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Enzyme Immunoassay for Cinobufagin

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A competitive enzyme immunoassay for cinobufagin with alkaline phosphatase-labeled cinobufagin and polyethylene glycol as a precipitant was developed. The anti-cinobufagin antisera produced in rabbits by immunization with cinobufagin 3-hemisuccinate–bovine serum albumin conjugate efficiently recognized 14β,15β-epoxy, 16β-acetoxy and 17β-six-membered unsaturated lactone structures, and therefore showed high affinity for cinobufagin and cinobufotalin. The range of cinobufagin measurable by the enzyme immunoassay was 10—500 pg/tube. Plasma cinobufagin levels of dogs and cats were determined after *i.v.* and *p.o.* administrations.

Keywords——cinobufagin; enzyme immunoassay; alkaline phosphatase; polyethylene glycol; plasma level

Introduction

Cinobufagin is one of the active components extracted from Senso, the dried venom of Chinese toad. It has been proved that cinobufagin shows digitalis-like cardiac action,[1] respiratory-stimulating action and a blood pressure-increasing effect.[2] With regard to the pharmacokinetics of cinobufagin, only data on the binding of plasma protein are available.[3] It is important to develop an assay method with high sensitivity for the study of the pharmacokinetics. An immunoassay was considered to be suitable for the analysis of biological materials because of its high sensitivity and simple pretreatment.

In this paper, we report a competitive enzyme immunoassay (EIA) for cinobufagin with alkaline phosphatase as the label and polyethylene glycol to separate antibody-bound from free compound. The EIA method was applicable to measurement of the plasma cinobufagin levels of dogs and cats after *i.v.* and *p.o.* administrations.

Materials and Methods

Materials——Alkaline phosphatase (ALP) from calf intestine for enzyme immunoassay (grade I) was purchased from Boehringer Mannheim, Mannheim, West Germany, silica gel plates for preparative thin layer chromatography (TLC) (layer thickness, 2 mm) from E. Merck, Darmstadt, West Germany, and bovine serum albumin (BSA) from Sigma Chemical Company, St Louis, U.S.A. Polyethylene glycol 6000 was purchased from Wako Pure Industries, Ltd., and digitoxin from Tokyo Chemical Industry Co., Ltd. All other reagents employed were of analytical grade.

Buffers——Buffer A, 0.05 M phosphate buffer, pH 8.0; buffer B, buffer A containing 0.1% gelatin, 0.9% NaCl and 0.1% NaN_3; buffer C, 0.1 M glycine buffer (pH 10.5) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$; substrate buffer, buffer C containing 1.8 mM *p*-nitrophenyl phosphate.

Cinobufagin Standard Solution——A stock solution of cinobufagin in methanol (2 μg/ml; 0.1 ml) was dried under a stream of nitrogen gas. The residue was dissolved in 20 ml of buffer A and serially diluted.

Preparation of Cinobufagin 3-Hemisuccinate——Cinobufagin (I) (230 mg) was added to a solution of succinic anhydride (800 mg) in the mixture of triethylamine (2 ml) and acetone (15 ml), and the whole was stirred for 10 min then allowed to stand for 5 d at room temperature. After evaporation of the acetone, the residue was diluted with AcOEt,

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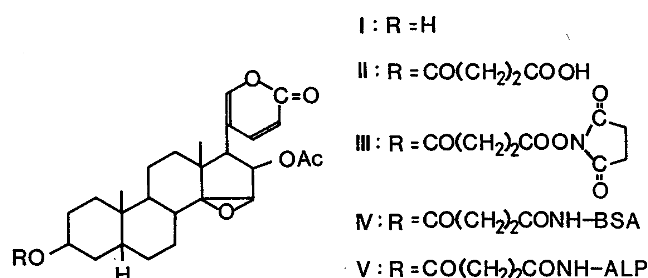

Fig. 1. Chemical Structures of Cinobufagin and Its Derivatives

Cinobufagin (I) is 3β-hydroxy-16β-acetoxy-14β,15β-epoxy-5β-bufa-20,22 dienolide: ring fusion; A/B *cis*, B/C *trans*, C/D *cis*.

and washed with 1 N HCl then with H_2O. The organic layer was dried over anhydrous $MgSO_4$ and evaporated. The residue was dissolved in $CHCl_3$, the solution was filtered, and the filtrate was subjected to preparative TLC using $CHCl_3$–acetone–hexane (3 : 3 : 4) as the developing solvent. The adsorbent corresponding to the spot of cinobufagin 3-hemisuccinate was eluted with EtOH. The eluate was evaporated, the residue was dissolved in $CHCl_3$, and the solution was washed with 0.5 M KH_2PO_4. Evaporation of the $CHCl_3$ layer and recrystallization of the residue from $CHCl_3$–hexane gave cinobufagin 3-hemisuccinate (II) (117 mg). mp 262—264 °C, $[\alpha]_D^{20}$ +1.28 ° (c=0.10, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 2.65 (4H, s, –CO$\underline{H}_2$C$\underline{H}_2$CO–). Other proton signals were assignable to cinobufagin.[4)]

Preparation of Cinobufagin–BSA Conjugate (BSA Conjugate) and Cinobufagin–Alkaline Phosphatase Conjugate (ALP Conjugate)——*N*-Succinimidyl ester of cinobufagin (III), BSA conjugate (IV) and ALP conjugate (V) were prepared by the method described by Hosoda *et al.*[5)]

N-Succinimidyl ester of cinobufagin was coupled with BSA in 50% pyridine and with ALP in 17% dioxane at a molar ratio (hapten/protein) of 30. BSA conjugate was purified by precipitation with acetone and dialysis, then lyophilized. ALP conjugate was stored in buffer A at a concentration of 200 μg per ml at 4 °C after dialysis. It was diluted with 4000 vols. of buffer A containing 0.5% normal rabbit serum and 0.25% BSA just before use.

Immunization of Rabbits——Anti-cinobufagin antiserum was obtained from all three rabbits immunized with BSA conjugate by the procedure described previously.[6)] The antiserum was used as anti-cinobufagin antibody after removal of anti-BSA antibody with BSA–Sepharose 4B. It was diluted with 20000 vols. of buffer B before use.

Procedure for the EIA——All standards and samples were assayed in duplicate.

Diluted antiserum (1 : 20000) (0.1 ml) and diluted ALP conjugate solution (1 : 4000) (0.1 ml) were added to sample solution (0.1 ml) and buffer A (0.1 ml). Standard cinobufagin solution (0.1 ml, 0—500 pg) and buffer B (0.1 ml) were used instead of sample solution and buffer A for the standard curve. The mixture was allowed to stand at 8 °C for 1 h. After addition of 50 μl of normal rabbit serum and 1 ml of 15% polyethylene glycol aqueous solution to each tube, the resulting solution was mixed and centrifuged at 3000 rpm for 20 min. The supernatant was decanted off and the inner wall of the tube was wiped with tissue paper. The precipitate was dissolved in 1 ml of buffer C. The solution was preincubated for 15 min and incubated with 0.5 ml of substrate buffer at 37 °C for 1 h. After addition of 0.25 ml of 1 N NaOH to each tube, followed by centrifugation, the absorbance of the supernatant was measured at 405 nm using a Shimadzu UV-150-02 spectrophotometer.

Measurement of Plasma Cinobufagin Levels——Cinobufagin was administered to dogs and cats intravenously at 0.05 mg/kg as a solution of EtOH–saline (1 : 8) or orally at 0.6 mg/kg as a capsule. Blood was withdrawn at appropriate times and plasma levels were determined by the EIA with or without extraction.

i) Direct Method: Plasma samples after *i.v.* or *p.o.* administration were diluted with 20 or 4 vols. of buffer B, respectively. For the standard curve, cinobufagin-free plasma diluted with buffer B in the same way was used instead of buffer B.

ii) Extraction Method: Ether–AcOEt (9 : 1) (3 ml) was added to 0.5 ml of a plasma sample diluted with buffer B. The mixture was shaken for 10 min and centrifuged at 3000 rpm for 10 min. The organic layer (2.5 ml) was transferred to another test tube and dried under a stream of nitrogen gas. The residue was dissolved by adding 0.3 ml of buffer A, and 0.1 ml of the solution was subjected to the EIA.

Results and Discussion

Cinobufagin 3-hemisuccinate was coupled with BSA and ALP by the *N*-succinimidyl ester method. The number of cinobufagin molecules linked to a BSA molecule was determined to be 19.7 by ultraviolet (UV) spectrophotometric analysis of the BSA conjugate. The anti-cinobufagin antisera were obtained from all three rabbits by immunization with the BSA conjugate. The enzyme activity and the immunoreactivity of the ALP conjugate were 500 U/mg and 46%, respectively.

Polyethylene glycol was used as a precipitant to separate antibody-bound and free

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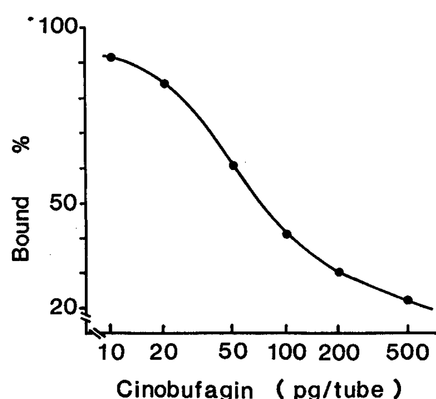

Fig. 2. Standard Curve for Cinobufagin

Bound % is the percentage of bound ALP conjugate with cinobufagin.

TABLE I. Cross Reaction of Bufadienolides

Compound	% cross-reactivity (50%)
Cinobufagin	100
Cinobufotalin	89.7
Bufotalin	0.7
Resibufogenin	0.4
Desacetylcinobufagin	<0.1
3-Epidesacetylcinobufagin	<0.02
Bufalin	<0.002
Gamabufotalin	<0.002

TABLE II. Precision of the EIA

Intra-assay		Inter-assay	
Cinobufagin (pg)	CV[a] (%)	Cinobufagin (pg)	CV[a] (%)
139.0	10.6	120.0	20.1
44.8	17.0	53.3	10.8
23.1	13.8	16.9	44.3

($n=10$)

a) Coefficient of variation.

TABLE III. Recovery of Cinobufagin from Plasma

Added (pg)	Expected (pg)	Found (pg)	Recovery (%)
0	—	28.3	—
50	78.3	86.7	117
100	128.3	138.3	110
200	228.3	218.3	95

($n=3$)

steroid in the EIA with horseradish peroxidase (M_r 40000).[7] Because this separation method is less laborious and much quicker than other methods, polyethylene glycol was applied for the EIA with alkaline phosphatase in the same manner. It was possible to separate antibody-bound ALP conjugate from free ALP conjugate, though the molecular weight of ALP is about 100000.

Figure 2 shows a typical standard curve for the EIA with the diluted antiserum (1 : 20000), the diluted ALP conjugate (1 : 4000) and polyethylene glycol. The percentage of enzyme conjugate bound was plotted against the logarithm of the amount of cinobufagin. With this curve, the amount of cinobufagin could be determined in the range of 10—500 pg/tube.

The cross reactivities of the antiserum with other bufadienolides are shown in Table I. The antiserum showed high affinity for both cinobufagin and cinobufotalin, because the difference in structure between them is only a 5-hydroxy group. Alteration of ring D structure resulted in a substantial decrease of reactivity with the antiserum, as seen with bufotalin, resibufogenin, bufalin and gamabufotalin. Desacetylcinobufagin and 3-epidesacetylcinobufagin (metabolites of cinobufagin in rat plasma)[8] showed negligible cross reactivities. Digitoxin, which is a 3β,14β-dihydroxy-5β-card-20,22-enolide having the same ring fusion as bufadienolide, showed 59% inhibition even at 10 μg as digitoxigenin. Corticosterone, cortisol and cholesterol, which have C/D *trans* structure and no six-membered unsaturated lactone, inhibited the reaction between antiserum and ALP conjugate by 35, 20 and 0%, respectively, even at 10 μg. Thus, the antiserum was specific for 14β,15β-epoxy, 16β-acetoxy and 17β-unsaturated lactone structures, which are apart from the binding site with BSA at C-3.

Three plasma samples containing various cinobufagin levels were examined to assess intra- and inter-assay variations in the EIA. The intra- and inter-assay coefficients of variation

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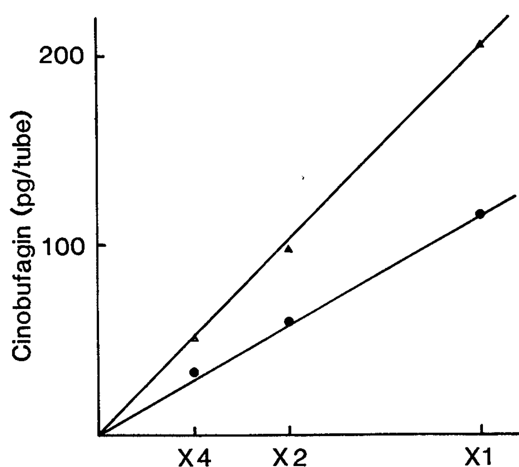

Fig. 3. Dilution Curves for Plasma

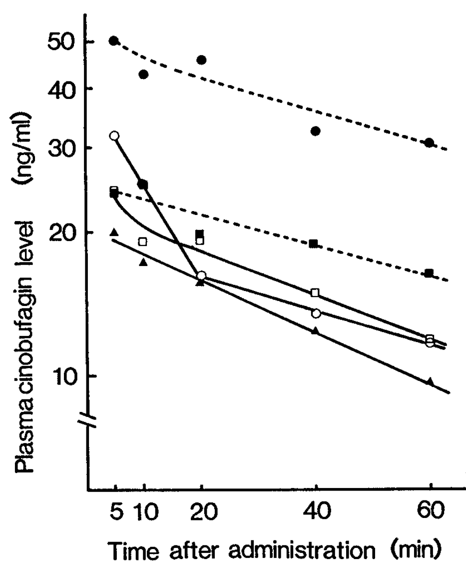

Fig. 4. Time Courses of Cinobufagin Levels in Plasma of Dogs and Cats after *i.v.* Administration at 0.05 mg/kg

----, dog; ——, cat; by the direct method.

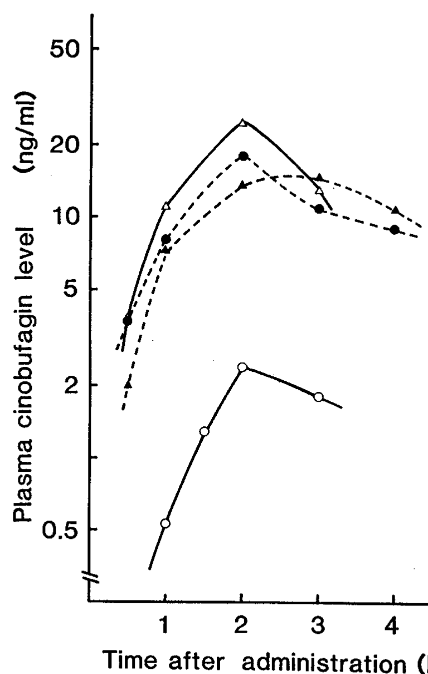

Fig. 5. Time Courses of Cinobufagin Levels in Plasma of Dog and Cat after *p.o.* Administration at 0.6 mg/kg

●---●, dog; ○—○, cat; by the direct method.
▲---▲, dog; △—△, cat; by the extraction method.

were 10.6—17.0% ($n=10$) and 10.8—44.3% ($n=10$), respectively (Table II).

The percent recovery was determined by adding known amounts of cinobufagin to a plasma sample containing cinobufagin. As shown in Table III, the percent recoveries were 117, 110 and 95% ($n=3$) at 50, 100 and 200 pg of cinobufagin added, respectively. The effect of dilution was investigated to assess the accuracy of the EIA. Plasma samples containing a known amount of cinobufagin were diluted with 20 vols. of buffer B and then serially diluted with diluted cinobufagin-free plasma (1 : 20). As shown in Fig. 3, linear relationships were obtained.

Figure 4 shows the time courses of cinobufagin (including its metabolites) levels in plasma of dogs and cats after *i.v.* administration. These levels were determined by the direct method. No work on the metabolism of cinobufagin in the dog or cat has been reported. Only desacetylcinobufagin and 3-epidesacetylcinobufagin were identified as metabolites of cinobu-

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fagin in rat plasma.[8] However, from a study of digitoxigenin,[9] 3-keto, 5β-hydroxy and 6β-hydroxy derivatives of cinobufagin (or desacetylcinobufagin) can be speculated to be metabolites in addition to desacetylcinobufagin and 3-epidesacetylcinobufagin. The extent to which metabolites may be included in the apparent cinobufagin level in plasma is dependent on their cross-reactivities. However, desacetylcinobufagin and 3-epidesacetylcinobufagin show low cross-reactivities. Their existence in plasma is likely to be negligible. The plasma levels determined by the EIA with extraction were 52—120% of those determined by the direct method. The possible existence of metabolites with high polarity which are not extracted with ether–AcOEt (9 : 1) cannot be ruled out.

The cinobufagin levels in plasma of dog and cat after *p.o.* administration were determined by the direct and extraction method (Fig. 5). Dog plasma levels determined by the extraction method were 54—135% of those determined by the direct method. When cat plasma levels were determined by the direct method, however, they were very low. Furuta[3] reported that cinobufagin binds to plasma proteins (mainly albumin) and suggested that the binding sites of cinobufagin were likely to be the 17β-unsaturated lactone and 14β,15β-epoxide moieties. It was suggested that the main metabolite in plasma after *p.o.* administration has a higher affinity for cat plasma protein than for anti-cinobufagin antibody, but is easily extracted from the protein-metabolite complex by an organic solvent.

Though it is necessary to obtain detailed knowledge of the metabolites, the present EIA should be useful as an assay method in the study of the pharmacokinetics of cinobufagin because of its high sensitivity and specificity.

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