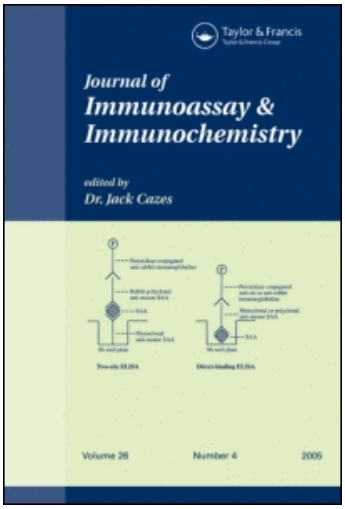

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DEVELOPMENT OF A SENSITIVE ENZYME IMMUNOASSAY FOR OPC-7251, A NOVEL ANTIMICROBIAL AGENT FOR PERCUTANEOUS APPLICATION

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ABSTRACT

A sensitive enzyme immunoassay for OPC-7251, a novel pyridone carboxylic acid antimicrobial agent, was developed and applied for the determination of human plasma levels. OPC-7251 was coupled to bovine serum albumin through a formation of N-hydroxysuccinimide ester. By immunization of rabbits, highly specific antiserum was raised. Using the antiserum and ß-D-galactosidase-labeled hapten, the homologous assay system allowed the detection of 2 pg of this compound. Plasma samples were precisely analyzed down to the minimum value of 200 pg/ml after heat treatment. The system was further validated by the recovery test and correlation with the HPLC analyses. Percutaneous application of 10 g of 1% OPC-7251 cream to healthy volunteers resulted in the peak plasma value of 1.6 ng/ml about 8 hours after dosing, indicating extremely low absorption efficiency through a transdermal system. (KEY WORDS: Enzyme immunoassay, ß-D-Galactosidase, OPC-7251, Antimicrobial agent, Percutaneous application, Polystyrene ball)

INTRODUCTION

OPC-7251, 9-fluoro-6,7-dihydro-8-(4-hydroxy-1-piperidyl)-5-methyl-1-oxo-1H,5H-benzo[ij]quinolizine-2-carboxylic acid, is a newly-synthesized antimicrobial agent for topical use (1). This

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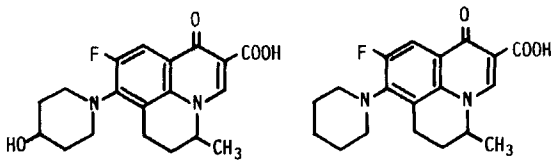
pyridone carboxylic acid compound has been reported to exhibit a potent and broad-spectrum antibacterial activity against aerobic Gram-positive and Gram-negative bacteria and anaerobic bacteria including Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa, and Propionibacterium acnes (2). In addition, this compound was demonstrated to be more effective than some potent antibiotics, such as erythromycin and gentamycin, on experimental infection models in mice (3). OPC-7251 is, therefore, expected to be one of the most promising drugs in clinical therapy for some infectious disorders, such as acne vulgaris. This antimicrobial agent exhibits a remarkable therapeutic potential through a transdermal system (3). Although this application system seems to be advantagous in carrying out an effective dosage regimen for patients, it has been described that only limited amounts of drugs are absorbed through a skin. A sensitive method to determine such low plasma levels of OPC-7251 has not yet been established. In this report, we describe a highly sensitive and specific enzyme immunoassay system for OPC-7251 and its availability for the determination of human plasma concentrations after percutaneous application.

MATERIALS AND METHODS

Materials

OPC-7251 and its structural analogue, OPC-7256, were generously donated from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Their chemical structures are illustrated in Figure 1. ß-D-Galactosidase (ß-Gal, from Escherichia coli,



OPC-7251 OPC-7256

FIGURE 1. Structures of OPC-7251 and OPC-7256.

grade Ⅷ, 580 units/mg of protein), bovine serum albumin (BSA), ovalbumin, and 4-methylumbelliferyl-ß-D-galactoside (4-MUG) were purchased from Sigma Chemical Co. (St. Louis, MO). N-Hydroxysuccinimide and dimethylformamide were from Ishizu Pharmaceutical Co. (Osaka, Japan) and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) was from Tokyo Chemical Industries (Tokyo, Japan). Goat immunoglobulin G (IgG) raised against rabbit IgG was purified in this laboratory by ammonium sulfate precipitation and ion-exchange chromatography and used as the second antibody. Peroxidase-labeled goat antibody specific to rabbit IgG was purchased from Cappel Products (Cochranville, PA). Freund's complete and incomplete adjuvants were products of Difco Labs. (Detroit, MI). Polystyrene balls (1/4 inch in diameter) were obtained from Immunochemical Co. (Okayama, Japan). All other reagents were of analytical grade.

Preparation of Immunogen

OPC-7251 was conjugated to BSA via the formation of its activated ester according to the method of Anderson et al. (4) with some modifications. Briefly, 5.5 mg (15.3 μmol) of OPC-

7251 was dissolved in 200 μl of anhydrous dimethylformamide. N-Hydroxysuccinimide (2.1 mg/50 μl) and EDC (3.2 mg/100 μl) dissolved in anhydrous dimethylformamide were successively added to the solution. The reaction mixture was gently stirred for 70 minutes at room temperature in the dark. The activated OPC-7251 was dried by N_2 gas flushing, redissolved again in 100 μl of anhydrous dimethylformamide, and then added dropwise, with continuous stirring, to the solution of BSA (10 mg, 153 nmol, in 1 ml of 0.1 M sodium carbonate buffer (pH 9.2). The reaction mixture was incubated for 2 hours at room temperature in the dark. The coupling conjugate was finally obtained after dialysis for 2 days with several changes of distilled water. The amount of OPC-7251 covalently bound to BSA was calculated from the spectral data by using the values of 2.45×10^4 and 1.32×10^4 as molar extinction coefficients at 295 and 325 nm, respectively. Protein concentration was determined by the method of Lowry et al. (5) with BSA as a standard. In this typical reaction, the molecule density of OPC-7251 bound to BSA was estimated to be 12.5.

Preparation of Antiserum

OPC-7251-BSA conjugate (0.5 mg) in Freund's complete adjuvant was injected intradermally into male white rabbits. Booster injections of the conjugate (0.2 mg) in Freund's incomplete adjuvant were given in the same sites at 4-week intervals. One week after the last injection, the rabbit was exsanguinated. The serum obtained was treated at 56°C for 30 minutes and stored at -20°C. The production of antibody

specific to OPC-7251 was determined by an enzyme-linked immunosorbent assay method using a 96-well flat-bottomed microtiter plate, as described previously (6). In this assay, each well was coated with OPC-7251-ovalbumin conjugate, which was prepared in the same way as described for the preparation of the immunogen and found to have an epitope density of 20.6.

Preparation of OPC-7251-ß-Gal Conjugate

The N-hydroxysuccinimide ester of OPC-7251 was obtained in the same way as described for the preparation of immunogen. Activated OPC-7251 (38 μl, 1.48 μmol) was added dropwise under continuous stirring to the ß-Gal solution (200 μg in 1 ml of 0.1 M sodium bicarbonate). In this reaction, molar ratio of OPC-7251 to ß-Gal was 1,000:1. After 2-hour incubation at room temperature in the dark, the reaction mixture was dialyzed at 4°C for 2 days with four changes of 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 M NaCl and 1 mM $MgCl_2$. Spectral analysis showed that 13.1 moles of OPC-7251 were bound to 1 mole of ß-Gal. The enzyme-hapten conjugate was mixed with an equal volume of glycerol and stored at -20°C until use. The enzyme was stable for more than 1 year under this storage condition.

Preparation of Assay Samples

All plasma samples were generously provided from Drug Safety Research Center, Otsuka Pharmaceutical Co., Ltd. and stored at -20°C until assay. Every sample was diluted with 9 volumes of 0.1 M potassium phosphate buffer (pH 7.0) containing

0.1 M NaCl and 1 mM $MgCl_2$ (EIA buffer), heated for 5 minutes in a boiling water, and then centrifuged at 12,000 × g for 30 minutes. The supernatant was subjected to enzyme immunoassay after an appropriate dilution with EIA buffer.

In another experiment, a series of OPC-7251 standards were prepared in normal rat plasma diluted by 1:10 with EIA buffer and they were subjected to either heat treatment at 100°C for 5 minutes or the extraction with 1 ml of chloroform. After heat treatment, the centrifugal supernatant was used for assay. The organic phase obtained was evaporated to dryness, reconstituted with the same volume of EIA buffer as that of starting sample, and used for assay.

Assay Procedure

The enzyme immunoassay of OPC-7251 was developed on the basis of the double antibody solid phase principle. The antiserum specific to OPC-7251 was diluted 1:800,000 with EIA buffer containing 1% BSA. The second antibody was immobilized on polystyrene balls as described previously (7). Standard solution of OPC-7251 was dissolved in methanol at 10 μg/ml and its exact concentration was spectrophotometrically determined by using its molar extinction coefficient. This solution was diluted to the indicated concentrations with EIA buffer. ß-Gal-labeled OPC-7251 was employed after dilution of 1:20,000 with EIA buffer containing 1% BSA.

In every triplicate assay, 100 μl of standard or sample solution was incubated at 4°C for 15 hours with 50 μl each of ß-Gal-labeled OPC-7251 and anti-OPC-7251 serum. Then, 200 μl

of EIA buffer and a second antibody-immobilized polystyrene ball were added to each assay tube and the mixtures were agitated at 15°C for 4 hours. The balls were thoroughly washed with 0.01 M potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, and 0.1% BSA, and transferred to another tube containing 300 μl of this buffer. The enzyme reaction was performed by incubating with 200 μl of 0.3 mM 4-MUG prepared in EIA buffer at 37°C for 2 hours, followed by termination with the addition of 2 ml of 0.1 M sodium carbonate. The fluorescence intensity generated was measured in a Shimadzu fluorospectrometer with the excitation at 360 nm and the emission at 450 nm. B/B_0 value (%) was calculated by the following equation;

$$\frac{B}{B_0}\ (\%) = \frac{B - Bl}{B_0 - Bl} \times 100$$

B: flourescence intensity of sample

B_0: fluorescence intensity of zero standard

Bl: fluorescence intensity of blank

RESULTS

Sensitivity and Specificity of the Proposed Enzyme Immunoassay

With an enzyme-linked immunosorbent assay using a microtiter plate, the production of antibody specific to OPC-7251 was observed in two rabbits' sera after the second booster injection. One antiserum having the necessary specificity and sensitivity was used to establish an enzyme immunoassay. In this homologous system, OPC-7251 was measurable over the range of 2.0 to 512 pg/tube (Figure 2). The linearity was assured

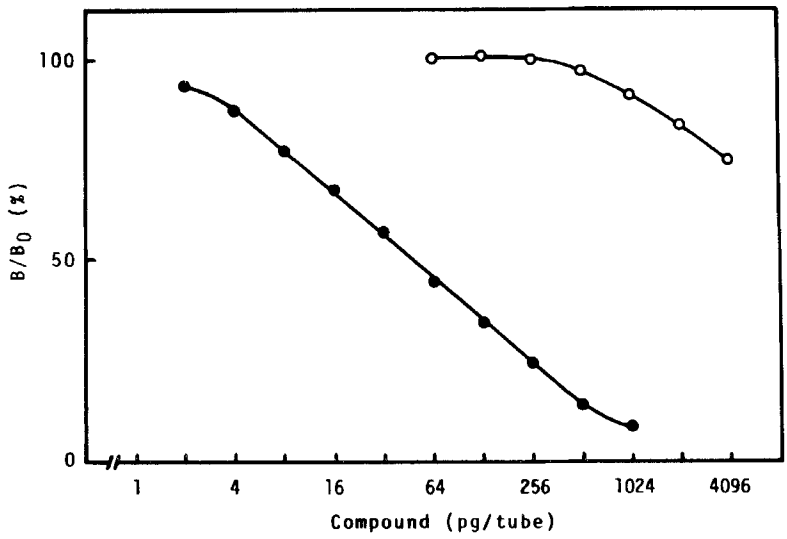


FIGURE 2. Typical calibration curves for OPC-7251 (●) and crossreactivity of OPC-7256 (○).

with a high average correlation coefficient of 0.996. Consequently, the lowest concentration of sensitivity for the standard OPC-7251 was 20 pg/ml. No metabolite of this drug has been identified in plasma or urine. The specificity of the antibody was examined by using OPC-7256, a chemically-derived dehydroxylated form of OPC-7251 (Figure 2). The antiserum was shown to be highly specific to OPC-7251 because of its low crossreactivity to this analogue.

Effect of Plasma Samples on the Sensitivity

The addition of normal rat plasma diluted with the buffer to the standard assay system reduced the fluorescence intensity

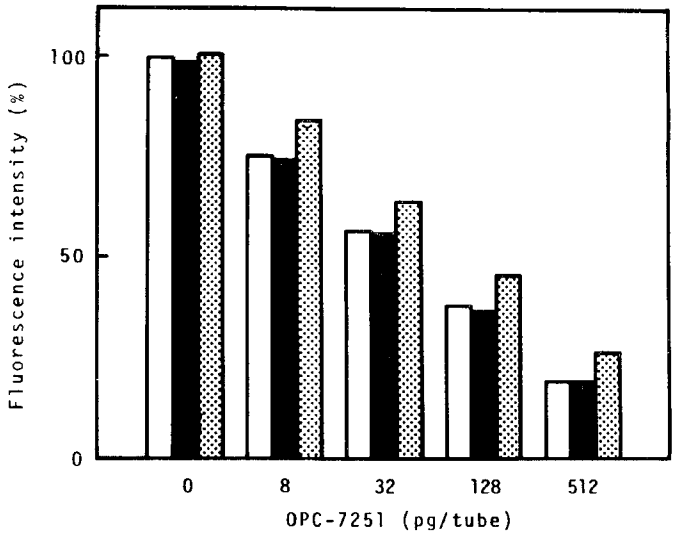


FIGURE 3. Effect of pretreatment of plasma samples for the determination of OPC-7251. A series of OPC-7251 standards were prepared in diluted normal rat plasma. The concentration of OPC-7251 was determined by EIA after heat treatment (closed column) or chloroform extraction (dotted column). The fluorescence intensity of zero standard in the buffer system (open column) was defined as 100% and relative values of standards or samples were calculated.

considerably, whereas the relative B/B_0 values were similar to those of the standards, indicating overlapping calibration curves (data not shown). However, this reduction may contain some errors in the estimation of exact plasma values of the drug. Therefore, two effective methods to alleviate the interference from plasma proteins in this assay system were examined (Figure 3). Fluorescence intensities for the samples after heat treatment were strictly consistent with those of the standards at any concentrations tested. OPC-7251 was, further-

TABLE 1

Between-Day Precision of Back-Calculated Values of OPC-7251 Standards

	Standard (pg/tube)						
Run	8	16	32	64	128	256	512
1	8.11	16.9	29.5	61.4	146.3	283.3	443.4
2	8.21	15.3	29.6	68.7	147.6	233.8	505.4
3	8.80	15.3	29.5	54.4	124.9	276.6	543.4
4	7.70	17.0	31.0	65.0	125.0	250.0	480.0
5	7.80	14.8	34.0	70.0	131.0	263.0	500.0
Mean	8.12	15.9	30.7	63.9	135.0	261.3	494.4
C.V.(%)	5.3	6.4	5.7	9.8	8.3	7.7	7.4
R.E.(%)	+1.0	-0.6	-3.5	-0.2	+5.5	+2.1	-3.4

C.V., coefficient of variation; R.E., relative error.

more, found not to be decomposed by heat treatment. On the other hand, fluorescence intensities after chloroform extraction became higher than those of the standards, resulting in the underestimation of plasma levels. This was due to the extraction efficiency of OPC-7251 by chloroform. Consequently, plasma proteins should be removed by heat denaturation prior to the assay. The assay system thus established made it possible to detect plasma OPC-7251 at 200 pg/ml.

Precision, Accuracy, and Validity

The presented method demonstrated high precision and accuracy in analyzing seven concentration standards (Table 1). The percent relative error varied only from -3.5 to +5.5%.

TABLE 2

Variation of OPC-7251 Determination by the Proposed EIA

	Intra-assay (n=5)		Inter-assay (n=4)	
Sample	Mean (pg/tube)	C.V.(%)	Mean (pg/tube)	C.V.(%)
1	17.6	12.2	18.3	10.2
2	47.6	4.9	45.2	7.3
3	110.0	8.2	109.4	8.3
4	370.8	8.7	379.0	5.0

Intra- and inter-assay variations were examined with rat plasma samples and they ranged from 4.9 to 12.2% and 5.9 to 10.2%, respectively (Table 2). Recovery tests were carried out with normal rat plasma containing the indicated amounts of OPC-7251 (Table 3). This indicated that OPC-7251 effectively remained in the supernatant after heat treatment.

To determine the validity of the assay method, rat plasma samples which were separately estimated for OPC-7251 contents by the HPLC method were analyzed by the proposed method (Figure 4). These plasma values obtained showed a good correlation with those by HPLC.

Plasma Levels of OPC-7251 in Healthy Volunteers

The time course of plasma levels of OPC-7251 after percutaneous application to six healthy volunteers were determined (Figure 5). After application of 10 g of 0.5% OPC-7251 cream (50 mg of this compound), the slow elevation of plasma concen-

TABLE 3

Recovery Test of OPC-7251 added in Rat Plasma by the Proposed EIA

OPC-7251 added (ng/ml)	OPC-7251 measured (ng/ml)	Recovery (%)
25	23.5	94
50	51.8	104
100	106.2	106
200	205.4	103
400	377.5	94
Mean	—	100.2

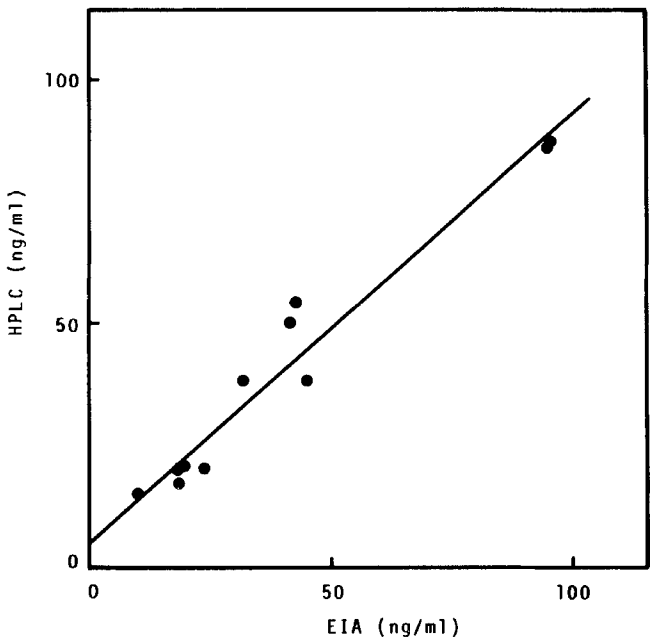


FIGURE 4. Correlation between EIA and HPLC analyses with rat plasma samples. Plasma concentrations of OPC-7251 were estimated by EIA just after heat treatment and further dilution. HPLC values were separately determined by applying chloroform-extracted samples on a reverse-phase column. The detectable limit of this compound by HPLC system was 5 ng/ml. Linear regression analysis gave the correlation coefficient; r= 0.974 and the linear regression equation; Y=0.869X + 5.62.

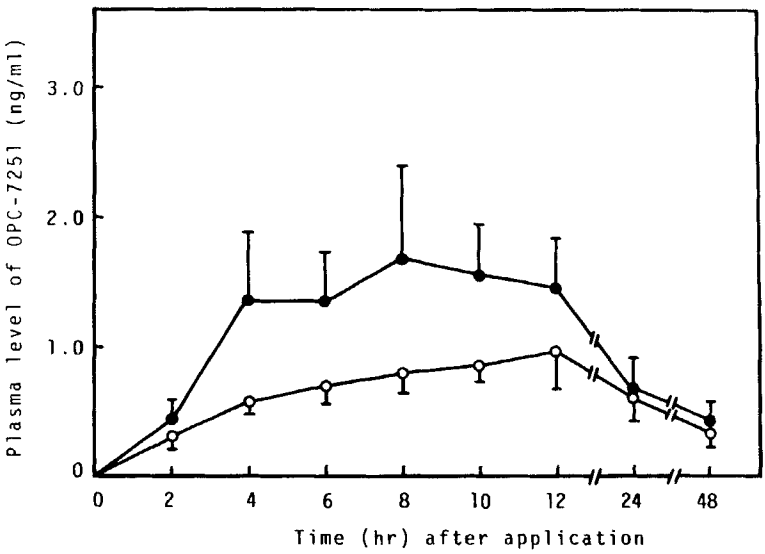


FIGURE 5. Plasma concentrations of OPC-7251 after percutaneous application of OPC-7251 creams to healthy volunteers. Ten g of 0.5% or 1.0% OPC-7251 cream was unifomly spread over the back skin (30 cm × 30 cm area) of six healthy volunteers with 2-week interval. The creams remained in contact with the skin for 8 hours. Plasma samples taken periodically were diluted by 1:10 and assayed by EIA. All values represent the mean ± S.E. of 6 experiments.

tration was observed and the peak value of 1.0 ng/ml was obtained 12 hours after dosing. In addition, the application of 10 g of 1.0% OPC-7251 cream (100 mg of this compound) to the same volunteers gave the maximum level of 1.6 ng/ml about 8 hours. Although dose-dependent profiles were observed between two groups, the penetration of this compound through the skin was considerably slow and little.

DISCUSSION

Preliminary experiments to examine the pharmacokinetics of OPC-7251 in rats have been carried out by the HPLC method (Dr. T. Shimizu, personal communication). The HPLC technique showed the low limit of detection at 5 ng/ml of plasma and required 1 ml of plasma only for one run even when samples obtained at the peak time should be measured. Therefore, this could be applied for the quantitation of plasma samples from experimental animals, but extremely difficult for human plasma samples. Since this drug would be applied for patients through a transdermal system, it may be detected in extremely low levels. To solve this problem, a highly sensitive enzyme immunoassay for OPC-7251 should be developed in lieu of the HPLC or other methods. The proposed method allowed the determination down to the level of 2 pg, namely 20 pg/ml of the standard solution. Although the assay was affected by the presence of intact plasma, the samples after removal of heat-denatured plasma proteins showed no interference for the quantitation of OPC-7251, similar to the case reported previously (8). Consequently, the limit of sensitivity of the assay results in 200 pg/ml of plasma, since appropriate dilution of the sample is necessary for this pretreatment. This means that the method described here has more than 25-fold higher sensitivity than the HPLC method. Moreover, it is noteworthy that 50 μl of plasma is enough in triplicate assay of this method.

The reliability of the assay method was confirmed by its precision, reproducibility, recovery efficiency, and corre-

lation with HPLC data. High sensitivity of this method is considered to be due to the following reasons: 1) The antiserum used had high specificity and affinity only to OPC-7251. This was confirmed with low crossreactivity of a structural derivative and high dilution rate of the antiserum. 2) The enzyme-hapten possessed high enzyme activity and moderate density of hapten. The amount of enzyme added in each assay tube could be estimated to be only 217 pg, to which 7.57 pg of OPC-7251 was bound (data not shown). This means that the enzyme retained its activity satisfactorily during the process of preparation of the enzyme-hapten.

In this study, the proposed assay was attempted to measure plasma levels of OPC-7251 in human subjects after percutaneous application of the cream. Although the absorption of this drug through a human skin was demonstrated to be extremely slow and poor, these levels were found to be measurable by the present enzyme immunoassay, but not by the HPLC method. OPC-7251 is a medicine for topical application to several kinds of skin infections (3). Therefore, it is advantageous that it hardly penetrates into the blood through a transdermal system, because such a drug would cause little side effect. The enzyme immunoassay for OPC-7251 would be useful to monitor plasma levels after dosing and elucidate its pharmacokinetics.

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