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High-performance liquid chromatographic determination of guanidino compounds by automated pre-column fluorescence derivatization

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SUMMARY

An automated pre-column derivatization approach was used to develop an high-performance liquid chromatographic method for the determination of endogenous guanidino compounds. A commercially available autoinjector which was capable of adding and mixing reagent solutions and timing the reaction prior to injection was used to generate a fluorescent product by reaction of guanidino compounds with alkaline ninhydrin. The fluorescent products were separated by reversed-phase chromatography and detected with a fluorometer. Optimization of the pre-column reaction conditions resulted in a simple, highly sensitive and specific analytical method for the determination of guanidino compounds with excellent reproducibility and linearity. Application of this methodology to the determination of methylguanidine in human plasma samples resulted in a limit of quantification of 1 ng/ml (13.7 pmol/ml). The method was successfully employed for the quantification of circulating levels of methylguanidine in normal human subjects and uremic patients. The methodology should be generally applicable to the detection of other guanidino compounds in biological fluids.

INTRODUCTION

Pre-column derivatization methods are often preferred over post-column reaction methods in high-performance liquid chromatography (HPLC) because the chromatographic system is less complex and higher sensitivity may be achieved due to the elimination of peak broadening, baseline noise and dilution effects resulting from the addition of post-column reagents and the volume of the reaction coil. Manual pre-column derivatization methods, however, also suffer from certain disadvantages such as requirements for precise control and reproducibility of reaction conditions and the generation, in most cases, of a single, stable reaction product. For example, several fluorescence reagents are difficult to utilize in a pre-column derivatization method since the reaction product has only limited stability. However, automation of this

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process so that pre-column derivatization occurs just prior to chromatographic analysis can eliminate these drawbacks. In the present paper, we describe such an automated pre-column derivatization approach for the fluorescence determination of several endogenous guanidino compounds.

Guanidino compounds, including methylguanidine, have been shown to be elevated substantially in the plasma of uremic patients compared to normal subjects[1–5] and are suspected renal toxins whose measurement provides an indication of renal status. These guanidino compounds react with ninhydrin under strongly alkaline conditions to generate a highly fluorescent, but unstable product and, consequently, a previously reported method utilized a combination of HPLC and post-column fluorescence derivatization[6] for the measurement of guanidino compounds. In our approach, guanidino compounds are reacted with ninhydrin just prior to HPLC analysis, by a commercially available autoinjector with sophisticated pre-column derivatization capabilities, and the derivatized products were analyzed by reversed-phase HPLC with fluorescence detection. Using this methodology, we developed a substantially simplified, specific and highly sensitive (1 ng/ml) analytical method for the measurement of methylguanidine in human plasma samples. This methodology has been shown to be suitable for the measurement of methylguanidine plasma levels in normal human subjects and those with renal insufficiency.

EXPERIMENTAL

Chemicals

L-Arginine (ARG) hydrochloride, guanidine (G) hydrochloride, guanidinoacetic acid (GAA), guanidinosuccinic acid (GSA), β-guanidinopropionic acid (GPA), γ-guanidinobutyric acid (GBA), methylguanidine (MG) hydrochloride and ethylguanidine (EG) hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Ninhydrin and trifluoroacetic acid (TFA) were obtained from Pierce (Rockford, IL, U.S.A.). HPLC-grade methanol and sodium acetate were purchased from J. T. Baker (Philipsburg, NJ, U.S.A.). Weak cation-exchange (carboxymethylhydrogen form, CBA) solid phase extraction columns (1 ml) and the Vac-Elut manifold were purchased from Analytichem (Harbor City, CA, U.S.A). All other chemicals were reagent grade and obtained from local sources.

Sample preparation

A CBA solid phase extraction column was conditioned by successive washings with 1 ml of 1% TFA in methanol, 1 ml of methanol and 2 ml of water. An aliquot of plasma (1 ml) was mixed in a 75 × 10 mm borosilicate tube with 50 μl of internal standard solution (ethylguanidine, 10 μg/ml). The pH of the plasma was adjusted to 11 by adding 26 μl of 1.0 *M* sodium hydroxide solution. The plasma sample was vortex mixed and the sample was then poured onto the CBA column and vacuum was applied. The column was washed with 3 ml of water and then with 1 ml of methanol. The sample was then eluted from the column with 2 ml of 1% TFA in methanol and the eluents were collected into a 100 × 75 mm borosilicate tube. The methanol was evaporated under a gentle stream of nitrogen at 40°C and the residue reconstituted in 200 μl of 20% aqueous methanol.

HPLC

The HPLC system consised of a Hitachi 665A-12 high-pressure gradient solvent delivery system (EM Science, Cherry Hill, NJ, U.S.A.), an autoinjector with pre-column reagent addition and mixing capabilities (Varian, Model 9090, Sunnyvale, CA, U.S.A.) and an Hitachi F-1000 fluorescence detector (EM Science). Chromatographic separation of methylguanidine and other endogenous guanidino compounds was achieved on a 22 cm × 4.6 mm I.D., 5-μm octadecyl silica column (Pierce) connected in-line with a 5 cm × 4.6 mm I.D. octyl silica guard column at a mobile phase flow-rate of 1 ml/min. The mobile phase consisted of 0.05 *M* sodium acetate buffer (pH 6) and methanol as the organic modifier. The mobile phase solvents were degassed by filtering through a 0.2-μm Nylon-66 filter before use. Following chromatographic separation, fluorescence detection of derivatized guanidino compounds was accomplished using excitation at 390 nm while monitoring the fluorescence emission with a 470-nm cut-off filter. The chromatographic data were collected with a computer automated laboratory system (CIS-Beckman, Berkely, CA, U.S.A.).

Automated pre-column derivatization

A portion (30 μl) of the sample extract or a standard solution was transferred to an autoinjector vial and loaded on the autoinjector. Sodium hydroxide solution (25 μl) was added and the resultant solution was mixed by syringe filling and expulsion (three cycles). Ninhydrin reagent solution (5 μl) was then added to the sample vial, mixed as above, allowed to react at room temperature for 5 min and then a portion of the final solution (20 μl) was injected for HPLC analysis. Low volumes of sodium hydroxide and ninhydrin reagent solutions were chosen to perform the pre-column derivatization in order to minimize dilution effects and thus maximize the overall sensitivity.

Optimization of the pre-column reaction proceeded from the initial conditions described above using a standard mixture of several endogenous guanidino compounds (containing GSA, GAA, GPA, ARG, GBA, G, MG and EG; 300 pmol per component). This standard mixture was repetitively derivatized using systematic alterations in the reagent concentrations and reaction time prior to HPLC separation and analysis. The fluorescence intensity for each component was monitored by measuring the resultant chromatographic peak height while keeping the HPLC conditions constant. Using this approach optimal conditions were determined to maximize the fluorescence intensity obtained following pre-column derivatization.

Standard curves for methylguanidine assay

To establish calibration curves, a series of methylguanidine standard solutions, containing 0, 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/ml, were prepared in plasma and 1 ml samples were processed by the extraction procedure described above. The peak height ratios of methylguanidine to internal standard were weighted by $1/y$ (based on analysis of residuals) and plotted against the concentrations of methylguanidine. Linear regression analysis gave a calibration line that was used to calculate the concentration of methylguanidine in unknown samples and seeded control samples.

RESULTS AND DISCUSSION

As mentioned earlier, several chromatographic methods using post-column fluorescence derivatization have been described for the determination of guanidino compounds in biological fluids[7–11]. Only one previous pre-column derivatization method has been described using the reaction of benzoin with guanidino compounds to generate a fluorescence product suitable for HPLC analysis[12,13]. However, this manual pre-column method suffers from several disadvantages including benzoin's lack of aqueous solubility, a complex number of reagent addition steps to perform the reaction and stabilize the resultant product and variability in the reaction time. In order to develop simplified methodology for the determination of guanidino compounds, we explored fluorescence derivatization methods for their applicability for automated pre-column derivatization. We found that ninhydrin was the reagent of choice due to its stability and solubility in aqueous solutions, low background fluorescence and the ability to carry out the reaction at room temperature in a period of time consistent with that for chromatographic analysis (< 15 min). The use of automated pre-column derivatization resolves the problem associated with the stability of the fluorescent product formed in the reaction of ninhydrin with guanidino compounds.

Optimization of the ninhydrin pre-column derivatization conditions was accomplished as described in the experimental section of the text and based upon measurement of chromatographic peak height. The derivatized guanidino compounds from the standard mixture were separated by gradient elution HPLC using an initial mobile phase composition of 0.05 *M* sodium acetate (pH 6)–methanol (80:20). Following the injection, the methanol concentration was held at 20% for 5 min, increased to 30% over 5 min, held at 30% for 5 min and then cycled back to the initial conditions in 2 min.

The effect of the reagent concentration on the fluorescence intensity observed following ninhydrin pre-column derivatization was examined by successively varying the base and ninhydrin concentrations to establish optimal concentrations for each reagent. Initially, the concentration of sodium hydroxide was varied from 0.1 to 1.0 *M* while maintaining the ninhydrin concentration at 0.6% (w/v) (Fig. 1). As can be observed from Fig. 1, the base concentration has a significant and similar effect on the fluorescence intensity observed with all the endogenous guanidino compounds examined here. Based on these results, a sodium hydroxide concentration of 0.8 *M* was determined to be optimal for routine use. Subsequently, the concentration of ninhydrin was varied from 0.05 to 1.0% (w/v) while maintaining the base concentration at 0.8 *M* (Fig. 2). As can be seen from Fig. 2, maximum fluorescence intensity was observed for all the guanidino compounds, with the exception of GSA, at a ninhydrin concentration of 0.8% (w/v).

Using the optimal reagent concentrations established above, the effect of the pre-column reaction time was examined by allowing the reaction to proceed for times ranging from 1 to 14 min. The results of this experiment are shown in Fig. 3. In order to optimize the fluorescence intensity for all the guanidino compounds examined, a reaction time of 10 min was chosen for the pre-column derivatization. At reaction times longer than 12 min, a loss of fluorescence intensity was routinely observed due to instability of the fluorescent product formed in the reaction.

Thus the optimal sodium hydroxide and ninhydrin concentrations and reaction

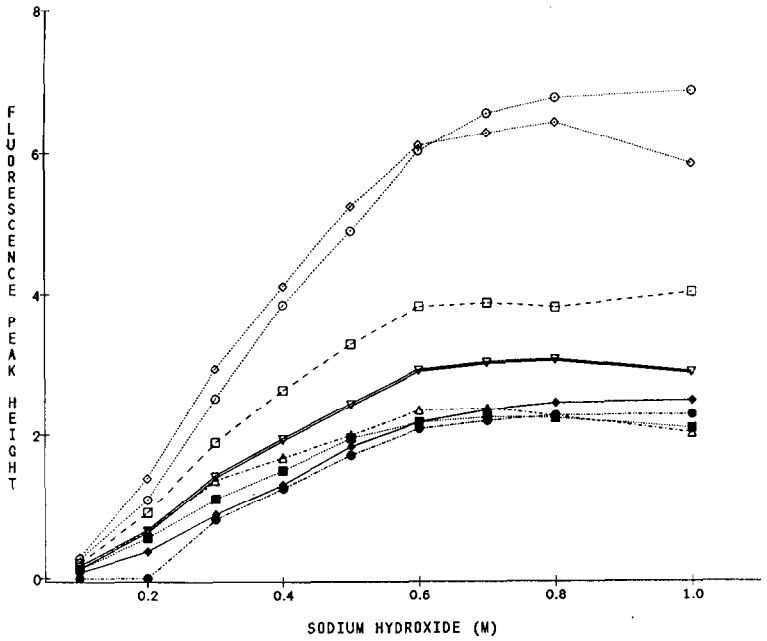

Fig. 1. Effect of sodium hydroxide concentration on the pre-column derivatization of guanidino compounds. The conditions are described in the text. Curves: ○ = GSA; ◇ = GAA; □ = GPA; △ = ARG; ▽ = GBA; ● = G; ◆ = MG; ■ = EG.

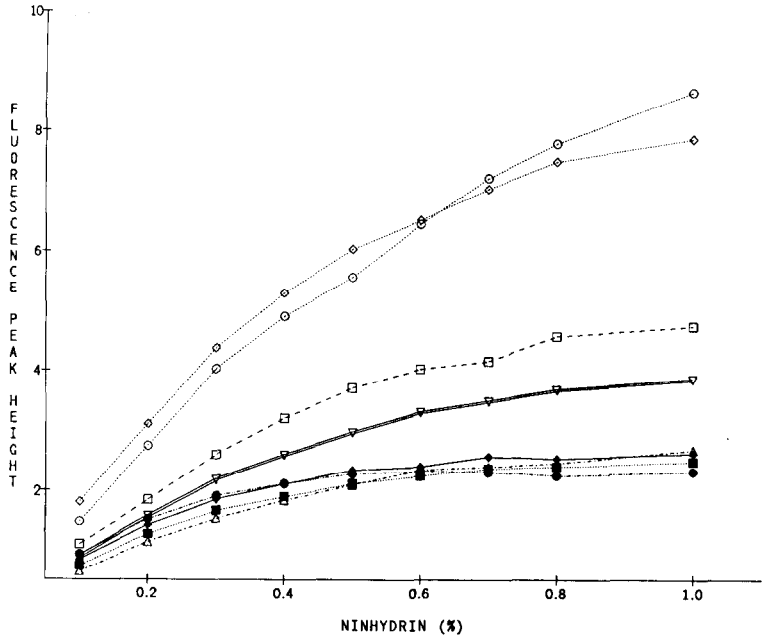

Fig. 2. Effect of ninhydrin concentration on the pre-column derivatization of guanidino compounds. The conditions are described in the text. See Fig. 1 for identification of curves.

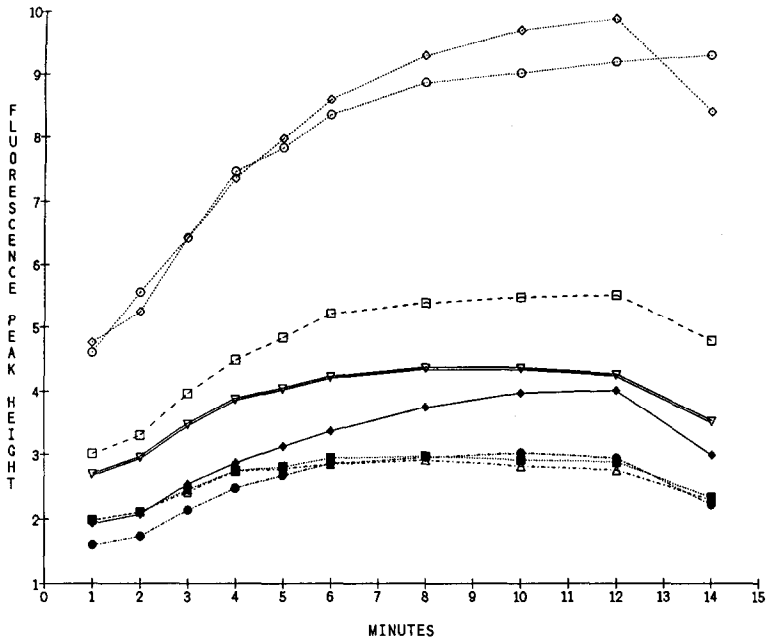

Fig. 3. Effect of reaction time on the pre-column derivatization of guanidino compounds. The conditions are described in the text. See Fig. 1 for identification of curves.

time for the pre-column derivatization of guanidino compounds with ninhydrin were determined to be 0.8 *M*, 0.8% (w/v) and 10 min, respectively. These conditions were used to obtain the remainder of the results described in this paper. Fig. 4 shows a chromatogram obtained from the analysis of a standard solution of eight guanidino compounds separated using the gradient elution conditions described above. As can be observed, separation of the guanidino compounds was achieved in less than 20 min.

In order to determine the applicability of this approach for routine quantification of guanidino compounds, the linearity and precision of the ninhydrin pre-column derivatization method were examined. The linearity was evaluated over the range from 9 to 3000 pmol by analysis of a series of standard solutions prepared by serial dilution of a guanidino stock standard solution (100 μmol per component). As shown in Fig. 5, the methodology demonstrated excellent linearity yielding linear responses for all guanidino compounds examined over the range of 9 to 3000 pmol. The curves were highly reproducible and correlation coefficients were typically greater than 0.999 for all guanidino components. The precision of the pre-column derivatization method was determined by repetitive analysis of a guanidino standard solution (containing 300 pmol per component) with measurement of the resultant chromatographic peak heights. The method displayed excellent chromatographic peak height reproducibility, yielding a coefficient of variation (C.V.) ranging from 0.8 to 4.2% for the guanidino compounds examined (Table I). The lower limit of detection (signal-to-noise ratio = 3) ranged from 1 to 8 pmol for the guanidino compounds examined. These results provide the necessary dynamic range for guanidino compounds in physiological samples.

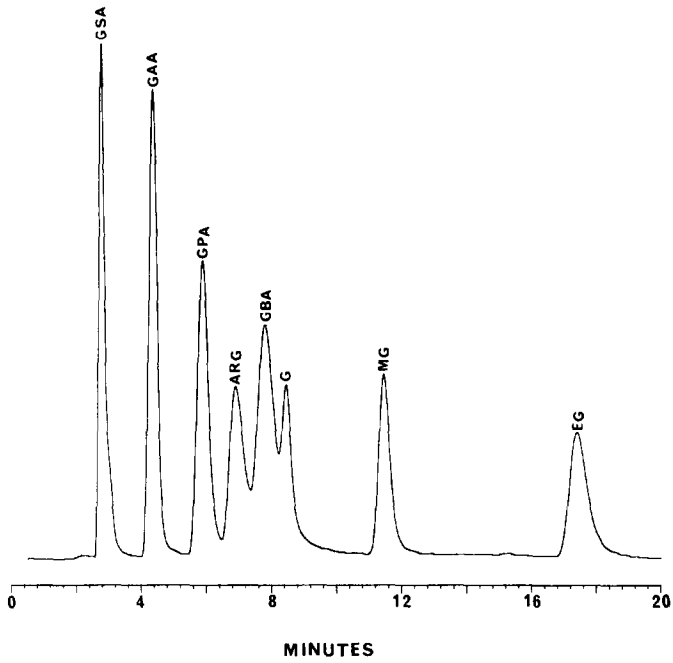

Fig. 4. Chromatogram of an aqueous standard solution of endogenous guanidino compounds separated by gradient elution. The chromatographic conditions are described in the text.

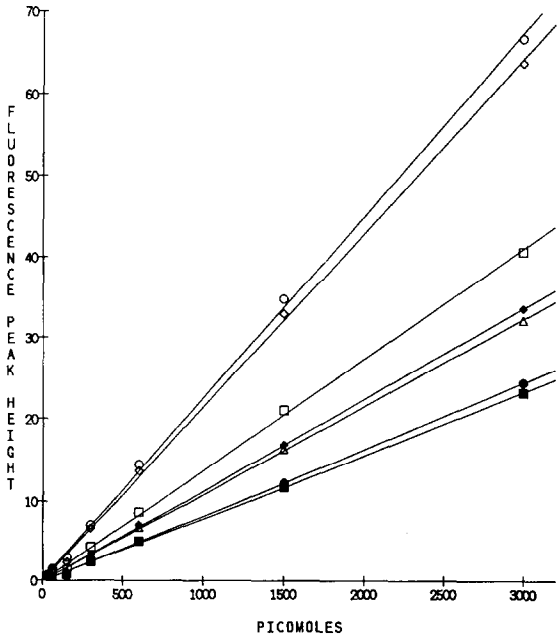

Fig. 5. Linearity of the pre-column derivatization HPLC method for the measurement of guanidino compounds. The conditions are described in the text. See Fig. 1 for identification of curves.

TABLE I

REPRODUCIBILITY OF THE PRE-COLUMN DERIVATIZATION HPLC METHOD

Injection number[a]	*Fluorescence intensity of guanidino compounds (peak heights)*							
	GSA	*GAA*	*GPA*	*ARG*	*GBA*	*GUA*	*MG*	*EG*
1	6.72	6.14	3.85	2.20	2.99	2.21	2.34	1.61
2	6.98	6.20	3.83	2.32	3.00	2.23	2.35	1.60
3	6.99	6.32	3.88	2.39	3.11	2.25	2.28	1.70
4	6.87	6.30	3.85	2.28	3.02	2.22	2.37	1.69
5	6.80	6.42	3.94	2.32	3.06	2.26	2.38	1.69
6	6.81	6.36	3.95	2.36	3.08	2.22	2.37	1.73
7	6.87	6.39	3.94	2.37	3.09	2.24	2.43	1.71
8	6.74	6.39	3.87	2.36	3.11	2.21	2.66	1.65
9	6.86	6.55	4.03	2.44	3.19	2.21	2.40	1.71
10	7.03	6.59	3.92	2.34	3.11	2.21	2.39	1.69
Mean	6.87	6.37	3.91	2.34	3.08	2.23	2.40	1.68
S.D.	0.11	0.14	0.06	0.07	0.06	0.02	0.10	0.04
C.V. (%)	1.54	2.18	1.57	2.79	1.98	0.83	4.20	2.66

[a] An aliquot (30 μl) of standard solution containing 300 pmol of each guanidino compound was repetitively analyzed using the methods described in the text and their fluorescence was measured.

The sensitivity, specificity, linearity and precision of the ninhydrin pre-column derivatization approach described here for the determination of guanidino compounds was clearly suitable for their quantitative analysis in biological fluids. In addition, this approach was equally sensitive to other methods utilizing post-column derivatization of guanidino compounds. Based upon these results, we applied this methodology to the development of a highly sensitive and specific HPLC method for the measurement of methylguanidine in human plasma samples. The method involves isolation of methylguanidine and an internal standard (ethylguanidine) from plasma by solid phase extraction prior to analysis by the combined pre-column derivatization and HPLC methodology described here. In order to optimize for the separation and measurement of methylguanidine, isocratic mobile phase conditions were employed. The isocratic mobile phase composition used was 0.05 *M* sodium acetate buffer (pH 6)–melthanol (75:25, v/v), at a flow-rate of 1 ml/min. These conditions provided highly reproducible chromatographic analysis providing the retention times of 10.3 and 20.2 min for methylguanidine and the internal standard, respectively. In addition, using these chromatographic conditions, methylguanidine and the internal standard were fully separated from other endogenous guanidino compounds. Typical chromatograms of plasma extracts obtained from a normal subject and a patient with renal insufficiency are shown in Fig. 6 A and B, respectively. Using this method, the limit of detection for methylguanidine in plasma samples was 1 ng/ml (signal-to-noise ratio = 3). Extraction recovery of methylguanidine and internal standard was determined by comparing the peak heights of the standards injected directly on the column with the response to standards extracted from plasma. The recoveries for methylguanidine and internal standard were quantitative. Calibration curves obtained for methylguanidine were linear with concentration from 1 to 1000 ng/ml in plasma samples. Correlation coefficients exceeded 0.999 for all plasma standard curves. The accuracy

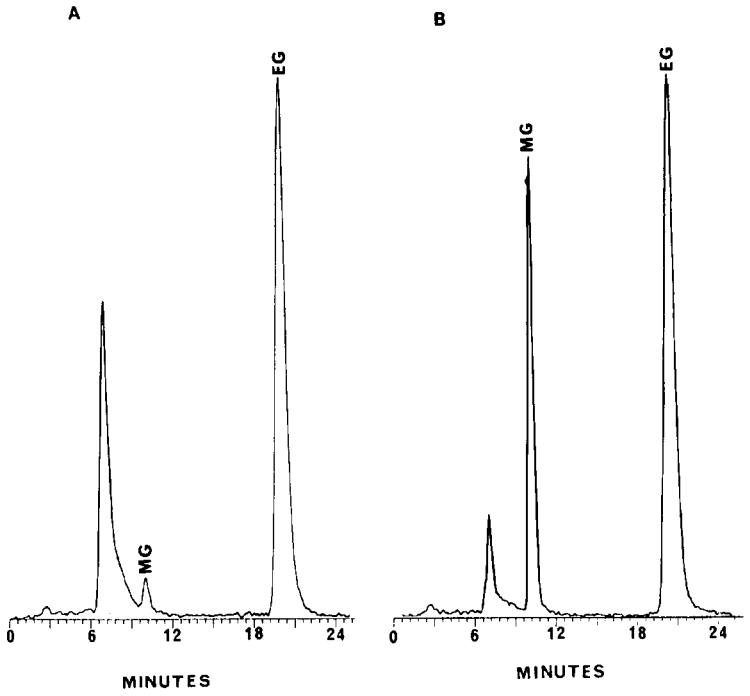

Fig. 6. Chromatograms of plasma extracts from a normal subject (A) and a subject with renal insufficiency (B). The conditions are described in the text. The concentrations of methylguanidine (MG) are 10.3 ng/ml in (A) and 186.6 ng/ml in (B), respectively. EG = Ethylguanidine.

and precision of the methylguanidine assay were within 11% across the calibration range. Table II summarizes the results of a three-day assay validation study in which six replicate seeded standards at three concentrations, 10, 100 and 1000 ng/ml were analyzed each day by this methodology. Clearly, the assay developed here for the measurement of methylguanidine in plasma samples was sufficiently accurate and precise for routine analysis of clinical samples.

TABLE II

ACCURACY AND PRECISION DATA FOR METHYLGUANIDINE IN PLASMA

Theoretical concentration (ng/ml)	*Assay concentration[a] (Mean ± S.D.) (ng/ml) (n = 6)*	*Coefficient of variation (%)*		*Accuracy (%)[d]*
		Intra-assay[b] (n = 6)	*Inter-assay[c] (n = 18)*	
10	9.27 ± 0.78	8.29	10.71	99.7
100	105.46 ± 2.08	1.97	6.74	99.5
1000	1014.60 ± 40.50	3.99	7.02	98.4

[a] Mean value on a single day.
[b] (S.D./mean) · 100 in same assay.
[c] (S.D./mean) · 100 in three different assays.
[d] Percent ratio of actual to theoretical concentration.

In conclusion, the methodology described here was simple, highly sensitive, specific and routinely useful for the quantification of methylguanidine in normal and uremic subjectgs. This automated pre-column derivatization methodology offers convenience and can be readily incorporated into existing laboratory HPLC systems. Moreover, this method offers a simple alternative for post-column reaction systems for the measurement of guanidino compounds without compromising the sensitivity and selectivity offered by such systems.

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