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Preparation of Specific Antisera to Digoxin Using Hapten-[C-17]- and [C-12]-Bovine Serum Albumin Conjugates[1)]

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The preparation and antigenic properties of etianic acid-[C-17]- and digoxin-[C-12]-bovine serum albumin (BSA) conjugates are described. The antisera raised against the conjugate having an [O-[N-(carboxymethyl)carbamoyl]methyl]oxime bridge at C-12 in rabbits were highly specific to digoxin, exhibiting no significant cross-reactions with digoxigenin, or its bis- and mono-digitoxosides. The specificity of antisera elicited is discussed in relation to the coupled position of the hapten with BSA.

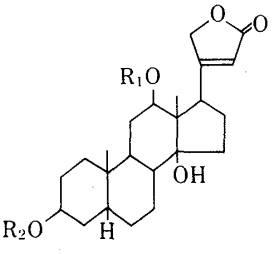
Keywords——digoxin; radioimmunoassay; anti-digoxin antiserum; etianic acid-[C-17]-BSA conjugate; digoxin-[C-12]-BSA conjugate

Cardiac glycosides obtained from various plant sources, notably digoxin (Ia) from digitalis leaf, are widely used for the treatment of congestive heart failure and of various disturbances of cardiac rhythym. The clinical use of this valuable, potentially dangerous, therapeutic agent is complicated by the fact that individual patients vary considerably both in the dosage required to produce a beneficial therapeutic response and in their sensitivity to the toxic effects of this drug. The ability to determine serum digoxin concentrations by radioimmunoassay has proved to be of considerable value in controlling the treatment of patients maintained on this cardiac glycoside and in the diagnosis of digoxin intoxication.[2,3)] The antibodies were elicited by immunization with the cardenolide-bovine serum albumin (BSA) conjugate. The immunogens were prepared by coupling either the 3-hydroxyl group of the aglycone[4)] or the sugar moiety of the glycoside to BSA.[5)] The antisera exhibited appreciable cross-reactivities with principal metabolites of digoxin (digoxigenin, its mono- and bis-digitoxosides and other related conjugates) having lower or no cardiotonic activities.[2,3)] Of the synthetic steroid hormones, spironolactone and canrenone were reported to exhibit cross-reaction with antisera to digoxin to some extent.[6)] The lack of methodological reliability for the monitoring of blood level may, thus, lead to clinical problems in the diagnosis of digoxin intoxication.

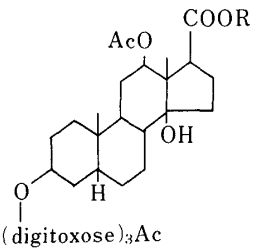
In the preceding papers,[7-9)] we reported the preparation of specific antisera to estrogen glucuronides by immunization with hapten-BSA conjugates having a bridge remote from the glucuronoside linkage. In the light of these results, the use of 3β,12β,14β-trihydroxy-5β,17β-etianic acid 3-tridigitoxoside-[C-17]- or digoxin-[C-12]-BSA conjugate appeared to be promising for obtaining specific antisera to digoxin. The present paper deals with the preparation and antigenic properties of the hapten-BSA conjugates in which the steroid is linked to the carrier through a bridge at C-17 or C-12 on the steroid nucleus.

Our effort was initially directed to the preparation of 3β,12β,14β-trihydroxy-5β,17β-etianic acid 3-tridigitoxoside-[C-17]-BSA conjugate. Digoxin pentaacetate (Ib)[10)] derived from digoxin (Ia) was taken as a starting compound. Oxidation of Ib with potassium permanganate in the usual manner[11)] provided the etianic acid derivative (IIa), which, on

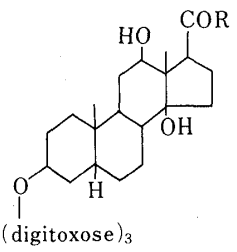
alkaline hydrolysis, was led to 3β,12β,14β-trihydroxy-5β,17β-etianic acid 3-tridigitoxoside (IIIa). Previously, we obtained antisera highly specific to estrogen glucuronides by immunization with hapten-BSA conjugates having a bridge of appropriate length.[12] Based upon these findings, the bridge in IIIa was further extended by coupling with glycine employing the mixed anhydride method. The resulting glycinate (IIIc), IIa and IIIa were each treated with diazomethane to give the methyl esters (IIb, IIIb and IIId), and their structures were confirmed by the usual criteria. Condensation of haptens (IIIa, IIIc) with BSA was readily attained by the mixed anhydride method, providing the [C-17]-BSA conjugates (IVa, IVb).



Ia : R_1=H, R_2=(digitoxose)$_3$
Ib : R_1=Ac, R_2=(digitoxose)$_3$Ac

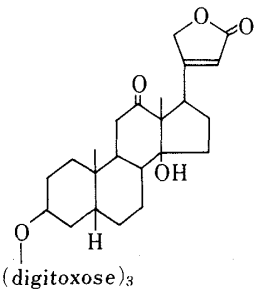


IIa : R=H
IIb : R=CH_3



IIIa : R=OH
IIIb : R=OCH_3
IIIc : R=gly·OH
IIId : R=gly·OCH_3

IVa : R=BSA
IVb : R=gly·BSA



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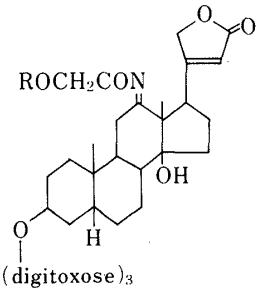

VIa : R=OH
VIb : R=OCH_3
VIc : R=gly·OH
VId : R=gly·OCH_3

VIIa : R=BSA
VIIb : R=gly·BSA

Chart 1

Next, our efforts were directed to the preparation of digoxin-[C-12]-BSA conjugate. 12-Dehydrodigoxin (V), obtainable from digoxin in three steps,[13] was treated with carboxymethoxylamine at 60 °C for 7 days to give the oxime (VIa). The glycine conjugate (VIc) was also prepared from VIa by the mixed anhydride method. Condensation of VIa and VIc with BSA was carried out in the same way as described for IVa. Spectrophotometric analysis revealed that a satisfactory number (>10) of steroid hapten molecules were incorporated into each BSA molecule.

Each immunogen thus obtained was used for producing antibodies in three rabbits. Several months after an initial injection of the antigen, some of the immunized rabbits produced antibodies exhibiting increased binding activity to digoxin. The preliminary test indicated that one of the three rabbits in each group immunized with IVa, IVb, or VIIa yielded anti-digoxin antisera of higher titer. In contrast, all the antisera raised against VIIb in three rabbits showed higher titer, and no substantial difference in affinity and specificity was observed among the antisera. Evaluation of the titer was carried out by incubating various

TABLE I. Per Cent Cross-Reactions of Anti-digoxin Antisera Raised against Hapten-BSA Conjugates with Selected Steroids

Steroid	% cross-reactivity (50%)			
	Hapten-BSA conjugate			
	IVa	IVb	VIIa	VIIb
Digoxin	100.0[a)]	100.0	100.0	100.0
Dihydrodigoxin	100.0	100.0	92.5	65.3
Digoxigenin bisdigitoxoside	23.5	8.0	15.1	0.6
Digoxigenin monodigitoxoside	1.4	0.3	0.3	0.2
Digoxigenin	0.04	0.01	0.01	<0.01
4'''-O-Methyldigoxin	0.06	<0.01	<0.01	<0.01
4'''-O-Acetyldigoxin	<0.01	<0.01	<0.01	<0.01
Digitoxin	37.5	42.5	32.6	29.1
Digitoxigenin bisdigitoxoside	22.7	5.0	11.1	0.6
Digitoxigenin monodigitoxoside	1.3	0.3	0.3	<0.01
Digitoxigenin	0.02	0.01	0.01	<0.01
Spironolactone	<0.01	<0.01	<0.01	<0.01
Canrenone	<0.01	<0.01	<0.01	<0.01

a) Values are calculated on a molar basis.

dilutions of antiserum with a constant amount of ^{3}H-labeled digoxin. The dilution of antiserum which was able to bind 50% of the labeled antigen was defined as a titer. The binding affinity was determined by incubating constant amounts of antiserum and labeled antigen with increasing amounts of the nonlabeled antigen. The ratio of bound to free antigen was plotted against the concentration of bound antigen. According to the Scatchard analysis,[14] the association constants of anti-digoxin antisera raised against IVa, IVb, VIIa, and VIIb were calculated to be 7.9×10^8, 3.4×10^8, 1.2×10^9, and 3.8×10^9 M^{-1}, respectively. The dose-response curves were constructed with 1 : 8000, 1 : 4000, 1 : 1000, and 1 : 8000 final dilutions of antisera. The plot of per cent bound radioactivity *vs.* logarithm of the amount of digoxin showed a linear relationship over the range of 250 pg to 5000 pg for antisera elicited with IVa and IVb, while the plot was linear over the range of 65 pg to 1000 pg for antisera elicited with VIIa and VIIb. These data showed that the 17β-butenolide ring of digoxin and an appropriate length of spacer between hapten and carrier may be necessary to obtain anti-digoxin antiserum of high affinity.

The specificity of antiserum was assessed by testing the ability of closely related compounds to compete for binding sites on the antibody. The per cent cross-reactions determined by the method of Abraham[15] with four antisera are collected in Table I. The antiserum elicited with VIIb possessed the highest specificity, exhibiting only minor cross-reactions with digoxigenin-bisdigitoxoside (0.6%) and -monodigitoxoside (0.2%), digoxigenin (<0.01%) and 4'''-substituted digoxin (<0.01%). Spironolactone and canrenone showed no significant cross-reactivities. To the best of our knowledge this is the first reported specific antiserum that is capable of recognizing the sugar moiety of digoxin. The marked cross-reaction with digitoxin (29.1%), that is 12-deoxydigoxin, can be reasonably explained on the basis of the structure of the hapten-BSA conjugate, having a linkage through the C-12 position. Dihydrodigoxin, one of the urinary metabolites formed from digoxin by gastrointestinal bacteria,[16] also showed considerable cross-reaction (65.3%). It is evident from the data that the antiserum is highly specific to the sugar part but not to the steroid moiety. With the aim of obtaining a more specific antiserum, a further detailed study on the design of a hapten-

BSA conjugate involving the linked position and the length of a spacer is to be undertaken.

Development of a simple and reliable radioimmunoassay system for digoxin in biological fluids using specific antiserum will be the subject of a future communication.

Experimental

Chemicals and Reagents——[12α-^{3}H]-Digoxin (13.0 Ci/mmol) was supplied by New England Nuclear (Boston, MA), and the radiochemical purity was checked by thin-layer chromatography (TLC) prior to use. Digoxin was kindly donated by Chugai Pharmaceutical Co. (Tokyo, Japan), and spironolactone and canrenone by Dr. S. Görög (Gedeon Richter Ltd., Budapest, Hungary). 4'''-*O*-Methyldigoxin (Lanirapid) was purchased from Yamanouchi Pharmaceutical Co. (Tokyo, Japan), BSA fraction V from Sigma Chemical Co. (St. Louis, MO), Amberlite XAD-4 resin from Rohm and Haas Co. (Philadelphia, PA), and digitoxin and complete Freund's adjuvant from Nakarai Chemicals Ltd. (Kyoto, Japan). Other cardiac steroids were prepared in this laboratory by known methods.[17]

Synthesis of Hapten-BSA Conjugates

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO model DIP-4 automatic polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded using tetramethylsilane as an internal standard on a JEOL Model FX-100 spectrometer at 100 MHz (^{1}H). Abbreviations used: s=singlet and m=multiplet. Silica gel 60 HF_{254} (E. Merck AG, Darmstadt) was used for preparative TLC and silica gel 60 (70—230 mesh) (E. Merck AG) for column chromatography.

12β-Acetoxy-3β,14β-dihydroxy-5β,17β-etianic Acid 3-Triacetyldigitoxoside (IIa)——A solution of digoxin pentaacetate (Ib)[10] (100 mg) in acetone (5 ml) was treated with $KMnO_4$ (40 mg), and the whole was allowed to stand at room temperature for 12 h. After addition of oxalic acid to decompose the excess $KMnO_4$ followed by removal of the resulting precipitate by filtration, the filtrate was extracted with ethyl acetate. The organic layer was washed with 5% $NaHSO_3$ and water, dried over anhydrous Na_2SO_4, and evaporated down. The residue was subjected to chromatography on a silica gel column (30 cm × 1 cm i.d.). Elution with chloroform–methanol (10:1) and recrystallization of the product from ether–hexane gave IIa (10 mg) as a colorless amorphous substance. mp 216—220 °C. $[\alpha]_D^{16}$ +40.0° (c=0.10, chloroform–methanol (1:1)). *Anal.* Calcd for $C_{48}H_{72}O_{19}\cdot H_2O$: C, 59.37; H, 7.68. Found: C, 59.04; H, 7.33. NMR (CD_3OD–$CDCl_3$) δ: 0.95 (6H, br s, 18-, 19-CH_3), 1.15 (9H, m, sugar-CH_3), 2.00 and 2.10 (3H and 12H, each s, 12-, sugar-$OCOCH_3$).

In order to elucidate the structure, IIa (5 mg) was dissolved in methanol (1 ml) and treated with ethereal diazomethane. Usual work-up gave the methyl ester (IIb) (3 mg) as a colorless oily substance. NMR ($CDCl_3$) δ: 0.92 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.25 (9H, m, sugar-CH_3), 2.00 and 2.10 (3H and 12H, each s, 12-, sugar-$OCOCH_3$), 3.75 (3H, s, $COOCH_3$).

3β,12β,14β-Trihydroxy-5β,17β-etianic Acid 3-Tridigitoxoside (IIIa)——Compound IIa (15 mg) in methanol (1.5 ml) was treated with 10% methanolic NaOH (0.5 ml) at room temperature for 12 h. The reaction mixture was diluted with water (10 ml) and concentrated under reduced pressure. The resulting solution was percolated through an Amberlite XAD-4 column (20 cm × 0.6 cm i.d.). The column was washed with water, then elution with methanol (100 ml) containing a drop of conc. NH_4OH gave IIIa (10 mg) as a colorless oily substance.

In order to elucidate the strucrure, IIIa was methylated with ethereal diazomethane in the manner described for IIb to give the methyl ester (IIIb) as a colorless oily substance. NMR ($CDCl_3$–CD_3OD) δ: 0.90 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 1.25 (9H, m, sugar-CH_3), 3.70 (3H, s, $COOCH_3$). Treatment of IIIb with acetic anhydride and pyridine in the usual manner gave IIb.

3β,12β,14β-Trihydroxy-5β,17β-etianoylglycine 3-Tridigitoxoside (IIIc)——Compound IIIa (12 mg) in dimethylformamide (0.8 ml) was treated with tri-*n*-butylamine (12 μl) and isobutyl chloroformate (6 μl) under ice-cooling, and the whole was then allowed to stand at 4 °C for 20 min. This mixture was added to an aq. solution of glycine (40 mg) (pH 10; adjusted with 0.1 N NaOH, 1 ml), and the whole was allowed to stand at 4 °C for 12 h. The solution was extracted with ether, and the aq. layer was percolated through an Amberlite XAD-4 column (20 cm × 0.6 cm i.d.). The column was washed with water, then the desired compound was eluted with methanol (100 ml) containing a drop of conc. NH_4OH. The eluate was subjected to preparative TLC using chloroform–methanol–water (80:20:2.5) as a developing solvent. Elution of the adsorbent corresponding to the spot (Rf 0.10) with chloroform–methanol–water (70:30:3) and recrystallization of the product from methanol–ether gave IIIc (5.5 mg) as a colorless amorphous substance. mp 180—184 °C. $[\alpha]_D^{12}$ +15.0° (c=0.10, methanol). Compound IIIc is unstable and no sample for analysis could be obtained.

In order to elucidate the structure, IIIc was methylated with ethereal diazomethane in the manner described for IIb to give the methyl ester (IIId) as a colorless oily substance. NMR (CD_3OD–$CDCl_3$) δ: 0.90 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 1.30 (9H, m, sugar-CH_3), 3.70 (5H, s, $COOCH_3$, NCH_2CO).

Etianic Acid Tridigitoxoside Derivative-BSA Conjugates (IVa, IVb)——Tri-*n*-butylamine (12 μl) and isobutyl chloroformate (8 μl) were added to a solution of IIIa or IIIc (7 mg) in dimethylformamide (0.5 ml) under ice-cooling, and the whole was stirred for 30 min. This mixture was added to an aq. solution of BSA (15 mg) (pH 10; adjusted with

0.1 N NaOH, 0.5 ml), and the whole was then stirred at 4 °C for 12 h. The resulting solution was dialyzed against cold running water for 48 h. Lyophilization of the solution gave IVa (21.5 mg) or IVb (20 mg) as a fluffy powder. The molar steroid: protein ratios of IVa and IVb were determined to be 20 and 25, respectively, by spectrometric analysis using sulfuric acid as a coloring reagent.[3]

12-Dehydrodigoxin 12-(*O*-Carboxymethyl)oxime (VIa)——(*O*-Carboxymethyl)hydroxylamine·1/2HCl (17 mg) and a solution of sodium acetate (24 mg) in water (0.2 ml) were added to a solution of 12-dehydrodigoxin (V)[13] (17 mg) in ethanol (2 ml), and the whole was allowed to stand at 60 °C for a week. The resulting solution was diluted with water and extracted with ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4, and evaporated down under reduced pressure. The oily residue obtained was subjected to preparative TLC using chloroform–methanol–water (80 : 20 : 2.5) as a developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.33) with chloroform–methanol–water (80 : 20 : 2.5) and recrystallization of the product from methanol–ether gave VIa (4 mg) as a colorless amorphous substance. mp 200—205 °C. $[\alpha]_D^{16}$ +57.5° (*c*=0.20, methanol). NMR ($CDCl_3$–CD_3OD) δ: 1.00 (3H, s, 19-CH_3), 1.05 (3H, s, 18-CH_3), 1.25 (9H, m, sugar-CH_3), 4.95 (5H, m, 21-CH_2, anomeric-H), 5.90 (1H, br s, 22-H). Compound VIa is unstable and no sample for analysis could be obtained.

In order to elucidate the structure, VIa was methylated with ethereal diazomethane in the manner described for IIb. Recrystallization of the product from methanol–ether gave the methyl ester (VIb) as colorless needles. mp 138—140 °C. $[\alpha]_D^{16}$ +70.0° (*c*=0.05, chloroform–methanol (1 : 1)). *Anal.* Calcd for $C_{44}H_{67}NO_{16}\cdot 3/2H_2O$: C, 59.18; H, 7.90; N, 1.57. Found: C, 59.04; H, 7.57; N, 1.53. NMR ($CDCl_3$) δ: 0.98 (3H, s, 19-CH_3), 1.05 (3H, s, 18-CH_3), 1.25 (9H, m, sugar-CH_3), 3.75 (3H, s, $COOCH_3$), 4.60 (2H, s, $NOCH_2$), 4.82 (5H, m, 21-CH_2, anomeric-H), 5.90 (1H, s, 22-H).

12-Dehydrodigoxin 12-[*O*-[*N*-(Carboxymethyl)carbamoyl]methyl]oxime (VIc)——Compound VIa (10 mg) in dioxane (1 ml) was treated with triethylamine (20 μl) and ethyl chloroformate (14 μl) under ice-cooling, and the whole was allowed to stand at 4 °C for 30 min. After addition of an aq. solution of glycine (20 mg) (pH 8; adjusted with 0.1 N NaOH, 0.4 ml), the whole was allowed to stand at 4 °C for 12 h, then diluted with water (10 ml) and extracted with ether. The aq. layer was percolated through an Amberlite XAD-4 column (20 cm × 0.6 cm i.d.). The column was washed with water, then elution with methanol gave VIc (6 mg) as a colorless oily substance. NMR ($CDCl_3$–CD_3OD) δ: 1.00 (3H, s, 19-CH_3), 1.05 (3H, s, 18-CH_3), 1.30 (9H, m, sugar-CH_3), 4.50 (2H, br s, $NOCH_2$), 4.90 (5H, br s, 21-CH_2, anomeric-H), 5.95 (1H, br s, 22-H).

In order to elucidate the structure, VIc was methylated with ethereal diazomethane in the manner described for IIb. Recrystallization of the product from methanol–ether gave the methyl ester (VId) as a colorless amorphous substance. mp 136—140 °C. $[\alpha]_D^{19}$ +40.0° (*c*=0.08, chloroform–methanol (1 : 1)). NMR ($CDCl_3$) δ: 1.00 (3H, s, 19-CH_3), 1.07 (3H, s, 18-CH_3), 1.25 (9H, m, sugar-CH_3), 3.70 (3H, s, $COOCH_3$), 4.00 (2H, m, NCH_2CO), 4.55 (2H, s, $NOCH_2$), 4.85 (5H, m, 21-CH_2, anomeric-H), 5.95 (1H, br s, 22-H). Compound VId is unstable and no sample for analysis could be obtained.

12-Dehydrodigoxin Derivative-BSA Conjugates (VIIa, VIIb)——A solution of VIa or VIc (12 mg) in dioxane (1 ml) was treated with triethylamine (30 μl) and ethyl chloroformate (20 μl) under ice-cooling, and the whole was stirred for 1 h. This mixture was added to an aq. solution of BSA (25 mg) (pH 8; adjusted with 0.1 N NaOH, 1.0 ml), and the whole was then stirred at 4 °C for 12 h. The resulting solution was dialyzed against cold running water for 48 h. Lyophilization of the solution gave VIIa (25 mg) or VIIb (39 mg) as a fluffy powder. The molar steroid: protein ratios of VIIa and VIIb were determined to be 15 and 11, respectively, by spectrometric analysis using sulfuric acid as a coloring reagent.

Immunization of Rabbits——Three male albino rabbits were used for immunization with each hapten-BSA conjugate. The antigen (0.5—1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into rabbits subcutaneously at multiple sites over the back and foot pads. The procedure was repeated at intervals of two weeks for a further 2 months and then once a month. The rabbits were bled 10 d after the booster injection. The sera were separated by centrifugation at 3000 rev./min for 10 min and stored at −20 °C. The antisera were thawed and diluted with phosphate saline buffer (0.15 M NaCl, 0.008 M K_2HPO_4 and 0.002 M NaH_2PO_4) (pH 7.4) containing 0.1% BSA for assay.

Measurement of Radioactivity——The samples were counted on a Beckman model LS 7000 liquid scintillation spectrometer employing toluene-Triton X-100 (tT 21) scintillant.[18]

Assay Procedure——A standard curve was constructed by setting up duplicate centrifuge tubes (10 ml) containing 0, 65, 125, 250, 500, 1000, 2000, and 5000 pg of non-labeled digoxin in assay buffer (0.1 ml) and ^{3}H-labeled digoxin (203 pg, 7500 dpm) in assay buffer (0.1 ml). After addition of assay buffer (0.1 ml) and the diluted antiserum (0.1 ml), the whole was incubated at 4 °C for 2 h. After addtion of dextran (0.05%)-coated charcoal (0.5%) in assay buffer (0.4 ml), the mixture was centrifuged at 3000 rev./min for 15 min. A 0.6 ml aliquot of each supernatant was transferred into a counting vial containing a scintillation cocktail (5 ml) and the radioactivity was counted.

Cross-Reaction Studies——The specificity of the antiserum obtained was tested by cross-reaction studies with 12 kinds of related steroids. The relative amounts required to reduce the initial binding of ^{3}H-labeled digoxin by half, where the mass of non-labeled digoxin was arbitrarily taken as 100%, were calculated by the standard curve.

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References and Notes

1) Part CIC of "Studies on Steroids" by T. Nambara; Part CLXXXXVIII: S. Miyairi, H. Shioya, M. Ebihara, H. Hosoda and T. Nambara, *Chem. Pharm. Bull.*, **32**, 1891 (1984).
2) S. Görög, "Quantitative Analysis of Steroids," Akademiai Kiado, Budapest, 1983, p. 372.
3) V. P. Butler, Jr. and D. Tse-Eng, "Methods in Enzymology," Vol. 84, ed. by S. P. Colowick and N. O. Kaplen, Academic Press, New York, 1982, p. 558.
4) G. C. Oliver, Jr., B. M. Parker, D. L. Brasfield and C. W. Parker, *J. Clin. Invest.*, **47**, 1035 (1968).
5) V. P. Butler, Jr. and J. P. Chen, *Proc. Nat. Acad. Sci.*, **57**, 71 (1967).
6) R. W. Thomas and R. R. Maddox, *Ther. Drug Monitor.*, **3**, 117 (1981).
7) T. Nambara, M. Numazawa, T. Tanaka and T. Ohkubo, *J. Pharm. Dyn.*, **1**, 55 (1978).
8) T. Nambara, M. Numazawa, T. Tanaka and T. Ohkubo, *J. Steroid Biochem.*, **9**, 785 (1978).
9) T. Nambara, K. Shimada, T. Ohkubo and T. Niwa, *J. Steroid Biochem.*, **16**, 533 (1982).
10) H.-W. Voigtländer and G. Balsam, *Arch. Pharm.*, **301**, 208 (1968).
11) O. Schindler, *Helv. Chim. Acta*, **39**, 1698 (1956).
12) T. Nambara, T. Ohkubo and K. Shimada, *J. Pharm. Dyn.*, **6**, 692 (1983).
13) A. V. Wartburg, F. Kalberer and J. Rutschmann, *Biochem. Pharmacol.*, **14**, 1883 (1965).
14) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
15) G. E. Abraham, *J. Clin. Endocr. Metab.*, **29**, 866 (1969).
16) J. F. Dobkin, J. R. Saha, V. P. Butler, Jr., H. C. Neu and J. Lindenbaum, *Science*, **220**, 325 (1983).
17) D. Satoh and K. Aoyama, *Chem. Pharm. Bull.*, **18**, 94 (1970).
18) M. S. Patterson and R. C. Greene, *Anal. Chem.*, **37**, 854 (1965).