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High-Performance Liquid Chromatographic Determination of Oxidized Derivatives of Tolbutamide in Rat Blood and Urine

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A sensitive and specific high-performance liquid chromatographic method for the determination of hydroxytolbutamide (HTB), formyltolbutamide (FTB), and carboxytolbutamide (CTB) in rat blood and urine is described. The ethanol-treated blood and urine were chromatographed on a reverse-phase column on a high-performance liquid chromatograph equipped with a ultraviolet absorbance detector. Quantitation of HTB, FTB, and CTB in blood samples containing less than 100 μg/ml of these compounds was achieved, using chlorpropamide as an internal standard. There were no interfering peaks due to other components of blood or urine.

Keywords——HPLC; hydroxytolbutamide; formyltolbutamide; carboxytolbutamide; tolbutamide; rat

Tolbutamide(1-butyl-3-(*p*-tolylsulfonyl)-urea) is one of the sulfonylurea derivatives used as oral hypoglycemic agents for the treatment of diabetes mellitus. The metabolism of tolbutamide has been extensively studied in man[1-4] and several laboratory animals.[2,4-6] Although pronounced species differences have been recognized, similar metabolic patterns were found in man, rabbit, and rat. McDaniel *et al.* indicated that tolbutamide is metabolized to hydroxytolbutamide(1-butyl-3-(*p*-hydroxymethylphenyl)sulfonylurea, HTB) and HTB is further metabolized to carboxytolbutamide (1-butyl-3-(*p*-carboxyphenyl)sulfonylurea, CTB), presumably by the way of formyltolbutamide(1-butyl-3-(*p*-formylphenyl)sulfonylurea, FTB), as shown in Chart 1.[7]

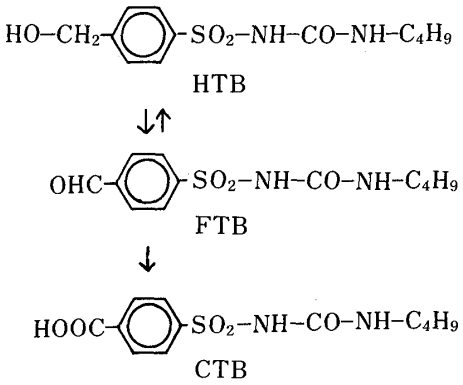

Chart 1. Possible Metabolic Pathway of FTB

The present report describes a high-performance liquid chromatographic (HPLC) method for the determination of HTB, FTB, and CTB in rat blood and urine.

Experimental

Materials——HTB,[8] FTB,[8] and CTB[9] were synthesized in this laboratory. Chlorpropamide was of phar-

maceutical grade. All other chemicals were of reagent grade.

Apparatus——The chromatographic apparatus(model 6000A pump, model U6K injector; Water Associates Inc., U.S.A.) was fitted with a ultraviolet (UV) absorbance detector(model UVILDG-5III variable-wavelength detector; Oyo-Bunko Kiki Co., Ltd., Japan). The column was a bonded octadecylsilane-silica gel type (Fine SIL C_{18}, Japan Spectroscopic Co., Ltd., Japan), 10 μm particle size, 4.6 × 250 mm internal dimensions. This column was used at room temperature.

Chromatographic Conditions——HPLC was performed with 43% acetonitrile, 57% water, and 1% acetic acid (volume ratio) as the mobile phase. At a flow rate of 1.0 ml/min, the back pressure was approximately 450 psi. The chromatographic mobile phase was filtered by passing it through a 0.45 μm pore size membrane filter (Toyo Roshi Co., Ltd., Japan).

Preparation of Standard Curves——Blood: A standard solution was prepared to contain 1 mg of HTB/ml, CTB/ml, and FTB/ml in ethanol. The internal standard solution was prepared to contain 100 μg of chlorpropamide/ml in acetonitrile. Pooled rat blood (0.5 ml) was added to 2.5, 5, 10, 20, 30, 40, and 50 μl of these solutions, and each was diluted to 0.55 ml with ethanol. The mixture was centrifuged for 5 min at 4000 rpm. The supernatant (0.2 ml) was mixed with 0.2 ml of the internal standard solution. The mixture was centrifuged for 5 min at 10000 rpm and an aliquot of the supernatant was chromatographed.

Urine: Urine was diluted to 50 times its original volume with water. The diluted urine (0.5 ml) was assayed as already described for the blood determination. Standard curves were prepared by using five samples at each concentration and plotting peak height ratio (HTB and CTB) or peak area ratio (FTB) *versus* drug concentration.

Animal Experiments——Male Wistar albino rats weighing 250—300 g were used. Animals were anesthetized with pentobarbital, given intraperitoneally. Twenty mg of FTB dissolved in propylene glycol was administered into a femoral vein by a single injection. Blood samples were obtained at 5 min after intravenous administration of the drug. These samples were assayed for HTB, FTB, and CTB levels using the standard procedures.

Results and Discussion

The chemical structures of HTB, FTB, and CTB are presented in Chart 1. HTB and CTB have been identified as metabolites of tolbutamide in man and several laboratory animals. In addition, McDaniel *et al.* suggested that FTB is a possible intermediate in the metabolic pathway of tolbutamide.[7] To achieve good separation of HTB, FTB, and CTB from each other, various chromatographic conditions were tried. The conditions finally chosen utilized a microparticulated bonded reverse-phase packing with a mobile phase consisting of acetonitrile, water, and acetic acid (43 : 57 : 1, volume ratio). HTB, CTB, FTB, and chlorpropamide showed absorbance maxima at 227, 235, 250, and 235 nm, respectively, when present in the mobile phase. A wavelength of 250 nm was selected as the standard. The retention times and capacity factors for HTB, CTB, FTB, and chlorpropamide are shown in Table I. All compounds tested have retention times different from chlorpropamide and thus do not interfere. Typical chromatograms are shown in Fig. 1 for a pooled blood sample containing no drug, pooled blood sample spiked with 80 μg of HTB/ml, 30 μg of CTB/ml, and 60 μg of FTB/ml, and a blood sample obtained from a rat 5 min after a 20 mg intravenous dose of FTB. Chlorpropamide was added to the latter two samples. HTB, CTB, FTB, and internal standard peaks were well resolved with retention times of 5.8, 6.5, 9.7, and 12.2 min, respectively, and no interfering peaks appeared in the blank blood. Figure 2 shows the

TABLE I. Retention Characteristics of HTB, CTB, FTB, and Chlorpropamide

Substance	k' [a]	t [b] (min)
Solvent front (t_0)	0	1.9
HTB	2.052	5.8
CTB	2.421	6.5
FTB	4.105	9.7
Chlorpropamide	5.421	12.2

a) $k'=(t-t_0)/t_0$, capacity factor. *b*) Retention time in min.

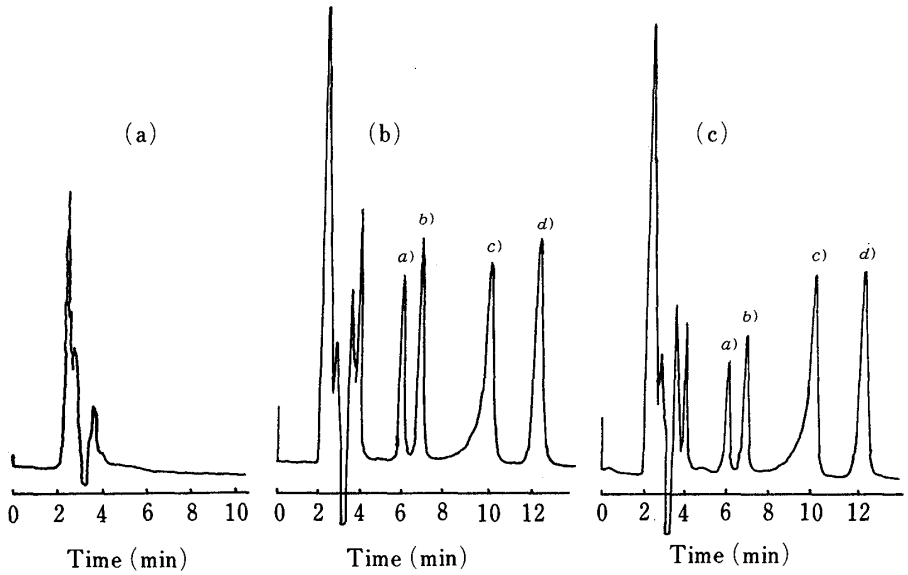

Fig. 1. Chromatograms of HTB, CTB, and FTB

Conditions for HPLC were as given in Experimental.
(a) Blank blood.
(b) Blank blood spiked with HTB, CTB, FTB, and chlorpropamide (internal standard).
(c) Blood sample obtained at 5 min after intravenous administration of FTB, with chlorpropamide added as an internal standard.
a) HTB. *b*) CTB. *c*) FTB. *d*) Chlorpropamide.

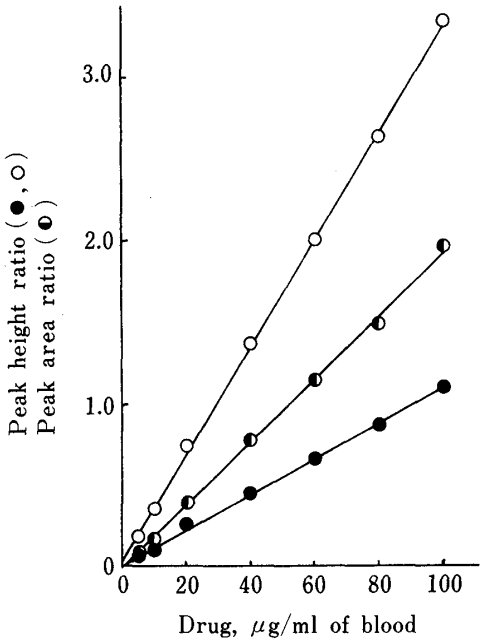

Fig. 2. Calibration Curves for HTB, CTB, and FTB in Blood

●, HTB; ○, CTB; ◐, FTB.

TABLE II. Statistical Parameters of the Calibration Curves for the Assay of HTB, CTB, and FTB

Sample	Drug	Correlation coefficient (r^2)	Regression	
			Intercept	Slope
Blood	HTB	0.998	0.0177	0.1084
Blood	CTB	0.999	0.0449	0.3281
Blood	FTB	0.998	−0.0059	0.1934
Urine	HTB	0.996	0.0195	0.0691
Urine	CTB	0.998	0.0425	0.2224

calibration curves for HTB, CTB, and FTB. The ratio of the peak height (HTB and CTB) or peak area (FTB) of each drug to that of the internal standard was linearly related to drug concentration. Similar linear relationships were obtained in the case of blank urine spiked with various amounts of the drugs. The statistical parameters of the calibration curves are summarized in Table II; the correlation coefficients were 0.99 or better, and the intercepts were negligible. The precision of the assay for HTB, CTB, and FTB was determined by the analysis of five samples at each concentration. The lower limits of sensitivity were 4 μg/ml for HTB, 2 μg/ml for CTB, and 3 μg/ml for FTB.

In conclusion, the HPLC procedure is fast, sensitive, and specific for the determination of HTB, CTB, and FTB. This method should be useful for pharmacokinetic studies on the metabolism of tolbutamide.

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