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Determination of guanidino compounds by anion-exchange chromatography and amperometric detection

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SUMMARY

Guanidino compounds were separated and determined by anion-exchange chromatography and electrochemical detection using a basic aqueous eluent and a nickel working electrode. It was found necessary to use a sample clean-up procedure prior to chromatographic analysis of uremic dialysate and serum samples. The effect of eluent hydroxide concentration on the retention of guanidino compounds was studied. Quantitative calibration showed that working curves were non-linear. Electrochemical detection for guanidino compounds with a nickel working electrode, while not selective, has high detection sensitivity. Detection limits for guanidino compounds ranged from 3 to 12 pmol.

INTRODUCTION

Guanidino compounds have been widely studied because of their importance as clinical markers in uremia. Since guanidines are ionic and do not possess native UV absorbance nor fluorescence, they are usually separated by ion exchange or ion-pairing chromatography and detected by pre- or post-column derivatization. The disadvantages of pre-column derivatization often include complicated chromatograms, lengthy preparation times and a low recovery of the desired constituents. Post-column derivation with phenanthrenequinone

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is sensitive and specific for guanidines, but extra pumps and complicated plumbing are necessary.

Recently, liquid chromatography (LC) with electrochemical detection (ED) has become very widely used in the analysis of biologically active compounds. Detection limits at the picomole level have been achieved for a number of oxidizable compounds. Since about 1970, the anodic oxidation of alcohols, amines, carbohydrates and other compounds using a nickel electrode in alkaline solution has been studied by a number of research groups [1–6]. Fleischmann et al. [1,2] and others have reported the mechanism of catalytic oxidation of organic compounds by an active nickel(III) oxide (NiOOH) formed in situ on the electrode surface at potentials near 0.45 V versus a saturated calomel electrode. Application of the nickel(III) oxide electrode to determination of amines, amino acids, ethanol and glucose by flow injection analysis [7–11] and the determination of amino acids by reversed-phase chromatography [12] and carbohydrates by anion-exchange chromatography [13] have been reported. Since the guanidino functionality responds to oxidation, it is possible to detect low concentrations of these compounds at a nickel(III) oxide electrode. In addition to cation-exchange separation, guanidines can also be separated by anion-exchange chromatography [14] with an alkaline eluent which is required for the formation of an active nickel(III) oxide electrode surface.

This paper describes an anion-exchange chromatographic procedure for guanidines using ED.

EXPERIMENTAL

Apparatus

The chromatographic system included a Waters 6000A pump, a Rheodyne 7125 injector and a Dionex AS-6 analytical column. The sample loop was 20 μl, unless indicated otherwise; a flow-rate of 1.0 ml/min was used. The detector was identical to the one originally designed by Reim and Van Effen [13]. An Ag/AgCl (3 *M* NaCl) reference electrode was purchased from Bioanalytical Systems. The working nickel electrode was operated at 0.45 V versus Ag/AgCl, unless indicated otherwise. The bore of the nickel electrode was polished with 80–200 mesh alumina daily. Potential control and current measurement were provided by an IBM EC/225 voltammetric analyzer. A Hewlett-Packard 3396A integrator was used for peak integration and for recording all chromatograms.

Chemicals and reagents

L-Arginine (ARG), creatinine (CRN), guanidinoacetic acid (GAA), β-guanidinopropionic acid (GPA) and guanidinosuccinic acid (GSA) were obtained from Sigma (St. Louis, MO, U.S.A.). Methylguanidine (MG), guanidine (G) and 4-guanidinobutyric acid (GBA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). MG, G, and ARG were obtained as the hydrochlo-

ride salts. All guanidino acids were obtained as the neutral compounds. Taurocyamine (TC) was synthesized as previously described [15]. The mobile phase was prepared daily from 50% (w/w) sodium hydroxide solution (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and LC-grade water. During use, the mobile phase was protected from carbon dioxide uptake by an Ascarite II trap (Fisher Scientific, Swedesboro, NJ, U.S.A.) on the mobile phase reservoir. AG 50W-X8 (100–200 mesh, hydrogen form) and AG 1-X8 (100–200 mesh, chloride form) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Hydrochloric acid (36.5–38.0% ACS reagent grade) was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). LC-grade glacial acetic acid was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Sep-Pak™ C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Samples of physiological fluids

Normal serum samples were obtained from healthy individuals. Blank dialysate, uremic dialysate and uremic sera were provided by The Dialysis Unit at Geisinger Medical Center (Danville, PA, U.S.A.). Uremic dialysates were obtained from four patients (one female, 67 years; three males, 43, 42 and 76 years) who had been dialyzed for 4 h. Uremic sera were obtained from two patients (females, 67 and 74 years).

Sample clean-up

Because of the presence of electroactive interferences in serum and urine, it was found necessary to use a sample clean-up procedure prior to chromatographic analysis of guanidine-containing physiological fluids. GSA was isolated prior to chromatography by use of a small gravity-feed anion-exchange column. A cation-exchange column was used to isolate the other guanidines.

Anion-exchange column. A 3.5 cm × 1.1 cm I.D. anion-exchange column (AG 1-X8), 100–200 mesh, chloride form) was prepared and converted first to the hydroxide form by washing with 25 ml of 2.5 *M* NaOH solution and then converted to the acetate form by washing with 25 ml of 10% acetic acid and 25.0 ml of 3.0 *M* sodium acetate. The column was stored in 1.0 *M* acetic acid when not in use. Before use, the resin was washed with 25 ml of 1 *M* acetic acid and equilibrated with 25 ml of 0.05 *M* acetic acid.

A 1-ml hemodialysate sample was diluted with ca. 4 ml of water and adjusted to pH 6.0 with 0.1 *M* acetic acid. The sample was passed through the column and washed with 30 ml of 0.05 *M* acetic acid. The anions retained on the column were then eluted with 20 ml of 1 *M* acetic acid. The effluent was collected and evaporated to dryness. The residue was dissolved in 1.0 ml of water.

Cation-exchange column. A 2 cm × 1.0 cm I.D. cation-exchange column (AG 50W-X8, 100–200 mesh, hydrogen form) was prepared and washed with 50 ml of 4 *M* HCl and stored in 1 *M* HCl. Before use, the resin was washed with 25 ml of 1 *M* HCl and equilibrated with 25 ml of 0.01 *M* HCl. A 1.0-ml sample of

hemodialysate was diluted with ca. 4 ml of water and acidified to pH 2 with 0.10 *M* HCl. The diluted sample was then passed through the column and washed with 50 ml of water. The cations absorbed on the column were then eluted with 50 ml of 4 *M* HCl. The effluent was collected and evaporated to dryness on a rotary evaporator. The residue was dissolved in 1 ml of water.

For serum analysis, 1.0 ml serum was diluted to 2 ml with water and passed through a Sep-Pak C_{18} cartridge. The cartridge was washed with water until 10 ml of effluent was collected. This sample was then treated according to the above anion- and cation-exchange procedures.

RESULTS AND DISCUSSION

Effect of hydroxide concentration

The effect of hydroxide concentration on guanidine retention was examined by measuring the capacity factors as a function of NaOH concentration in the eluent (0.01–0.2 *M*). For GSA, the NaOH concentration was varied from 0.15 to 0.4 *M*. Baseline currents were found to change with NaOH concentration. The more concentrated the eluent, the larger the baseline current. When 0.010 *M* NaOH solution was used as eluent, the baseline current became negative. Thus, in this case, measurements were made at 0.50 V.

The relationship between capacity factors for guanidino compounds and NaOH concentration is shown in Fig. 1. It is clear that capacity factors of ARG, GBA, GPA, GAA and CRN do not change substantially as the NaOH concentration increases. The capacity factors of G, MG, TC and GSA decrease markedly as the NaOH concentration increases.

GAA and CRN, as well as G and MG, were found to coelute at any NaOH concentration. When the eluent was more concentrated than 0.15 *M*, MG and GAA coeluted. Therefore 0.15 *M* NaOH solution was the best compromise between optimum separation and total analysis time for standard guanidines (except GSA). The choice of eluent concentration has to be determined by other components present in serum and/or dialysate. In this work, 0.4 *M* NaOH solution was chosen for GSA analysis, while 0.05 *M* NaOH solution was chosen for other guanidino compounds. Fig. 2 shows a chromatogram for a standard mixture of GAA, MG and TC.

The retention mechanism for CRN, G and MG cannot be anion-exchange because these compounds are not anionic. The pK_a values of CRN, G and MG are 5.02 [16], 13.4 and 13.6 [17], respectively. Thus, it appears that CRN exists as a neutral molecule in NaOH eluent, while G and MG exist as an equilibrium mixture of the cation and neutral species.

ARG, GAA, GPA and GBA each have a strongly basic guanidino group and a weakly acidic carboxylic acid group. Thus, it appears that the carboxylic acid groups play a minor role in their retention. Among these four guanidines, ARG is the strongest base. It eluted at essentially t_0 at any NaOH concentration.

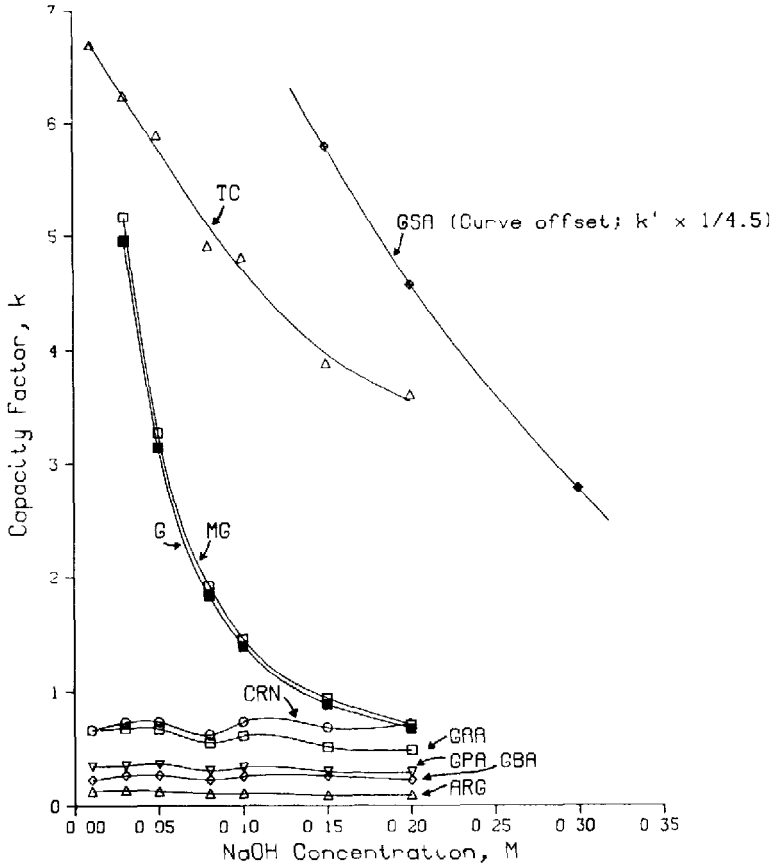

Fig. 1. Effect of NaOH concentration on retention of guanidino compounds. ARG = arginine; GAA = guanidinoacetic acid; GPA = guanidinopropionic acid; GBA = guanidinobutyric acid; GSA = guanidinosuccinic acid, CRN = creatinine; G = guanidine; MG = methylguanidine; TC = taurocyamine.

The carboxylic acid pK_a values of GAA, GPA and GBA were reported to be 2.86 [18], 3.5 and 3.8 [19]. The latter two values were inferred from experimental results previously published [19]. The order of their retention is apparently related to their pK_a values. The higher the pK_a value, the shorter the retention time. In addition to possessing a strongly basic group, TC also has a very strongly acidic anionic sulfonate functional group. Therefore it showed a significant retardation on an anion-exchange column.

GSA has two carboxy groups and a pI of 3.46 [20]. It showed very strong retention and could not be eluted with dilute NaOH eluent.

Calibration, repeatability and detection limits

Fig. 3 shows the quantitative calibration curves for seven guanidino compounds eluted with 0.050 M NaOH (CRN and GSA are not included). G, GPA

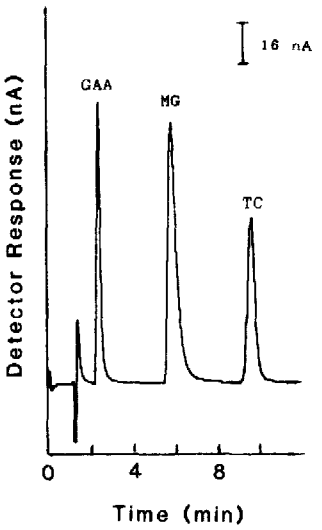

Fig 2. Chromatogram for a standard mixture of GAA, MG and TC. Eluent: 0.050 *M* NaOH; flow-rate: 1.0 ml/min. Peaks: GAA = guanidinoacetic acid (47 ng); MG = methylguanidine (44 ng); TC = taurocyamine (67 ng)

and GBA were plotted for concentrations of 1–100 μM. ARG, GAA, TC and MG were plotted for concentrations of 1–200 μM. All the calibration curves were non-linear. This result is similar to the work of Reim and Van Effen [13]. Since guanidine concentrations in physiological fluids are quite low over a limited analytical range, their response can be approximated as a linear function of concentration.

Kissinger [21] pointed out that a configuration of three electrodes in which the auxiliary electrode is placed downstream, "results in non-linear behavior when large samples are injected (typ. > 200 ng for compounds with small k' values), unless the ionic strength is very high". The results presented here are consistent with that observation. In this work, low-ionic-strength eluents were used, except for GSA. For GSA, the NaOH eluent concentrations was high (0.4 *M*). Therefore, when concentrations of GSA were lower than 50 μM, the calibration curve was observed to be a straight line. When the GSA concentration was as high as 100 μM, the uncompensated resistance was no longer negligible, causing the peak area to be less and the calibration curves non-linear.

The precision of the method was estimated by carrying out eight repetitive injections of guanidine solutions using a 0.40 *M* NaOH mobile phase for GSA and a 0.050 *M* NaOH mobile phase for the other guanidino compounds. The concentrations of the compounds were 20 μM, except for CRN which was 50 μM. The relative precision for these determinations at the 95% confidence

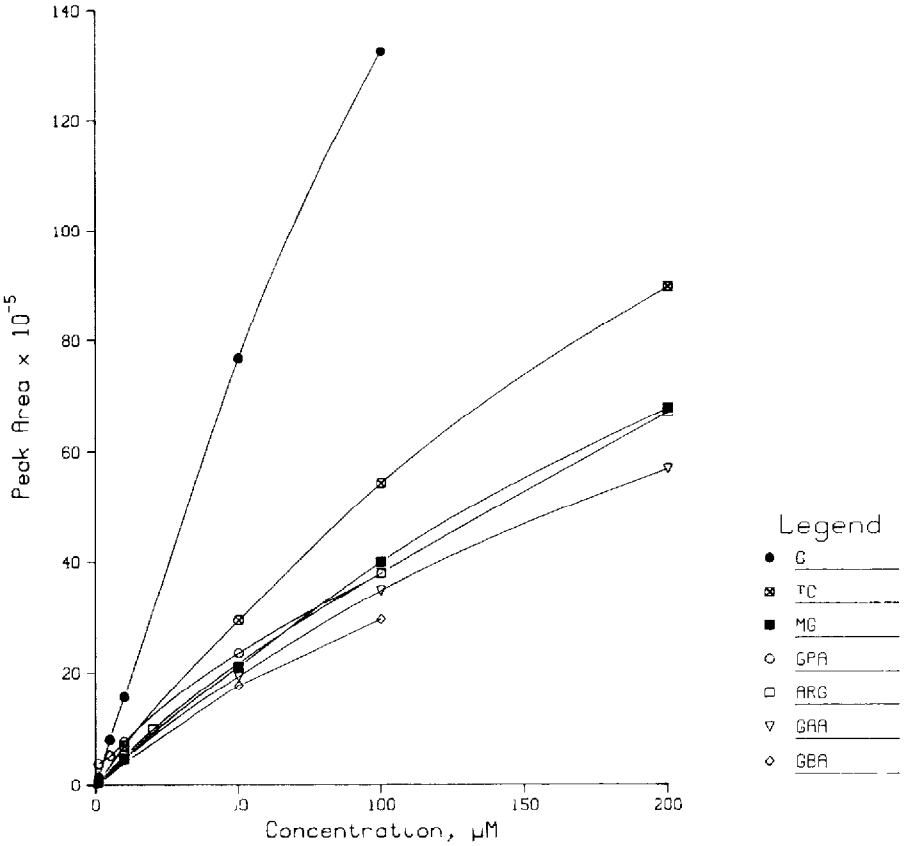

Fig. 3. Quantitative calibration curves for guanidino compounds. Eluent: 0.050 *M* NaOH; flow-rate: 1.0 ml/min. For abbreviations, see legend to Fig. 1.

level was found to be: GAA, 2.7%; MG, 2.8%; TC, 2.9%; GPA, 3.3%; G, 3.9%; GBA, 1.5%; ARG, 7.9%; CRN, 11.2%; GSA, 7.3%.

Detection limits for most guanidines were determined with 0.05 *M* NaOH as eluent at a signal-to-noise ratio of 3. The results were GAA, 3.5 pmol; MG, 5.1 pmol; TC, 6.2 pmol; GPA, 5.2 pmol; G, 3.2 pmol; GBA, 5.5 pmol; ARG, 3.2 pmol; CRN, 571.4 pmol. The detection limit for GSA was 12 pmol. Fig. 4 shows detector responses for trace amounts of GAA, MG and TC.

Table I represents a comparison of the detection limits obtained in this work with those reported previously using fluorimetric detection and post-column derivatization [19]. It can be seen that, with the exception of CRN, the amperometric detection limits are considerably lower than those previously reported. This observation supports the feasibility of amperometric detection for clinical analysis for guanidines. Unfortunately, in the method reported here,

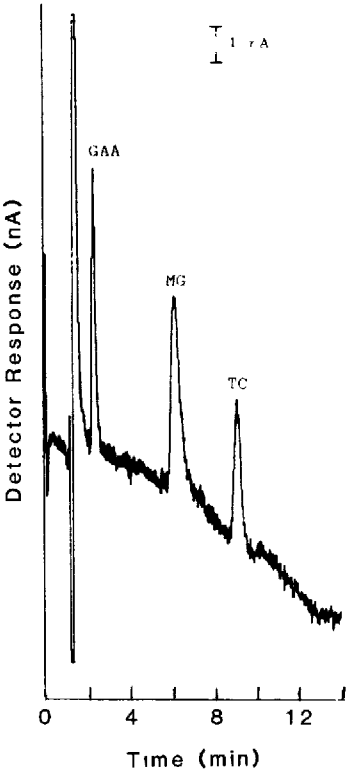

Fig. 4 Chromatogram showing detector response for trace amounts of GAA, MG and TC. Conditions are the same as in Fig. 2. Peaks: GAA = guanidinoacetic acid (2.3 ng), MG = methylguanidine (2.2 ng); TC = taurocyamine (3.3 ng).

TABLE I

DETECTION LIMITS OF GUANIDINO COMPOUNDS WITH FLUORIMETRIC [19] AND AMPEROMETRIC DETECTION

Compound	Limit of detection (ng)	
	Fluorimetric	Amperometric
ARG	55	0.7
GBA	60	0.8
GPA	5	0.7
GAA	2	0.4
MG	5	0.6
G	20	0.3
CRN	15	64 6
TC	1	1.0
GSA	5	2.1

TABLE II

RECOVERIES OF GUANIDINO COMPOUNDS USING CATION- AND ANION-EXCHANGE CLEAN-UP PROCEDURES

Compound	Clean-up procedure	Recovery ($n=3$) (%)
ARG	Cation-exchange	90.2 ± 4.3
GAA	Cation-exchange	94 9 ± 4.0
MG	Cation-exchange	93.3 ± 9.1
GSA	Anion-exchange	93.9 ± 3.3

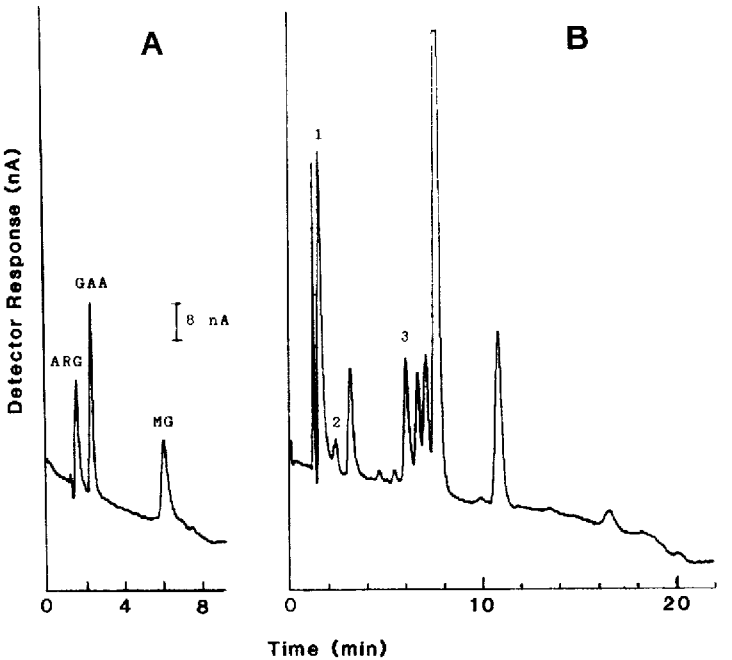

Fig. 5. Chromatograms for standards and dialysate fluid. Eluent: 0.050 *M* NaOH. Working electrode potential: 0.47 V vs. Ag/AgCl (A) Standards (21 ng ARG; 14 ng GAA, 11 ng MG). (B) Dialysate sample (female, age 67 years). For peak identification see text.

the response for CRN was insufficient for routine determinations of this compound.

Analysis of physiological fluid samples

It was found necessary to use a sample clean-up procedure (previously described) prior to chromatographic analysis of guanidine-containing physiological fluids. Recovery data for the clean-up procedure were obtained by adding

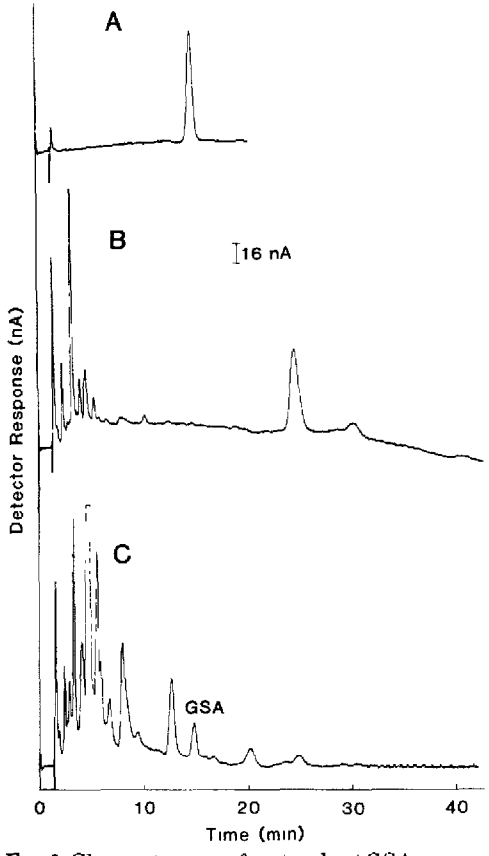

Fig. 6. Chromatograms for standard GSA, normal serum and uremic serum. Eluent 0.40 *M* NaOH. Working electrode potential: 0.45 V vs. Ag/AgCl. (A) Standard GSA (70 ng GSA). (B) Normal serum. (C) Uremic serum (female, age 74 years).

measured amounts of guanidines to blank hemodialysate and comparing peak heights of guanidines to those of the same concentration in standard guanidine solutions.

In this procedure 20.0 μl of 1.0 m*M* standard guanidino compounds were added to 1.0 ml blank dialysate. After the clean-up procedure, the volume was adjusted to 1.0 ml. Thus, the concentration of each guanidino compound was 20.0 μM. Table II shows that the recovery of four guanidino compounds ranges from 90 to 94%.

In order to identify guanidino compounds in physiological fluids, two different eluent strengths were chosen. Fig. 5 illustrates two chromatograms obtained with 0.050 *M* NaOH as eluent. Fig. 5A is the chromatogram of standard ARG, GAA and MG and Fig. 5B the chromatogram for a patient dialysate sample (female, age 67 years). From the retention times shown in the two

chromatograms and spiking experiments, we may infer that peak 1 (Fig. 5B) could be ARG and peak 2 is either GAA or CRN. Peak 3 is most likely MG.

It was found that trace amounts of GSA could be determined in uremic serum samples by eluting with 0.4 *M* NaOH. Fig. 6A shows a chromatogram for standard GSA. Fig. 6B is a chromatogram for normal serum; the data indicate that there is no evidence for GSA in normal serum. Fig. 6C is a chromatogram for a uremic serum sample with GSA eluting at 14.9 min. GSA concentrations for two uremic serum samples were found to be 6.24 and 8.24 μM, respectively. Additionally it was found that trace amounts of GSA could be determined in uremic dialysate samples by using a 100-μl sample loop and eluting with 0.40 *M* NaOH. GSA concentrations determined in four uremic dialysate samples were found to be 0.59, 1.65, 1.85 and 0.78 μM, respectively.

CONCLUSION

It has been shown that ED with a nickel working electrode has high detection sensitivity for guanidino compounds, although the response is not selective. For this reason, sample pre-treatment is necessary prior to LC analysis of physiological fluids for guanidino compounds. Other working electrodes have, thus far, not yet been examined; it is possible that with another electrode surface the electrochemical selectivity for guanidines could be improved.

Anion-exchange chromatography of guanidino compounds with an AS-6 column shows appreciable retention for G, MG, TC and GSA; however, ARG, GAA, CRN, GBA and GPA are only weakly retained. It was found that GSA is very strongly retained on anion-exchange columns but it can be eluted using separate chromatographic conditions.

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