DISPOSITION OF OXPRENOLOL IN SPONTANEOUSLY HYPERTENSIVE RATS AS DETERMINED BY AN ENZYME IMMUNOASSAY

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Specific antisera against oxprenolol, a beta adrenoceptor blocking agent, were produced in rabbits immunized with oxprenolol hemigluturate-bovine serum albumin conjugate. The antiseraum, beta galactosidase labelled oxprenolol and 4-methylumbelliferyl-β-D-galactoside as substrate were used to develop an enzyme immunoassay for oxprenolol. The assay can reliably detect as little as one ng of oxprenolol directly from plasma. The plasma concentrations of oxprenolol in spontaneously hypertensive rats were determined by an enzyme immunoassay after a single and multiple oral doses for 14 consecutive days. Oxprenolol was rapidly absorbed from the gastrointestinal tract and was quickly eliminated from plasma. Plasma concentrations-time data conformed to equations describing one-or two-compartment open kinetic model. No significant differences in pharmacokinetic parameters were observed between after a single and multiple doses.

Keywords — oxprenolol; enzyme immunoassay; beta galactosidase; pharmacokinetics; single dose; multiple doses; SHR rats

INTRODUCTION

The methods for the determination of oxprenolol, a beta adrenoceptor blocking agent, are gas-liquid chromatography and thin layer chromatography. All these methods require time consuming extraction of the drug from plasma and derivative formation before the final determination step. This paper reports development of a simple and specific enzyme immunoassay for oxprenolol which can determine as little as one ng of the drug directly from plasma. Plasma concentrations of oxprenolol in spontaneously hypertensive (SHR) rats after a single or multiple oral doses were determined by this procedure and no significant differences were found in pharmacokinetic parameters between the treatments.

MATERIALS AND METHODS

Chemicals and Drugs

Oxprenolol hydrochloride and N-dealkylated oxprenolol were supplied by Chiba-Geigy (Japan), Takarazuka. Bovine serum albumin (BSA) was purchased from Sigma, St. Louis, Mo., U.S.A. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI) hydrochloride was obtained from Protein Research Foundation, Osaka. Complete Freund's adjuvant was from Difco, Detroit, Mich., U.S.A. Beta galactosidase from E. coli with specific activity of 50 U/mg (25°C, o-nitrophenyl-β-D-galactopyranoside as substrate) and 4-methylumbelliferyl-β-D-galactoside were purchased from P-L Biochemicals, Milwaukee, Wis., U.S.A. Isobutyl chlorocarbonate was obtained from Eastman, Rochester, N.Y., U.S.A. Sephadex G-50 (fine) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Immunobeads® with goat antirabbit immunoglobulins was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Propranolol hydrochloride, atenolol, sotalol hydrochloride and pindolol were obtained from respective producers. All other chemicals used were of reagent grade.

Preparation of Immunogen

(1) Synthesis of Oxprenolol Hemigluturate
—Glutaric anhydride (3 mmol) was added to a mixture of one mmol of oxprenolol hydrochloride and 3 ml of triethylamine. The reaction was allowed to proceed for 2 days at room temperature under constant magnetic stirring (Fig. 1A). Triethylamine was removed from the reaction mixture by evaporation. The residue was dissolved in 10 ml of distilled water. The solution was adjusted to pH 5.0 with 0.5 N NaOH and extracted with ethyl acetate. The organic layer was washed with distilled water and evaporated to dryness under vacuum. The dried material had a pale yellowish color. Oxprenolol hemigluturate was characterized by nuclear magnetic resonance and infrared spectral analyses.

(2) Conjugation of Oxprenolol Hemigluturate to BSA —Oxprenolol hemigluturate (20 mg) and EDCI hydrochloride (100 mg) were added to a BSA (100 mg) solution in 10 ml of 0.2 M phosphate buffer, pH 6.0. The reaction was allowed to

A. Synthesis of oxprenolol-hemigluturate

\[
\begin{array}{c}
\text{O} \\
\text{C} - \text{CH}_2 \text{CH}_2 \\
\text{O} \\
\text{C} - \text{CH}_2 \\
\text{O} \\
+ \\
\text{OH} \\
\text{OCH}_2 \text{CH} - \text{CH}_2 \text{NH} - \text{CH} \text{CH}_3 \\
\text{O} - \text{CH}_2 \text{CH} - \text{CH}_2 \text{NH} - \text{CH} \text{CH}_3 \\
\text{O} - \text{CH}_2 \text{CH} - \text{CH} = \text{CH}_2 \\
\end{array}
\]

B. Conjugation of oxprenolol-hemigluturate to bovine serum albumin

\[
\begin{array}{c}
\text{BSA} - \text{NH}_2 \\
\text{+} \\
\text{COOH} \\
\text{(CH}_2)_3 \\
\text{C} = \text{O} \\
\text{O} \\
\text{OCH}_2 \text{CH} - \text{CH}_2 \text{NH} - \text{CH} \text{CH}_3 \\
\text{O} - \text{CH}_2 \text{CH} - \text{CH}_2 \text{NH} - \text{CH} \text{CH}_3 \\
\text{O} - \text{CH}_2 \text{CH} - \text{CH} = \text{CH}_2 \\
\end{array}
\]

FIG. 1. Preparation of Oxprenolol Immunogen

EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; BSA, bovine serum albumin.
proceed for 2 hours at room temperature and overnight at 4°C (Fig. 1B). The reaction mixture was dialyzed against distilled water for 2 days at 4°C. Oxprenolol-BSA conjugate was lyophilized and stored at -20°C.

Immunization — Six male albino rabbits were immunized with one mg of oxprenolol-BSA conjugate once a week for the first 2 weeks and then once every month. The immunogen was dissolved in distilled water (2 mg/ml), and emulsified with an equal volume of complete Freund’s adjuvant. The emulsion was injected into the four foot pads and subcutaneously into several sites on the back. Rabbits were bled from the central ear artery 7 to 10 days after the booster injection. The blood was centrifuged at 2000 × g for 15 min to separate the serum.

Enzyme Immunoassay

(1) Preparation of beta Galactosidase Labelled Oxprenolol — Oxprenolol hemisuccinate was prepared from oxprenolol and succinic anhydride with the same procedures for the synthesis of oxprenolol hemiglutathate. Oxprenolol hemisuccinate (0.2 mg) was conjugated to 10 mg of beta galactosidase by the mixed anhydride technique with isobutyl chloroformate and triethylamine as reported for the preparation of chlorpromazine immunogen.\(^6\) The reaction mixture was dialyzed against 0.01 M phosphate buffer, pH 7.0, containing 0.001 M MgCl₂ and 0.1 M NaCl for 2 days. Oxprenolol-beta galactosidase conjugate (2 mg) was applied to Sephadex G-50 (fine) column chromatography (9 × 500 mm) for purification.\(^7\)

(2) Buffer — Working buffer for enzyme immunoassay consisted of 0.01 M phosphate buffer, pH 7.0, containing 0.1% BSA, 0.001 M MgCl₂ and 0.1 M NaCl.\(^7\)

(3) Titration of Antisera — The antisera were diluted with the working buffer. To a 12 × 70 mm plastic tube containing 200 μl of working buffer were added with 100 μl of various dilutions of antisera and 50 μl of beta galactosidase labelled oxprenolol (0.5 μU). The tubes were left overnight at 4°C. To separate the antibody-bound enzyme labelled oxprenolol from the free drug, each tube was added with 400 μl of Immuno-beads\(^5\) with goat antirabbit immunoglobulins. The tubes were incubated for 2 hours at 30°C and centrifuged at 2000 × g for 10 min. The supernatant was removed by aspiration. After a washing of the precipitate with 800 μl of working buffer, the tubes were added with 5 μg of 4-methyllumbiferyl-β-D-galactoside in 400 μl of working buffer and incubated for one hour at 30°C. The reaction was terminated by adding 3 ml of ice-cold 0.1 M glycine-NaOH buffer, pH 10.5. After centrifugation at 2000 × g for 10 min, fluorescence intensity of the supernatant was determined with a fluorometer (MPF-4, Hitachi).

(4) Standard Curve and Specificity — A standard curve was prepared by adding various amounts of oxprenolol (1–300 ng) in 10 μl to the tubes containing 100 μl of diluted antiserum (1:8000, final dilution 1:28000), 50 μl of the enzyme labelled oxprenolol (0.5 μU) and working buffer to bring the volume up to 350 μl. The tubes were incubated overnight at 4°C and were processed as described above.

Specificity of the antiserum for oxprenolol, its metabolite and other beta adrenoceptor blocking agents was estimated by determination of the amount of each compound required to inhibit the enzyme labelled oxprenolol-antibody complex formation by 50% (ID₂₀).\(^9\)

(5) Enzyme Immunoassay of Plasma Oxprenolol — To the assay tubes containing 100 μl of diluted antiserum (1:8000) and 50 μl of the enzyme labelled oxprenolol (0.5 μU) was added 20 or 40 μl of plasma sample. An equal volume of control plasma was added to the standard curve tubes. Appropriate volume of working buffer was added to each tube to make a final incubation volume of 350 μl. The tubes were handled as described above.

Treatment of SHR Rats — Male SHR rats with ages of 12 to 15 weeks were treated with either a single oral dose (50 mg/kg) or multiple doses (50 mg/kg per day) of oxprenolol for 14 consecutive days. In rats treated with a single dose, blood was drawn from an aortic cannula before and at 0.5, 1, 2 and 4 hour after the dosing. In subchronically treated rats, blood was collected
before and at the designated times after the dosing on the 14th day of experiment. Plasma samples were obtained by centrifugation and stored at 
\(-20^\circ\text{C}\) until assayed.

**Analyzes of Data** — Graphic simulation of plasma oxprenolol concentration-time data was done by application of the one- or two-compartment open model.\(^8\) The calculated pharmacokinetic parameters were: \(T_{\text{max}}\), time to maximum plasma concentration; \(C_{\text{max}}\), maximum plasma concentration; \(K_a\), absorption constant; \(K_e\), elimination constant; \(T_{1/2}\), biological half-life; \(\text{AUC}\), area under the plasma concentration-time curve from time 0 to infinity.

Significance of differences between means of pharmacokinetic parameters from rats treated with a single dose and multiple doses was calculated with Student's \(t\)-test.

**RESULTS**

**Titration of Antisera**

A typical antiserum dilution curve is shown in Fig. 2. The percentage of the enzyme activity bound to the antibody relative to the total amount of the activity added was calculated and plotted against the final dilution of the antiserum. A final dilution of the antiserum of 1:28000 was chosen for an enzyme immunoassay. This dilution bound about 38\% of the enzyme activity added to the incubation mixture.

**Sensitivity**

A typical standard curve for oxprenolol is shown in Fig. 3. The limit of the assay was about one ng/tube. The standard curve was linear up to 300 ng when plotted on a logit-log scale.

**Specificity**

 Specificity of the antiserum used for the assay is shown in Fig. 4. A ID\(_{50}\) value for oxprenolol was 19.89 ng. N-Dealkylated oxprenolol, one of the metabolites of oxprenolol\(^{3,9-11}\) had ID\(_{50}\) of 1108 ng showing a negligible cross-reactivity with the antiserum. The antiserum did not show any significant cross-reactivities with other beta adrenoceptor blocking agents.

**Pharmacokinetics of Oxprenolol**

Oxprenolol was rapidly absorbed from the

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**FIG. 2. Oxprenolol Antiserum Dilution Curve**

The percentage of beta galactosidase labelled oxprenolol bound to antibody is plotted against the final dilution of the antiserum represented on a semilog scale.

**FIG. 3. Standard Curve for Oxprenolol**

The percent inhibition of beta galactosidase labelled oxprenolol bound to the antibody in the presence of various amount of unlabelled oxprenolol is plotted on a logit-log scale. Points are means \(\pm\) S.E.M. for 4 assays.
FIG. 4. Specificity of Oxprenolol Antiserum

The amount of a compound required to inhibit the enzyme labelled oxprenolol binding to antibody by 50\% (ID$_{50}$) was calculated by interpolation from the logit-log amount fitted line.

TABLE I. Pharmacokinetic Parameters of Oxprenolol in Spontaneously Hypertensive Rats after a Single Oral Dose and Multiple Doses for 14 Consecutive Days

<table>
<thead>
<tr>
<th></th>
<th>T$_{max}$ (h)</th>
<th>C$_{max}$ (ng·ml$^{-1}$)</th>
<th>K$_a$ (h$^{-1}$)</th>
<th>K$_e$ (h$^{-1}$)</th>
<th>T$_{1/2}$ (h)</th>
<th>AUC (ng·ml$^{-1}$·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single (5)</td>
<td>0.49</td>
<td>1952</td>
<td>5.21</td>
<td>1.23</td>
<td>0.78</td>
<td>2764</td>
</tr>
<tr>
<td>(50mg/kg)</td>
<td>±0.11</td>
<td>±456</td>
<td>±1.78</td>
<td>±0.31</td>
<td>±0.04</td>
<td>±310</td>
</tr>
<tr>
<td>Multiple (7)</td>
<td>0.58</td>
<td>2433</td>
<td>6.66</td>
<td>1.46</td>
<td>1.25</td>
<td>3054</td>
</tr>
<tr>
<td>(50mg/kg/day)</td>
<td>±0.02</td>
<td>±324</td>
<td>±0.57</td>
<td>±0.23</td>
<td>±0.21</td>
<td>±398</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for the number of animals indicated in parentheses.
gastrointestinal tract and the maximum plasma concentrations were observed about 30 min after the dosing in SHR rats. The drug was quickly eliminated from plasma. Because of rapid absorption and elimination, no residual concentrations were detected in plasma samples obtained from subchronically treated rats before dosing of oxprenolol on the 14th day of experiment.

The plasma oxprenolol concentration-time data were fit to equations describing one- and two-compartment open kinetic models. The appropriate model was selected for each set of data. Pharmacokinetic parameters after a single and multiple oral doses for 14 consecutive days in SHR rats are presented in Table I. Although all the parameters after multiple doses were somewhat larger than those after a single dose, the differences were statistically insignificant ($P > 0.05$).

**DISCUSSION**

An enzyme immunoassay which can reliably determine as little as one ng of oxprenolol has been developed with specific antiserum and the beta galactosidase labelled drug. The sensitivity of the assay is comparable to those of gas-liquid chromatography$^{1-4}$ and thin layer chromatography.$^5$ However, this enzyme immunoassay has the distinct advantages over other methods, in that oxprenolol can be measured directly from a small volume of plasma without the need for extraction and derivative formation. The sensitivity of the assay could be further improved by reducing the total amount of enzyme activity to be added to the incubation mixture.

The sensitivity of an enzyme immunoassay appears to be influenced by the length of spacer arms of bridges through which ligands are conjugated to enzyme and carrier protein molecules as immunogen. Hosoda *et al.* have found that sensitivity of the assay is significantly improved by using beta galactosidase labelled cortisol via a spacer arm with a shorter length than that used for immunogen preparation.$^{12}$ However, they have found no increase in sensitivity when a longer spacer arm is used for conjugation of cortisol to the enzyme.$^{12}$ Thus, in the present study, oxprenolol hemiglutamate and oxprenolol hemisuccinate were used for preparation of the immunogen and the enzyme labelled oxprenolol, respectively.

Several metabolites of oxprenolol have been identified in rats,$^9$ dogs$^{10}$ and man.$^{11}$ Only one metabolite, N-dealkylated oxprenolol was tested for cross-reactivity with the antiserum, because others were not available. However, considering the degree of cross-reactivity of N-dealkylated oxprenolol and chemical structure of the immunogen, ring hydroxylated and O-dealkylated metabolites do not appear to cross-react extensively with the antiserum.

Rapid absorption and clearance of oxprenolol in SHR rats is in agreement with the observations in man.$^{13-15}$ However, apparent elimination half-lives in SHR rats after a single dose and multiple doses (0.78 and 1.25 hour, respectively) are somewhat shorter than those observed in man (1.33, 1.69, 1.94, and 1.98 hour).$^{15}$ This could be due to species difference in elimination process. High peak plasma concentrations observed in SHR rats could be due to less susceptibility of oxprenolol to first pass hepatic elimination. Because of short half-life and rapid elimination, oxprenolol did not accumulate in plasma after multiple doses. This is confirmed by no significant differences in pharmacokinetic parameters after a single dose and multiple administrations.

Plasma concentrations of oxprenolol were well correlated with suppression of stress-induced tachycardia, but dissociated with the hypotensive effect in SHR rats.$^{16}$

In conclusion, a sensitive and specific enzyme immunoassay for oxprenolol has been developed. The assay does not require extraction and derivative formation steps. With this procedure, plasma concentrations of oxprenolol in SHR rats were determined after a single and multiple oral doses. No significant differences in pharmacokinetic parameters were observed between the treatments.

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REFERENCES