Micellar Electrokinetic Capillary Chromatography Determination of Guanidino Compounds in Serum of Uremic Patients by using Pyridoin as Derivatizing Reagent.

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Summary: An analytical procedure has been developed for the determination of 5 guanidino compounds; guanidine (G), guanidinoacetic acid (GAA), guanidinopropionic acid (GPA), guanidinobutyric acid (GBA), and guanidinosuccinic acid (GSA) by micellar electrokinetic capillary chromatography (MEKC) within 5 min. using pyridoin as derivatizing reagent. Sodium dodecyl sulfate (SDS) (0.03 M) was used as a micellar medium in sodium tetraborate (0.1 M) buffer at pH 9. Uncoated fused silica capillary was used with effective length 39 cm and 75 µm id. Applied voltage was 25.2 kV and photo diode array detection was at 228 nm. Linear calibrations were obtained from 0.70 to 122 µmol/L and limits of detection (LODs) were within 0.023-0.032 µmol/L. The derivatization and separation were repeatable with relative standard deviation (RSDs) within 1.2-3.1 %. Serum of healthy volunteers and uremic patients was analyzed and amounts found in uremic patients were G 1.71-2.64, GAA 4.56-6.61, GPA 1.15-1.94, GBA 1.25-1.98, and GSA 6.81-8.56 µmol/L with RSD (n=4) within 1.2-3.3 %. The amount of guanidino compounds in uremic patients was found higher than healthy volunteers.

Keywords: MEKC, Pyridoin, Guanidino compounds, Uremic patients, Human serum, Photodiode array detection.

Introduction

Guanidino compounds are small water soluble solutes to which neurotoxic effects have been attributed [1]. These are excreted from the healthy human body by healthy kidneys. However, the concentration of guanidino compounds, especially guanidine (G), and guanidinosuccinic acid (GSA), is highly increased in uremic biological fluids and tissues [2]. GSA is related to uremic bleeding diathesis and uremic encephalopathy [3, 4]. The compounds, GSA, and G are suggested to cause chronic and generalized seizers after systemic and intracerebroventricular administration in mice [5-7]. Guanidino compounds do not show a similar kinetic behavior as urea, with easy removal by dialysis strategy. Eloot *et al*. [2] observed that G, GSA and 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. Therefore it requires monitoring of the guanidino compounds in the biological fluids of uremic patients for clinical purposes [8].

The analytical procedures reported for the determination of the guanidino compounds are based on high performance liquid chromatography (HPLC), [9-23] gas chromatography (GC) [24, 25] and capillary electrophoresis (CE) [26-29]. 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 [21], benzoin [15-19], anisoin [9], furoin [10], pyridoin [20], 9, 10-phenanthrenequinone [13] and 9, 10-phenanthrenequinone-3-sulfonate [14]. The detection is usually reported by spectrophotometry or spectrofluorimetry [22]. The chromatography of guanidino compounds has been reviewed [23]. Yonekura *et al* have examined benzoin and its analogues including pyridoin as chemiluminogenic reagents for arginine (Arg) containing peptides [30].

Capillary electrophoresis (CE) is increasingly being used as an alternate separation technique in chromatography, because of its short analysis time and less running cost due to low sample and solvent consumption. Capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) have been used for the determination of creatine and creatinine from blood sera and urine (26-28). MEKC has been used for the separation of seven guanidino compounds using pre-capillary derivatization with benzoin and photodiode array detection at 228 nm [29].

The work examines pyridoin [1, 2-di-2-pyridyl-2-hydroxyethanone] (Fig. 1) as pre-capillary derivatizing reagent for the separation and determination of five guanidino compounds: G, GAA, GPA, GBA, and GSA by MEKC. The reagent

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pyridoin contains same functional group as benzoin, but is substituted with two (2-pyridyl) groups instead of two phenyl groups. The work also examines the effect of pyridyl group's substitution on the separation and determination of guanidino compounds. The separation parameters are optimized for its applications in biological fluids. The conditions for the derivatization and separation of guanidino compounds are optimized and examined in the terms of linearity, limits of detection (LODs), limits of quantitation (LOQs), repeatability (inter and intra-day) and accuracy. The results obtained are compared with reported procedures.

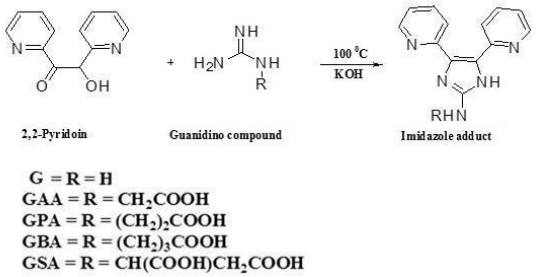

Fig. 1: Derivatization reaction of Guanidino compounds with pyridoin.

Results and discussion

The derivatization reaction of guanidino compounds with pyridoin was carried out as reported [20]. The reactions were carried out by warming the reaction mixture under alkaline conditions in the presence of β- mercaptoethanol and sodium sulfite as for related derivatizing reagents for guanidino compounds [15, 16]. The pH after derivatization was adjusted to 8.8-9.0 for maximum absorbance. A turbid solution was observed at the end of reaction, but a clear solution was obtained when volume was made up to mark with acetonitrile-methanol-water (40:40:20 v/v/v). The derivatization was repeatable (n=4) with relative standard deviation (RSD) within 3.8 % in the measurement of absorbance at 258 nm for each of the guanidino compounds separately by spectrophotometer.

Optimization of CE Conditions

The electrophoretic mobility was examined using different buffer solution within pH 1-10 for G, GAA, GPA, GBA, and GSA as derivatives of pyridoin formed as 2-substituted amino-4, 5-di (2-pyridyl) imidazoles (Fig. 1) [15, 16-20]. The examination of different buffer systems for electrophoretic mobilities of the guanidino compounds indicated that sodium tetraborate buffers gave some electrophoretic mobility to the derivatives, but the peak shape remained distorted. The surfactant sodium dodecyl sulfate (SDS) above critical micellar concentration (CMC) was added to run buffer solution to enhance solubility and electrophoretic mobility of derivatized organic molecules. An improvement in peak shape, resolution, selectivity and sensitivity was observed as reported for benzoin as derivatizing reagent [29]. A systemic study was carried out to optimize experimental conditions for the MEKC separation and determination of guanidino compounds.

The effect of pH within 7.0-10.0 using borate buffers in the presence of SDS was examined. All the compounds eluted as a single peak at pH 7.0, 7.5, 8.0 and 8.5. A separation was observed within 8.5-9.5 with a maximum at pH 9.0. Again at pH 9.5 and 10.0 a single peak was observed. Thus borate buffer pH 9.0 was used during the study (Fig. 2).

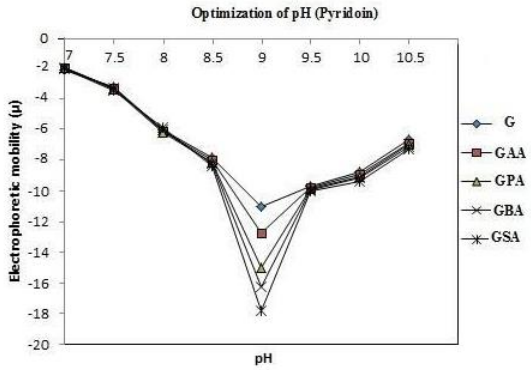

Fig. 2: Variation in electrophoretic mobilities of guanidino compounds versus pH as derivatives of pyridoin on uncoated fused silica capillary with total length 50 cm, effective length 39 cm, 75 µm id at 25ºC. Run buffer tetraborate (0.1 M), SDS (0.03 M) (2:1 v/v), voltage 25.2 kV, pH 9 and photodiode array detection at 228 nm. G = guanidine, GAA = guanidinoacetic acid, GPA = guanidinopropionic acid, GBA = guanidinobutyric acid, and GSA = guanidinosuccinic acid.

The effect of the buffer and SDS concentration on the separation was examined. The ratio of tetraborate buffer (0.1 M) and SDS (0.03 M) was varied from 1:1 to 4:1 and 1:4 v/v at pH 9.0. A maximum separation was observed with buffer-SDS 2:1 v/v and was selected. The concentration of borate buffer was varied from 0.01 to 0.15 M at an interval of 0.01 M and SDS from 0.01 to 0.08 M at an interval of 0.01 M, keeping the buffer-SDS ratio 2:1 v/v at pH 9.0. Symmetrical peaks with complete separation

were observed with 0.1 M borate buffer and 0.03 M SDS for all the five guanidino compounds and derivatizing reagent (Fig. 3).

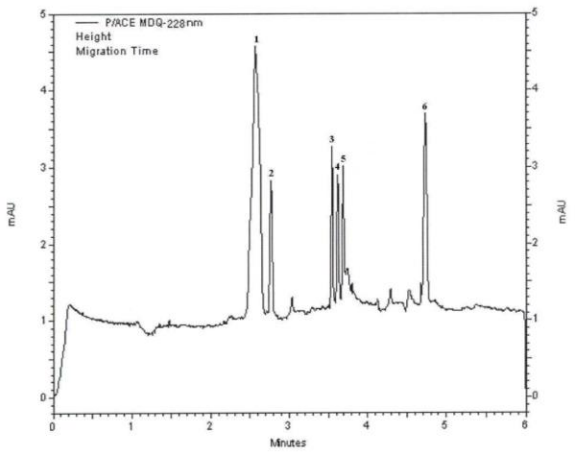

Fig. 3: MEKC Separation of (1) reagent pyridoin, (2) G, (3) GAA, (4) GPA, (5) GBA and (6) GSA as derivatives of pyridoin on uncoated fused silica capillary. Conditions as Fig. 2

The applied voltage was varied from 15 to 30 kV at an interval of 2 kV. As voltage was increased, the migration time decreased without affecting the separation, but to keep the Ohmic resistance within the limits by using high voltage, an applied voltage of 25.2 kV was selected with the total run time of 5 min.

Different solvent systems were tried to dissolve the turbidity obtained at the end of derivatizing reaction. The solvent system comprising methanol-acetonitrile-water proved better solvents with compositions (40:40:20 v/v/v), performing better in terms of electrophoretic mobility and was selected. The electrophoretic mobility's (μ) at optimized conditions for G, GAA, GPA, GBA and GSA as derivatives of pyridoin was observed within the range -18.3 to -11.5 cm^2 /V.s (Table-1).

The wave lengths for UV detections were scanned at the peak maximum between 200-300 nm using photodiode array detection and maximum responses were obtained at 280, 254, 228 nm, 211nm and 203 nm. The quantitative determinations of serum were carried out at 228 nm to avoid possible interfering effects of the matrix.

Validation of Quantitative Determination

The linear calibration curves were obtained by recording average peak height/peak area (n=4) versus concentration at the optimized operating conditions within 0.70-122 μmol/L for G, GAA, GPA, GBA, and GSA compounds with a coefficient of determination (R^2) with ten calibrators within 0.9980-0.9993. The (LODs) and limit of quantitation (LOQs) measured as S/N (3:1) and (10:1) were within 0.023-0.032 μmol/L and 0.07-0.095 μmol/L (Table-2). The reproducibility in terms of migration time and peak height was examined for all 5 compounds at inter (n=4) and intra-day (n=6) variations in concentration 2.6-7.2 μmol/L. The changes in peak height inter and intra-day was observed with RSD 1.2-3.6 % and 1.4-2.6 % and corresponding RSDs in migration times was 1.1-3.2 % and 1.5-2.8 % respectively.

Effect of Additives

The interfering effects of additives and some amino-acids on the determination of the guanidino compounds were examined. The compounds lactose, glucose monohydrate, starch, magnesium stearate, methylparaben, talc, gum acacia, β-alanine and L-cystine were added twice the concentration of G and their effect on separation, migration and average peak height (n=4) was examined. The responses were compared with standard solutions of the guanidino compound derivatives. The relative error was observed within ± 2.2 %. Five different test solutions covering the calibration range of G, GAA, GPA, GBA, and GSA were analyzed and relative error was observed within ± 3. 5 %.

Sample Analysis

The method developed was examined for the determination of G, GAA, GPA, GBA, and GSA from deproteinized serum of healthy volunteers and uremic patients. The average amounts (n=4) from six healthy volunteers within the age of 18-26 years were observed μmol/L G 0.14-0.32, GAA 0.68- 0.87 and GSA 0.73-0.95 with RSD within 1.3-3.5 % (Table-2). The average amounts (n=4) from eight uremic patients within the ages 46-63 years indicated μmol/L G 1.71-2.64, GAA 4.56-6.61, GPA 1.15-1.94, GBA 1.25-1.98 and GSA 6.73-8.56 with RSD within 1.2-3.3 % (Table-2) (Fig. 4a). A sample of uremic patient was spiked with G and GSA and response obtained increased at a corresponding migration time with calculated recovery of 98.2 %, and 98.7 % with RSD 2.1 % and 2.5 % respectively (Fig. 4b). Thus it was observed that deproteinized serum matrix of uremic patients did not affect the determination of G and GSA. The concentrations of the guanidino compounds were observed higher in uremic patients than healthy volunteers and agreed with earlier reported results [16-20].

Table-1: Quantitative data of guanidino compounds by MEKC 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)	Linear regression equations	Mobilities $cm^2/V.s$
1	G	0.7-90	0.07	0.023	0.9991	Y=97.73×+74.04	-11.5
2	GPA	0.74-94	0.074	0.025	0.9993	Y=85.06×+121.7	-13.2
3	GAA	0.82-103.5	0.082	0.027	0.9988	Y=140.9×+82.03	-15.5
4	GBA	0.77-98.5	0.077	0.026	0.9980	Y=111.0×+122.2	-16.7
5	GSA	0.95-122	0.095	0.032	0.9990	Y=174.8×+155.2	-18.3

Table-2: Analysis data of guanidino compounds in deproteinized serum 1 to 6 in healthy volunteers and 7 to 15 uremic patients by MEKC µmol/L.

S.No.	Age/Sex	G µmol/L (RSD %)	GAA µmol/L (RSD %)	GPA µmol/L (RSD %)	GBA µmol/L (RSD %)	GSA µmol/L (RSD %)
1	22 M	0.25 (2.3)	0.81 (1.9)	B/DL	B/DL	0.86 (1.8)
2	18 F	0.27 (1.9)	0.74 (2.3)	B/DL	B/DL	0.79 (2.4)
3	23 M	0.14 (2.7)	0.68 (2.9)	B/DL	B/DL	0.73 (2.5)
4	26 M	0.24 (3.1)	0.85 (2.8)	B/DL	B/DL	0.89 (2.9)
5	21 F	0.19 (1.4)	0.79 (1.7)	B/DL	B/DL	0.82 (1.6)
6	24 M	0.32 (2.3)	0.87 (2.5)	B/DL	B/DL	0.95 (2.1)
7	58 M	2.58 (1.4)	5.87 (1.6)	1.38 (1.5)	2.15 (1.3)	8.16 (1.2)
8	63 M	2.64 (2.7)	6.61 (2.9)	1.94 (2.6)	1.98 (3.0)	8.56 (2.8)
9	49 F	2.31 (2.6)	5.91 (3.3)	1.80 (2.7)	1.78 (3.1)	8.34 (2.8)
10	55 F	2.41 (2.6)	4.56 (2.9)	1.54 (2.4)	1.82 (2.5)	7.15 (2.8)
11	62 M	2.25 (2.2)	6.53 (1.8)	1.46 (2.1)	2.05 (2.0)	7.35 (1.7)
12	53 F	2.05 (2.3)	5.15 (2.2)	1.82 (2.5)	1.71 (2.4)	8.21 (1.9)
13	57 M	2.29 (1.9)	5.12 (2.7)	1.49 (2.3)	1.27 (2.1)	6.73 (2.2)
14	60 M	1.94 (2.6)	4.61 (2.4)	1.15 (2.7)	1.25 (2.5)	6.81 (2.8)
15* Spiked sample	50 F	1.71(2.4) 1.75 (2.1)	4.68 (2.8)	1.35 (2.2)	1.35 (2.6)	7.18 (2.7) 7.23 (2.5)

Bellow Detection Limits=BDL

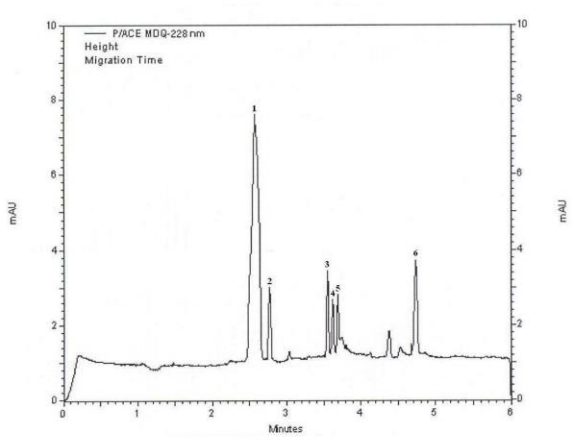

Fig. 4a: The sample analysis by MEKC of serum of uremic patient (Table-2) for (1) reagent pyridoin, (2) G, (3) GAA, (4) GPA, (5) GBA and (6) GSA a derivatives of pyridoin Conditions as Fig 2.

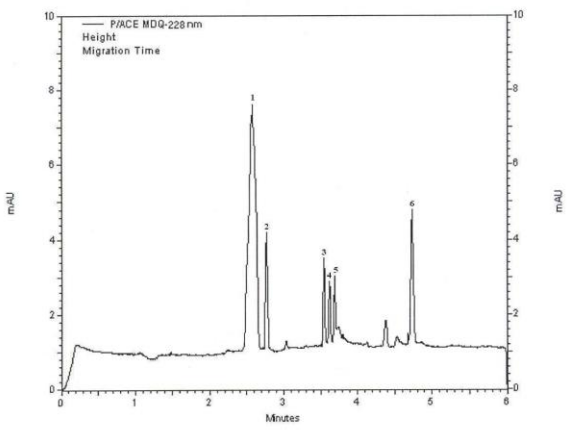

Fig. 4b: Sample as Fig. 4a after spiking with 0.5 ml each of G (10 µmol/L) and GSA (15 µmol/L). Conditions as Fig. 2.

Now comparing the results with HPLC reported procedures [20] using same derivatizing reagent (pyridoin), the HPLC procedure is based on

the separation of eight guanidino compounds with a flow rate of 1 ml/min within 25 min. The present pyridoin MEKC method separates 5 guanidino compounds within five min with insignificant consumption of solvent > 0.2 ml per analysis.

The results obtained were also compared with MEKC method using benzoin as derivatizing reagent [29]. The results indicated a similar sensitivity and selectivity using UV detection for the determination of guanidino compounds and substitution of 2-pyridyl for phenyl groups does not have adverse effect, and could be used for clinical analysis of guanidino compounds.

Experimental

Chemicals and solutions

2-Pyridinecarboxaldehyde (Fluka, Switzerland), G, GAA, GPA (Sigma-Aldrich Louis, USA), GBA (Sigma-Aldrich, Switzerland), GSA (Sigma GmbH, Germany), methanol (RDH, Germany), 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 of guaranteed reagent grade from E. Merck, Germany.

The stock solutions of guanidino compounds containing 70-122 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 [31] from 2-pyridinecarboxaldehyde, following benzoin type condensation in pyridine-water (30:70 v/v) as solvent. The derivatizing reagent pyridoin solution (4 mmol/L) was prepared in dimethyl formamide-methanol (1:4 v/v), where pyridoin (85 mg) was dissolved in 20 ml of dimethylformamide and the volume adjusted to 100 ml with methanol. The solutions of β-mercaptoethanol (0.1 M), sodium sulphite (0.2 M) and potassium hydroxide (2 M) were prepared in distilled water.

Buffer solutions (0.1 M) between pH 1-7 at unit interval and between 6.5-10.0 at 0.1 intervals were prepared from the following: hydrochloric acid and potassium chloride (pH 1-2), acetic acid and sodium acetate (pH 3-6), ammonium acetate and acetic acid (pH 7), boric acid and sodium tetraborate (pH 6.5-10) and sodium bicarbonate and sodium carbonate (pH 9) and ammonium chloride and ammonia solution (pH 10).

Equipment

The pH measurements were carried out with an Orion 420A pH meter (Orion Research Inc. Boston, USA) with combined glass electrode and reference internal electrode. 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 range 4000-660 cm^{-1}.

The capillary electrophoresis (CE) system consisted of Beckman Coulter P/ACE MDQ Instrument (Beckman Instruments Inc. Fullerton CA) equipped with auto sampler, photo-diode array detector and a data system comprising of an IBM Personal computer and P/ACE system MDQ (32 karat) software. Uncoated fused silica capillaries were obtained from Beckman Instruments Inc. with total length 50 cm, effective length 39 cm, 75 µm id and 375 µm OD. Capillary and sample was thermostated at 25 ºC.

Prior to sample run the capillary was regenerated and conditioned with methanol for 1 min., followed by water for 0.5 min., hydrochloric acid (0.1 M) for 2 min., water for 0.5 min., sodium hydroxide (0.1 M) for 2 min., water for 0.5 min. and then running buffer for 2 min. Each sample run was interspersed by capillary washing with sodium hydroxide (0.1 M) for 2 min; water for 1.0 min; later equilibrated with running buffer for 2 min.

Analytical Procedure MEKC

The derivatization procedure was followed as reported [20]:

In 5 ml volumetric flask placed in ice cold water was transferred 0.5 ml of an aqueous solution of guanidino compounds containing (µmol/L) G, GAA, GPA, GBA, and GSA within calibration range (Table-1) were treated with 0.5 ml pyridoin solution (4 mmol/L), 0.3 ml β-mercaptoethanol (0.1 M), 0.3 ml sodium sulphite (0.2 M), and 0.5 ml potassium hydroxide (2 M). The mixture was heated in boiling water bath for 5 min., cooled in ice water for 2 min. and then added 0.5 ml sodium tetraborate buffer pH 9.0 (0.1 M). The contents were mixed well and the volume of the slightly turbid solution formed was adjusted to mark with solvent system acetonitrile: methanol: water (40:40:20 v/v/v). An aliquot (1.5 ml) was placed in septum capped sample vial and the solution was injected by autosampler with a pressure of 0.5 Psi for 5 sec. The electrophoretic migration was affected with boric acid-sodium tetraborate

buffer pH 9.0 (0.1 M) and SDS (0.03 M) (2:1 v/v) with applied voltage of 25.2 kV. The power supply was 2.5-2.7 W with a current of 99.3-104 µA and UV detection with a photo diode array was at 228 nm.

Analysis of Guanidino Compounds from Serum

The blood sample (5 ml) collected from healthy volunteers and uremic patients in EDTA tubes were kept at room temperature (30 ^{0}C) for 1 h and centrifuged at 3000×g for 30 min. The supernatant layer of serum (2.5 ml) was separated, and 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, 0.5 ml solution was transferred to a 5 ml volumetric flask, and the analytical procedure MEKC was followed. The quantification was carried out from linear regression equation Y= mx + b derived from the external calibration curve.

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

A blood sample (5 ml) collected from uremic patient (No. 15**,** Table-2) was treated as analysis of guanidino compounds from serum. Serum (0.5 ml) in duplicate was taken after deproteinization with methanol and one of the parts was added with G 0.5 ml (10 µmol/L) and GSA 0.5 ml (15 µmol/L). Both the parts were processed as analytical procedure MEKC. The quantification was carried out from increase in response from added standard and linear calibration curve.

The electrophoretic mobility for each of the derivatives at optimized conditions and at different pH was calculated from the observed migration times as reported [29].

Blood samples of uremic patients with verbal consent were collected in sterilized EDTA tubes from the medical wards at Liaquat University of Medical and Health Sciences Hospital, Jamshoro, and Hyderabad, Pakistan, by vein puncture. The blood samples of healthy volunteers who had not been taking any medicine for at least one preceding week were collected with verbal permission from students and employee of Institute of Advanced Research Studies in Chemical Sciences. The collected samples were quickly analyzed for guanidino compounds.

Conclusions

A simple and inexpensive method has been developed for the determination of five guanidino compounds using MEKC within 5 min. Sensitivity and selectivity of the method have been achieved by using pyridoin as derivatizing reagent. Calibrations were linear over three orders of magnitude within 0.70-122 µmol/L and (LODs) between 0.023-0.032 µmol/L. The method has been used for the determination of guanidino compounds from the serum in uremic patients and healthy volunteers. The deproteinized serum matrix did not interfering the determination of the guanidino compounds.

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