DETERMINATION OF PROSCILLARIDIN IN PLASMA BY RADIOIMMUNOASSAY

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The development of a sensitive and selective radioimmunoassay for determination of proscillaridin in plasma is described. Antiserum against proscillaridin was obtained from guinea pig immunized with an immunogen prepared by conjugating methylproscillaridin, 14 β -hydroxy-3 β -[(4-*O*-methyl- α -L-rhamnosyl)oxy]bufa-4,20,22-trienolide to bovine serum albumin. Methyl *N*-[3-[(14 β -hydroxybufa-4,20,22-trienolid-3 β -yl)oxy-carbonyl]propanoyl]-L-[3',5'-¹²⁵I₂]diiodotyrosinate ([¹²⁵I] SST) was used as a radioactive ligand. [¹²⁵I] SST and antiserum were added to the recovered sample from plasma using a Bond Elut[®] column. After overnight incubation, the antibody-bound and free [¹²⁵I] SST were separated using polyethylene glycol. The mean coefficients of variation of intra and inter-assay at four different plasma concentrations were 4.0 and 5.0%, respectively. The assay was able to determine as little as 125 pg/ml of proscillaridin in plasma by using 1.5 ml of sample.

Beagle dogs were orally administered one tablet (Talusin[®]) containing 0.25mg of proscillaridin. Proscillaridin was rapidly absorbed, exhibiting plasma maximum concentration of 2.06 ng/ml at 20 min. Thereafter, the plasma level declined biphasically with half-lives of 0.6 and 25.4 h.

Keywords — proscillaridin; radioimmunoassay; Bond Elut[®] column; plasma concentration; beagle dog; proscillaridin cross-reactivity; Talusin[®]

Proscillaridin, 14-hydroxy-3 β -[(α -L-rhamnosyl)oxy]bufa-4,20,22-trienolide is used in the treatment of cardiac failure. The most commonly used method for measuring proscillaridin in plasma is the ⁸⁶Rb-uptake assay of Belz *et al.*,¹⁾ but it is not specific for proscillaridin since inhibition of membrane-ATPase activity brought about by cardiac glycosides and/or their active metabolites interferes with the assay. Thus, the ⁸⁶Rb-uptake assay reflects one of the pharmacodynamic effects produced by the glycosides, and in certain circumstances, also by their metabolites. Belz *et al.*²⁾ have reported that the plasma concentration of proscillaridin by a single oral dose showed double peaks. Similar results have been obtained by Andersson *et al.*³⁾ They have suggested that the substances responsible for the second peak were metabolites with cardioactivity. Although the metabolites of proscillaridin have not been known, metabolic pathways of methylproscillaridin have been reported by Weymann *et al.*⁴⁾ Thus, metabolites of methylproscillaridin were 3 α -scillarenin, 3 β -scillarenin, scillarenon and 12 β -OH-3 α -scillarenin *via* the proscillaridin formed by demethylation. On the basis of the structural

analogy, it may be considered that the metabolites of proscillaridin are the same as methylproscillaridin. The structure of these compounds are shown in Chart 1. The present report describes the development of a sensitive and selective radioimmunoassay for proscillaridin, using a Bond Elut[®] column to separate proscillaridin from four putative metabolites.

EXPERIMENTAL

Chemicals and Reagents — Proscillaridin and 12 β -OH-3 α -scillarenin were provided by Knoll AG Co., Ltd. (Germany). Talusin[®] was provided by Dainippon Pharmaceutical Co., Ltd. (Osaka). 3 α -Scillarenin, 3 β -scillarenin and scillarenon were synthesized from proscillaridin in our laboratories. Bovine serum albumin (BSA) and bovine γ -globulin (BG) were purchased from Sigma Chemical Co., Ltd. (St. Louis, Mo., U.S.A.), Freund's complete adjuvant from Difco Laboratories (Detroit, Mich., U.S.A.), polyethylene glycol (PEG, molecular weight 6000) from Nakarai Chemical Co., Ltd. (Japan), and the Bond Elut[®] column from Analytichem International (Harbor City, CA., U.S.A.). All other chemicals were of analytical reagent grade.

CHART 1. Chemical Structures of Proscillaridin, Methylproscillaridin and Their Metabolites

Preparation of Radioactive Ligand — Radioactive ligand was prepared as shown in Chart 2.

a) 3 β -(3-Carboxypropanoyl)oxy-14 β -hydroxybufa-4,20,22-trienolide: A mixture of 3 β -schillarenin (1 g, 2.6 mmol), succinic anhydride (1.25 g, 12.25 mmol) and anhydrous pyridine (20 ml) was stirred at 110 °C for 4 h. After cooling, the mixture was poured into 100 ml of 10% H₂SO₄ in an ice bath. The resulting precipitates were collected by filtration and washed with water. The precipitates were shaken with a mixture of ethyl acetate (100 ml) and 1.5% Na₂CO₃ (120 ml). The aqueous phase was acidified with 5% HCl and extracted with ethyl acetate. The ethyl acetate extract was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The residue was chromatographed on silica gel column. The fractions eluted with 10–30% acetone-CH₂Cl₂ were collected and the crystals obtained with methylisobutylketone were 3 β -(3-carboxypropanoyl)oxy-14 β -hydroxybufa-4,20,22-trienolide (1.2 g, 97% yield) as colorless prisms, mp 192–194 °C. *Anal.* Calcd for C₂₈H₃₆O₇: C, 69.40; H, 7.49. Found. C, 69.58; H, 7.56. ¹H-NMR (δ in DMSO-*d*₆) 0.64 (3H, s, C₁₈-CH₃), 0.98 (3H, s, C₁₉-CH₃), 2.46 (4H, s, COCH₂CH₂COO), 5.18 (2H, bs, 3- and 4-H), 6.28 (1H, d, *J*=9 Hz, 23-H), 7.50 (1H, m, 21-H), 7.92 (1H, dd, *J*=2, 9 Hz, 22-H).

b) Methyl *N*-[3-[14 β -Hydroxybufa-4,20,22-trienolid-3 β -yl]oxycarbonyl]propanoyl]-L-tyrosinate: Triethylamine (0.1 ml, 0.71 mmol) was added to a solution of 3 β -(3-carboxypropanoyl)oxy-14 β -hydroxybufa-4,20,22-trienolide (339 mg, 0.70 mmol) in anhydrous CH₂Cl₂ (14 ml). The mixture was stirred at room temperature for 50 min and cooled to 0 °C. Isobutyl chloroformate (0.1 ml, 0.77 mmol) was added to the mixture. After stirring at 0 °C for 1.5 h, a solution of methyl-L-tyrosinate (140 mg, 0.72 mmol) in anhydrous tetrahydrofuran (40 ml) was added dropwise to the mixture at 0 °C. After stirring at 0 °C for 1 h, the mixture was stirred at room temperature overnight. The reaction mixture was filtered. The filtrate was evaporated to give a residue which was dissolved in CH₂Cl₂ (50 ml). The CH₂Cl₂ phase was washed with water. The organic phase was dried over anhydrous Na₂SO₄ and evaporated. The residue was purified by means of silica gel column chromatography. The fractions eluted with 7–20% acetone-CH₂Cl₂ were collected and evaporated. From the residue, the crystals obtained with acetone-diethylether-*n*-hexane were methyl *N*-[3-[(14 β -hydroxybufa-4,20,22-trienolid-3 β -yl)oxy-carbonyl]propanoyl]-L-tyrosinate (370 mg, 79% yield) as a colorless crystalline powder, mp 120–122 °C. *Anal.* Calcd for C₃₈H₄₇NO₉.

1/4H₂O: C, 68.50; H, 7.19; N, 2.10. Found. C, 68.46; H, 7.33; N, 2.08. ¹H-NMR (δ in CDCl₃): 0.72 (3H, s, C₁₈-CH₃), 1.02 (3H, s, C₁₉-CH₃), 2.55 (4H, m, COCH₂CH₂CON), 3.03 (2H, d, *J* = 6 Hz, CHCH₂ph), 3.70 (3H, s, COOCH₃), 4.83 (1H, q, t on deuteration, NHCHCH₂ph), 5.24 (1H, bs, 4-H), 5.24 (1H, m, 3-H), 6.17 (1H, d, NH, this proton signal disappeared by addition of D₂O), 6.17 (2H, d, *J* = 8 Hz, aromatic H), 6.25 (1H, d, *J* = 9 Hz, 23-H), 6.74 (2H, d, *J* = 8 Hz, aromatic H), 7.23 (1H, m, 21-H), 7.82 (1H, dd, *J* = 2, 9 Hz, 22-H).

c) [¹²⁵I] SST: [¹²⁵I] SST was prepared by a modification of the chloramine-T method of Oliver⁵) as follows: Two hundred microliters of 0.1 N NaOH containing Na¹²⁵I (1 mCi/ml) and 100 μl of KI solution (2.4 μg/ml) were mixed with 20 μl of 1 N HCl. Then, 50 μl of 8% ethanol solution containing methyl *N*-[3-[(14β-hydroxybufa-4,20,22-trienolid-3β-yl)oxy-carbonyl]propanoyl]-L-tyrosinate (10 μg/ml), 50 μl of 0.5 M phosphate buffer (pH 7.4) and 10 μl of chloramine-T solution (10 mg/ml) were added,

and the mixture was stirred at 0 °C for 5 min. After 5 min, 100 μl of Na₂S₂O₅ solution (1.5 mg/ml) were added. After stirring at 0 °C for 2 min, 450 μl of ethanol were added, and the reaction mixture was passed through a column of Amberlite IRA 400 (500 mg, Cl⁻form). The 50% ethanol eluate having radioactivity was collected. The radiopurity of this fraction was more than 95%.

Preparation of Immunogen — Conjugates of BSA with methylproscillaridin were prepared by a periodate oxidation method^{6,7}) as follows: Two hundred milligrams of methylproscillaridin were dissolved in 14 ml of ethanol. Then, 14 ml of 0.1 M NaIO₄ were added, and the mixture was stirred at 0 °C for 20 min. After 20 min, 0.4 ml of 1 M ethylene glycol was added, and the reaction mixture was added to 370 mg of BSA in 13 ml of water. The mixture was adjusted to pH 9.4 with 5% K₂CO₃ and was stirred at 0 °C for 1 h with dropwise additions of 5% K₂CO₃ to maintain the pH at 9.4. After 1 h, 200 mg of NaBH₄ in 13 ml of H₂O were added, and the

CHART 2. Synthetic Route of [¹²⁵I] SST

reaction mixture was stirred at 0 °C for 3 h. About 6 ml of 1 M formic acid were added to lower the pH to 6.5. After 1 h, 0.7 ml of 1 M NH_4OH was added to raise the pH to 8.5. The mixture was dialyzed overnight against running tap water at 4 °C. Then, the suspension was concentrated to 15 ml by ultrafiltration and the conjugates were passed through a column of Sephadex G-50 and lyophilized to give a fluffy powder. Spectrophotometric analysis of the conjugate of BSA with methylproscillaridin indicated that about 9 mol of methylproscillaridin were covalently coupled to 1 mol of BSA.

Immunization — Three male Hartley guinea pigs, weighing about 400 g, were immunized with the conjugates. Two milligrams of the immunogen were dissolved in 1 ml of saline and emulsified with an equal volume of Freund's complete adjuvant. One milliliter of the emulsion was injected into the guinea pig, intradermally, at several sites on the back and in the foot pads. Booster injections were given once every two weeks for six weeks and then every three weeks for an additional six weeks. Blood was collected by cardiac puncture five days after each injection or after the final booster injection, and the serum was separated and stored at -20 °C.

Antiserum Titer Determination — For antiserum titer determination, 0.05 ml of [^{125}I] SST (2.0×10^4 cpm, 50 pg) solution containing 2% BSA, 0.1 ml of 0.05 M borate buffer (pH 8.6) and 0.05 ml of diluted antiserum ($\times 100$, 500, 1000, 2000, 4000 or 8000) were added to 0.4 ml of buffer solution consisting of methanol and 0.05 M borate buffer (1:10, v/v). The contents of the tube were mixed and incubated at 4 °C overnight (20 to 22 h). After addition of 0.1 ml of 2% BG solution, the antibody-bound [^{125}I] SST was separated from free [^{125}I] SST by adding 0.7 ml of 35% PEG solution. After centrifugation ($1600 \times g$, 20 min), the supernatant fluid was aspirated and the radioactivity in the precipitates was measured in a gamma scintillation spectrometer (Shimadzu, RIAC-200A).

Chromatographic Separation of Proscillaridin — Bond Elut[®] column containing 500 mg of bonded-phase cyanopropyl was dry packed into a 2.8 ml polypropylene column. The column was washed with 6 ml of distilled water followed by 4 ml of chloroform and 6 ml of methanol. The plasma sample (1.5 ml) was mixed in 1.5 ml of 0.05 M borate buffer (pH 8.6) and was passed

through the column, and the column was successively washed thrice with 2 ml of distilled water. When the top of the final water wash reached the solid phase bed, the column was rinsed with 3 ml of solvent consisting of chloroform and *n*-hexane (2:1, v/v) and the effluent was discarded. The column was eluted with 3 ml of solvent which consisted of chloroform and methanol (1:1, v/v). The effluent was collected in a 10-ml centrifuge tube and concentrated by evaporation under a reduced pressure using a centrifugal evaporator (Yamato, Model RD-21).

Assay Procedure — The following stock solutions were prepared for the assay: standard solutions containing 0, 0.125, 0.25, 0.5, 1, 2 and 4 ng of proscillaridin in 1 ml of 0.05 M borate buffer; a solution of [^{125}I] SST (2.0×10^4 cpm/0.05 ml) in 0.05 M borate buffer containing 2% BSA; and antiserum diluted 1:2500 with 0.05 M borate buffer.

One point five milliliters of sample or control plasma were mixed in 1.5 ml of 0.05 M borate buffer and submitted to Bond Elut[®] column chromatography according to the method described for chromatographic separation of proscillaridin. The resultant samples were vortexed first in 0.1 ml of methanol, and then in 1 ml of 2% BSA. The extraction solution, 0.4 ml, was transferred into plastic assay tubes. To the assay tubes, 0.05 ml of [^{125}I] SST (2.0×10^4 cpm, 50 pg) solution containing 2% BSA, 0.1 ml of 0.05 M borate buffer and 0.05 ml of diluted antiserum were added. The procedures, Thereafter, were the same as described for antiserum titer determination.

A standard curve was obtained by a logit-log plot of the relative percentage (B/B_0) of bound [^{125}I] SST against the amount of proscillaridin added. All assays were made in duplicate.

The specificity of the antiserum to proscillaridin and its metabolites was evaluated by determining the amount of each compound needed to inhibit [^{125}I] SST-antibody complex formation by 50%. The cross-reactivity of each compound was expressed as percentage ($X/Y \times 100$) of the 50% inhibitory amount of proscillaridin (X) to the 50% inhibitory amount of the test compound (Y).

Animal Experiments — Six male beagle dogs (body weight 9.0–12.3 kg) were fasted overnight. Each dog received one tablet of Talusin[®] containing 0.25 mg of proscillaridin with 60 ml

of water.

Blood samples were drawn before and at various times after oral administration. Samples were centrifuged immediately to obtain plasma fractions which were stored at -20°C until analyses were performed.

RESULTS

Antibody Titer

A typical antiserum titer curve is shown in Fig. 1. The dilution of the antiserum chosen for the radioimmunoassay was 1:30000 in final dilution, which gave about 43% binding.

Sensitivity of Assay

Figure 2 represents the standard curve obtained with different concentrations in terms of bound radioactivity/bound radioactivity without proscillaridin $\times 100$ versus the logarithm of

the dose. As little as 125 pg/ml of proscillaridin can be determined by this procedure.

Specificity of Assay

The cross-reactions of the antiserum with the four putative metabolites have been studied using the $[^{125}\text{I}]$ SST. The results are shown in Table I. Data are expressed in terms of percentage cross-reactivity. The cross-reactivity of the antiserum with the eluate after chromatographic separation of plasma containing four putative metabolites was found to be less than 0.1%. Thus, the radioimmunoassay was highly specific for proscillaridin and the major metabolites do not interfere with the assay.

FIG. 1. Dilution Curve of Antiserum

The percentage of $[^{125}\text{I}]$ SST bound to the antibody is plotted against the dilution of the antiserum represented on a semilogarithmic scale.

FIG. 2. Standard Curve of Proscillaridin

The relative percentage of $[^{125}\text{I}]$ SST bound to the antibody in the presence of various amount of proscillaridin is plotted on a logit-log scale.

TABLE I. Specificity of Antiserum before and after Chromatographic Separation

Compound	Cross reactivity (%)	
	Before	After
Proscillaridin	100	100
3 α -Scillarenin	93	<0.1
3 β -Scillarenin	109	<0.1
Scillarenon	72	<0.1
12 β -OH-3 α -Scillarenin	16	<0.1

The values are expressed in percentage cross reactivity which is defined as $X/Y \times 100$, where X is the amount of proscillaridin and Y is the amount of the test compound required to produce 50% inhibition of binding of $[^{125}\text{I}]$ SST to the antiserum.

TABLE II. Recoveries at Four Different Plasma Concentrations of Proscillaridin Extracted with Solvent Consisting of Chloroform and Methanol (1:1, v/v) Using a Bond Elut® Column

Concentration (ng/ml)	Recovery (%)
0.13	105.3
0.25	86.8
1.0	80.2
4.0	81.2

Chromatographic Separation

The chromatographic separation between proscillaridin and the metabolites, except for 12 β -OH-3 α -scillarenin, was successfully obtained. The recovery by elution of 3 α -scillarenin, 3 β -scillarenin and scillarenon by a solvent consisting of chloroform and *n*-hexane (2:1, v/v) was approximately 100%. The separation between proscillaridin and 12 β -OH-3 α -scillarenin, however, was not sufficient for a given column. On the other hand, no proscillaridin was eluted in a solvent consisting of chloroform and *n*-hexane but was easily eluted in a solvent consisting of chloroform and methanol (1:1, v/v). The mean recovery at four different plasma concentrations of proscillaridin was about 88% as shown in Table II.

Precision of Assay

Intra and inter-assays of the radioimmunoassay were examined by four repeated determinations at four different plasma concentrations of proscillaridin. The results are shown in Table III. The mean coefficients of variation of intra and

inter-assays were 4.0 and 5.0%, respectively.

Concentration in Plasma Samples

Figure 3 represents the mean plasma concentration-time profile of proscillaridin after oral administration of a Talusin® in fasting dogs. Proscillaridin from the orally administered Talusin® was absorbed rapidly. The mean peak plasma concentration of proscillaridin occurred at 20 min after oral administration and was 2.06 ng/ml. The apparent half-lives of α and β phases, estimated graphically from the mean plasma concentration-time profile, were 0.6 and 25.4 h, respectively.

DISCUSSION

The present paper reports on a sensitive and selective radioimmunoassay method for proscillaridin. The antiserum produced by using the

FIG. 3. Mean Plasma Concentration-Time Curve for proscillaridin after Oral Administration of a Talusin® to Fasting Dogs
Plottings are means $\pm$ S.E. of six dogs.

TABLE III. Precision of Assay

Number of assay	Mean concentration (ng/ml)	Coefficient of variation (%)
1) Intra-assay variation		
4	0.12	2.3
4	0.20	0.9
4	1.04	7.7
4	3.86	5.0
2) Inter-assay variation		
4	0.13	3.7
4	0.23	9.2
4	1.01	4.4
4	3.96	2.6

methylproscillaridin-BSA conjugates showed significant cross-reactivities with four putative metabolites of proscillaridin (3α -scillarenin, 3β -scillarenin, scillarenon and 12β -OH- 3α -scillarenin). However, problems of the cross-reactivity were solved by using a solid-phase extraction column (Bond Elut®) procedure to separate proscillaridin from its putative metabolites (Table I). The chromatographic separation of proscillaridin from metabolites, except for 12β -OH- 3α -scillarenin, was successfully obtained. Although 12β -OH- 3α -scillarenin was insufficiently separated from proscillaridin, the minimal degree for cross-reactivity of 12β -OH- 3α -scillarenin with proscillaridin, in this assay, allows the detection of very small quantities of the proscillaridin. Therefore, the radioimmunoassay has specificity for proscillaridin. The precision of this radioimmunoassay for proscillaridin was good in both intra and inter-assays (Table III). With this procedure, it is possible to determine as little as 125 pg/ml of proscillaridin in plasma by using 1.5 ml of sample.

In dogs, as shown in the present study, proscillaridin was very rapidly absorbed and a double peak of plasma concentration was not found. Thereafter, the plasma level declined biphasically, and the half-lives of elimination, estimated from 20 min to 2 h and from 4 to 24 h, were 0.56 and 25.4 h, respectively.

This method may be useful to investigate the pharmacokinetics of proscillaridin in humans.

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