ENZYME IMMUNOASSAY OF CYCLAZOCINE USING PEROXIDASE AS LABEL

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A sensitive enzyme immunoassay for the determination of serum or urinary cyclazocine was developed. Horseradish peroxidase was used as the labelling enzyme; it was conjugated with cyclazocine derivatives by the mixed anhydride method. After the immune reaction, bound and free fractions were separated by a double-antibody solid-phase method, using Sepharose 4B gel coupled with purified IgG from goat anti-rabbit IgG serum. The enzyme activity was measured fluorophotometrically, with p-hydroxyphenyl propionic acid and hydrogen peroxide as substrates. The correlation coefficients verified that there was excellent agreement between the results obtained by the new enzyme immunoassay and those of radioimmunoassay (r = 0.92) and gas chromatography-mass spectrometry (r = 0.94).

Keywords—cyclazocine; enzymeimmunoassay; serum cyclazocine level urinary cyclazocine level; cyclazocine-HRP conjugate

INTRODUCTION

Cyclazocine, 3-(cyclopropylmethyl)-1,2,3,4,5,6-hexahydrro-6,11-dimethyl-2,6-methano-3-benzazocine-8-ol, is a synthetic analgesic of the benzomorphan series.1 It is a potent, relatively longacting narcotic antagonist, with a low potential for habituation. At doses as low as 0.1–0.25 mg, cyclazocine effectively alleviates pain in man,2 however, a clinically applicable, sensitive measurement method remained to be developed. Cyclazocine has been measured by thin-layer (TLC)3 and gas chromatography (GC)4 methods, however, their sensitivity was not sufficiently high to determine low serum cyclazocine levels, and required assay time was long. Radioimmunoassays (RIAs) using 3H-dl-cyclazocine5 or 125I-labeled cyclazocine6 have been reported; their sensitivity was 3 ng/ml serum and 20 pg/ml serum, respectively. We previously presented RIA using 3H-cyclazocine for the determination of serum cyclazocine7 and a gas chromatography-mass spectrometry method (GC-MS) using trifluoroacetyl (TFA) derivatives of cyclazocine.8

Although, RIAs are sensitive and simple, they may involve some health hazards and disposal, due to the use of radioisotopes as label, is problematic. The use of enzyme labels instead of radioisotopes for the measurement of antigen, antibodies and haptens has stimulated interest in enzyme immunoassays (EIAs).9 We now report a highly sensitive EIA with peroxidase as label and 3-(p-hydroxyphenyl) propionic acid (HPPA)-H2O2 as substrate, for the determination of serum and urinary cyclazocine.

EXPERIMENTAL

Material and Methods

Cyclazocine and norcyclazocine and its tritiated compounds were supplied by Torii Pharmaceutical Co., Ltd. (Tokyo). Horseradish peroxidase (HRP) (290 U/mg) was purchased from Sigma Chemical Co. (USA). Biogel P-60 and Sepharose 4B were from Bio Rad Laboratories and Seikagaku Kogyo Co., respectively. HPPA was from K & K Co. (USA). Other chemicals were of the highest commercially available purity. 0.05M phosphate buffer (pH 7.0) containing 0.1 % gelatin and 0.9 % NaCl was the assay buffer. Anti-cyclazocine antisera used were prepared as described previously.7 Goat anti-rabbit IgG anti-
serum was generously supplied by Fuji-Zoki Pharmaceutical Co. (Tokyo) and purified by affinity chromatography using Sepharose 4B gel coupled with normal rabbit IgG as previously described.9

HRP-cyclazocine conjugate was prepared as previously described7 by the mixed anhydride method from the cyclazocine derivatives listed in Table I. Cyclazocine-HRP conjugates were purified by gel chromatography on a Bio gel P-60 column eluted with 0.05M phosphate buffer saline (PBS, pH 7.0).

For double antibody solid phase (DASP), the insolubilized second antibody was prepared by the method of Axén et al.,10 using purified goat anti-rabbit IgG antiserum and Sepharose 4B.11 The enzyme immunoassay procedure was as follows:

(1) Standard Procedure: sample solution or standard solution (100 μl) containing 0—1000 pg of cyclazocine was incubated with 500 μl of diluted anti-cyclazocine serum (1:2000) and 500 μl of DASP (1:50) at 4°C overnight, using an immuno-rotor. After adding 50 μl of HRP-cyclazocine conjugate solution (1:100), the mixture was incubated for 3 h at room temperature and then the reaction mixture was centrifuged at 1000×g for 5 min at 4°C. The supernatant was aspirated off, the precipitated DASP was washed twice with 2 ml of saline and HRP activity was measured by the fluorophotometric method described below.

(2) Direct Method for the Determination of Serum dl-Cyclazocine: Human serum samples were assayed as described above, using 300 μl of serum samples and 500 μl of PBS buffer as a blank.

(3) Extraction Method for the Determination of Serum and Urinary dl-Cyclazocine: To measure low cyclazocine concentrations, serum (0.5—2.0 ml) or diluted or undiluted urine samples (1.5 ml) were introduced into extraction

<table>
<thead>
<tr>
<th>Cyclazocine derivative in immunogen</th>
<th>I-1~BSA</th>
<th>I-2~BSA</th>
<th>II-2~BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclazocine derivative in HRP conjugate</td>
<td>Dilution of antiserum</td>
<td>Midpoint</td>
<td>Dilution of antiserum</td>
</tr>
<tr>
<td>I-1~HRP</td>
<td>1000</td>
<td>1.8 ng</td>
<td>2000</td>
</tr>
<tr>
<td>I-2~HRP</td>
<td>1000</td>
<td>−</td>
<td>2000</td>
</tr>
<tr>
<td>II-1~HRP</td>
<td>1000</td>
<td>−</td>
<td>2000</td>
</tr>
</tbody>
</table>

—: no binding.

R1
O
CH3
\[N-R_2\]
\[R_1\]
\[CH_2\]
\[CH_3\]

<table>
<thead>
<tr>
<th>R1</th>
<th>R2</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>-CH_3COOH</td>
<td>I-2</td>
<td>-CH_2CH_2COOH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH_2</td>
</tr>
<tr>
<td>I-2</td>
<td>-CH_2CH_2COOH</td>
<td>I-2</td>
<td>CH_2CH_2CH_2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH_2</td>
</tr>
<tr>
<td>II-1</td>
<td>-CH_3CH_2COOH</td>
<td></td>
<td>-H</td>
</tr>
</tbody>
</table>
tubes (10 × 100 mm), 0.2 ml of 0.1M sodium carbonate buffer (pH 9.8) and 10 ml of isopropyl ether chloroform (7:3, v/v) were added and mixed for 2 min on a Vortex type mixer. The tubes were then centrifuged for 5 min at 1000 × g and an aliquot of the organic phase was transferred to another assay tube. The organic solvent was evaporated to dryness under a nitrogen gas stream, the resulting residue was dissolved by adding 400 μl of assay buffer and 200 μl of the resultant solution were assayed by the standard procedure described above.

Assay of HRP Activity: Assay buffer (0.8 ml), 0.01 % H₂O₂ solution (50 μl) and 0.5 % HPPA solution (50 μl) were added to the washed immunosorbent in the assay tube and mixed well. After 30 min incubation at room temperature, the reaction was stopped by adding 50 μl of 1.25 % KCN solution and 50 μl of 0.5N NaOH solution. After 5 min centrifugation, the fluorescence intensity of the supernatant was measured at an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Peroxidase activity was also measured by the previously described chemiluminescence method. Urinary cyclazocine was assayed according to Tanaka et al.

RESULTS AND DISCUSSION

As the clinical dose of cyclazocine is very small, we set out to develop a highly sensitive EIA for measuring low serum and urinary cyclazocine levels. The sensitivity of EIA depends on various factors, e.g. the quality of anti-serum, the immunoreactivity of the enzyme-antigen conjugate, the order in which the reagent are added and the incubation time. The hapten EIA in particular presents problems not encountered in hapten RIAs using ³H-label or in EIAs of protein antigen and antibody. In RIAs using ³H-label, the affinity of the labeled hapten to the antibody is almost the same as that of unlabeled hapten. On the other hand, in the case of EIAs, the antibody has a higher affinity for the enzyme-labeled than the native hapten, because the structural difference between the compound to be assayed and the enzyme-labeled hapten is always large. In EIAs of steroids, the system in which the same steroid derivatives are used for preparing the steroid-carrier protein and the steroid-enzyme conjugate, is referred to as "homologous" in contrast to the "heterologous" system, in which different derivatives are used. Van Weemen and Schuurs suggested that the sensitivity of EIA for estrogen could be considerably improved if heterologous systems are used and Numazawa et al. and Arakawa et al. made similar suggestions with respect to EIA of estradiol, cortisol and dehydroepiandrosterone. Therefore, site- and bridge-heterologous systems were examined, using various cyclazocine derivatives to devise a highly sensitive EIA for cyclazocine. As shown in Table I, the most sensitive combination was between antibody (I-2), and the bridge-heterologous HRP-conjugate (I-1). Although the homologous HRP-conjugate (I-2) and hapten heterologous HRP-conjugate (II-1) manifested considerably higher affinity for the antibody (I-2) and their midpoints are more than 100 ng/tube. Therefore, the combination of these HRP-conjugates with antibody (I-2) could not be used for the assay. On the other hand, the homologous combination of antibody (I-1) and HRP-conjugate (I-1) was effective, and the other HRP-conjugates (I-2 and

![Graph](image_url)

**FIG. 1. Standard Curves obtained by the Fluorophotometric (●) and Chemiluminescence Method (■)**
II-1) did not bind to antibody (I-1). Based on these results, in the assays we used the combination of antiserum (I-2) and HRP-conjugate (I-1). In previously reported studies, performed in ours (9, 11, 12) and other laboratories (17, 18), the DASP method was employed to separate free and bound fractions after immune reaction, because this method was more sensitive and precise compared with insolubilized first antibody method. Sepharose 4B beads and polystyrene tubes were used as the solid phase. As the DASP-tube method was less sensitive than the DASP-

**TABLE II. Recovery of dl-Cyclazocine added to Serum and Urine**

<table>
<thead>
<tr>
<th>Sample and Method</th>
<th>Added (pg)</th>
<th>Found (pg)</th>
<th>Recovery (%)</th>
<th>n</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (Direct method)</td>
<td>100</td>
<td>117.9</td>
<td>117.9</td>
<td>5</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>224.0</td>
<td>89.6</td>
<td>5</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>355.0</td>
<td>71.0</td>
<td>5</td>
<td>2.7</td>
</tr>
<tr>
<td>Serum (Extraction method)</td>
<td>100</td>
<td>107.2</td>
<td>107.2</td>
<td>5</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>256.5</td>
<td>102.6</td>
<td>5</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>406.5</td>
<td>81.3</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>Urine (Extraction method)</td>
<td>250</td>
<td>252.0</td>
<td>100.8</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>493.0</td>
<td>98.6</td>
<td>5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1077.0</td>
<td>107.7</td>
<td>5</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*Amounts added per assay tube are listed.*

**FIG. 2. Comparison of Urine Cyclazocine Levels determined by EIA and RIA**

\[ Y = 0.84X + 8.33 \]

\[ r = 0.918 \]

\[ n = 24 \]

**FIG. 3. Comparison of Urine Cyclazocine Levels determined by EIA and GC-MS**

\[ Y = 0.98X + 4.8 \]

\[ r = 0.94 \]

\[ n = 49 \]
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Sepharose method, we chose the latter for the new EIA.

Typical standard curves, obtained by using the fluorophotometric and chemiluminescence methods for assaying peroxidase activity bound to DASP-Sepharose, are shown in Fig. 1.

Both methods had almost the same detection limit (ca. 50 pg/assay tube) and the shape of the curves was similar. The coefficients of variation of intraassay in the range of 25–1000 pg/assay tube were 2.2–6.3% for the fluorophotometric, and 2.2–5.3% (n=5) for the chemiluminescence method, respectively. The chemiluminescence method was interfered with various compounds in the serum and urine. Therefore, the fluorophotometric method, using HPPA and H₂O₂ as substrates, was chosen for this study.

The specificity of the heterologous combination between antiserum (I-2) and HRP-conjugate (I-1) was assessed by measuring their cross-reactivity with dl-cyclazocine, d-cyclazocine, l-cyclazocine, nor-cyclazocine, and dl-pentazocine. The percent cross-reactivity was calculated at 50% displacement of dl-cyclazocine-HP conjugate (I-1). Antiserum (I-2) manifested 156% cross-reactivity with d-cyclazocine, 4% with l-cyclazocine, less than 0.5% with dl-nor-cyclazocine and 12.7% with dl-pentazocine.

The mean recovery of dl-cyclazocine from serum and urine samples is shown in Table II.

In serum assays, somewhat better results were obtained with the extraction method than the direct method. The reliability of new EIA was then evaluated by comparing the results obtained by RIA7 and GC-MS8 using urine samples. As shown in Fig. 2 and 3, the EIA results were in excellent agreement with those obtained by the other two methods, the correlation coefficients were 0.92 with RIA and 0.94 with GC-MS. Although the EIA values tended to be slightly higher than those obtained by RIA and GC-MS, this presents no problem in routine cyclazocine assays.

In conclusion, a highly sensitive and practical EIA for the determination of serum and urinary cyclazocine has been developed. The new EIA provides clinicians with a valuable analytical tool for the determination of cyclazocine levels in biological fluids.

REFERENCES


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