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Note

Determination of serum sulphonylureas by high-performance liquid chromatography with fluorimetric detection

AYUMI IGAKI*, KUNIO KOBAYASHI, MASAKO KIMURA, TAKAFUMI SAKOGUCHI and AKIRA MATSUOKA

Department of Clinical Pathology and Clinical Laboratories, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya 663 (Japan)

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Oral hypoglycemic agents, acetohexamide, chlorpropamide, tolbutamide and gliclazide, are widely used in the management of blood glucose control in non-insulin-dependent diabetes mellitus (NIDDM). Conventional determination of the serum sulphonylureas has been performed by colorimetry [1], gas chromatography [2–7], gas chromatography–mass spectrometry [8], radioimmunoassay [9,10] and high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [11–13]. Previously, we reported a fluorimetric method for the determination of the serum gliclazide level, based on dansylation of *p*-toluenesulphonamide produced by gliclazide degradation [14]. However, these methods require time-consuming, complicated procedures (extraction and derivatization) and a large amount of sample [15–17]. The total and free gliclazide levels in serum are below 10 and 0.4–0.6 μg/ml, respectively, when an effective therapeutic dose (80 mg per day) is taken by diabetic patients [18]. Therefore, a more sensitive method is required for the determination of the free gliclazide level in serum.

Fluorimetric determination of the serum sulphonylurea level has not been reported because it is difficult to fluorinate directly a sulphonylurea-type compound. 7-Fluoro-4-nitrobenzo-1,3-diazole (FNBD) has been used as an excellent fluorogenic reagent for the fluorination of amines, particularly secondary amino acids such as proline and hydroxyproline [19], in terms of reactivity under moderate conditions. In the present study, we successfully developed a

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sensitive and precise HPLC method for determining the serum sulphonylurea after fluorination with FNBD. Simple extraction and fluorination of the drug, and rapid HPLC analysis, should make the method useful for routine clinical application.

EXPERIMENTAL

Chemicals

Pure reference samples of gliclazide, acetohexamide, chlorpropamide and tolbutamide were obtained from Dainippon Pharmaceutical (Osaka, Japan), Shionogi (Osaka, Japan), Pfizer & Taitoh (Tokyo, Japan) and Hoechst Japan (Tokyo, Japan), respectively. FNBD was supplied by Dojindo Labs. (Kumamoto, Japan). Acetonitrile and acetone were of HPLC grade (Wako Pure Chemicals, Osaka, Japan). All other chemicals were of analytical reagent grade (Wako Pure Chemicals). All aqueous solutions containing reagents for HPLC analysis were passed through a filter (Millipore types cs, 0.22 μm, Millipore, Bedford, MA, U.S.A.) before use.

Apparatus and HPLC conditions

A high-pressure liquid chromatograph (Model TWINCLE, Japan Spectroscopic, Tokyo, Japan) equipped with a spectrofluorometer, Model FP-550F, and a reversed-phase column, resin particle size 10 μm (Finepak SIL C_{18}, 250 mm$\times$4.6 mm I.D., Japan Spectroscopic) was used. HPLC conditions were as follows: column temperature, ambient (25–27°C); detection wavelength, emission 534 nm and excitation 470 nm; mobile phase, acetonitrile–water (60:40, v/v); flow-rate, 1.0 ml/min; sample volume injected, 20 μl. All eluting solutions were filtered and degassed before use. Ultrafiltration was performed using a membrane filter (Centrisalt, I, cut-off 20 000; Sartorius, Göttingen, F.R.G.).

Extraction of serum sulphonylurea and fluorination

A mixture of serum sample (0.2 ml), 2 ml of 0.1 *M* phosphate buffer (pH 3.0) and 4 ml of chloroform was shaken vigorously for 15 min. The mixture was centrifuged for 5 min at 1400 *g*, then 3.0 ml of the chloroform layer were evaporated to dryness under a stream of nitrogen gas at 40°C. The residue was redissolved in 20 μl of 0.05 *M* carbonate buffer (pH 8.5). FNBD solution (40 μl, 5 mg/ml of acetone) was added and then the mixture was heated at 60°C for 15 min. After cooling on ice-water, 40 μl of 0.01 *M* hydrochloric acid was added to the reaction mixture. A 20-μl portion of the solution was injected onto the HPLC column. An internal standard was not used. The serum concentration of sulphonylurea was calculated based on the relative fluorescence intensities (peak heights) of sample and standard solutions containing authentic concentrations of sulphonylurea. Free gliclazide in serum was separated by

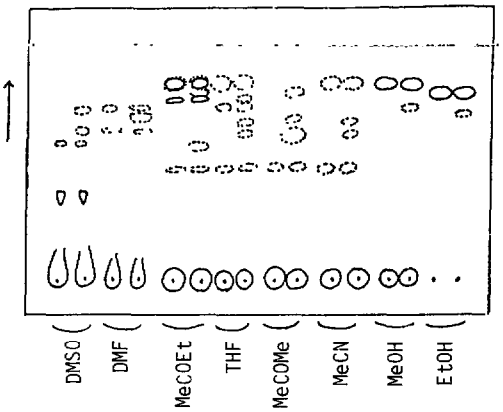

Fig. 1. TLC profiles of fluorinated products from gliclazide with FNBD. Right, with gliclazide; left, without gliclazide. Solid lines indicate UV absorption and broken lines fluorescence detection. The following solvents were tested for fluorination of gliclazide with FNBD: DMSO, dimethyl sulphoxide; DMF, dimethylformamide; MeCOEt, methyl ethyl ketone; THF, tetrahydrofuran; MeCOMe, acetone; MeCN, acetonitrile; MeOH, methyl alcohol; EtOH, ethyl alcohol.

ultrafiltration using a filter membrane. Free gliclazide in the filtrate was measured by the same procedure as total gliclazide.

Thin-layer chromatographic analysis

Thin-layer chromatographic (TLC) analysis of the reaction mixture containing fluorinated sulphonylurea with FNBD was performed on Kieselgel 60 plates with a solvent of dichloromethane–acetonitrile (10:1, v/v). Spots due to fluorinated products showing UV absorption and fluorescence were detected using a UV lamp (250–350 nm) (Fig. 1).

RESULTS AND DISCUSSION

The fluorinated products from the reaction of gliclazide with FNBD and their yields showed a large variation depending on the reaction conditions. Of the many solvents tested, the acetone solution of FNBD gave a fluorinated product (NBD-gliclazide) with the strongest fluorescence intensity and the highest yield. Moreover, when the volume ratio of acetone to buffer solution (carbonate or borate buffer) was 1:2, the highest yield of NBD-gliclazide was obtained (Fig. 2A).

Several conditions (reaction time, concentrations of acetone, buffer and FNBD and pH of medium) for the fluorination of gliclazide with FNBD were examined (Fig. 2B–E). As shown in Fig. 2A, the highest relative fluorescence intensity (RFI) was obtained in the reaction medium containing 67% (v/v) acetone. The fluorination reaction was accelerated by a higher reaction temperature, i.e. the RFI more quickly reached a plateau level at 60°C (Fig. 2B).

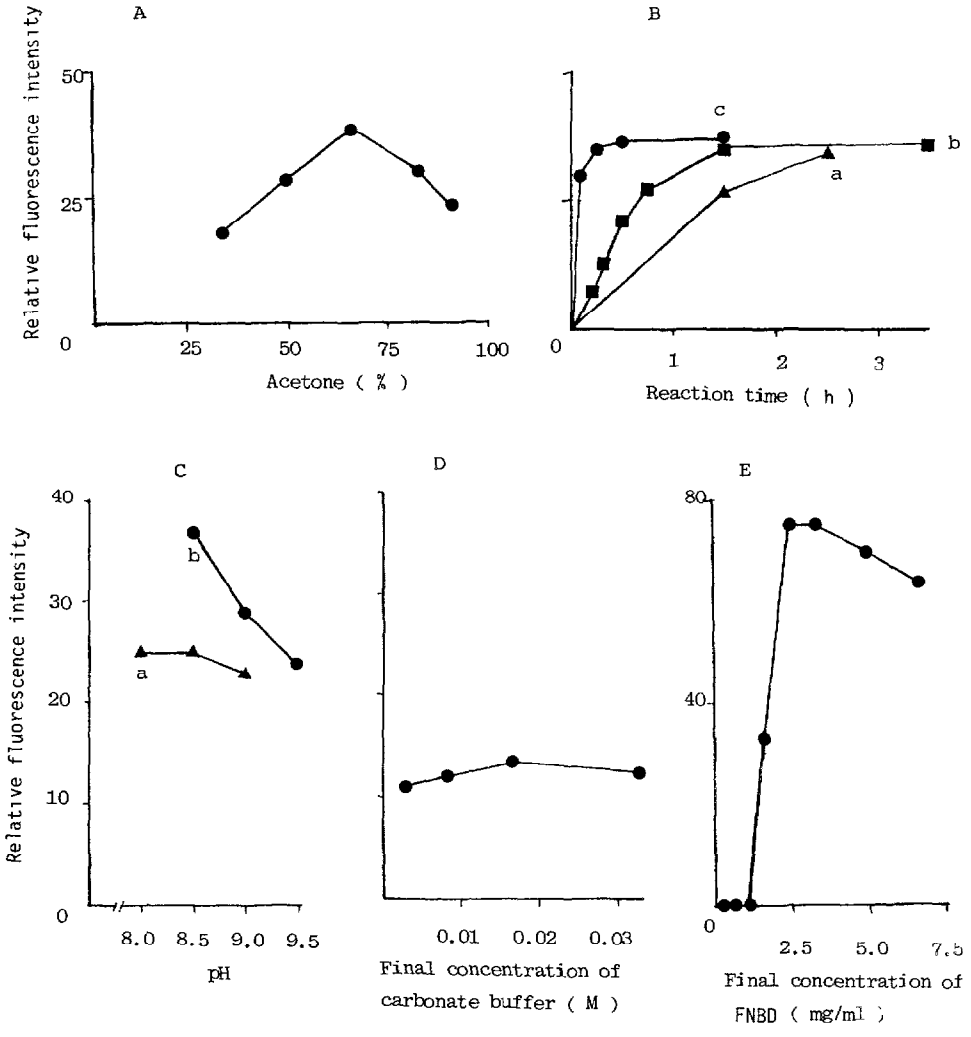

Fig. 2. Evaluation of reaction conditions. The experimental conditions, except for the parameter tested, are as described in Experimental. The sample is serum with gliclazide added. (A) Volume of acetone; (B) reaction time at (a) 4°C, (b) room temperature (26–27°C), (c) 60°C; (C) pH value in (a) borate buffer, (b) carbonate buffer; (D) concentration of buffer solution (medium); (E) concentration of FNBD.

The RFI showed a plateau level that was independent of the concentration of the carbonate buffer (pH 8.5) in the range 0.017–0.033 *M* (Fig. 2D). Interestingly, the fluorination of gliclazide with FNBD did not occur at a lower concentration than 1.25 mg/ml FNBD, but it increased rapidly at a higher con-

centration of FNBD (1.25 mg/ml), reaching a peak at 2.5–3.3 mg/ml, then gradually declined. Consequently, we adopted the following as the optimal conditions: 67% (v/v) acetone, 3.3 mg/ml FNBD and 0.017 *M* carbonate buffer (pH 8.5). The incubation was carried out at 60°C for 15 min.

The HPLC profile of the reaction mixture of gliclazide with FNBD gave three peaks, with retention times of 6.15, 8.50 and 9.25 min. The peak with a retention time of 6.15 min and the highest RFI was used for the determination of serum gliclazide. The fluorophores derived from other sulphonylureas each gave a single peak with retention times of 8.25 (acetohexamide), 5.75 (chlor-

TABLE I

RETENTION TIMES FOR FLUORINATED SULPHONYLUREAS (NBD-SULPHONYLUREAS) IN HPLC ANALYSIS

Retention time of ghost peaks in serum was less than 4.5 min.

Sulphonylurea	Retention time (min)
Gliclazide	6.15
Acetohexamide	8.25
Chlorpropamide	5.75
Tolbutamide	6.75

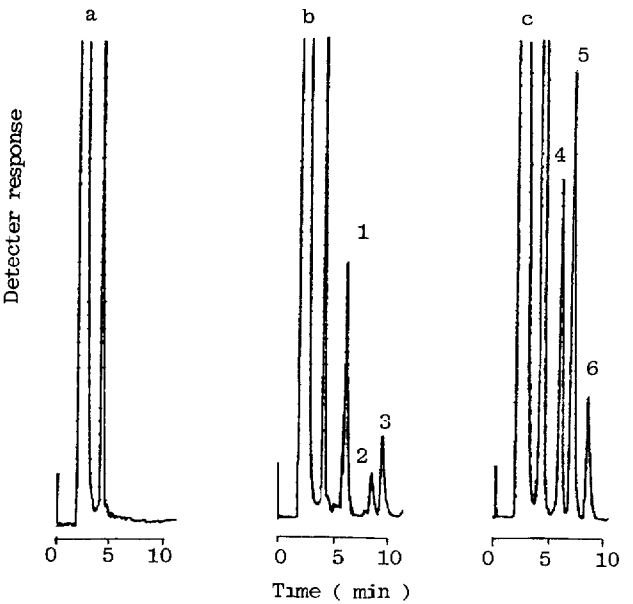

Fig. 3. Chromatogram of NBD-gliclazide and other sulphonylureas. (a) Serum blank; (b) serum with NBD-gliclazide; (c) serum with NBD-chlorpropamide, NBD-acetohexamide, and NBD-tolbutamide. Peaks: 1-3 = NBD-gliclazide; 4 = NBD-chlorpropamide; 5 = NBD-tolbutamide; 6 = NBD-acetohexamide.

TABLE II

INTRA-ASSAY PRECISION AND ANALYTICAL RECOVERY FOR ASSAY OF SERUM SULPHONYLUREA

Each sample tested was prepared by adding sulphonylurea to the pooled human serum; $n = 10$.

Sulphonylurea	Concentration (μg/ml)		C.V. (%)	Accuracy (%)
	Added	Found (mean ± S.D.)		
Gliclazide	0.2	0.18 ± 0.01	5.6	90.0
	1.0	0.92 ± 0.04	4.3	92.0
	5.0	4.93 ± 0.18	3.7	98.6
Acetohexamide	1.0	1.06 ± 0.07	6.6	106.0
	10.0	10.94 ± 0.57	5.2	109.4
	50.0	50.58 ± 1.46	2.9	101.2
Chlorpropamide	1.0	1.12 ± 0.05	4.5	112.0
	10.0	10.10 ± 0.52	5.1	101.0
	50.0	48.18 ± 2.15	4.5	96.4
Tolbutamide	1.0	0.95 ± 0.04	4.2	95.0
	10.0	9.25 ± 0.40	4.3	92.5
	50.0	52.31 ± 2.00	3.8	104.6

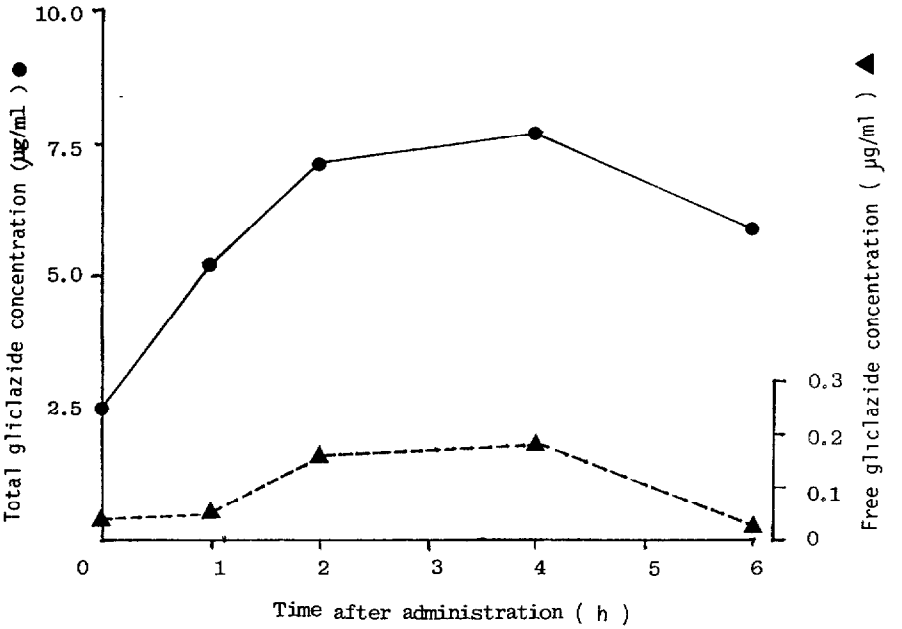

Fig. 4. Decay curves of total (●) and free (▲) gliclazide levels in a diabetic patient. Each point represents the mean of duplicate assays for a diabetic patient who was successively administered gliclazide (40 mg per day).

propamide) and 6.75 min (tolbutamide) (Table I). Thus, three fluorinated sulphonylureas other than gliclazide were simultaneously analysed and all peaks were eluted within 10 min (Fig. 3). Calibration curves for the HPLC assays of serum gliclazide and other sulphonylureas were straight lines with coefficients of regression of 0.99–1.00 over the ranges 0.1–20 and 0.5–100 μg/ml, respectively. The detection limits of gliclazide and other sulphonylureas were estimated to be 0.1 and 0.5 μg/ml in serum, based on a peak height of twice the baseline noise. Intra-assay coefficients of variation (C.V.) and accuracy (recovery) are listed in Table II.

Decay curves of total and free serum gliclazide levels in diabetic patients who had been successively administered the drug (40 mg per day) are shown in Fig. 4. The total gliclazide level reached a maximum (ca. 7.5 μg/ml) at 3–4 h after successive drug administration. The decay curve for the free gliclazide level paralleled that for the total gliclazide level. Both decay curves for the total and free gliclazide levels had the same profile as those reported in our previous paper [18].

CONCLUSIONS

Fluorimetric determination of serum sulphonylureas utilizing HPLC separation was developed following the fluorination of sulphonylureas with FNBD. Compared with the UV detection method [12], the present method is more rapid and sensitive and enables the assay of a large number of samples because of the single extraction, short fluorination time and short retention time. The determination limit of serum gliclazide in this method is lower than in the UV detection method [12]. Other sulphonylureas could also be more sensitively determined than by other methods [15–17]. Because sulphonylureas, except for gliclazide, used here display high serum levels for a large dosage, their serum levels can be determined by the present method.

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