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SIMULTANEOUS DETERMINATION OF BUNITROLOL AND ITS METABOLITE IN BIOLOGICAL FLUIDS, PLASMA AND URINE

AKIHITO NAGAKURA and HIROSHI KOHEI*

Department of Biochemistry, Nippon C.H. Boehringer Sohn Co., Ltd., Kawanishi Pharma Research Institute, Yato, Kawanishi, Hyogo 666-01 (Japan)

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SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of bunitrolol (Koe 1366) and its metabolite, *p*-hydroxybunitrolol (Koe 1801) has been developed. Using the method, the sensitive and selective determination of Koe 1366 and Koe 1801 can be performed with a simple extraction with diethyl ether and spectrofluorometric detection. The detection limits of Koe 1366 and Koe 1801 in plasma are both less than 2 ng using 1-ml samples.

This method was applied to human and rabbit plasma samples collected after oral administration of bunitrolol tablets (20 mg). The results show the species difference in the metabolism of bunitrolol.

INTRODUCTION

O-[3-(*tert.*-Butylamino)-2-hydroxypropoxy]-benzonitrile hydrochloride (Koe 1366 Cl), a β-adrenergic blocking agent, is highly metabolized in rats [1] and man [2]. Propranolol, one of the β-blocking agents, is also metabolized in the liver in rats, dogs and man [3, 4]. The main metabolites of Koe 1366 are Koe 1801 and its glucuronide.

It is necessary to determine the plasma levels of Koe 1366 and its metabolites for pharmacokinetic study of Koe 1366 in man. Previously the plasma level of Koe 1366 was determined by gas chromatography—mass spectrometry (GC—MS) [5]. However, this technique can measure only Koe 1366 and the analytical procedure is complicated because of the necessity for derivatization.

Recently we developed a simple, sensitive method for the determination of both Koe 1366 and Koe 1801 by high-performance liquid chromatography (HPLC). We could determine the plasma levels of Koe 1366, Koe 1801 and conjugated Koe 1801 in man and rabbits.

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MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade purity. Diethyl ether used for extraction was distilled. DL-Propranolol HCl was purchased from Sigma (St. Louis, MO, U.S.A.). β-Glucuronidase (100 U/ml) was purchased from Boehringer (Mannheim, G.F.R.). Methanol, hydrochloric acid, Na_2HPO_4, and *d*-camphor sulfonic acid were purchased from Wako Pure Chemicals, Osaka, Japan.

HPLC conditions

The HPLC system consisted of a Shimadzu LC-3A constant flow pump with an injector (Shimadzu, SIL-1A) and a column oven (Shimadzu, CTO-2A), and two fluorescence detectors (Shimadzu RF-510 LC fluorescence spectromonitor). The two detectors were connected in series directly after the column. The column was Zorbax ODS (5 μm) prepacked column (150 mm × 4.6 mm I.D., Du Pont). The temperature of the column was set at 40°C. The mobile phase for HPLC was the mixture of 350 ml of water, 650 ml of methanol and 1 g of *d*-camphor sulfonic acid. The flow-rate was 1 ml/min. The first fluorescence detector (D_1) was set for detection of Koe 1366 and propranolol (excitation wavelength, 295 nm; emission wavelength, 330 nm). The second detector (D_2) was set for detection of Koe 1801 (excitation, 323 nm; emission, 371 nm). The signals from the two detectors were led to a two-pen recorder and the chromatogram was drawn. Two digital integrators (Chromatopac E1A, Shimadzu) were also connected to the respective detectors (Fig. 1).

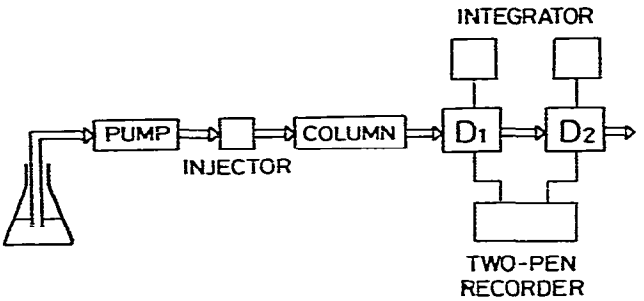

Fig. 1. Flow diagram. D_1 = fluorescence detector for Koe 1366; D_2 = fluorescence detector for Koe 1801.

Extraction procedure

A 0.1-ml volume of propranolol HCl aqueous solution (200 ng/ml for plasma, 10 μg/ml for urine) was added to an aliquot of plasma or urine in a 10-ml glass-stoppered centrifuge tube. To the sample was added 0.5 ml/ml sample of aqueous 1 *M* Na_2HPO_4 solution. Then extraction with 5 ml of diethyl ether was done twice. The ether layers were combined and transferred to another centrifuge tube. A 0.25-ml (1.0 ml in the case of urine) volume of 0.01 *M* HCl was added to the ether. After vigorous shaking and centrifugation, the tube was immersed in a water bath at 40°C to evaporate the ether. An aliquot of the residue (10—100 μl) was injected into the chromatograph.

Preparation of samples

Koe 1366 and free Koe 1801 in plasma (urine). We used 1--2 ml of plasma (1 ml of urine). Koe 1366 and Koe 1801 were extracted by the method described above.

Koe 1801 glucuronide in plasma (urine). A 0.1-ml volume of β-glucuronidase was added to 1 ml of plasma (urine). The mixture was incubated at 37°C for 2 h. The subsequent procedure was the same as that for free Koe 1801.

Calibration curves

Standard curves were constructed with 1 ml of blank plasma containing 0, 2.5, 5, 10, 20, 40 and 80 ng of both Koe 1366 Cl and Koe 1801 Cl; 20 ng of propranolol HCl were added to the sample as internal standard. These samples were extracted by the method described above. Standard curves for urine samples were constructed with 1 ml of water containing 0.25, 0.5, 1.0, 2.0 and 4.0 μg of both Koe 1366 Cl and Koe 1801 Cl. One microgram of propranolol HCl was added to the sample and then extracted. Peak heights of Koe 1366, Koe 1801 and propranolol were measured. These standard curves were obtained by linear regression.

Plasma levels and renal excretion of Koe 1366 and Koe 1801 in man

Two healthy volunteers took 20 mg of Koe 1366 Cl tablets. Then 7 ml of blood were collected at 0.5, 1, 1.5, 2, 4, 6 and 8 h after administration. A 20-μl volume of heparin solution (1000 U/ml) was added to the blood and the blood was centrifuged immediately to separate the plasma. The plasma samples were stored at −20°C until analyzed.

Urine was collected over the periods 0—3, and 3—6 h after administration. Urine samples were stored at −20°C until analyzed.

Plasma levels of Koe 1366 and Koe 1801 in rabbits

Two Himalayan rabbits (male, 2 kg body weight) were used; 20 mg of Koe 1366 Cl (5 ml) were administered by a stomach tube after 16 h fasting. Blood was collected at 0.5, 1, 1.5, 2, 3 and 4 h after administration. After the addition of heparin, the blood was immediately centrifuged to separate the plasma. The plasma samples were stored at −20°C until analyzed.

RESULTS AND DISCUSSION

Chromatography

We can determine the plasma concentration of both Koe 1366 and Koe 1801 simultaneously using fluorescence detectors. Fig. 2 shows the chromatograms obtained from extracts of standard plasma (B, C) and a human plasma sample (D). Chromatogram A was obtained from control plasma. Peak heights of the substances were calculated by digital integrators. A fluorometric method has been used for analysis of propranolol. Maximum fluorescence of propranolol which is dissolved in the mobile phase is observed at 295 nm (excitation wavelength) and 340 nm (emission wavelength); 84% of maximum fluorescence is obtained in this analytical system for Koe 1366 when propranolol is injected into the system. The relative fluorescence intensity of propranolol is 1.9

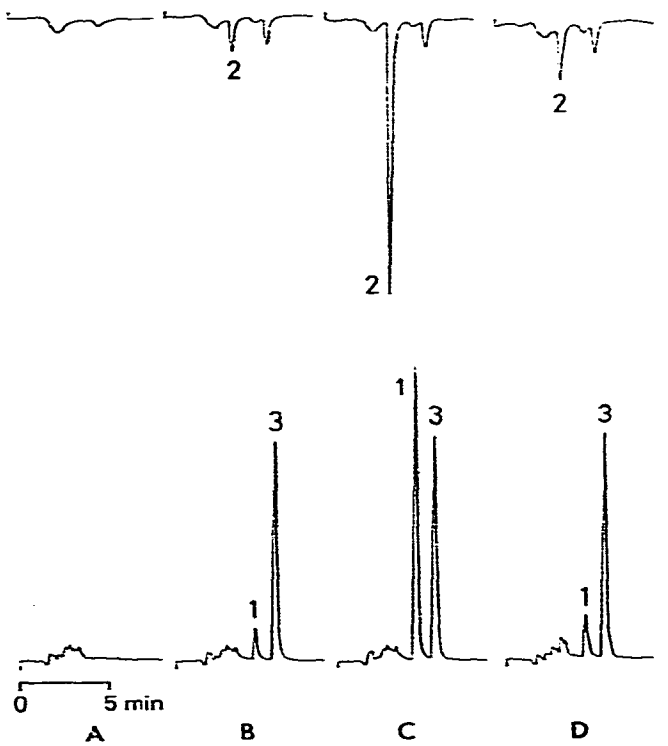

Fig. 2. HPLC chromatograms of control plasma (A), standard plasma (B, C) and a human plasma sample (D). Standard plasma B contains 5 ng of both Koe 1366 and Koe 1801, and C contains 40 ng of both. Propranolol, 20 ng, was added to the plasma as internal standard. 1 = Koe 1366; 2 = Koe 1801; 3 = propranolol.

(Koe 1366 = 1.0) in this condition. We used the peak height of propranolol from the chromatogram of detector D_1 to calculate the Koe 1366 and Koe 1801 peak height ratio.

TABLE I

PRECISION AND ACCURACY

	Concentration (ng/ml)	*n*	Peak height ratio (mean ± S.D.)	Accuracy (ng/ml) (mean ± S.D.)	C.V. (%)
Koe 1366	2.5	4	0.0870 ± 0.0027	2.6 ± 0.10	3.1
	5	4	0.1683 ± 0.0016	5.2 ± 0.05	1.0
	10	4	0.3145 ± 0.0074	9.8 ± 0.24	2.3
	20	4	0.6321 ± 0.0116	19.8 ± 0.36	1.8
	40	4	1.2693 ± 0.0158	40.0 ± 0.51	1.2
	80	4	2.5397 ± 0.0608	80.1 ± 1.89	2.3
Koe 1801	2.5	4	0.0622 ± 0.0045	2.5 ± 0.26	7.2
	5	4	0.1219 ± 0.0053	5.1 ± 0.25	4.3
	10	4	0.2410 ± 0.0012	10.1 ± 0.08	0.5
	20	4	0.4749 ± 0.0073	20.0 ± 0.33	1.5
	40	4	0.9432 ± 0.0069	39.8 ± 0.29	0.7
	80	4	1.8963 ± 0.0242	80.1 ± 1.02	1.3

Calibration curves, precision and accuracy

In the range of 2.5–80 ng/ml, the calibration curves for Koe 1366 and Koe 1801 in plasma showed excellent linearity ($r = 0.999$). Calibration curves for Koe 1366 and Koe 1801 in urine were also linear in the range 0.25—4 μg/ml.

Table I shows the results of precision and accuracy tests. High levels of precision and accuracy were obtained by this method. The recoveries of Koe 1366 and Koe 1801 by this extraction method were 95.5 ± 3.7% (S.D., $n = 24$) and 63.0 ± 2.3% (S.D., $n = 24$), respectively.

Plasma levels and renal excretion in man and rabbits

Plasma levels and renal excretion of Koe 1366, free Koe 1801 and total Koe 1801 after administration of 20 mg of Koe 1366 Cl were studied in two healthy volunteers. Figs. 3 and 4 show plasma levels of Koe 1366 and its major

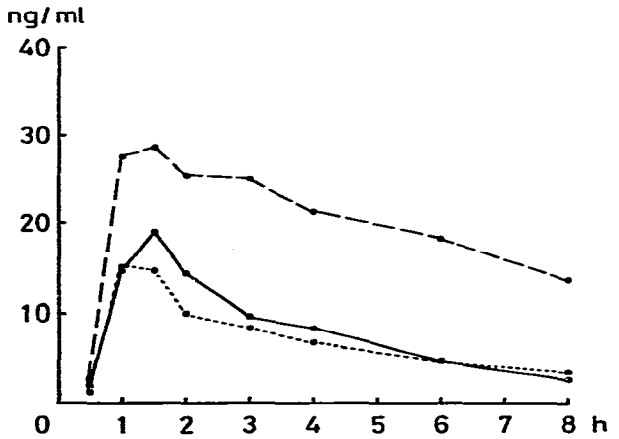

Fig. 3. Plasma levels of Koe 1366 (——), free Koe 1801 (- - - - -) and total Koe 1801 (— — — —) after administration of bunitrolol tablets (2 × 10 mg) in man. Total Koe 1801 means the concentration after enzymatic hydrolysis. Subject: H.K.

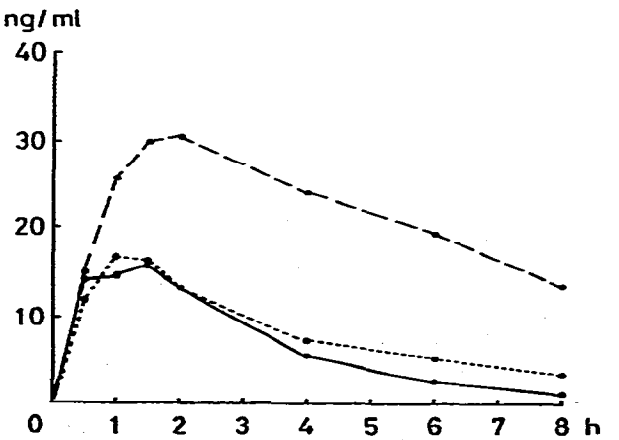

Fig. 4. Plasma levels of Koe 1366 (——), free Koe 1801 (- - - - -) and total Koe 1801 (— — — —) after administration of bunitrolol tablets (2 × 10 ng) in man. Subject: M.I.

TABLE II

URINARY EXCRETION

Subject	Dose (mg)		Amount excreted (μg)	
			0—3 h	3—6 h
H.K.	20	Koe 1366	145	64
		Free Koe 1801	1255	615
		Koe 1801 glucuronide	<10	103
M.I.	20	Koe 1366	378	166
		Free Koe 1801	1222	1104
		Koe 1801 glucuronide	<10	52

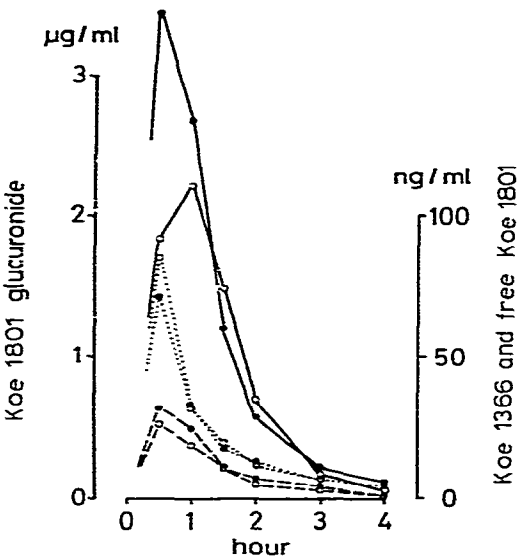

Fig. 5. Plasma levels of Koe 1366 (- - - -), free Koe 1801 (— — — —) and Koe 1801 glucuronide (——) after administration of 20 mg of bunitrolol in rabbits (n = 2).

metabolite, Koe 1801. Plasma concentration of Koe 1366 reached a maximum at 1.5 h after administration. The concentration of free Koe 1801 was about the same as that of Koe 1366.

Table II shows the urinary excretion of Koe 1366 and its major metabolites. It is interesting that only a small amount of Koe 1801 glucuronide was found in urine. However, it is not clear at this stage whether Koe 1801 glucuronide was not excreted in urine. It can be considered that Koe 1801 glucuronide may be hydrolyzed by glucuronidase existing in the urine.

Fig. 5 shows the plasma levels of Koe 1366 and Koe 1801 in rabbits. The plasma levels of Koe 1366 and free Koe 1801 reached a maximum within 30 min after administration. The maximum plasma level of total Koe 1801 reached was about 100 times higher than that of free Koe 1801. This indicates that the metabolic ability of Koe 1366 is much higher in rabbits than in man.

CONCLUSIONS

A sensitive and selective HPLC method was developed for the determination of Koe 1366 and its major metabolite, Koe 1801, in plasma and urine. This simple extraction method permits the analysis of the large number of samples required for pharmacokinetic studies. About 40 samples can be analyzed by this method in a day.

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