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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GUANIDINO COMPOUNDS USING BENZOIN AS A PRE-COLUMN FLUORESCENT DERIVATIZATION REAGENT

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SUMMARY

A high-performance liquid chromatographic method is described for the simultaneous separation of nine guanidino compounds of biological importance. Guanidino compounds are converted into the corresponding fluorescent derivatives by reaction with benzoin, and separated within 25 min on a reversed-phase column, μBondapak Phenyl, with linear gradient elution using aqueous methanol containing a Tris–hydrochloric acid buffer (pH 8.5). The method is simple, rapid and sensitive; the lower limits of detection for the guanidino compounds are 20–100 fmol in a 100-μl injection volume.

INTRODUCTION

Many chromatographic methods have been reported for the determination of guanidino compounds based on ion-exchange[1–7], thin-layer[8–10], paper[11,12] and gas chromatography[13–15] owing to the fact that compounds such as methylguanidine, guanidinosuccinic acid and taurocyamine have been implicated as uremic toxins in uremic syndrome[16–19]. Among the methods, ion-exchange chromatographic methods using improved techniques of amino acid analysis, including high-performance liquid chromatography (HPLC), are the most popular. These are based on post-column derivatization of the guanidino compounds by means of the Sakaguchi or Voges–Proskauer colour reaction[1–4], and the fluorescence reaction with ninhydrin or 9,10-phenanthraquinone[5–7]. However a pre-column derivatization method for guanidino compounds in liquid chromatography has not been presented.

Recently, we have developed a sensitive fluorimetric method for the selective determination of guanidino compounds, based on their reaction with benzoin in an aqueous methylcellosolve–potassium hydroxide solution in the presence of β-mercaptoethanol (a stabilizer of the resulting fluorescent product) and sodium sulphite (a suppressor of blank fluorescence)[20,21]. The reaction gives a single fluorescent prod-

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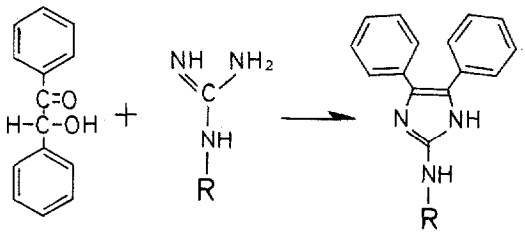

Benzoin Fluorescent product

uct, a 2-substituted amino-4,5-diphenylimidazole, from a guanidino compound[22]; the product retains the substituted group of the original guanidino compound (R in the scheme). Therefore, this reaction may be employed for both pre- and post-column derivatization in the HPLC of guanidino compounds.

This study aims to apply the fluorescence reaction to the pre-column derivatization of a variety of guanidino compounds of biological importance and to establish the conditions of rapid mutual separation of their fluorescent derivatives by HPLC with fluorescence detection. Nine guanidino compounds (guanidine, methylguanidine, guanidinoacetic acid, guanidinopropionic acid, guanidinobutyric acid, guanidinosuccinic acid, arginine, taurocyamine and phenylguanidine) were employed as representative compounds for the investigations.

EXPERIMENTAL

Materials and reagents

All chemicals were of analytical-reagent grade, unless otherwise stated. Distilled water was used. Taurocyamine was kindly supplied by Professor A. Mori (Institute for Neurobiology, Okayama University Medical School, Okayama, Japan). Tris(hydroxymethyl)aminomethane (Tris) (Wako, Osaka, Japan) was recrystallized from aqueous 60% methanol to remove fluorescent impurities. Argininosuccinic acid (barium salt, 90% purity; Sigma, St. Louis, MO, U.S.A.) was purified by cation-exchange chromatography according to the procedure of Sherwin and Natelson[23]. The solutions used for the fluorescent derivatization were prepared as follows[21].

Benzoin solution (4.0 mM). Benzoin (85 mg) was dissolved in 100 ml of methylcellosolve.

β-Mercaptoethanol (0.1 M)–sodium sulphite (0.2 M) solution. β-Mercaptoethanol (0.78 g) and sodium sulphite (2.52 g) were dissolved in water (80 ml) and diluted with water to 100 ml.

Hydrochloric acid (2 M)–Tris–hydrochloric acid buffer (0.5 M, pH 9.2) mixture. Equal volumes of 4.0 *M* hydrochloric acid and 1.0 *M* Tris–hydrochloric acid buffer (prepared by dissolving 12.11 g of Tris in 80 ml of water, adjusting the pH to 9.2 with concentrated hydrochloric acid and diluting the solution to 100 ml with water) were mixed together.

Fluorescent derivatization

A 200-μl portion of sample solution was placed in a test tube, to which was added 100 μl each of benzoin solution and β-mercaptoethanol–sodium sulphite solution and 200 μl of aqueous 2.0 *M* potassium hydroxide with cooling in ice–water.

The mixture was heated in a boiling water-bath for 5 min, cooled in ice–water for *ca.* 2 min and then 200 μl of hydrochloric acid–Tris–hydrochloric acid buffer mixture was added. A 100-μl portion of the final mixture was used for HPLC.

HPLC apparatus and conditions

The HPLC system consisted of a Hitachi 635 high-pressure pump, a Shimadzu SIL-1A syringe-loading sample injector and a Jasco FP-110 HPLC Fluorescence spectrophotometer equipped with a xenon lamp. The fluorescence of the eluate was monitored at 425 nm emission against 325 nm excitation.

Three reversed-phase columns [μBondapak Phenyl (300 × 3.9 mm I.D.; particle size, 10 μm; Waters Assoc., Milford, MA, U.S.A.), LiChrosorb RP-18 (150 × 4.0 mm I.D.; particle size, 5 μm; Japan Merck, Tokyo, Japan) and μBondapak CN (300 × 3.9 mm I.D.; particle size, 10 μm; Waters Assoc.)] were used for isocratic elution with aqueous methanol containing 0.5 *M* Tris–hydrochloric acid buffer (pH 8.5) as mobile phase. For HPLC with solvent gradient elution, the μBondapak Phenyl column was used. Gradient elution with methanol in the mobile phase described above was carried out using a Shimadzu GRE-2B solvent gradient device. The column temperature was ambient (23–27°C) and the flow-rate was usually 0.8 ml/min.

RESULTS AND DISCUSSION

The benzoin derivatives of some guanidino compounds fluoresce intensely in polar solvents, such as methanol, and in aqueous solution, particularly so in buffers at weakly alkaline pH[21,22], rather than in non-polar solvents. The derivatives (except for those of methylguanidine and phenylguanidine) are strongly retained when applied onto silica gel and alumina columns and are not readily eluted from these columns with methanol, ethyl acetate tetrahydrofuran, acetonitrile, chloroform or hexane. From these observations, the simultaneous separation of the benzoin derivatives was studied with reversed-phase HPLC.

Isocratic elution was first examined using μBondapak Phenyl, LiChrosorb RP-18 and μBondapak CN columns. The mobile phase was aqueous methanol containing 15% 0.5 *M* Tris–hydrochloric acid buffer (pH 8.5). The most favourable concentrations of methanol for the separation were 57% for μBondapak Phenyl, 55% for LiChrosorb RP-18 and 52% for μBondapak CN. The excitation and emission maxima of the fluorescence for all the guanidino compounds tested were around 325 and 435 nm, respectively, in the mobile phase containing 52–57% methanol. The chromatograms thus obtained are shown in Figs. 1–3. The best separation of the derivatives was achieved on the μBondapak Phenyl column, although the derivatives of guanidine, methylguanidine and phenylguanidine showed relatively long retention times. The LiChrosorb RP-18 and μBondapak CN columns did not permit satisfactory resolution of the peaks for taurocyamine, guanidinoacetic acid and guanidinopropionic acid. Acetonitrile and tetrahydrofuran, which have been used previously as mobile phase components for reversed-phase HPLC, were examined for possible use in place of methanol in the mobile phase at various concentrations, but no improved resolution of the peaks was observed. Incomplete resolution of the peaks was also obtained even on the μBondapak Phenyl column, when the Tris buffer in the mobile phase was replaced with a phosphate buffer (0.2 *M*, pH 8.5). The μBondapak

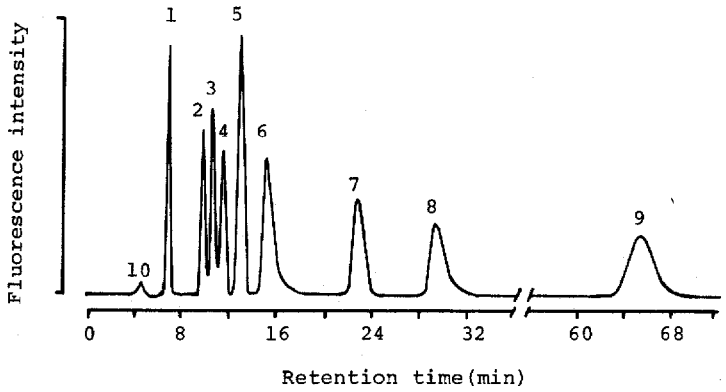

Fig. 1. Isocratic elution of the benzoin derivatives of guanidino compounds on the μBondapak Phenyl column. HPLC conditions: mobile phase, methanol–water–0.5 *M* Tris–hydrochloric acid buffer (pH 8.5) (57:28:15); other conditions, see text. Peaks and amounts of guanidino compounds (per injection volume of 100 μl): 1 = guanidinosuccinic acid (250 pmol); 2 = taurocyamine (125 pmol); 3 = guanidinoacetic acid (250 pmol); 4 = guanidinopropionic acid (250 pmol); 5 = guanidinobutyric acid (250 pmol); 6 = arginine (250 pmol); 7 = guanidine (250 pmol); 8 = methylguanidine (250 pmol); 9 = phenylguanidine (60 pmol); 10 = reagent blank.

Phenyl column and a mixture of aqueous methanol and Tris–hydrochloric acid buffer as mobile phase were thus used for further investigations.

Gradient elution with methanol served to minimize the retention times for the benzoin derivatives. Fig. 4 shows a chromatogram obtained by a linear gradient elution with a methanol concentration of between 50 and 80% in the mobile phase. The change in methanol concentration actually had no effect on the wavelengths and intensities of the fluorescence excitation and emission maxima for the guanidino com-

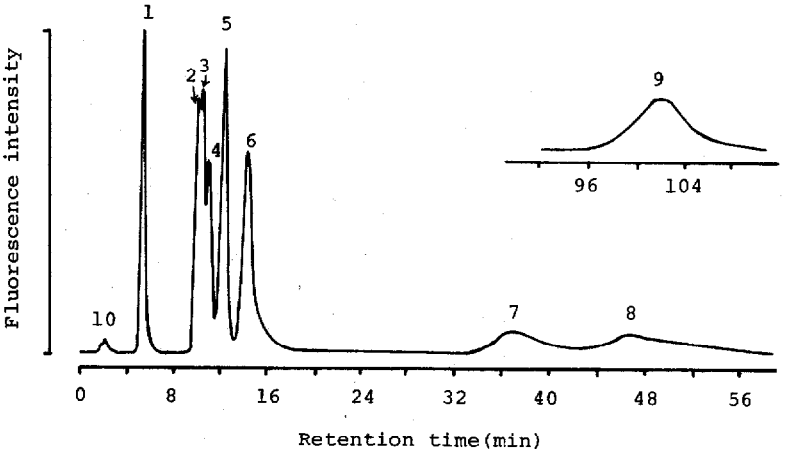

Fig. 2. Isocratic elution of the benzoin derivatives of guanidino compounds on the LiChrosorb RP-18 column. HPLC conditions: mobile phase, methanol–water–0.5 *M* Tris–hydrochloric acid buffer (pH 8.5) (55:30:15); other conditions, see text. For peak numbers and amounts of guanidino compounds, see Fig. 1.

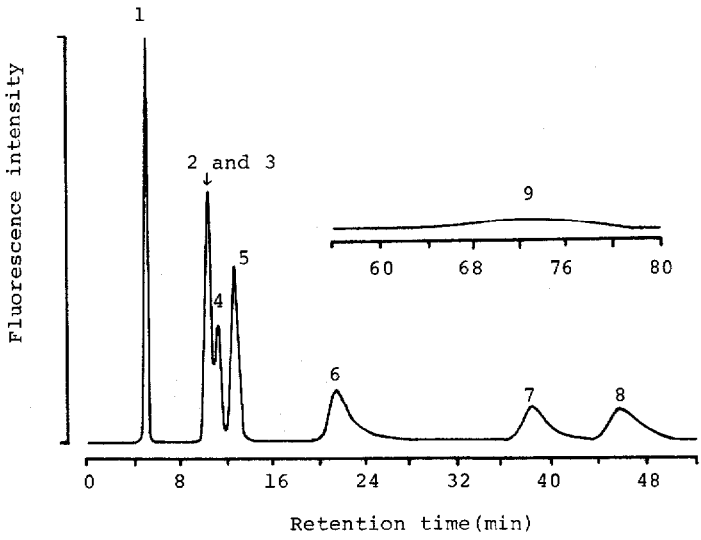

Fig. 3. Isocratic elution of the benzoin derivatives of guanidino compounds on the μBondapak CN column. HPLC conditions: mobile phase, methanol–water–0.5 *M* Tris–hydrochloric acid buffer (pH 8.5) (52:33:15); other conditions, see text. For peak numbers and amounts of guanidino compounds, see Fig. 1.

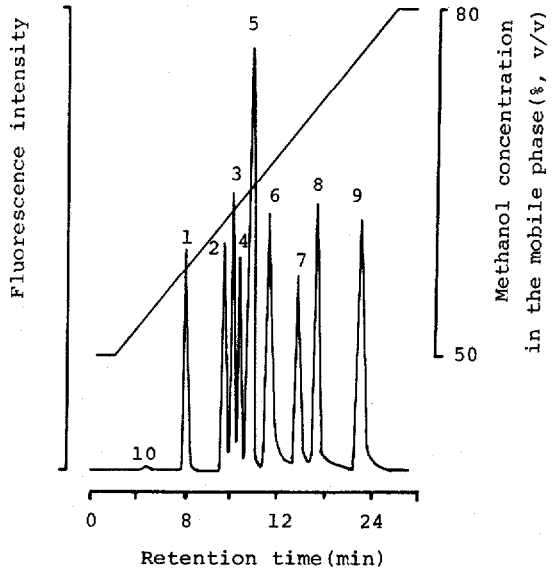

Fig. 4. Linear gradient elution of the benzoin derivatives of guanidino compounds on the μBondapak Phenyl column. HPLC conditions: mobile phase, methanol–water–0.5 *M* Tris–hydrochloric acid buffer (pH 8.5) (initial, 50:35:15; final, 80:5:15); other conditions, see text. For peak numbers and amounts of guanidino compounds, see Fig. 1.

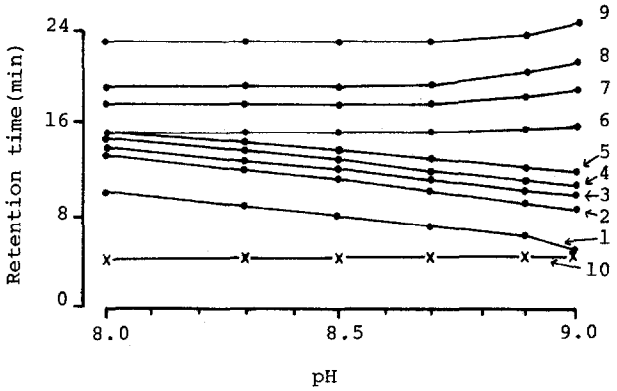

Fig. 5. Influence of the pH of the Tris–hydrochloric acid buffer in the mobile phase on the retention times of the benzoin derivatives of guanidino compounds. For HPLC conditions and curve numbers, see Figs. 4 and 1, respectively.

pounds. All the peaks corresponding to the derivatives were completely separated within 25 min, the peaks becoming sharp, particularly for derivatives eluting late. This therefore can be used as a highly sensitive fluorimetric detection method for these derivatives.

The pH of the Tris buffer in the mobile phase affected the separation of the derivatives as shown in Fig. 5. Although the peaks for arginine and guanidinobutyric acid overlapped at pH 8.0, a complete separation could be attained at pH 8.3–8.9; pH 8.5 was selected for the procedure. The Tris concentration in the buffer did not have any significant effect on the peak separation of over the range 0.4–0.8 *M*; 0.5 *M* was used for convenience.

The μBondapak Phenyl column can be used for more than 300 injections with only a small decrease in the theoretical plate number, provided it is washed with water–methanol (2:8) for *ca.* 20 min (flow-rate, 0.8 ml/min) after everyday analysis.

The conditions of the derivatization procedure are optimal as reported previously[21]. When a mixture of the guanidino compounds, excluding guanidine itself (for the amounts subjected to HPLC, see Fig. 1), was treated according to the derivatization procedure and the resultant reaction mixture subjected to HPLC on the μBondapak Phenyl column with gradient elution, a small peak was unexpectedly observed in the chromatogram at exactly the same retention time as that for guanidine. The peak height was approximately 20% of that obtained by 60 pmol of phenylguanidine. This peak was also observed when the reaction mixture was applied onto LiChrosorb RP-18 or μBondapak CN columns (for HPLC conditions, see Figs. 2 and 3). The same was also true when each guanidino compound separately was treated in the same way, although the peak height was less than 1% of that given by each parent compound. This suggests that the peak is due to guanidine derived from the other guanidino compounds under the derivatization conditions and that the coexistence of the various guanidino compounds promotes this derivation. The peak was not eliminated by changes in heating time (2–20 min), temperature (60–100°C) or concentration of potassium hydroxide (0.5–4.0 *M*), or the presence or absence of β-mercaptoethanol and/or sodium sulphite during the derivatization procedure.

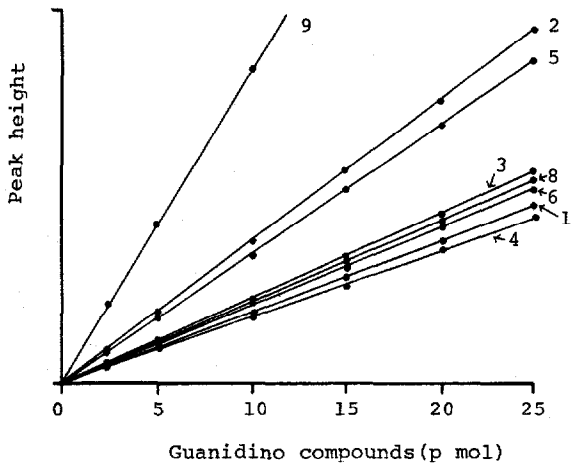

Fig. 6. Relationship between the peak heights and the amounts of guanidino compounds subjected to HPLC. The HPLC conditions are the same as those in Fig. 4. All correlation coefficients (r) of the curves are greater than 0.9998. For the HPLC conditions and the curve numbers, see Figs. 4 and 1, respectively.

Thus, the present derivatization procedure is unsuitable for the quantitative determination of guanidine.

The relationship between the peak heights and the amounts of the guanidino compounds, excluding guanidine itself, obtained by HPLC with gradient elution are linear, in amounts ranging from 2.5 pmol to at least 25 pmol per injection volume (100 μl) (Fig. 6). The lower limits of detection are 20 fmol for phenylguanidine, 50

TABLE I

RETENTION TIMES OF THE BENZOIN DERIVATIVES OF GUANIDINO COMPOUNDS AND PEPTIDES

Portions (200 μl) of 1 μmol/ml solutions of creatine, creatinine and argininosuccinic acid, and 10 nmol/ml solutions of the other compounds were used for the fluorescence derivatization. HPLC conditions are the same as those described in Fig. 4.

Compound	*Retention time (min)*	*Compound*	*Retention time (min)*
Guanidinosuccinic acid	8.0	Guanidine	18.0
Taurocyamine	11.5	Methylguanidine	19.0
Guanidinoacetic acid	12.0	Phenylguanidine	23.0
Creatine	12.0	Agmatine	26.0, 31.0
Creatinine	12.0	Tuftsin	15.0
Guanidinopropionic acid	13.0	Angiotensin III	14.0–26.0*
n-α-Acetylarginine	13.2	Angiotensin II	18.0–20.0*
Guanidinobutyric acid	13.8	Bradykinin	14.2, 18.0–23.5*
Arginine	15.0	Angiotensin I	19.0–24.0*
Argininosuccinic acid	4.2, 15.0	Neurotensin	15.0
Homoarginine	16.5	LH-Releasing hormone	14.5–18.5*
Canavanine	17.8		

* Broad and/or several peaks were observed in the chromatogram.

fmol for taurocyamine and guanidinobutyric acid and 100 fmol for guanidinoacetic acid, methylguanidine, arginine, guanidinosuccinic acid and guanidinopropionic acid. The limit is defined as the amount in the injection volume giving a signal-to-noise ratio of 2. The sensitivity is at least 100 times higher than those of other HPLC methods employing post-column fluorescent derivatization[5,7].

The retention times for various biogenic guanidino compounds and peptides with arginyl residues obtained by HPLC with gradient elution are listed in Table I. The fluorescent derivatives from the peptides give broad and/or several peaks which overlapped with some peaks from the other guanidino compounds. This is because the peptides are probably cleaved into several fragments during the fluorescence derivatization.

The presented method may be used for the determination of biogenic guanidino compounds, particularly taurocyamine, guanidinosuccinic acid and methylguanidine which are of special interest in our studies. However, some clean-up procedures would be required to remove those peptides containing arginyl residues by using an ion-exchange chromatographic or ultrafiltration technique. This work is in progress in our laboratory.

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