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FLUOROMETRIC DETERMINATION OF BIGUANIDES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH REAGENT-CONTAINING MOBILE PHASE

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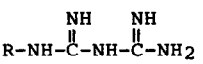
SUMMARY

A simple post-column derivatization method for the fluorometric determination of biguanides (buformin and phenformin) in serum by high-performance liquid chromatography is described. The serum was treated with 4% perchloric acid to precipitate proteins, and the supernatant was directly injected into the column. Synthesized 9,10-phenanthrenequinonesulphonate (PSQ) was used as a fluorogenic reagent and added to the mobile phase. Biguanides were separated within 10 min on a Radial-Pak μBondapak C_{18} cartridge (10 μm, 10 cm×8 mm I.D.) by reversed-phase ion-pair chromatography. They were then allowed to react with PQS in an alkaline stream and detected fluorometrically. This method was applied to the analysis of serum from patients with diabetes mellitus.

INTRODUCTION

Buformin (N-butylbiguanide) and phenformin (N-2-phenethylbiguanide) (Fig. 1) are used clinically as oral hypoglycemic agents for the treatment of dia-



Buformin : R=-$(CH_2)_3CH_3$

Phenformin : R=-$(CH_2)_2C_6H_5$

Fig. 1. Structures of buformin and phenformin.

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betes mellitus. High concentrations of biguanides in serum have been associated with the development of lactic acidosis [1]. Monitoring of biguanides levels in serum is necessary for safe and effective therapy.

Various chromatographic methods for the determination of biguanides in biological fluids have been reported. Gas chromatography requires the tedious extraction and derivatization [2–6], so it is unsuitable for routine clinical analysis. High-performance liquid chromatography (HPLC), however, is generally suitable for the clinical laboratory. In published methods, biguanides in serum have been detected by spectrophotometry [7–9] or fluorometry [10]. Spectrophotometric detection was inferior to fluorometric detection in both sensitivity and specificity. Recently, Tanabe et al. [10] reported a pre-column derivatization method for the fluorometric determination of biguanides using 9,10-phenanthrenequinone (PQ), which reacts specifically with biguanides in an alkaline medium and results in high-intensity fluorophores [13]. Tanabe's method requires troublesome pretreatment, and they did not use PQ for post-column derivatization because the reagent is practically insoluble in water and would cause precipitation in the flow system. On the other hand, 9,10-phenanthrenequinonesulphonate (PQS) is highly soluble in water and forms the same type of fluorophores as PQ.

We have already reported a new post-column derivatization system, in which a fluorogenic reagent was dissolved in the mobile phase, for the determination of guanidino compounds [11,14,15] and streptomycin [16,17]. The system was simplified because the fluorogenic reagent was contained in the mobile phase. This report describes the determination of biguanides in serum by post-column derivatization system using a mobile phase containing PQS. This method was applied to the analysis of serum from diabetes mellitus patients on biguanide medication.

EXPERIMENTAL

Apparatus

The chromatographic system was constructed from a Model 600 multisolvent-delivery system and a radial compression separation unit, which consisted of a Radial-Pak μBondapak C_{18} cartridge (10 μm, 10 cm×8 mm I.D.) and a Model M600 module for compressing the cartridge (Waters Assoc., Milford, MA, U.S.A.). The column effluent was introduced into a Soma S-3800 reaction system (Soma Optics, Tokyo, Japan) equipped with a mixing tee, a reciprocating pump, a pulse-dampening device and a reaction coil consisting of a stainless-steel tube (15 m×0.5 mm I.D.) in a heating bath at 70°C. A Soma S-3350 FL spectrophotometer was used to detect the fluorophore, with the excitation wavelength set at 300 nm and the emission wavelength at 500 nm. The detection signal was recorded with a recorder (National Pen Recorder, Matsushita Communication Industrial, Osaka, Japan) and a integrator (Waters 740 data module).

Reagents and chemicals

Buformin and phenformin were generously provided by Dr. Shinzo Tanabe (Meiji College of Pharmacy, Yato, Tanashi, Tokyo, Japan). PQS was synthesized

according to the reported method [12]. Sodium hexanesulphonate was obtained from Aldrich (Milwaukee, WI, U.S.A.). Water and acetonitrile used were of liquid chromatographic grade. All the other chemicals were of reagent grade.

The mobile phase was prepared to contain 5 m*M* sodium hexanesulphonate and 2 m*M* PQS in water–acetonitrile (80:20, v/v for buformin; 75:25, v/v for phenformin), adjusted to pH 4.0 with acetic acid.

The alkaline solution was 0.3 *M* sodium hydroxide.

Serum samples were obtained from Kitasato University Hospital.

Preparation of serum

A 100-μl serum sample in a 1.5-ml tapered polypropylene centrifuge tube was vortex-mixed with 100 μl of 4% perchloric acid solution for a few seconds. The mixture was centrifuged at 10 000 *g* for 1 min. At 100-μl aliquot of the supernatant was injected into the chromatograph.

Standard sera, supplemented with various known amounts of buformin or phenformin, were prepared and analysed. Peak-height measurements were performed to construct the calibration curve. Every serum was analysed in duplicate.

RESULTS AND DISCUSSION

Three kinds of fluorogenic reagents (ninhydrin, 1,2-naphthoquinone-4-sulphonate and PQS) that dissolve in the mobile phase were tested. These reagents reacted with buformin in an alkaline medium. PQS was superior to the other two reagents in stability of baseline and sensitivity. Therefore, PQS was chosen as a fluorogenic reagent.

Optimization of the analytical chromatographic system

The optimum reaction conditions for the post-column derivatization system were examined by injecting 10 μl of a standard solution of buformin (3 μg/ml) into the chromatograph. The flow-rate of the mobile phase was 2.0 ml/min and the flow-rate of the alkaline solution was 0.5 ml/min.

Reaction coils (0.5 mm I.D.) of various lengths with a spiral diameter of 5.0 cm were studied to find the optimal fluorescence intensity for buformin (Fig. 2). The maximum intensity was obtained with a coil, 15 m long, and at this length the peak did not broaden.

The effect of temperature on the reaction of buformin with PQS in the alkaline stream was studied in the range 60–80°C (Fig. 3). The fluorescence intensity reached a plateau at ca. 70°C, so this temperature was used for the reaction.

Fig. 4A and B show the effect of the sodium hydroxide concentration and the PQS concentration on the fluorescence intensity of buformin, respectively. The maximum intensity was obtained at 0.3–0.4 *M* sodium hydroxide solution, and it increased as the PQS concentration rose to ca. 2 m*M*, followed by a gradual decrease above this concentration. Therefore, 0.3 *M* sodium hydroxide solution and 2 m*M* PQS solution were used.

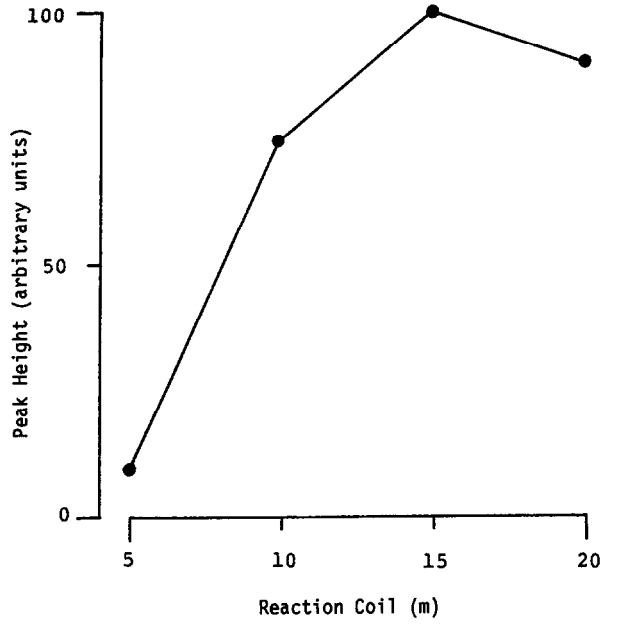

Fig. 2. Effect of reaction coil length on the fluorescence intensity of buformin.

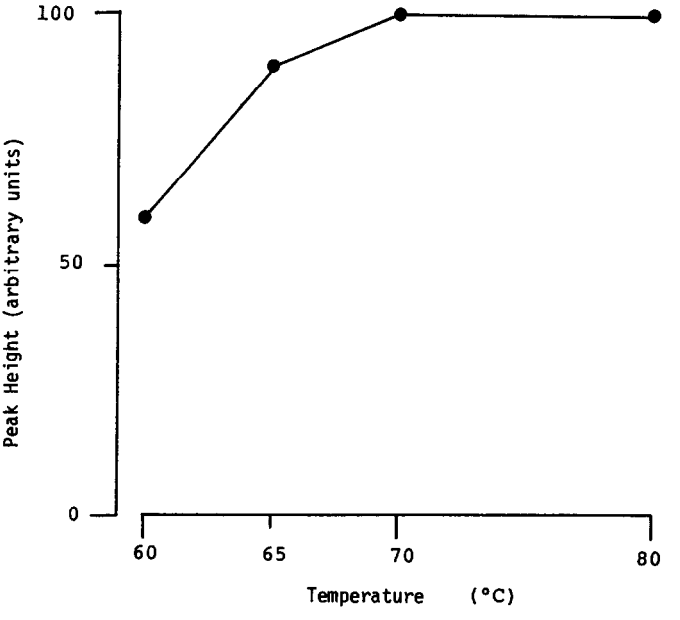

Fig. 3. Effect of reaction temperature on the fluorescence intensity of buformin.

Chromatographic separation and quantitation response

Fig. 5 shows chromatograms obtained from the standard solution of buformin, buformin-free serum and patient serum. No interfering peaks were observed close to the peak of buformin. Fig. 6 shows chromatograms obtained from the standard

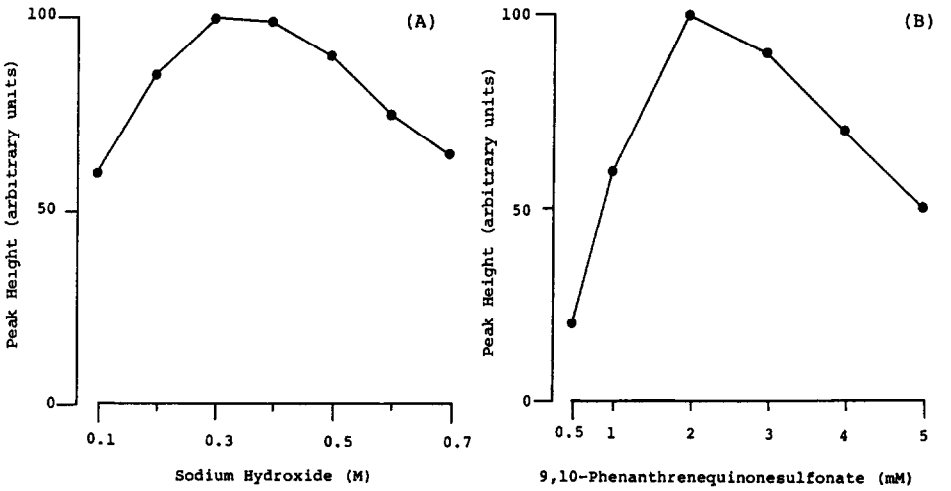

Fig. 4. Effect of alkaline concentration (A) and PQS concentration (B) on the fluorescence intensity of buformin.

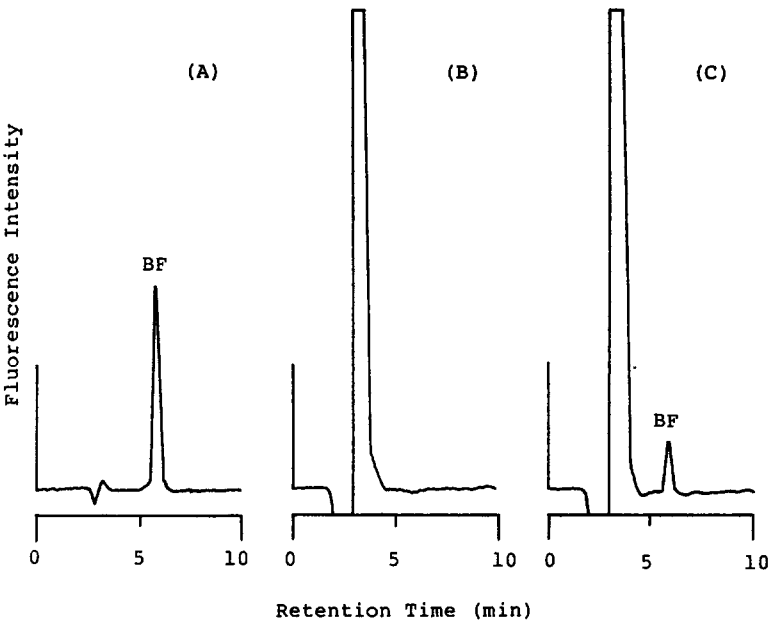

Fig. 5. Chromatograms obtained from a standard solution of buformin (40 ng) (A), buformin-free serum (B) and patient serum (0.2 μg/ml) after the administration of buformin (C). Peak BF = buformin.

solution of phenformin, phenformin-free serum and phenformin-spiked serum. No interfering peaks were observed close to the peak of phenformin. The separation of biguanides from interferences in serum has been performed by reversed-phase ion-pair chromatography.

A linear regression analysis of the calibration curve yielded the equation

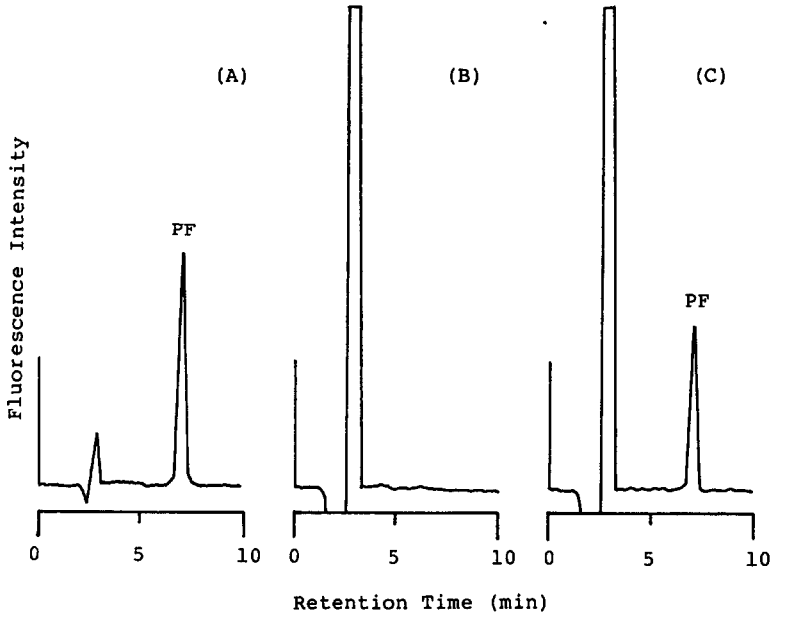

Fig. 6. Chromatograms obtained from a standard solution of phenformin (68 ng) (A), phenformin-free serum (B) and phenformin-spiked serum (1 μg/ml) (C). Peak PF = phenformin.

TABLE I

RESULTS OF DETERMINATION OF BUFORMIN IN PATIENTS SERUM

B.H. = body height; B.W. = body weight.

Patient	Sex	Age	B.H. (m)	B.W. (kg)	Serum concentration (μg/ml)
Administration of 150 mg/day					
A	M	50	1.64	60	0.19
B	M	59	1.60	63	0.01
C	M	56	1.82	53	0.22
D	M	30	1.78	95	0.01
E	F	52	1.57	53	0.17
F	F	22	1.65	67	0.09
G	F	48	1.57	63	0.43
H	F	47	1.50	45	0.12
Administration of 300 mg/day					
I	M	48	1.76	105	0.32
J	F	52	1.57	53	0.60
K	F	55	1.55	58	0.07

$y = 2.43x + 0.270$ ($r = 0.998$) for the buformin-spiked sera (0.1–8.0 μg/ml) and $y = 3.00x + 0.180$ ($r = 0.997$) for the phenformin-spiked sera (0.1–4.0 μg/ml). Good linearity was obtained between the peak height (y) and the biguanide concentra-

tion (x) in serum. The limit of detection was 20 ng/ml for both buformin and phenformin.

The coefficients of variation (within-run, $n=10$) for the assay of buformin- and phenformin-spiked sera (0.6 μg/ml) were 2.84 and 2.37%, respectively. Even though no internal standard was used in the method, high precision was obtained because a simple one-step pretreatment (deproteinization) was used.

Application

Sera were collected from patients on buformin medication (300 mg/day or 150 mg/day) and analysed by this method. As shown in Table I, the buformin concentration showed a wide variation between patients on the same dosage. Therefore, it will be clinically useful to monitor biguanides for the prevention of lactic acidosis.

CONCLUSION

We established an HPLC method for the determination of buformin in serum with a very simple pretreatment. The analysis time, including sample pretreatment and chromatographic detection, was less than 15 min for each specimen. This method can be used for pharmacokinetic studies and for routine therapeutic monitoring of biguanides in serum.

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