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High-performance liquid chromatography with chemiluminescence detection of penbutolol and its hydroxylated metabolite in rat plasma

Keiko Funato[a], Teruko Imai[b,*], Kenichiro Nakashima[c], Masaki Otagiri[b]

[a]*Aventis Pharma Ltd., 3-2, Minamidai, 1-chome, Kawagoe, Saitama, 350-1165, Japan*
[b]*Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto, 862-0973, Japan*
[c]*School of Pharmaceutical Science, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki, 852-8131, Japan*

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Abstract

This paper describes a new method of high-performance liquid chromatography with chemiluminescence detection for the analysis of penbutolol (PB) and its main metabolite, 4-hydroxy penbutolol (4-OH PB) in rat plasma. 4-Dimethylaminosulfonyl-7-(*N*-chloroformylmethyl-*N*-methyl) amino-2,1,3-benzoxadiazole (DBD-COCl) was used as a fluorogenic labeling reagent. A mixture of hydrogen peroxide and bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate (TDPO) in acetonitrile was used as a post-column chemiluminogenic reagent. The derivatives of PB and 4-OH PB with DBD-COCl were separated by isocratic effluent with 0.01 *M* imidazole buffer (pH 7.0)–acetonitrile within 10 min. The detection limits of the proposed method for PB and 4-OH PB were 9.9 and 15 fmol on column, respectively. After intravenous administration of PB in rats, its plasma concentration profiles of PB and 4-OH PB were determined by the proposed method. PB was demonstrated to be rapidly metabolized to 4-OH PB at the same rate as cardiac output. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Penbutolol, [1-*t*-butylamino-3-(2-cyclopentylphenoxy)propan-2-ol] (PB, Fig. 1), is a non-cardioselective β-adrenoreceptor blocking agent and widely used for the treatment of patients with hypertension [1]. PB is mainly metabolized to 4-hydroxy penbutolol (4-OH PB) in human [2]. However, the detail pharmacokinetics of PB has not been clarified in human and experimental animals. A number of analytical methods for PB and/or its related compounds have been developed: gas chromatography (GC) [3], mass spectrometry (MS) [4], gas chromatography–mass spectrometry (GC–MS) [5–7], thin layer chromatography (TLC) [8,9], radio immunoassay, spectrophotometry [10] and high-performance liquid chromatography(HPLC) [11–15]. Among these methods, the GC–MS method is highly sensitive, but it needs special and expensive equipment. On the other hand, the HPLC method with chemiluminescence detection (HPLC–CL) also has

*Corresponding author. Tel.: +81-96-371-4626; fax: +81-96-371-4639.

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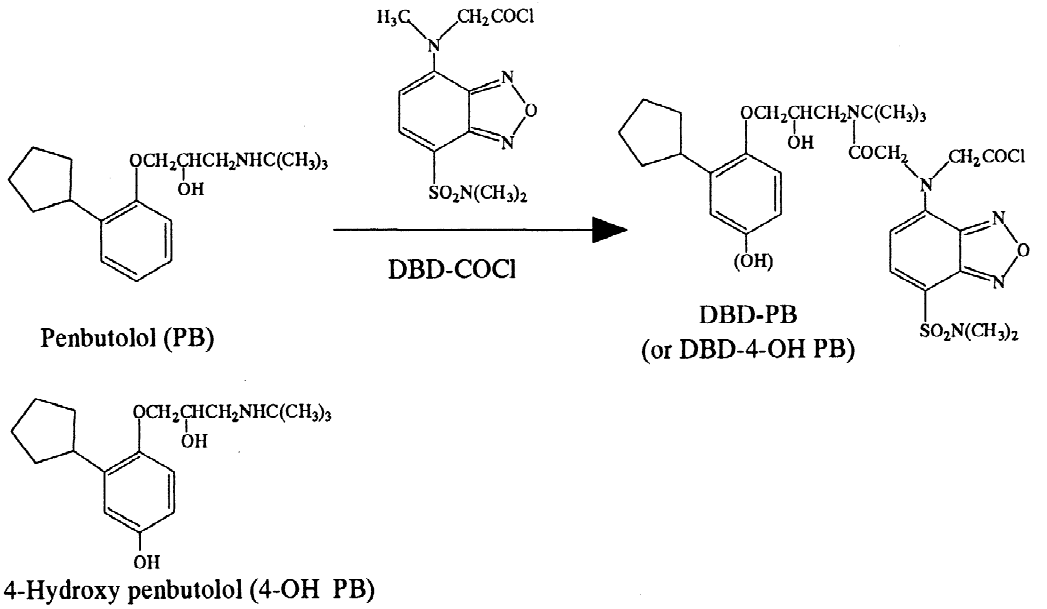


Fig. 1. Structure of PB and its metabolite (4-OH PB), and reaction of PB with DBD-COCl.

high sensitivity and selectivity that are comparable to those of the GC–MS method.

In previous studies [16–19], we had developed sensitive, selective and simple HPLC–CL methods for the determination of sympathomimetic agents by using *N*-(4-aminobutyl)-*N*-ethylisoliminol (ABEI) as a precolumn chemiluminescence labeling reagent and bis [4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)-phenyl] oxalate (TDPO) and hydrogen peroxide as postcolumn peroxylate CL reagents. Furthermore, in the HPLC postcolumn CL (HPLC–PO-CL) method [19] using 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) developed as a fluorogenic reagent for thiols or amines [20,21], high sensitive detections of β-adrenoreceptor blocking agents such as metoprolol and propranolol were possible with limits being from 1.4 to 2.6 fmol on column [22].

In the present study, we described a HPLC–PO-CL method for the analysis of PB and 4-OH PB, by using a newly developed electrophilic fluorogenic reagent having a benzofurazan moiety [23], 4-dimethylaminosulfony-7-(*N*-chloroformylmethyl-*N*-methyl)amino-2,1,3-benzoxadiazole (DBD-COCl) [24] instead of DBD-F. Application of the method in determinations of PB and 4-OH PB after intravenous administration of PB in rat is also described.

2. Experimental

2.1. Materials and reagents

DBD-COCl, TDPO and TCPO were purchased from Tokyo Kasei Kogyo Co.(Tokyo, Japan). Imidazole and hydrogen peroxide (30% v/v) were purchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). PB and 4-OH PB were kindly provided by Nippon Hoechst Marion Roussel Ltd. (Present: Aventis Pharma Ltd., Tokyo, Japan). Water, acetonitrile and chloroform were of HPLC grade (Merck, Darmstadt, Germany). Chloroform was dehydrated with molecular sieve. All other chemicals used were of analytical grade.

2.2. Apparatus

An 850 fluorescence spectrophotometer (Hitachi Seisakusho Co. Ltd. Tokyo, Japan) with a 10-mm quartz cell was used for fluorescence measurement.

A HPLC system consisted of two HPLC pumps (880-PU and BIP-1; JASCO, Tokyo, Japan), an autosampler (717; Nippon Waters Ltd. Tokyo, Japan), a GL-PACK separation column (Nucleosil ODS 100-5C_{18}, 150×4.6 mm I.D., 5 μm, GL Science Inc., Tokyo, Japan), an 825 CL detector (JASCO) and an integrator (HP3390; Hewlett-Packard, USA). A schematic diagram of the HPLC system is shown in Fig. 2.

2.3. Procedure of the determination of PB and 4-OH PB in rat plasma

To 50 μl of rat plasma, 10 μl each of PB and 4-OHPB working solution (10.5 ng/ml–2.97 μg/ml in phosphate buffer, pH 7.4) and 10 μl of 0.2 *M* NaOH were added. After mixing with a vortex-mixer, 1 ml of chloroform was added. The solution was further vortex-mixed for 1 min and then centrifuged at 2000 *g* for 5 min. The organic phases collected were dried up with a flow of nitrogen gas. The residue was dissolved in 50 μl of anhydrous chloroform followed by the addition of an aliquot of 1 m*M* DBD-COCl in anhydrous chloroform (50 μl) and mixed. The resultant solution was left at room temperature for 40 min. After cooling with ice water, 900 μl of acetonitrile was added to the solution. An aliquot of the solution (50 μl) was injected onto the HPLC column.

2.4. Administration of PB to rats

PB sulfate dissolved in PBS (1 mg/kg) was administered intravenously to male rats (Wistar, 200–230 g) through a cannula inserted into the femoral vein. At fixed intervals, blood was taken from the femoral artery through the cannula.

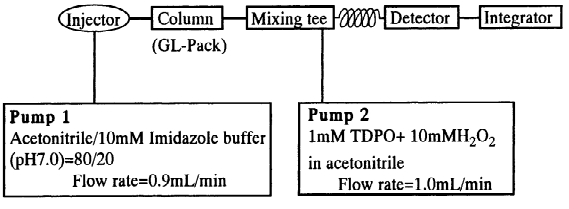


Fig. 2. Schematic flow diagram of HPLC–CL system.

3. Results and discussion

3.1. Labeling reaction of PB and 4-OH PB with DBD-COCL

DBD-F and Dansyl chloride had been used as labeling reagents [9] but no fluorescent derivatives of PB could be obtained owing to the presence of a bulky tertiary butyl residue at the secondary amino group of PB (Fig. 1). On the other hand, DBD-COCl was able to form fluorescent derivatives of PB and 4-OH PB with maximum fluorescence excitation and emission wavelengths of 430 and 545 nm, respectively. The fluorescent property of DBD is retained in both derivatives [24], by a spacer (–$COCH_2$–) between two amino moieties (Fig. 1). Moreover, the presence of linkage between the secondary amino group of PB and the carbonyl group of DBD-COCl has been confirmed by ^{1}H-NMR.

The conditions for the labeling reaction with DBD-COCl were examined using a mixture of PB and 4-OH PB in anhydrous chloroform. As a post-column chemiluminogenic reagent, a mixture of TDPO and hydrogen peroxide in acetonitrile was used [25,26]. Imidazole buffer (10 m*M*, pH 7) was used as an effluent because the pH dependency of an eluent for the HPLC showed maximum peak area at pH 7. Therefore, a mixture of imidazole buffer (10 m*M*, pH 7)–acetonitrile (20:80) was used as mobile phase of pump 1 in the subsequent experiment.

As shown in Fig. 3, the reaction rate increased at high temperature, but the peak areas for both compounds treated at 60°C over 30 min were nearly the same as those treated at room temperature. Then, the effect of solvent was examined with benzene, dimethylformamide, dimethylsulfoxide and chloroform. The reaction fluorescent intensities of these solvents as percentages of the fluorescent intensity of chloroform being set at 100 were 30, 55 and 75%, respectively. Maximum peak area could be obtained when chloroform was used as a solvent. In addition, the effect of concentration of DBD-COCl on the reaction yields was examined. As shown in Fig. 4, the optimal concentration of DBD-COCl was 1 m*M* for both PB (17.6 pmol) and 4-OHPB (26.4 pmol). From these data, the reaction conditions for labeling were decided where PB and 4-OH PB were allowed

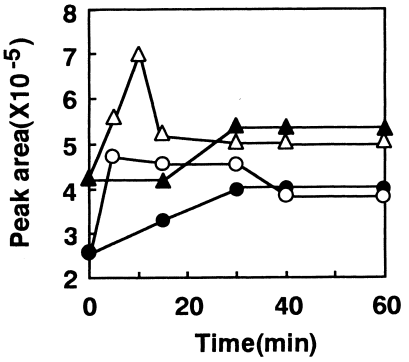


Fig. 3. Effect of reaction time and temperature on chemiluminescence intensities of DBD-derivatives. Sample amount on column was 5.5 pmol of PB and 11.4 pmol of 4-OH PB. PB (circle) and 4-OH PB (triangle) was reacted with DBD-COCl (1 m*M*) at room temperature (closed symbol) or at 60°C (open symbol). TDPO (1 m*M*) and H_2O_2 (1 m*M*) were used as chemiluminogenic agents.

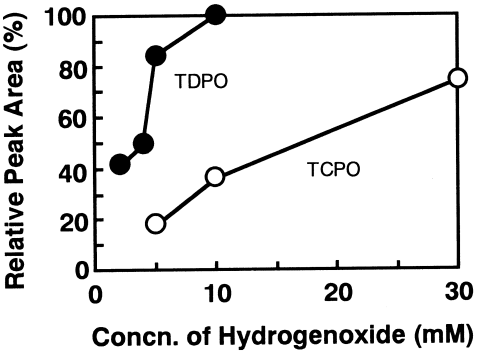


Fig. 5. Effect of TDPO and TCPO in the presence of various concentration of hydrogen peroxide on chemiluminescence intensities of PB derivative with DBD-COCl. Symbols show TDPO (closed circle) and TCPO (open circle). PB (17.6 pmol) was reacted with DBD-COCl (1 m*M*) for 40 min at room temperature. Relative peak area is represented as percentage of the peak area at condition of 1 m*M* TDPO and 10 m*M* H_2O_2.

to react with 1 m*M* DBD-COCl in chloroform for 40 min at room temperature.

3.2. Chemiluminogenic reagents for DBD-labeled PB and 4-OH PB

The components of post-column chemiluminogenic reagent were examined in order to identify the reagent that gives a high peak area. The effect of TDPO in the presence of hydrogen peroxide on chemiluminescence detection of PB was compared with that of bis (2,4,6-trichlorophenyl) oxalate (TCPO). TDPO and TCPO were used at 1 m*M* in acetonitrile. As shown in Fig. 5, TDPO always showed greater peak area for PB than TCPO at various concentrations of hydrogen peroxide, although the peak area of PB increased with the increase of hydrogen peroxide concentration in both cases. From this result, 1 m*M* TDPO with 10 m*M* hydrogen peroxide was chosen as the chemiluminogenic reagent.

In addition, the flow-rate of the chemiluminogenic reagent was found to affect the intensity and a flow-rate of 1 ml/min gave maximum peak areas. The length of the delay coil (I.D. 0.2 mm) also would affect the chemiluminogenic intensity and the maximum intensity was obtained at 15 cm. Typical chromatograms of control rat plasma and the plasma sample spiked with PB and 4-OH PB at limited concentration are shown in Fig. 6. By using the proposed method, DBD derivatives of PB and 4-OH PB were well separated within 10 min. In control rat plasma, no interfering peaks were observed at the retention times corresponding to those of the DBD derivatives of PB and 4-OH PB. The extracted and derivatized sample was stable (>95%) for 2 days at room temperature, although the CL intensity decreased to 80% after 3 days.

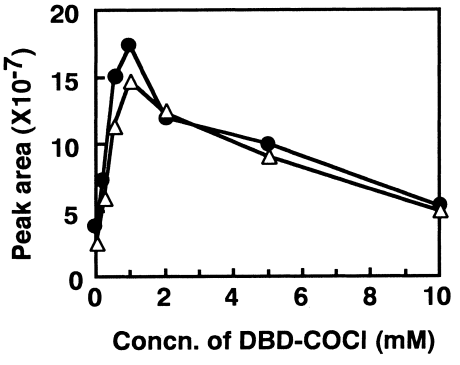


Fig. 4. Effect of DBD-COCl concentration on chemiluminescence intensities of DBD-derivatives. The reactions of PB (circle, 17.6 pmol) and 4-OH PB (triangle, 26.4 pmol) with DBD-COCl were performed in chloroform for 40 min at room temperature.

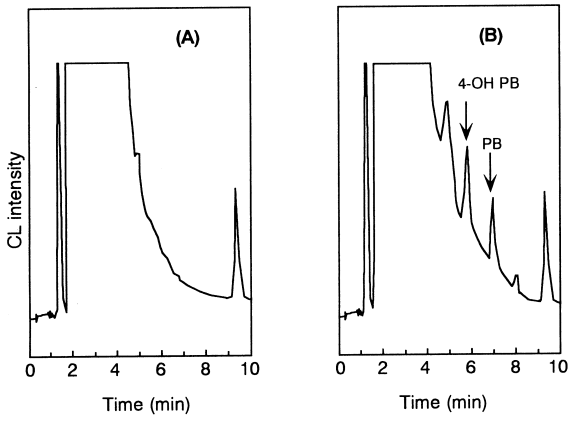


Fig. 6. Typical chromatograms of (A) control rat plasma and (B) spiked plasma by PB and 4-OH PB. Amount of PB and 4-OH PB in plasma were 1.0 ng/ml (9.9 fmol on column) and 1.4 ng/ml (15 fmol on column), respectively. The samples were reacted with DBD-COCl (1 m*M*) and their chemiluminescence were detected under the condition of 1 m*M* of TDPO and 10 m*M* H_2O_2.

3.3. Standard curves and its accuracy

A calibration curve was prepared by plotting the peak area of PB or 4-OH PB against their concentrations; a linear relation obtained over the range of 2.19–594 ng/ml plasma for PB ($r=0.999$) and 3.1–438 ng/ml plasma for 4-OH PB as shown in Table 1. The detection limits for PB and 4-OH PB were 1.0 ng/ml (9.9 fmol on column) and 1.4 ng/ml (15 fmol on column), respectively. As shown in Table 2, the recovery rates of PB and 4-OH PB in 50 μl of rat plasma at four different concentrations were between 95 and 101% and coefficient of variation were 2.4–11.0% ($n=3$). Regarding the precision of the procedure, the within- and inter-day coefficient of variation (C.V.s) were determined. The C.V.s for the within-day study of six replicate measurements in spiked plasma samples were 1.3% for PB (8.7 ng/ml) and 2.2% for 4-OH PB (32 ng/ml). The inter-day precision was determined by analyzing three times over 3 different days. The C.V.s for inter-day runs were 3.2% for PB (10.8 ng/ml) and 3.4% for 4-OH PB (32 ng/ml). Although the extraction procedures did not include an internal standard, the small C.V. value showed the reproducibility of the employed extraction and derivation. The yields of extraction for both PB and 4-OH PB were more than 96%. Considering that the limit of quantitation of spiked PB by fluorescence are reported to be 2–5 ng/ml using plasma [13–15], the proposed

Table 1
Calibration curves of PB and 4-OH PB[a]

Compound	Concentration of standard (ng/ml plasma)	Equations
PB	594, 216, 108, 21.6, 2.1	$Y=1.7\times10^4X-4877$ ($r=0.9999$)
4-OH PB	438, 308, 61.6, 30.8, 3.1	$Y=1.0\times10^4X-3956$ ($r=1.0000$)

[a] *Y*, peak area; *X*, concentration (ng/ml).

Table 2
Recovery rate and quantitation of PB and 4-OHPB in spiked rat plasma

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)	Recovery rate (%)
PB	1188	1183±13	11.0	99.6±1.1
	108	106±5	4.71	98.4±5.0
	21	20±2	10.0	95.3±3.0
	4.3	4.4±0.1	2.44	95.8±3.0
4-OHPB	4380	4346±107	2.46	99.2±2.4
	308	301±13	4.32	97.8±4.2
	30.8	30.7±1.9	6.19	99.7±6.1
	3.2	3.2±0.2	6.25	100.8±7.3

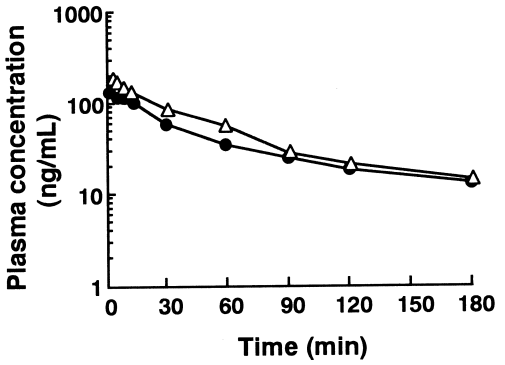


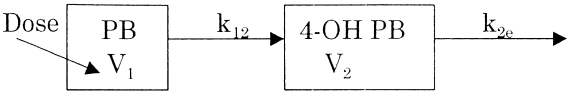
Fig. 7. Plasma concentration–time curves of PB and 4-OHPB after intravenous administration of PB to rat. Symbols show PB (circle) and 4-OH PB (triangle).

assay with PO-CL detection is two to five times more sensitive than the conventional method. Furthermore, the present assay requires only small amount of plasma (50 μl).

3.4. Plasma concentration of PB and 4-OH PB after intravenous administration of PB to rat

Fig. 7 shows the plasma concentration–time profiles of PB and 4-OH PB following intravenous administration of 1 mg/kg PB sulfate to rat. It has been reported that PB is excreted as 4-OH PB, 1″,2″-dihydroxy PB and their conjugate in urine and feces [2]. However, the other metabolites except 4-OH PB were not observed in plasma. These data suggested that the main metabolite after intravenous administration of PB into rat was 4-OH PB, although the reactivity of other metabolites were unknown. 4-OH PB was detected just after the administration, and its concentration was higher than PB. Table 3 lists the pharmacokinetic parameters for PB and 4-OH PB. The parameters were determined by the simultaneous fitting of two kinds of one-exponential equations which expressed individual plasma concentration–time course data for PB and 4-OH PB obtained after intravenous administration of PB. Since the model containing other excretion of PB in addition to the metabolism to 4-OH PB lead to negative value of the excretion parameter of PB except of hydroxylation, we used the model which PB was directly transformed to 4-OH PB. The parameters showed a large distribution volume of PB and dramatically fast metabolizing clearance of PB. The clearance of PB was 3-fold faster than the hepatic blood flow of rat (58 ml/min/kg) and comparable to the cardiac output (178 ml/min/kg) [27]. These data suggested that PB was rapidly metabolized to 4-OH PB in the liver and other organs such as kidney and lung, and that PB is a typical drug with high clearance and large distribution volume. In contrast, the distribution volume of 4-OH PB (36 ml/kg) was nearly the same as plasma volume (41 ml/kg) in rats. The 4-OH PB was rapidly eliminated with a half-life of 12.4 min (k_{2e} = 3.36±0.12 min^{-1}). These data suggests that 4-OH PB presents only in the blood vessel and is being rapidly eliminated.

Table 3
Pharmacokineic parameters of PB and 4-OH PB after administration of PB (1 mg/kg) to rat



	PB	4-OH PB
k_{12} (min^{-1})	0.0203±0.005	
k_{2e} (min^{-1})		3.36±0.121
V_1 (ml/kg)	8049.6±1427.0	
V_2 (ml/kg)		35.76±9.13
AUC (ng·hr/ml)	8.97±1.06	12.90±1.95
CL (ml/min/kg)	159.6±31.3	

4. Conclusion

This method is very sensitive, selective and simple. Only 50 μl of plasma sample is required for measurement. The detection limits of the proposed method for PB and 4-OH PB are very low and comparable to those of the other β-adrenoreceptor blocking agents, metoprolol and propranolol, by HPLC–PO-CL with DBD-F.

This shows DBD-COCl is a useful fluorogenic labeling reagent of PB which has a bulky tertiary butyl group on its secondary amino group. Thus, this method would be useful for the assay of secondary amines which could not be determined by using DBD-F or Dansyl chloride as a labeling reagent.

It is obvious from the experimental data that PB is a typical drug with large distribution volume and high clearance due to rapid metabolism to 4-OH PB in liver and other organs.

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