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QUANTITATIVE ANALYSIS OF METHYLGUANIDINE AND GUANIDINE IN PHYSIOLOGIC FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—FLUORESCENCE DETECTION METHOD

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method has been developed for the quantitative analysis of methylguanidine and guanidine in physiological fluids. These guanidino compounds are separated on a 6 × 0.23 cm cation-exchange column with 0.5 M sodium hydroxide solution. The guanidino compounds are detected with a fluorometer, which monitors the fluorescent guanidine derivatives produced by the reaction of the eluted constituents with 9,10-phenanthrenequinone. Sensitivity to sub-nanomole levels of methylguanidine and guanidine is demonstrated. The method was successfully applied to physiological fluids such as serum and cerebrospinal fluid from uremic patients.

INTRODUCTION

Methylguanidine levels have been demonstrated to be elevated significantly in sera of uremic patients [1—5], suggesting it is one of the toxic substances in uremia [6]. Therefore, a routine method for the quantitative analysis of methylguanidine in physiological fluids is strongly desired for clinical purposes.

The conventional column chromatographic method for the analysis of methylguanidine is time-consuming and needs a large blood sample [1—6]. Furthermore, this method does not resolve methylguanidine from guanidine, which leads to errors in the estimation of methylguanidine. Recently, all guanidino compounds in physiological serum, including methylguanidine, were analyzed using a modified automatic amino-acid analyzer [7,8]. However, methylguanidine and guanidine contents were not measured with accuracy, even for the uremic serum because of the low sensitivity of the method.

The purpose of this study was to develop a rapid and sensitive method for

the quantitative analysis of methylguanidine and guanidine. This paper describes the development of a high-performance liquid chromatographic (HPLC) procedure for separating guanidine, methylguanidine and other guanidino compounds, along with a means of detecting these guanidino compounds fluorometrically by utilizing the fluorophor produced by the reaction of the guanidines with 9,10-phenanthrenequinone. 9,10-Phenanthrenequinone has been reported to form a highly fluorescent product with guanidino compounds in alkaline solution [9].

EXPERIMENTAL

Chemicals

Methylguanidine hydrochloride and guanidine hydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). Other guanidino compounds were purchased from Sigma and Pierce (Rockford, Ill., U.S.A.). 9,10-Phenanthrenequinone was obtained from Tokyo Chem. Co. (Tokyo, Japan). N,N-dimethylformamide was purchased from Nakarai Chem. Co. (Kyoto, Japan). Durrum DC-4A cation-exchange resin was obtained from Durrum (Palo Alto, Calif., U.S.A.).

Chromatographic system

Fig. 1 is a schematic presentation of the high-performance liquid chromatograph which was used in these experiments. Two minipumps (Milton-Roy) served to pump the column eluent and the reagent solution through the system. The column eluent was pumped through a 6-port sample injection valve supplied with a 100-μl sample loop. A jacketed, stainless-steel column, 6 × 0.23 cm
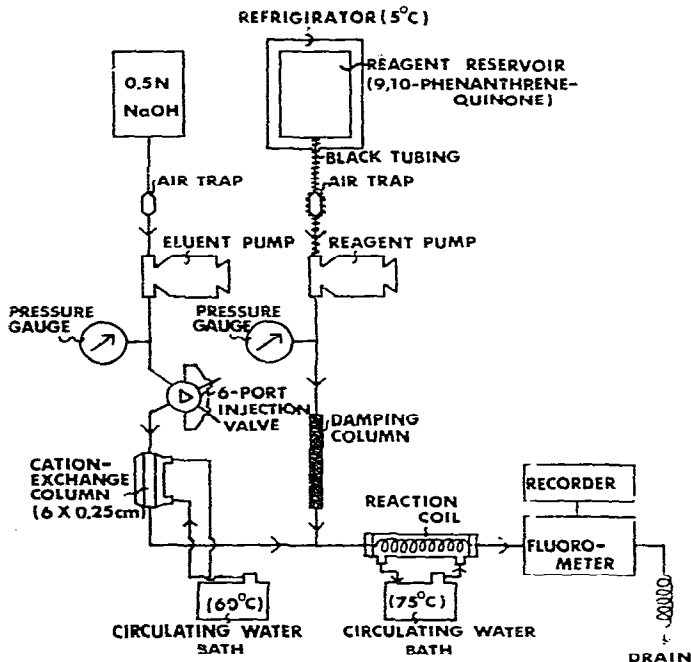

Fig. 1. Schematic diagram of high-performance liquid chromatograph for the separation and fluorescence detection of guanidine and methylguanidine.

I.D., was used in this investigation. The column was slurry-packed with Durrum DC-4A resin (8 ± 2 μm), which was supported by a 1/8- to 1/16-in. reducer union containing 1 μm stainless-steel frit. The column was operated at 60° utilizing a Haacke constant-temperature circulator. The reservoir of 9,10-phenanthrenequinone reagent solution was stored in a refrigerator at 5° to keep the reagent from degradation. The PTFE tubing connecting the reservoir to the reagent pump was covered with black tubing to prevent the exposure of the reagent to light. A pulse-damping device, a 30 × 0.23 cm I.D. column packed with Zipax (Du Pont), was inserted between the reagent pump and the mixing tee. The eluent from the chromatographic column was mixed with a stream of 9,10-phenanthrenequinone in a heated stainless-steel reaction coil, 153 × 0.05 cm I.D. At a total flow-rate of 18 ml/h, dwell time within the reaction coil was ca. 1 min. A JASCO FP-100 FluoroMonitor (Japan Spectroscopic Co., Tokyo, Japan) was used to detect the fluorescent guanidine derivatives produced by the reaction of 9,10-phenanthrenequinone with the constituents from the column. The FluoroMonitor was equipped with a high-pressure mercury lamp which emits strong 365 nm light and with primary and secondary filters to detect visible light beyond 460 nm emitted from the fluorescent compounds in the flow cell.

Operation of the chromatographic system

Methylguanidine, guanidine and other guanidino compounds were separated on cation-exchange resin using a 0.5 M sodium hydroxide solution. A 100-μl sample was introduced into the column using a 6-port sample injection valve. The column was operated at a flow-rate of 12 ml/h and a column inlet pressure of 10 kg/cm^2. A 2.5 mM solution of 9,10-phenanthrenequinone was prepared in the reagent reservoir by dissolving the reagent in dimethylformamide. The reagent solution was pumped into the reaction coil at a flow-rate of 6 ml/h. As mentioned later, the reaction coil was heated up to 75° to attain the highest detectability.

Preparation of physiologic fluid samples

Serum and cerebrospinal fluid samples from uremic patients were used for the analysis of guanidine and methylguanidine. A 1-ml aliquot from each serum sample was centrifuged at 80 g for 2 h in a CF-25 Centriflo® membrane [10] (Amicon, Lexington, Mass., U.S.A.), yielding approximately 0.5 ml of ultrafiltrate. A 0.1-ml sample of the deproteinized ultrafiltrate was applied to the column of the chromatographic system. Cerebrospinal fluid sample was used directly for the analysis without any deproteinization treatment.

RESULTS

Effect of alkalinity of mobile phase on retention time and resolution

Because of the strong basicity of guanidine and methylguanidine they are not eluted with basic buffers but with strong alkaline solutions. The effect of the alkalinity of the mobile phase on the retention time was tested in the range of 0.1—1.0 M NaOH. A plot of retention times and resolution ratios against the concentration of sodium hydroxide is shown in Fig. 2. Here, the resolution

ratio is the standard quantity R_S. Increasing alkalinity tends to produce a faster chromatogram of guanidine and methylguanidine. On the other hand, the resolution ratio increased with increasing alkaline concentration up to a maximum around 0.4 M, followed by a gradual decrease at higher alkaline concentration.

Using these data, it was determined that 0.5 M sodium hydroxide solution should be used as a column eluent to obtain optimum separation results (resolution and analysis time).

Fluorescence properties of 9,10-phenanthrenequinone derivatives of guanidine and methylguanidine

9,10-Phenanthrenequinone derivatives of guanidine and methylguanidine show fluorescence maxima at 525 nm and 510 nm, respectively. Excitation maxima are observed at 386 nm and 298 nm for guanidine derivative, and at 378 nm and 298 nm for methylguanidine derivative.

The effect of temperature on the reaction of the guanidines with 9,10-phenanthrenequinone was studied in the range of 30—95°. Fig. 3 shows a plot of the fluorescence intensity as a function of reaction coil temperatures after passing through the coil for 1 min. It can be seen that the degree of conversion to fluorescent derivatives shows a maximum at 75° for methylguanidine and at 80° for guanidine and that the response decreases at higher temperatures. This is presumably due to a breakdown of the reagent or the product at the higher temperatures. The fluorescence intensity of guanidine and methylguanidine derivative, obtained at 75° is 7—8 times greater than that at room temperature.

Accordingly, the reaction coil temperature utilized in the chromatographic system for the analysis was 75°.
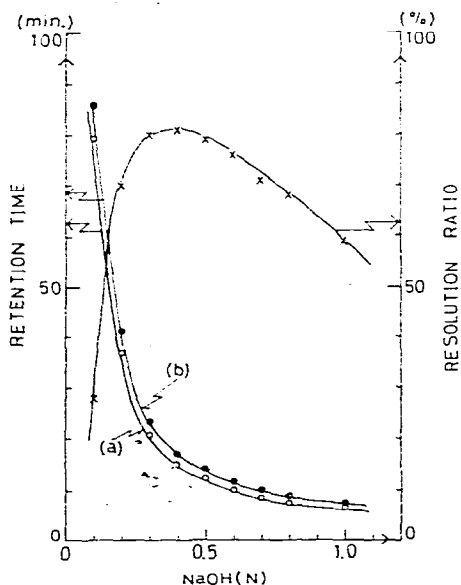

Fig. 2. Effect of alkaline concentration of mobile phase on retention time and resolution of guanidine and methylguanidine. (a) Guanidine, (b) methylguanidine.
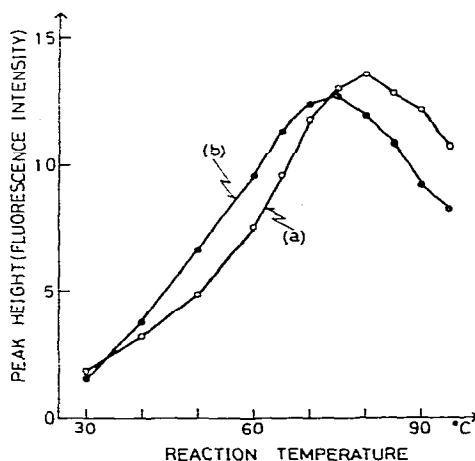
Fig. 3. Effect of reaction coil temperature on the reaction of conversion to fluorescent guanidine derivatives. (a) Guanidine, (b) methylguanidine.

Chromatographic separation and quantitative response

Fig. 4 shows a typical separation of a standard mixture of guanidine, methylguanidine and other guanidino compounds, each at a concentration of 1 nmole per 100 μl. All guanidino compounds except for guanidine and methylguanidine are eluted as a group in the first 7 min. Guanidine and methylguanidine are eluted at 12.4 min. and 14.1 min., respectively, showing satisfactory resolution. The entire analysis can be completed in less than 15 min.

The linear range of the analytical system was evaluated from 0.025 nmoles to 2.5 nmoles of guanidine and methylguanidine, and the relative weight response of each compound remained essentially constant, indicating good linearity. The minimum detectable quantity for these compounds was found to be 0.49 ng for guanidine and 0.63 ng for methylguanidine. In order to evaluate the precision and accuracy of the analytical method a recovery study was done using 20 serum samples from uremic patients. The recoveries of guanidine and methylguanidine are 98.5 ± 2.3% and 97.3 ± 3.2%, respectively and seem to be essentially quantitative.

Analysis of physiologic fluids

Several serum and cerebrospinal fluid samples were analyzed for guanidine and methylguanidine. Typical results for samples from uremic patients are shown in Fig. 5. The chromatogram in Fig. 5A represents a serum sample from a uremic patient. The major peak, eluting early in the chromatogram, contains other less basic guanidino compounds such as guanidinosuccinic acid, guanidinoacetic acid, creatinine and arginine. The labelled peaks were identified from the retention times as compared to those of a standard mixtures and estimated to be 0.47 nmoles for guanidine and 1.18 nmoles for methylguanidine. The contents of guanidine and methylguanidine were determined as 28.1 μg/dl and 86.5 μg/dl, respectively. The chromatogram in Fig. 5B shows a cerebrospinal fluid sample from a uremic patient. The labelled peaks of guanidine and methylguanidine were estimated to be 0.108 nmoles and 0.228 nmoles, respectively. The contents of guanidine and methylguanidine in this cerebrospinal fluid sample were determined to be 6.40 μg/dl and 16.7 μg/dl, respectively.

DISCUSSION

The conventional method for the analysis of methylguanidine is based on the colorimetric procedure after the isolation of methylguanidine by means of adsorption of charcoal [2] or ion-exchange column [3—6]. This method requires large sample amounts (usually 5—20 ml), and prior to the colorimetric measurement the fractionated sample is concentrated by the evaporation procedure. A recent analytical method using a modified automatic amino-acid analyzer [8,9] also requires concentration techniques such as lyophilization prior to application to the chromatographic column. Nevertheless, methylguanidine is sometimes not determined even for the uremic serum because of the lower sensitivity of this method. In experiments, not less than 1 ml of serum was passed through an ultrafiltration membrane and then 0.1 ml of the deproteinized sample was directly applied to the analytical column. Our new sensitive analytical method is advantageous particularly when only a limited sample is
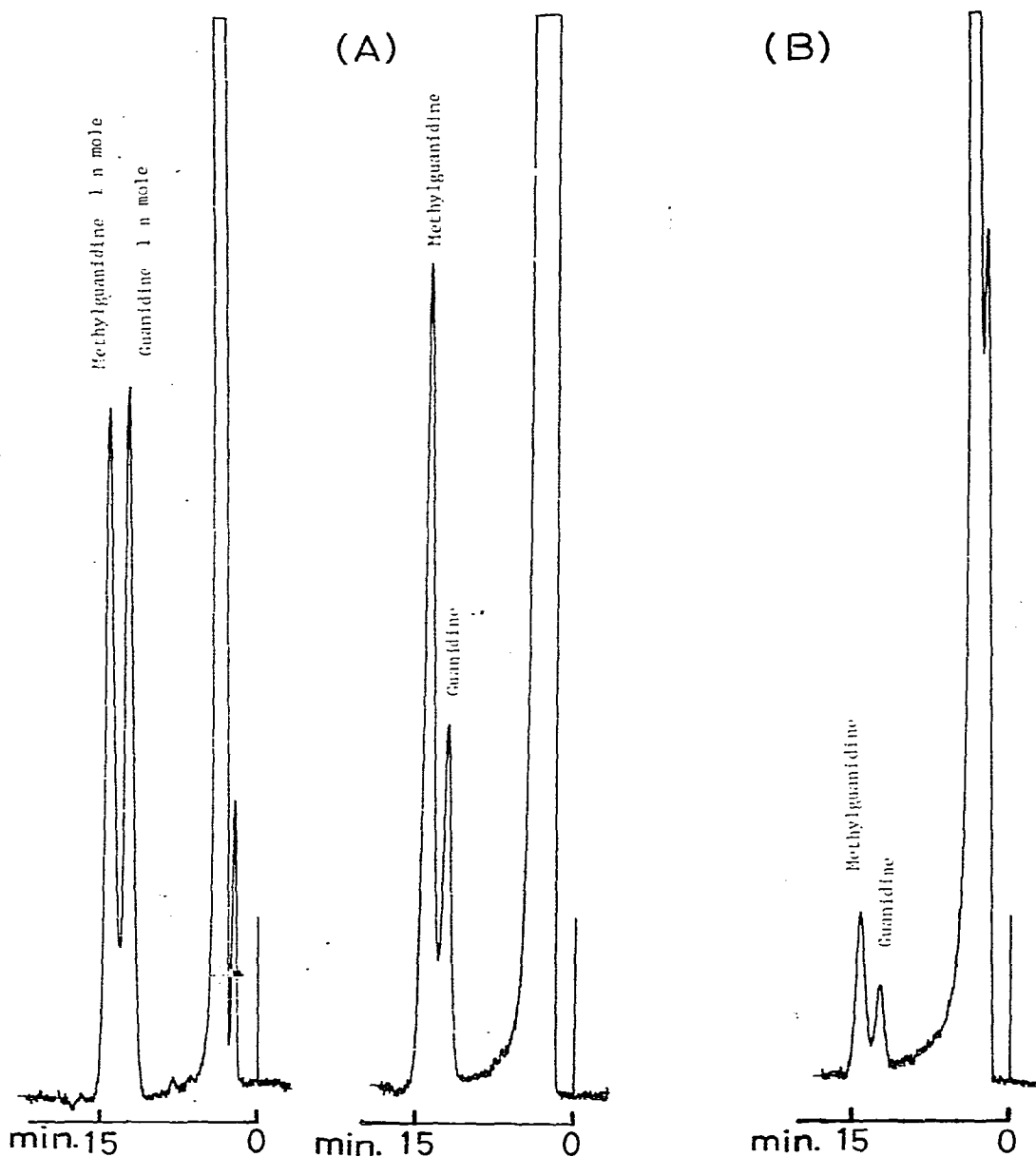
Fig. 4. Chromatogram of a standard mixture of guanidine, methylguanidine and other guanidine compounds, each at a concentration of 1 nmole/100 μl.

Fig. 5. (A) Analysis of a serum sample from a uremic patient. (B) Analysis of a cerebrospinal fluid sample from a uremic patient.

available, i.e. sampling of the cerebrospinal fluids or repetitive sampling in patients or in small animals. In the case of cerebrospinal fluid samples, only 0.2 ml of sample is required for analysis because deproteinization treatment is not required. Use of such small samples is possible because our fluorometric detection method is two orders of magnitude more sensitive than the conventional colorimetric procedure [1—9].

The conventional method did not separate methylguanidine from guanidine. Therefore, the results obtained showed artificially high values of methylguanidine. The use of an automatic amino-acid analyzer for the analysis of guanidino compounds [8,9] enabled the separation of most of the guanidino compounds in physiological fluids. Shainkin et al. [9] reported that guanidine and methylguanidine were found in small amounts only in uremic plasma. However, the amount of these guanidino compounds was not determined with accuracy because of the limitation in sensitivity of their method. With our analytical method, the cerebrospinal as well as serum levels of guanidine and methylguanidine were easily determined for all samples from uremic patients.

A large number of uremic serum and cerebrospinal fluid samples are currently being analyzed, and the results obtained from our analytical method will be the subject of a later report.

CONCLUSION

A simple and direct HPLC system has been described for the quantitative analysis of guanidine and methylguanidine in physiological fluids.

Our new analytical method has several distinct advantages:

(a) only a small amount of physiological fluid sample, i.e. no more than 1 ml of serum or 0.2 ml of cerebrospinal fluid, is needed for analysis.

(b) the method is so sensitive that no concentration techniques such as lyophilization or evaporation are required.

(c) the separation of guanidine and methylguanidine is satisfactory and the simultaneous quantitative analysis is easily performed.

(d) the entire analysis can be completed in less than 15 min, so that a large number of samples can be analyzed in reasonable time.

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