Determination of Dihydroergot Alkaloids in Serum by Radioimmunoassay

Shigeru Inada, Kenzo Kajii, Mitsuhiro Takenaga, Masataka Fujita, Kenji Kawamura and Noboru Yanaihara*
Pharmaceutical Development Laboratories, Meiji Seika Kaisha, Ltd.
580, Horikawa-cho, Saiwai-ku, Kawasaki-shi 210, Japan
*Shizuoka College of Pharmacy
2-2-1, Osika, Shizuoka-shi 422, Japan
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A radioimmunoassay for dihydroergotoxine was developed using antiserum from the rabbit injected with a dihydromethylergometrine-bovine serum albumin conjugate. The antiserum was found to be highly specific to dihydroergot alkaloids. The minimum detectable amount of dihydroergotoxine methanesulfonate with this assay system was about 100 pg/ml in serum. Practical applicability of the assay system was proved by measuring dog serum concentrations after oral administration of dihydroergotoxine methanesulfonate.

Key Words: radioimmunoassay, ergot alkaloid, serum concentration

1. Introduction
The methanesulfonate salt of dihydroergotoxine (DHET methanesulfonate), which is a mixture of approximately equal amounts of dihydroergocristine, dihydroergocryptine and dihydroergocornine, is used in the treatment of peripheral and cerebral vascular diseases (Fig. 1). Monitoring of the serum concentration of DHET is required to determine the proper dose for the desired therapeutic effect. There were recently reported several analytical methods for determining blood concentrations of ergot alkaloids: measurement of radioactivity in plasma after administration of tritium-labeled drugs, GC/MS (gas chromatography/mass spectrometry) analysis of plasma extract and radioimmunoassay of blood sample.

We describe in this communication development of a new sensitive radioimmunoassay for DHET and application of the assay system to measurement of dog serum concentrations after oral administration of DHET methanesulfonate.

2. Materials and Methods
2-1 Synthesis of Hapten-bovine serum albumin (BSA) conjugate
Methylergometrine maleate (2.0 g) was hydrogenated over palladium-carbon (0.5 g) in methanol-water (1:1) (200 ml). The mixture was filtered and the filtrate evaporated to give dihydromethylergometrine (2.02 g), 1.93 g of which was refluxed in pyridine (200 ml) with succinic anhydride (1.30 g) for 16 hr. The solution was then evaporated and the residue purified by Sephadex LH-60 column chromatography (500 ml) with methanol as mobile phase. The eluent containing the object substance was evaporated and the residue was recrystallized from methanol to give dihydromethylergometrine hemisuccinate (0.99 g). Triethylamine (0.04 ml) and isobutyl chloroformate (0.02 ml) were added to a solution of dihydromethylergometrine hemisuccinate (0.06 g) in dimethylformamide (2.0 ml) at 10°C and the mixture was stirred for 30 min at the same temperature. The solution was added to a solution of BSA (0.09 g) in a mixture of water (7.2 ml)-dimethylformamide (1.4 ml)-1N sodium hydroxide (0.08 ml) at 0°C. The mixture was stirred overnight at

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Fig. 1 Structures of several ergot alkaloids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>9, 10</th>
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<tr>
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<td>&quot;</td>
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<tr>
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<td>&quot;</td>
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</tr>
<tr>
<td>α-Ergocryptine</td>
<td>CH₂CH(CH₃)₂</td>
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<td>Dihydro</td>
</tr>
<tr>
<td>β-Ergocryptine</td>
<td>CH(CH₃)₂CH₂CH₂</td>
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<td>Dihydro-β-ergocryptine</td>
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<tr>
<td>Ergocornine</td>
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<td>Dihydroergocornine</td>
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</table>

Compounds are shown with their structures.

0°C and then dialyzed in Visking's tube against cold distilled water overnight. The pH of the solution was adjusted to 4.5 with 1N hydrochloric acid to give a white precipitate, which was collected by centrifugation and dissolved in 5% sodium bicarbonate (2 ml). The solution was dialyzed in the same manner and lyophilized to give dihydromethylergometrine-BSA conjugate (0.088 g) as a white powder. The number of dihydromethylergometrine molecules linked to one molecule of BSA was found to be 34 by UV spectrometric analysis.

2.2 Immunization
The dihydromethylergometrine-BSA conjugate (0.1 mg) was dissolved in 0.05M tris buffer pH 7.0 (0.5 ml) and emulsified with an equal volume of complete Freund's adjuvant. The emulsion was injected intracutaneously to 6 rabbits at multiple sites. The injections were given at 2 week intervals for 3 months. The antiserum (MS-NO. 4) used in this study was obtained from one of the rabbits 10 days after the 6th immunization and stored at -20°C.

2.3 Labelled antigen
9,10-3H-Dihydro-α-ergocryptine (New England Nuclear) (1143.3 GBq (30.9 Ci)/mM) was used.

2.4 Radioimmunoassay
The radioimmunoassay was carried out by the delayed addition method. Ethanol (6.0 ml) was added to dog (or human) serum (2.0 ml) containing standard DHET methanesulfonate or to unknown dog (or human) serum sample (2.0 ml) and mixed vigorously for 20 min. The mixture was centrifuged at 3,000 rpm for 20 min. The supernatant (2.0 ml) was evaporated to dryness in the test tube. To the residue were added standard diluent (0.5 ml), i.e. 0.01 M phosphate buffer (pH 7.4) containing 0.5% BSA, 0.025 M ethylenediaminetetraacetic acid and 0.14 M sodium chloride, and antiserum (1,000 fold diluted) (0.1 ml). The mixture was kept at 5°C for 24 hr and then the 3H-labelled antigen (=3,000 cpm/0.1 ml), was added. After 48 hr incubation at 5°C, dextran-coated charcoal in the standard diluent (0.5 ml), comprising Dextran T-70 (0.25 mg) and Norit "SX-II" (2.5 mg) per ml, was added. The mixture was incubated at 5°C for 20 min and then centrifuged at 3,000 rpm for 15 min. The supernatant was separated by decantation and counted with a liquid scintillation counter.

2.5 Characterization of antiserum
Crossreactivities of several ergot alkaloids were tested in the radioimmunoassay system.
The alkaloids examined were dihydroergotoxine methanesulfonate, dihydroergocristine methanesulfonate, dihydroergocryptine methanesulfonate, dihydroergocornine methanesulfonate, ergotoxine methanesulfonate, ergotamine tartrate, ergometrine maleate, methylergometrine maleate and dihydroergotamine tartrate.

2.6 Administration of DHET methanesulfonate tablets to dogs

Six female beagles (8.9–10.1 kg weight) were fasted overnight and each was given a tablet containing 1 mg of DHET methanesulfonate orally. Approximately 10 ml blood samples were taken 0.25, 0.5, 1, 2, 4, 6 and 8 hr afterwards. The blood samples were centrifuged at 3,500 rpm for 15 min and the sera were separated.

3. Results and Discussion

In order to develop a radioimmunoassay for DHET which can monitor the fate of the drug in blood, we first attempted to produce antisera specific to DHET. The haptenic antigen purposely selected was a derivative of the lysergic acid moiety of the alkaloids, methylergometrine, since the 3 components of DHET share the moiety as common structure. The BSA conjugate of the haptenic antigen served as efficient immunogen to produce in the rabbit immunized an antiserum highly sensitive and specific. The antiserum (MS-NO. 4) was able to bind 50% of the labeled antigen added at a final dilution as high as 1:7,000 and was found to recognize the saturated bond between positions 9 and 10 (see Fig. 1). Figure 2 shows the crossreactivities of several ergot alkaloids with the antiserum. Formation of a double bond between 9 and 10 considerably reduced crossreactivity with the antiserum. In fact, ergotoxine and other unsaturated compounds showed only 14–4.6% activities, while dihydroergotamine was almost as active as DHET in this assay system. The result indicated that antiserum (MS-NO. 4) was highly specific to dihydroergot alkaloids.

Interference of blood constituents with antigen-antibody interaction involved in radioimmunoassay could be avoided in the present manipulation by extraction of blood samples with ethanol. For separation of free and bound labeled antigens, the dextran-coated charcoal method was employed in the present assay system, which gave more satisfactory result than the double-antibody method in terms of sensitivity. The minimum detectable dose of DHET methanesulfonate added in dog serum was 100 pg. The intra- and interassay coefficients of variation of the assay system with antiserum (MS-NO. 4) were 3.6–8.6% (n=8) and 6.7–11.6% (n=5), respectively (Table 1), proving satisfactorily high reproducibility.

Table 1 Coefficients of intra- and interassay variations

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<th>Average of C.V.%</th>
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After oral administration of DHET methanesulfonate in dogs, the sera were examined with the radioimmunoassay system. Figure 3 repre-
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Dihydroergotoxine methanesulfonate(ng/ml)

Fig. 3 Standard curve for dihydroergotoxine radioimmunoassay in dog serum.

Fig. 4 Serum concentration of dihydroergotoxine immunoreactivity in dogs after a single oral administration of dihydroergotoxine methanesulfonate 1 mg/Tab. (average of n=6)

presents a standard curve for DHET radioimmunoassay in dog serum. As can be seen in Fig. 4, radioimmunologically measurable materials were found to exist in the sera and the concentration of the immunoreactive materials reached its peak about 1 hr after administration of the drug. The average peak level was 16.7±7.8 ng/ml (mean±S.D., n=6). The concentration was then decreased to 1.0±0.47 ng/ml after 8 hr. Although the immunoreactive materials in the sera were not yet structurally defined, the result supported usefulness of the present assay system to following up the fate in blood of dihydroergot alkaloids administered.

In the present radioimmunoassay, the dose-response curve of DHET methanesulfonate in human serum was also linear in logit-log scale between 39 pg/ml and 10 ng/ml and the ratio of B/Bo was reduced by 10% with 260 pg of DHET methanesulfonate (Fig. 5). This manifested applicability of the present assay system to measurement of immunoreactive materials related to dihydroergot alkaloids in human blood.

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ラジオイムノアッセイによるジヒドロエルゴット
アルカロイド類の血清中濃度測定

稲田 茂，楢井健造，竹長光博，藤田正敬，川村研治，矢内原 昇*
(株)明治製薬薬品開発研究所製剤開発室 210 川崎市幸区堀川町580
*静岡薬科大学生物薬品化学教室 422 静岡市清水2-2-1

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