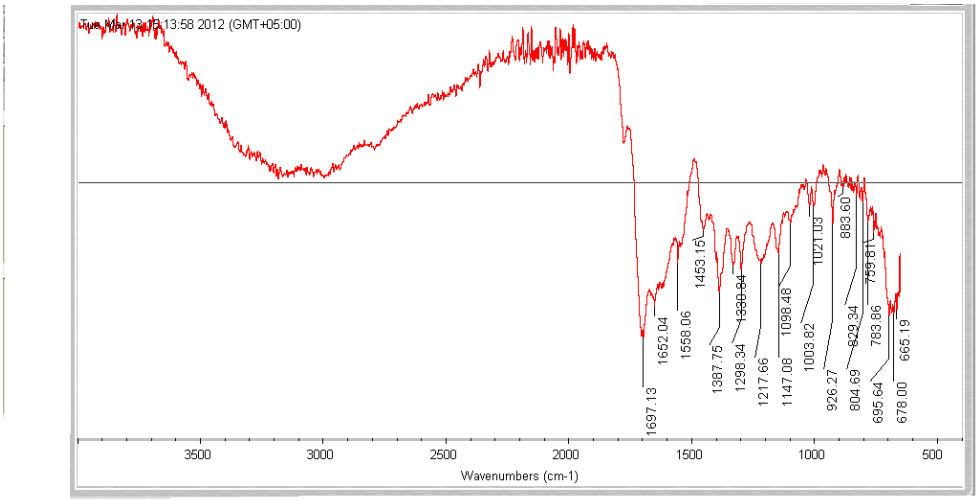

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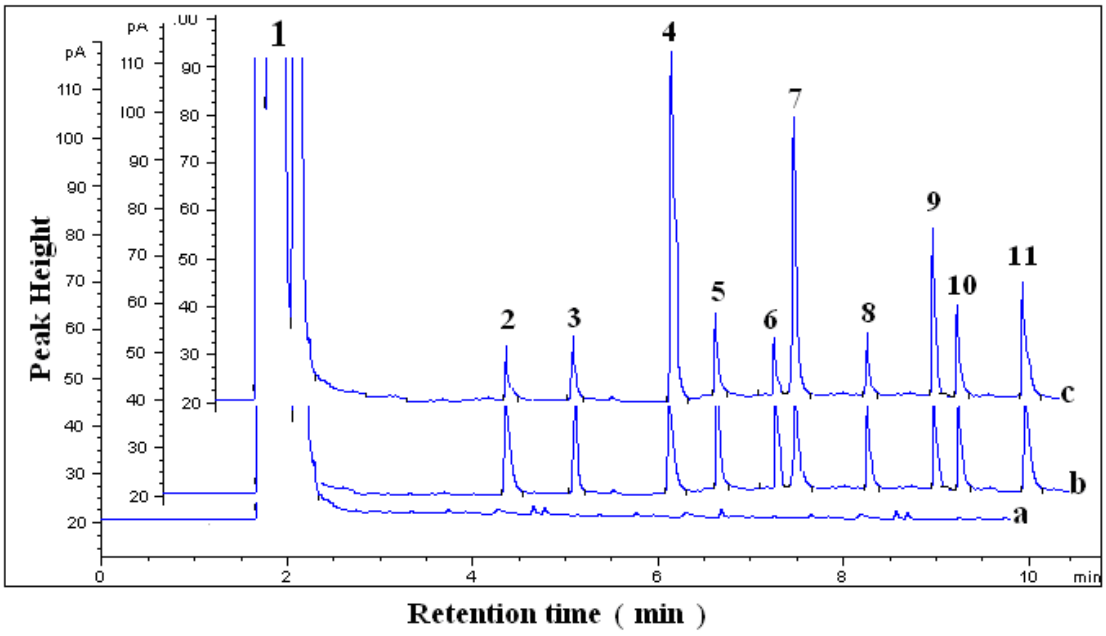

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440 Fig. 4.

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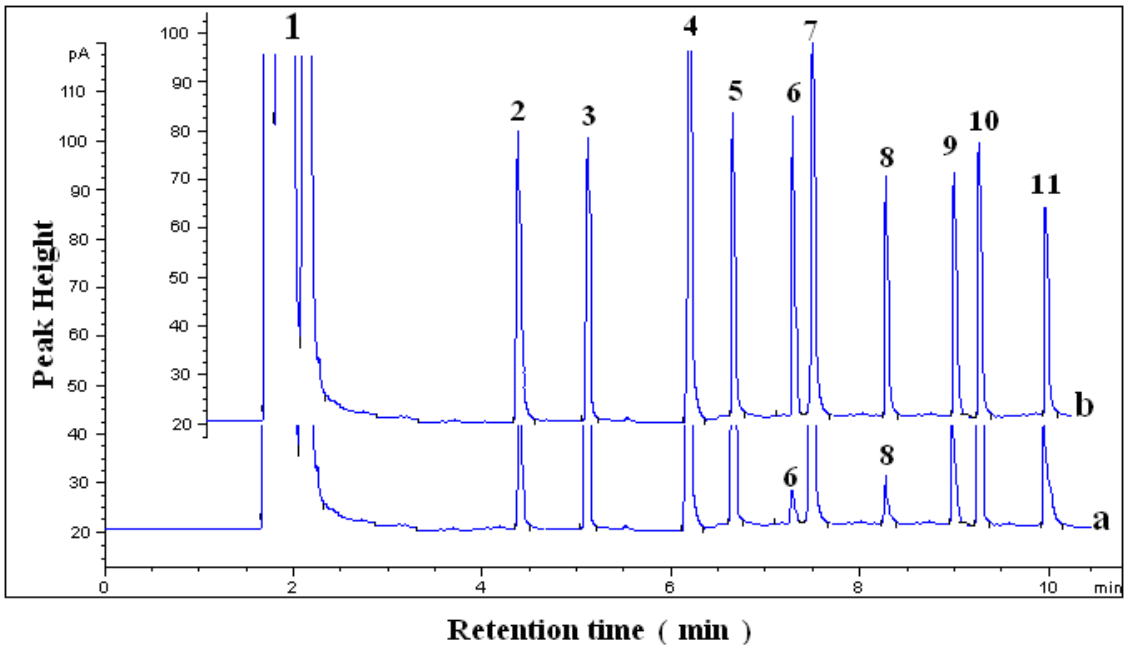

Fig. 5

Table1. Analytical parameters for the analysis of guanidino compounds as derivatives of MGo and ECF

Compound	Calibration range (μmol/L)	LOD (μmol/L)	LOQ (μmolL)	Coefficient of Determination (r^2)	Linear Regression Equation
G	0.2-20	0.016	0.048	0.9989	$Y = 26.697x + 6.132$
MG	0.2-20	0.015	0.045	0.999	$Y = 33.922x + 2.609$
GAA	0.2-20	0.014	0.042	0.9982	$Y = 42.8754x – 5.964$
GBA	0.2-20	0.014	0.042	0.9997	$Y = 31.565x + 8.171$
GPA	0.2-20	0.015	0.045	0.9989	$Y = 27.522x + 3.514$
GSA	0.2-20	0.02	0.06	0.9978	$Y = 51.872x + 3.969$
CTN	0.2-20	0.017	0.051	0.9992	$Y = 31.586x - 1.134$
CT	0.2-20	0.018	0.056	0.9973	$Y = 45.983x - 13.390$
Arg	0.2-20	0.027	0.081	0.9984	$Y = 26.657x + 7.690.$

Table 2. Concentration of guanidino compounds (μmol/L) in serum and urine of healthy volunteers and uremic patients.

	Healthy Volunteers				Uremic Patients			
	Serum		Urine		Serum		Urine	
	Average *n* = 16(SD)	Min - Max	Average *n* = 16 (SD)	Min - Max	Average *n* = 12(SD)	Min - Max	Average *n* = 12(SD)	Min - Max
Age Years	25.5	24-28	25.5	24-28	55.3	52-60	55.3	52-60
GSA	1.25(0.09)	1.1-1.4	24.2(0.8)	22.9-25.3	7.9(0.2)	7.5-8.1	131.7(4.8)	126-139
GAA	1.15(0.22)	0.9-1.5	337.25(7.3)	323-346	4.7(0.2)	4.5-5.0	33.9(1.9)	31-36.2
GPA	0.24(0.05)	0.17-0.3	0.035(0.04)	BDL-0.08	1.0(0.1)	0.8-1.1	0.025(0.04)	BDL-0.08
CTN	83.5(1.37)	82-86	6017(537)	4942-6518	470(30)	433-502	6699(167)	6425-6903
GBA	0.22(0.06)	0.1-0.29	0.4(0.02)	0.38-0.43	1.1(0.2)	0.8-1.4	1.56(0.2)	1.34-1.81
Arg	32.6(1.7)	30-35	45(8.1)	39-65	87.5(1.9)	85-90	117.3(2.1)	114-120.8
G	0.22(0.06)	0.1-0.28	14.9(0.7)	13.8-16	3.2(0.5)	2.7-4.0	22.3(1.3)	20.4-23.9
MG	0.36(0.04)	0.3-0.41	1.6(1.3)	BDL-3	1.9(0.1)	1.8-2.1	37(1.6)	34.8-39.2
CT	40.8(4.7)	34-48	2387(69)	2297-2512	42.2(3.7)	38-48	2808(72)	2697-2897

464 Table 3. Comparison of present method with reported methods

	Procedure	Derivatizing Reagent	Elution time	Detection	Calibration range	No of guanidino compounds separated	Ref
1	HPLC gradient elution	Benzoin	24 min	Fluorometric	2.5-25 pmol injection^{-1} (100 µL)	9	12
2	HPLC gradient elution	Ninhydrin	30 min	Fluorometric	20 µg/L to 2 mg L^{-1}	5	16
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5	GC	Hexafluoroacetylacetone and monotrimethylsilyltrifluoroacetamine	5 min	Mass spectrometry	38-7325 µmol L^{-1}	2	24
6	GC	Glyoxal	11 min	FID	0.1-20µmol L^{-1}	6	26
7	GC	Methylglyoxal and ethyl chloroformate	11 min	FID	0.1-20µmol L^{-1}	9	Present method

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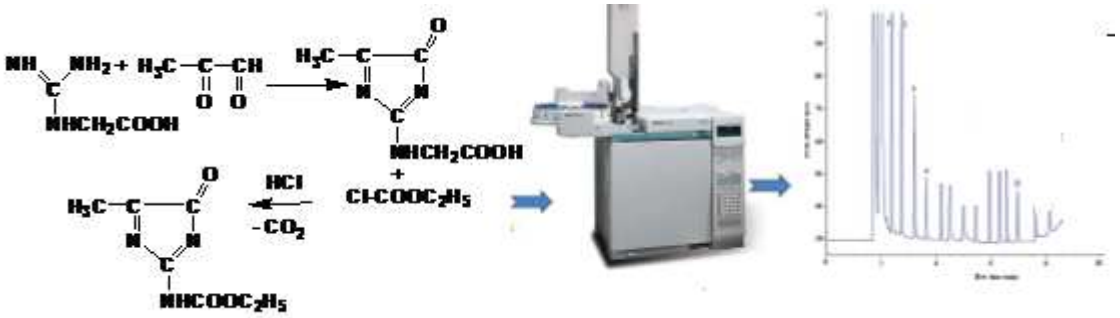

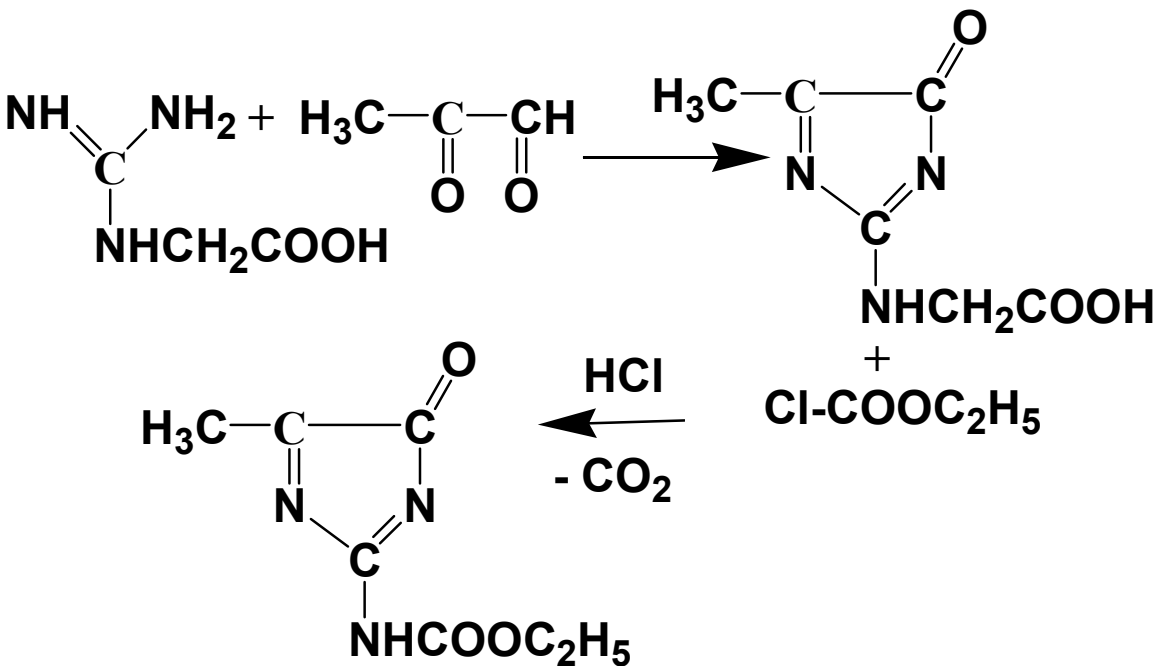

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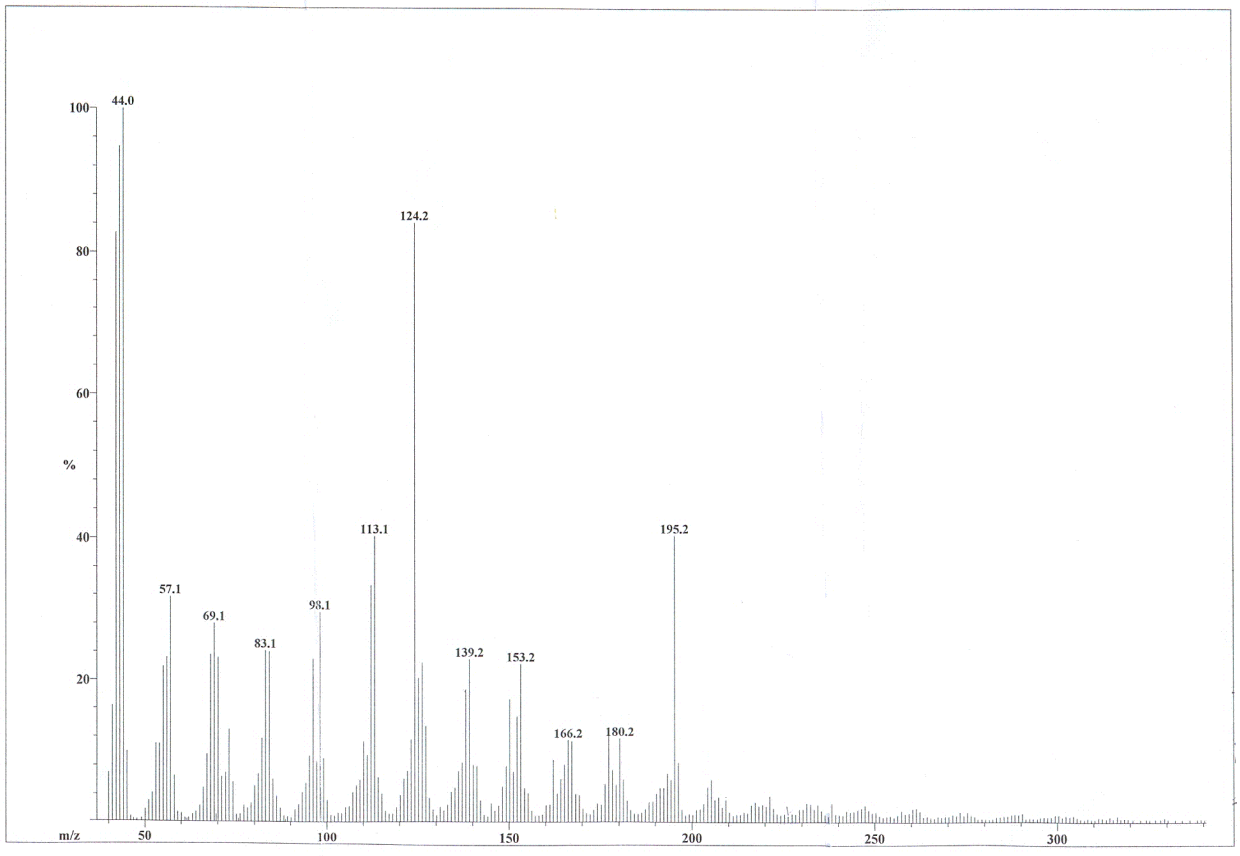

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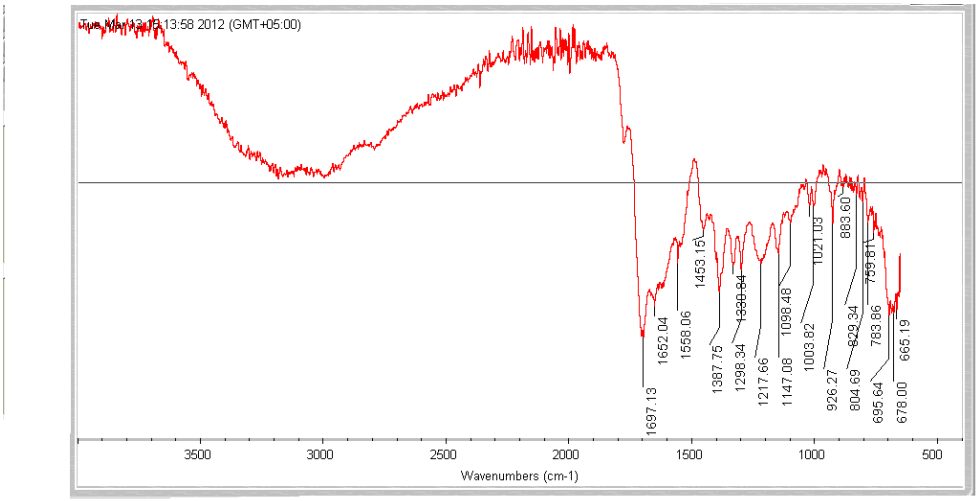
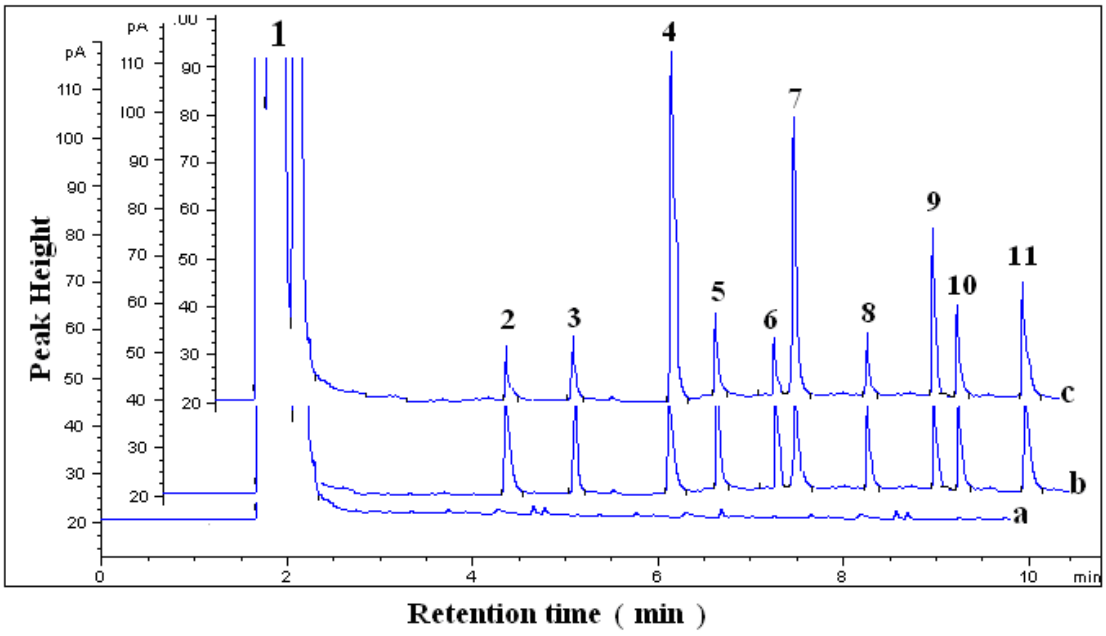


Fig3.



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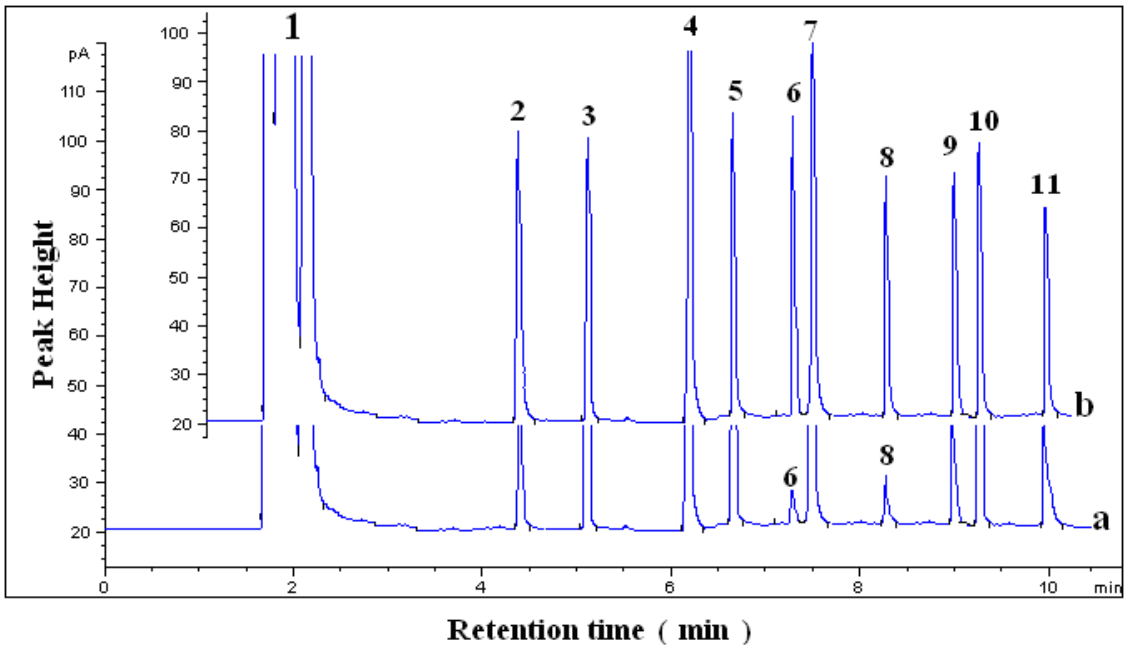

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