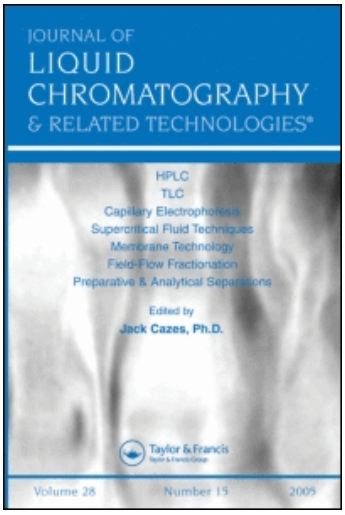

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PEROXYOXALATE CHEMILUMINESCENCE DETECTION OF SYNTHETIC PEPTIDE, EBIRATIDE

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ABSTRACT

Ebiratide [L-methionyl-L-glutamyl-L-histidyl-L-phenyl alanyl-D-lysyl- N-(8-aminooctyl)- L- phenylalanin -amide-S,S-dioxide] is one of the synthetic analogues of ACTH4-9 for Alzheimer disease. High performance liquid chromatography with peroxyoxalate chemiluminescence detection (HPLC-PO-CL) has been examined for the analysis of this compound. The fluorogenic reaction product of ebiratide with DBD-F [4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole] was detected by the chemiluminescence generated with bis(2,4,6-trichlorophenyl)oxalate (TCPO) or bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate (TDPO) in the presence of hydrogen peroxide. TDPO gave about five times higher CL intensity than TCPO using 100mM hydrogen peroxide. The

detection limit of DBD derivative of ebiratide was 25 fmol on column with TDPO. The HPLC-PO-CL was applied to the determination of ebiratide extracted from rat plasma by the solid phase extraction with Bond Elute C18. The method gave a good linearity over the range from 0.25 to 10 pmol on column with the recovery of 85% for the spiked rat plasma and was able to estimate the time course of ebiratide at a sub-pmol/mL level.

INTRODUCTION

A specific and sensitive determination method for trace amount of drug substance is important in the development of pharmaceutical products. Such a method is also important for the assay of clinical samples. For example, decrease in the amount of blood needed for biochemical examination will give great advantages for patients.

Ebiratide is produced by Hoechst AG as a therapeutic drug relating to the brain functional learning and memory.[1] This compound was injected up to 600μg/day in clinical studies.[2] Its concentration in the blood was expected to be at a low level; consequently, it has been necessary to develop a specific and sensitive determination method.

We have reported HPLC-fluorescence detection of ebiratide using precolumn derivatization with DBD-F,[3,4] and achieved the detection limit of 250 fmol on the column.[5] This paper describes the application of HPLC-PO-CL method to the determination of a fluorescent DBD-ebiratide with the combination of a solid phase extraction (sorbent extraction) for the rat plasma after administration (i.v.).

EXPERIMENTAL

Chemicals

Ebiratide was synthesized by Hoechst AG (Frankfurt, Germany). DBD-F and imidazole (fluorometric grade) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). TCPO, TDPO, hydrogen peroxide (30%), and nitric acid were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Acetonitrile, methanol, and distilled water for HPLC grade, were obtained from Kanto Chemicals Industries (Tokyo, Japan). Other chemicals were of analytical reagent grade.

Standard Solutions

Standard solutions to validate the method were prepared as follows; appropriate amounts of ebiatide were dissolved in water to obtain a 100 μM solution. The solution was diluted with 0.1 M borate buffer solution (pH 9.0) to obtain 10 pM to 10 μM of ebiratide stock solutions. Appropriate amounts of stock solutions were added to 400 μL of plasma to obtain spiked plasma samples of 0.5 to 50 ng/mL. Control solutions were prepared with 400 μL of water in the same manner.

Extraction Procedure of Ebiratide from Plasma

A rat plasma sample (400 μL) was diluted with 800 μL of water and applied to a cartridge of Bond Elute C18 column containing 100 mg of ODS (Varian Associates, Inc., Harbor City, CA, USA), which was activated in advance by a successive washing with each 2 mL of methanol and water. After the matrix was washed out with 1 mL of water, ebiratide was eluted and collected into a polypropylene reaction tube (2 mL, ASSIST, Tokyo, Japan) containing 1 mL of 0.005M HCl (in 30% aqueous methanol). The eluent was dried by a vacuum centrifuge freeze dryer (Tokyo Rika Co. Ltd., Tokyo, Japan), and the resultant residue was redissolved in 100 μL of 0.1M borate buffer solution (pH 9.0) for fluorescence derivatization.

Fluorescence Derivatization of Ebiratide with DBD-F

The sample solution and 100 μL of DBD-F acetonitrile solution were mixed in the reaction tube and kept standing at 50°C for 30 min. The resultant mixture was used for HPLC-PO-CL analysis.[5]

HPLC-PO-CL Apparatus

The system (Fig.1) consists of a Hewlett Packard Model 1090L HPLC system with a Vydac protein & peptide column 5C18 (150 x 4.6 mm I.D., The Separations Group, Hesperia, CA, USA), for analytical separation with an injection amount of 100 μL and a flow rate of 1.0 mL/min, a JASCO BIP-100 pump (Japan Spectroscopic Co. Ltd., Japan) for the delivery of chemiluminescence reagent through an Inertsil column ODS (150 x 4.6 mm

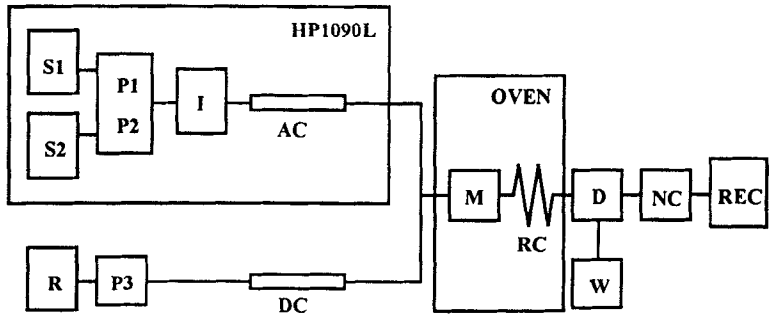

Figure 1. Schematic diagram of HPLC-PO-CL system. S1:50mM Imidazole nitrate (pH6.0):organic solvent*(3:2v/v), S2: 50mM Imidazole nitrate (pH6.0); organic solvent*(2:3v/v),; R, PO-CL reagent consisting of oxalate/hydrogen peroxide; P1, P2 and P3, pumps; I, injector; AC, analytical column; DC, dummy column; M, tee mixer; RC, reaction coil; OVEN was maintained at 30°C; D, detector; NC, noise cleaner; REC, recorder; W, waste. *organic solvent is a mixture of acetonitrile and methanol (2:1v/v)

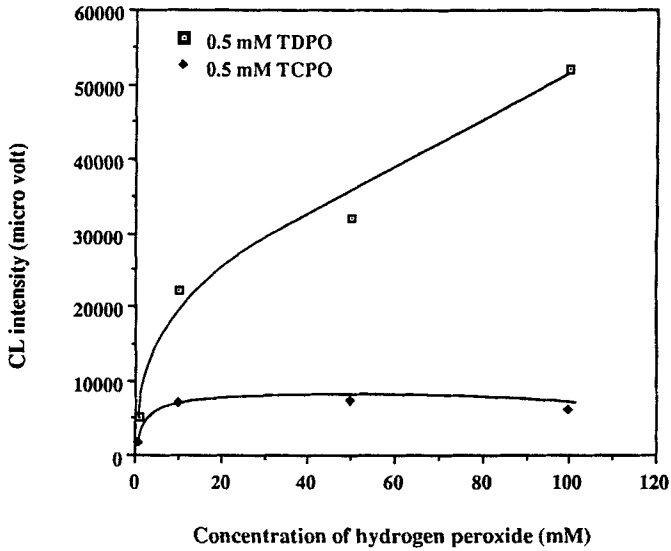

Figure 2. Effect of the hydrogen peroxide concentration on chemiluminescence. CL intensity was obtained with injected 2.5 pmol of ebiratide using 0.5 mM oxalate.

Table 1

Chemiluminescence Signal and Noise from Ebiratide in TDPO or TCPO System*

Oxalate	H_2O_2[mM] [mM]	Signal [micro volt]	Noise [micro volt]	Signal/ noise
	100	52140	202	258
TDPO	50	32140	165	195
system	10	22164	43	237
	1	4942	35	258
	100	5908	41	144
TCPO	50	7161	63	116
system	10	6893	40	171
	1	1734	44	39

*Each system consists of 2.5 mM TDPO or TCPO and Hydrogen peroxide. DBD-Ebirate was injected pmol as ebiratide on column. HPLC conditions: a flow rate of 1.0 mL/min using a mixture of solvent S1 and S2 (50:50). The flow rate of PO-CL reagent was maintained at 1.5 mL/min.

I.D., GL Science, Tokyo, Japan) for pressure control with a flow rate of 1.5 mL/min. A JASCO 825CL chemiluminescence detector (Japan Spectroscopic Co. ltd.) and a Type 3066 Pen recorder (Yokogawa Hokushin Electric, Tokyo, Japan) with a noise cleaner UNI-1(UNION, Tokyo, Japan).

RESULTS AND DISCUSSION

Chemiluminescence Intensity with TCPO and TDPO

Using 0.5 mM TCPO or TDPO and 1,10,50, and 100 mM hydrogen peroxide, the effect of the concentration of hydrogen peroxide on the PO-CL intensities of DBD-ebiratide derivative was studied. As shown in Fig.2 and Table 1, both the CL intensities of DBD-ebiratide and the noise in a TDPO system increased with the concentration.

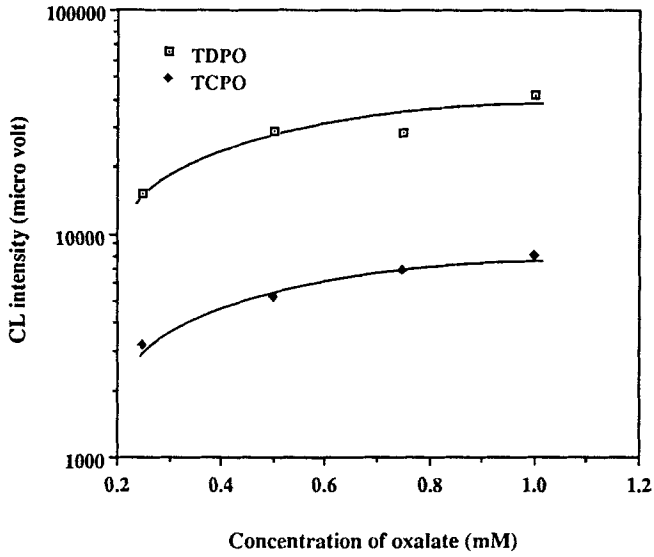

Figure 3. Effect of the oxalate concentration on chemiluminescence intensity. CL intensity was obtained with injected 2.5 pmol of ebiratide using 100 mM hydrogen peroxide.

On the other hand the noise level in a TCPO system was almost constant from 1 to 100 mM investigated. Figure 3 shows the effect of the concentration of oxalate on chemiluminescence intensity. DBD-ebiratide in a TDPO system gave about 5 times higher CL intensity than that in a TCPO system over the range of 0.25 to 1.0 mM of oxalates with 100 mM hydrogen peroxide.

HPLC-PO-CL detection is based on the reaction of aryloxalate with hydrogen peroxide, followed by the generation of dioxetanedione transferring its energy to fluorescent compounds to yield emission. Although both TCPO and TDPO might generate dioxetanedione sufficiently to excite the fluorescent compound injected, and thus was expected to give the same CL intensity, there were differences in both their signal and back ground noise of chemiluminescence.

The results showed that chemiluminescence generated in a TCPO system decreased with the high concentration of hydrogen peroxide, while in a TDPO system such a depression of chemiliminescence was not observed. This phenomenon was reported by Orlovic et al.[6] on the chemiluminescence from 9, 10-diphenylanthracene using TCPO, hydrogen peroxide and imidazole.

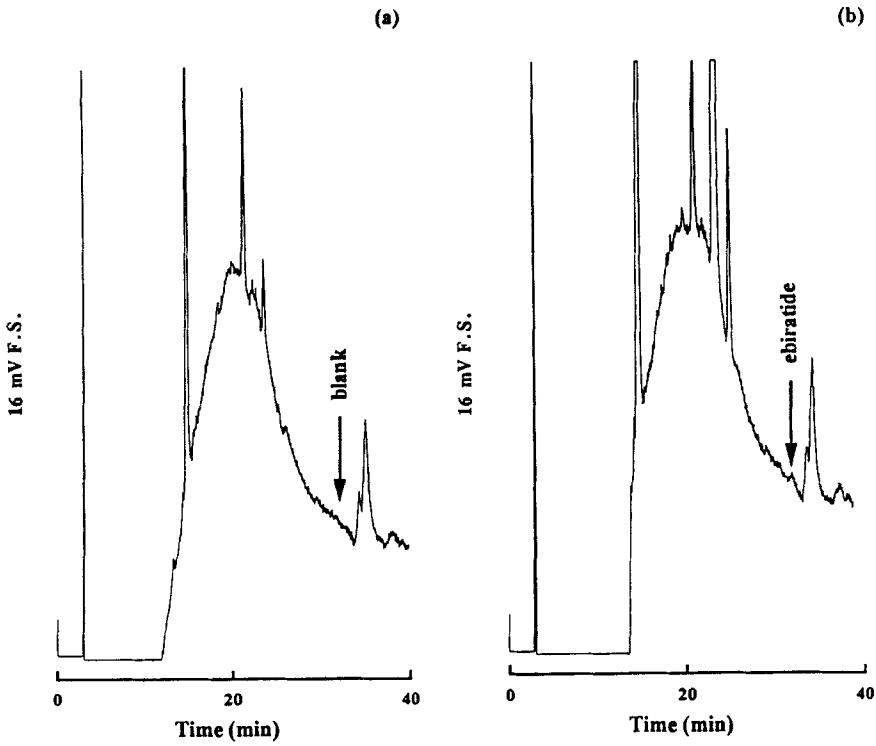

Figure 4. HPLC-PO-CL chromatogram of ebiratide-DBD. (a) Blank, and (b) ebiratide 25 fmol. HPLC conditions: Linear gradient at a flow rate of 1.0 mL/min using a mobile phase starting with a 100:0 mixture of solvent S1 and S2 changing to the mixing ratio to 55:45 and 30:70 in 15 and 60 min respectively, followed by 15 min initialisation with S1. The flow rate of PO-CL reagent was maintained at 1.2 mL/min.

It was reported that the PO-CL reaction with DFPO gave the reaction products of its hydroxyperoxy oxalate ester, half ester, and phenol.[7] TCPO was also considered to form such components, especially 2,4,6-trichlorophenol, which decreases PO-CL intensity by easily getting the energy of dioxetanedione. The results suggested that the differences in CL intensities contributed to the decomposed products from those oxalates. In practical use of HPLC-PO-CL a high intensity of chemiluminescence was required to obtain a high sensitivity.

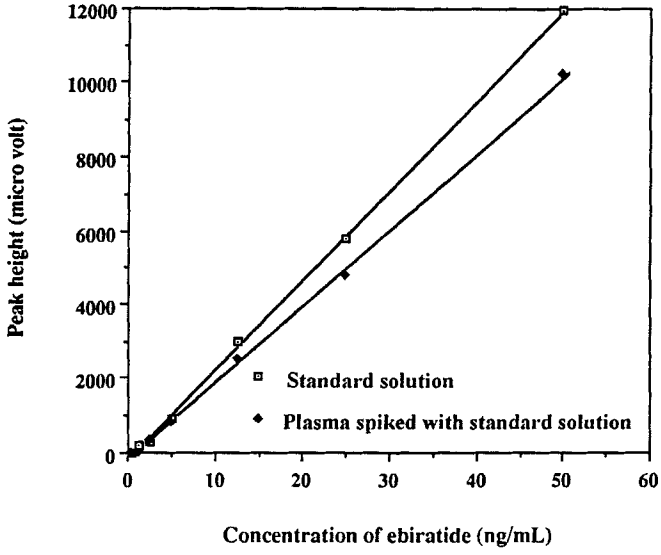

Figure 5. Absolute recovery study of ebiratide.

Therefore, the PO-CL reagent for ebiraitde was finally decided to consist of 0.5 mM TDPO and 100 mM hydrogen peroxide. TDPO was actually a preferable oxalate for this study to give the high sensitivity with the detection limit of ebiratide of 25 fmol on column (signal-to-noise ratio of 2) as shown in Fig. 4 (a) and 4 (b).

Biomedical Application of HPLC-PO-CL

In order to extract the peptide from plasma sample, sorbent extraction with Bond Elute C18 using 0.005 mol/L HCl in aqueous methanol as an extracting solvent was applied. The peptide is known to be stable around pH 3.[5] The residue obtained by centrifuge freeze drying was redissolved in 100 μL of 0.1 mol/L borate buffer solution for fluorescence derivatization.

The absolute recovery of ebiratide was calculated by the ratio of the slopes of linear regression lines obtained from the standard solution ($y = 242.66x - 198.46$, $r^2 = 0.999$, $n = 12$) and the plasma spiked with standard solutions treated with Bond Elute C18 sorbent extraction ($y = 206.40x - 190.67$, $r^2 =$

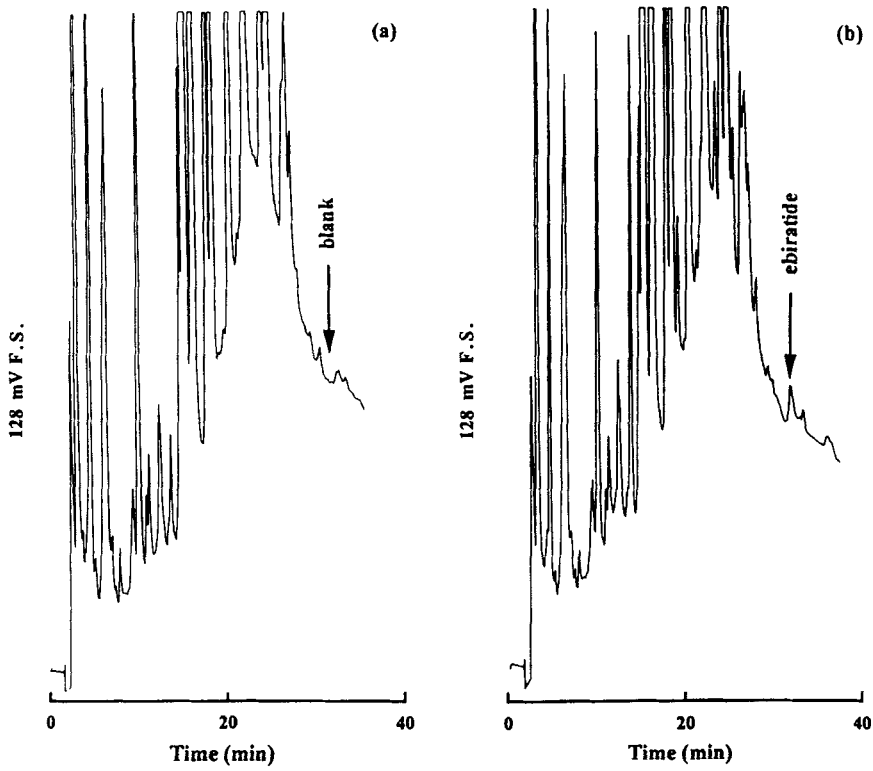

Figure 6. HPLC-PO-CL chromatogram of ebiratide-DBD. (a) Blank sample extracted from control rat plasma. and (b) ebiratide extracted from rat plasma sample (5 min after administration). HPLC conditions: Linear gradient at a flow rate of 1.0 mL/min using a mobile phase starting with a 100:0 mixture of solvent S1 and S2 changing to the mixing ratio to 55:45 and 30:70 in 15 and 60 min respectively, followed by 15 min initialisation with S1. The flow rate of PO-CL reagent was maintained at 1.2 mL/min.

0.999, n = 12) (Fig. 5). This method gave a good linearity over the range from 0.25 to 10 pmol on column with the recovery of 85% for the spiked rat plasma. Ebiratide was administered (i.v.) at the dose of 10 mg/kg to SD rat (body weight of 500 g, n = 3).

The blood was collected at 5, 30, 60, and 120 min after injection and centrifuged at 3000 rpm for 10 min at 5°C to collect plasma. Each sample was derivatized according to the procedure mentioned in the EXPERIMENTAL

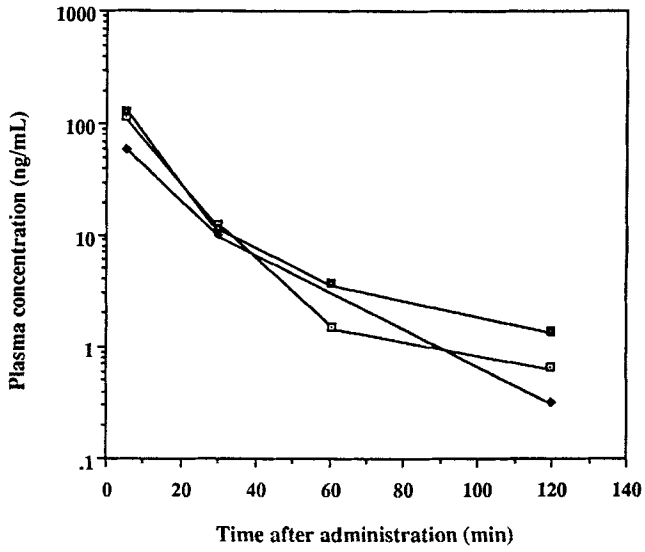

Figure 7. Plasma concentration of ebiratide (10mg/kg, i.v., n=3).

and analyzed by HPLC-PO-CL. As shown in Figs. 6 (a) and 6 (b), DBD-ebiratide was separated from the components of control plasma sample. Figure 7 shows the time course of the mean plasma concentration of ebiratide after injection.

It was found that HPLC-PO-CL, with the combination of sorbent extraction, was able to be applied to the determination of ebiratide in the rat plasma at the concentration of 0.25 pmol on column. On the other hand, the detection by the HPLC-Fluorescence method[5] gave 10 pmol on column under the same HPLC separation conditions as the HPLC-PO-CL method.

CONCLUSIONS

Although radioimmunoassay is known as a highly sensitive analytical method for ebiratide,[8] it requires specific antibodies and special facilities and is restricted in its application. The application of HPLC with fluorescence detection to plasma sample was difficult because of its deficiency in sensitivity.[5] On the other hand, the proposed HPLC-PO-CL is a very specific and sensitive method for the determination of ebiratide with the detection limit of 25 fmol on

column. The sensitivity is 10 times higher than that of HPLC-fluorescence detection.[5] The proposed HPLC-PO-CL method has successfully been applied to the determination of ebiratide in rat plasma with sorbent extraction which would be useful to the other peptide analyses.

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