[Chem. Pharm. Bull. 26(11)3347—3352(1978)]

UDC 612.017-08 : 547.586.2.09

Preparation of Specific Antibodies to Catecholamines and L-3,4-Dihydroxyphenylalanine. III.[1] Preparation of Antibody to Epinephrine for Radioimmunoassay

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(Received March 22, 1978)

Preparation of the antibody for radioimmunoassay of epinephrine was examined and an antibody with proper titre was obtained. A procedure for radioimmunoassay of epinephrine was developed by use of a membrane filter to separate bound and free hapten. The dose-response curves of analogs of epinephrine obtained by this method showed that the specificity of the antibody to epinephrine was sufficiently high: The 50% inhibition value of epinephrine was 1 pmol, those of metanephrine and synephrine were 80 pmol and those of norepinephrine and dopamine were 6×10^3 pmol. The result indicates that the antibody recognizes not only the side chain but also the catechol moiety of the hapten. The sensitivity of this radioimmunoassay was high enough to determine 0.1 pmol of epinephrine.

Keywords——catecholamine; epinephrine; radioimmunoassay; antibody; membrane filter

In the previous papers,[3] we developed a method for preparation of the antigens of catecholamines and L-3,4-dihydroxyphenylalanine, which were able to elicit the antibodies to these compounds.

This investigation was undertaken to prepare the antibody to epinephrine with higher titre and examine the conditions of radioimmunoassay (RIA) with the antibody.

Experimental

Materials——L-Epinephrine was obtained from Merck AG. D-Epinephrine was a gift from Winthrop Laboratories. L-Norepinephrine, dopamine hydrochloride, DL-metanephrine hydrochloride and DL-normetanephrine hydrochloride were obtained from Nakarai Chemicals Co., Ltd. L-DOPA, T-14893 [*trans*-2-methylamino-1,5,6-trihydroxy-1,2,3,4-tetrahydro-naphthalene][4] and MK-486 [L-α-hydrazino-α-methyl-β-(3,4-dihydroxybenzene)propanoic acid][5] were gifts from Daiichi Seiyaku Co., Ltd. (Tokyo), Dr. M. Nishikawa of Takeda Chemical Industries, Ltd. (Osaka) and Nippon Merck Banyu Co., Ltd. (Tokyo) respectively. Homovanillic acid and vanilmandelic acid were obtained from Tokyo Chemical Industries Co., Ltd. 3,4-Dihydroxyphenylacetic acid, DL-isoprenaline hydrochloride and DL-synephrine hydrochloride were obtained from Sigma Inc. Adrenalone hydrochloride was obtained from Fluka Inc. Complete and incomplete Freund's adjuvants were obtained from Difco Laboratories. Bovine serum albumin (BSA) and rabbit serum albumin (RSA) were obtained from Miles Laboratories Inc. DL-Epinephrine [7-^{3}H(N)], 11 Ci/mmol, was obtained from New England Nuclear Inc. 5-(Ethylamino)methyl-4-methylcatechol (HC-EA) was prepared from 4-methylcatechol and ethylamine by the Mannich reaction.[1] Other chemicals of reagent grade were obtained from Kanto Kagaku Co., Ltd. (Tokyo) or Nakarai Chemicals Co., Ltd. (Tokyo).

Millipore Filter, Micro Filter and Nuclepore Filter were obtained from Millipore Corp., Fuji Photo Film Co., Ltd. (Tokyo) and Nuclepore Corp. respectively.

1) Part II: A. Miwa, M. Yoshioka, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **26**, 2903 (1978).
2) Location: *Hongo, 7-3-1, Bunkyo-ku, Tokyo* (Address to M. Yoshioka).
3) *a*) A. Miwa, M. Yoshioka, A. Shirahata, Y. Nakagawa, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 1422 (1976); *b*) A. Miwa, M. Yoshioka, A. Shirahata, and Z. Tamura, *ibid.*, **25**, 1904 (1977).
4) M. Nishikawa, M. Kanno, H. Kuriki, H. Sugihara, M. Motohashi, K. Itoh, O. Miyashita, Y. Oka, and Y. Sanno, *Life Sci.*, **16**, 305 (1975); Y. Oka, M. Motohashi, H. Sugihara, O. Miyashita, K. Itoh, M. Nishikawa, and S. Yurugi, *Chem. Pharm. Bull.* (Tokyo), **25**, 632 (1977).
5) R.J. Baldessarini, *J. Pharm. Pharmacol.*, **24**, 78 (1972).

Procedure of RIA——A buffer solution (PBS-A: 0.15 M NaCl, 0.01 M KH_2PO_4, 0.01 M ascorbic acid, pH 7.0) was flushed with nitrogen and chilled in ice, then used for preparation of the diluted antisera, sample solutions and ^{3}H-epinephrine solution. A PBS-A solution of ^{3}H-epinephrine was prepared to contain 10 mM 4-methylcatechol for prevention of the non-specific binding of epinephrine to the serum proteins.[6]

All procedures were performed in a stream of nitrogen and samplings were carried out with some adequate microsyringes.

In a siliconized micro test tube, 210 μl of a mixture of the diluted antiserum and the sample solution was added to 10 μl of ^{3}H-epinephrine solution corresponding to 5000 dpm (0.21 pmol) as the total counts (T). After the substitution of the air in the tube with nitrogen, the tube was sealed with Parafilm. The solution was shaken well and incubated for 14 hr at 37° in the dark. Then the solution was chilled in ice and filtered through a Millipore Filter DAWP (15 mm). The filter was washed with 0.3 ml of chilled PBS-A and dissolved in 10 ml of modified Bray's solution[7] lacking POPOP and ethylene glycol. The radioactivities of the solutions were measured by a scintillation spectrophotometer, Packard Model 3255 Tri-carb System on-line programmed.

Preparation of Antisera to Epinephrine for RIA——The antisera to epinephrine for RIA were prepared by immunization of rabbits according to a following protocol. The primary injection was performed in the foot pads of a rabbit with a w/o emulsion prepared by mixing 5 mg of the antigen with complete Freund's adjuvant. Booster injections were performed subcutaneously in the back of the rabbit, in which earlier five injections were done with the same emulsion as in the primary injection at an interval for ten days, and the subsequent injections with a w/o emulsion prepared with 1—2 mg of the antigen and incomplete Freund's adjuvant at a monthly interval.

Results

In this investigation, Millipore Filter, Micro Filter and Nuclepore Filter were examined in their feasibility for separation of the bound (B) and free hapten (F), and it was found that Millipore Filter alone was suitable for the purpose. The adequate pore size and diameter of the filter were examined at the range of 0.22—1.2 μ and 13—20 mm respectively. The results showed that the pore size of the filter did not affect the B value, while the filter of larger diameter had the larger capacity of adsorption of B. The pore size of 0.65 μ and the diameter of 15 mm were chosen for easy manipulation. The washing of the filter was sufficiently done with 0.3 ml of PBS-A.

The method for preparation of antisera with higher titre was examined under several conditions, and the protocol described in Experimental was adopted. Typical responses of four rabbits immunized are shown in Fig. 1. The antiserum feasible for RIA was found to be obtained in a rabbit (a) for months after 8 weeks.

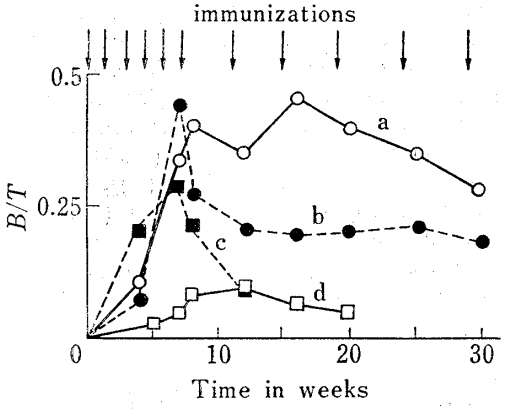


Fig. 1. Responses of Rabbits (a—d) to Immunization with Epinephrine-BSA Conjugate

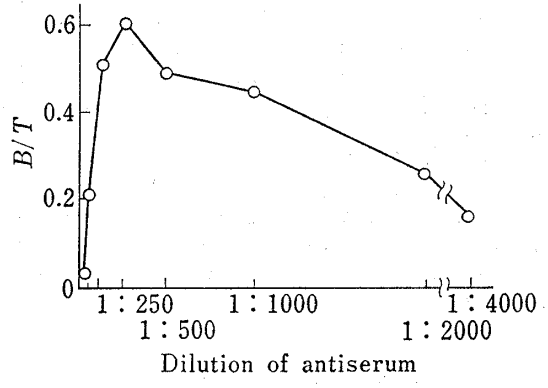


Fig. 2. Dilution Curve of the Antiserum to L-Epinephrine

6) G. Powis, *Biochem. Pharmacol.*, **24**, 707 (1975).
7) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).

The antiserum dilution curve (Fig. 2) showed that the highest value of B/T was obtained at 1: 250, and that the B/T value of 0.5, which was most suitable for RIA,[8] was obtained at 1: 500—1000. Thus the diluted antiserum (1: 1000) was used for this RIA. The unusual decrease of the B/T values at lower dilution of antisera would be caused by the presence of other proteins in the antisera. Actually the addition of RSA more than 1 mg per tube to the diluted antiserum (1: 1000) brought about the decrease of the B/T values. Since the blank values obtained by PBS–A were equal to those by the diluted normal serum (1: 1000), the formers were used for RIA.

When an antioxidant was not added to the assay system, the B/T values decreased during incubation. Sodium sulfite and sodium metabisulfite decreased the B/T values, while ascorbic acid showed no influence on the values. Thus 10 mM ascorbic acid was chosen as the antioxidant in this RIA.

The optimum pH for binding was between 7.0 and 7.5, so the pH of PBS–A was adjusted to 7.0 (Fig. 3).

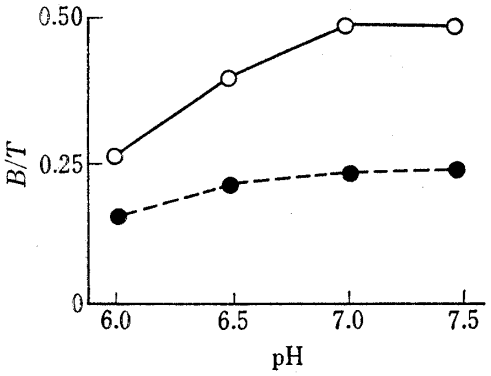


Fig. 3. Influence of pH on B/T Value

The B/T value was determined in the absence (○——○) or in the presence (●----●) of 1 pmol of cold L-epinephrine.

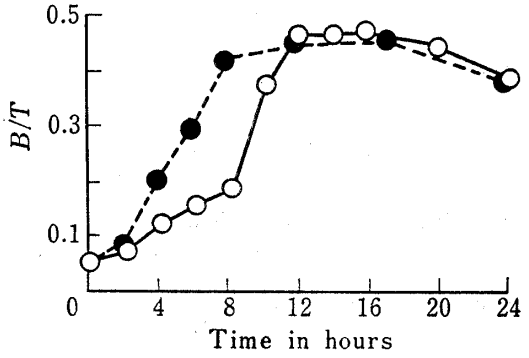


Fig. 4. Time Course of Binding

^{3}H-Epinephrine as T, 5000 (○——○) and 9000 dpm (●----●), were added to the solution. Then the solution was incubated as described in Procedure of RIA.

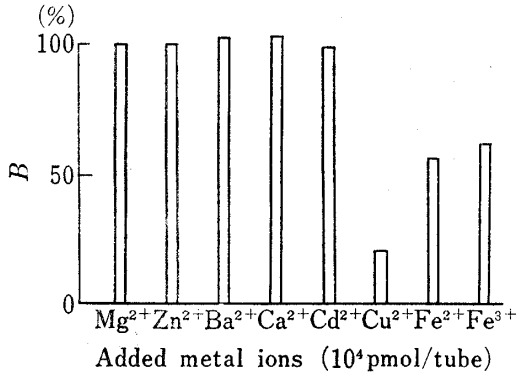


Fig. 5. Influence of Metal Ions on the Binding of ^{3}H-Epinephrine to the Anti-epinephrine-antibody

Ten μl of 1 mM $MgCl_2$, $ZnCl_2$, $BaCl_2 \cdot 2H_2O$, $CaCl_2$, $CdCl_2 \cdot 2\ 1/2H_2O$, $CuCl_2$, $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ each was added to the tube. B at zero dose is controlled as 100% binding.

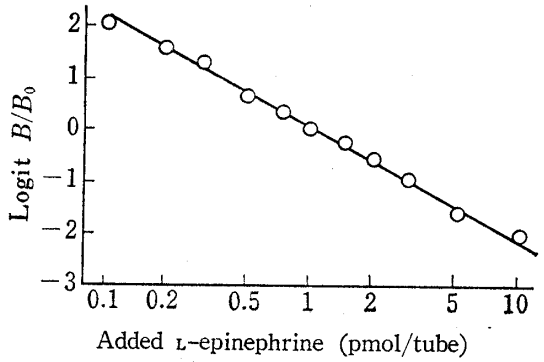
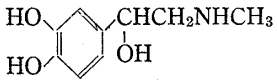
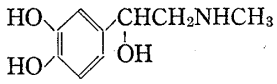
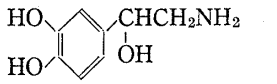
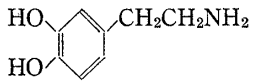
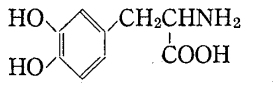
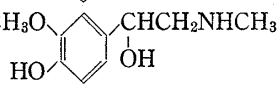
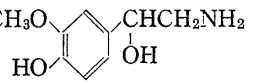
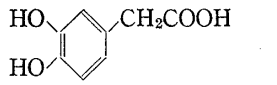
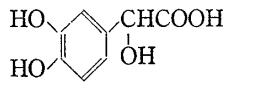
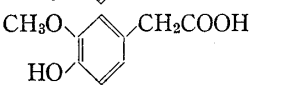
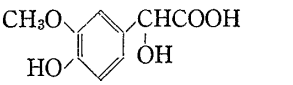
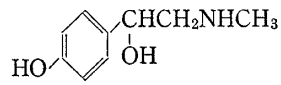
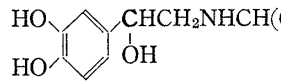
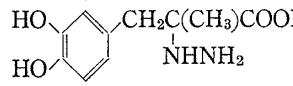
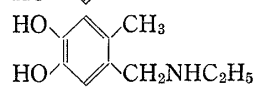
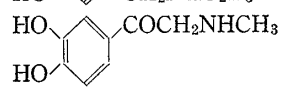
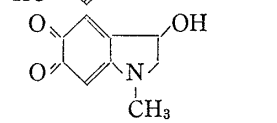
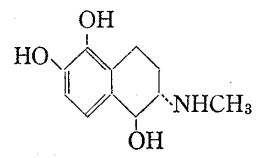


Fig. 6. Standard Curve of L-Epinephrine

B_0 is B at zero dose.

8) W.D. Odell, G.A. Abraham, W.R. Skowsky, M.A. Hescox, and D.A. Fisher, "Principles of Competitive Protein-Binding Assays," ed. by W.D. Odell and W.H. Daughaday, J.B. Lippincott Co., Philadelphia, 1971, Chapter III.

Table I. The Structures and 50% Inhibition Values of the Analogs

Analog (abbreviation)	Structure	Amount for 50% inhibition (pmol/tube)
L-Epinephrine (E)		1
D-Epinephrine		1
L-Norepinephrine (NE)		6×10^3
Dopamine (DA)		6×10^3
L-3,4-Dihydroxyphenylalanine (DOPA)		$>10^4$
DL-Metanephrine (MN)		80
DL-Normetanephrine (NMN)		$>10^4$
3,4-Dihydroxyphenylacetic acid (DOPAC)		$>10^5$
DL-3,4-Dihydroxymandelic acid (DOMA)		$>10^5$
Homovanillic acid (HVA)		$>10^5$
Vanilmandelic acid (VMA)		$>10^5$
DL-Synephrine (SN)		80
DL-Isoprenaline (IPN)		2×10^3
MK-486 (MK)		4×10^3
HC-EA		10^3
Adrenalone (ADL)		3×10^2
Adrenochrome (ADC)		$>10^3$
T-14893 (T_1)		$>10^3$

Under these conditions the incubation for 12—18 hours at 37° was required to give the maximum B/T values (Fig. 4). However, at 4° the maximum value was not attained even after 7 days.

The metal ions may influence on the binding reaction of antibody and hapten,[9] through the formation of chelates[10] or the oxidation[11] of catecholamines. Therefore the influences of the metal ions on the assay system were examined. Ten nanomoles of metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+} and Ba^{2+} showed no significant influences on the B/T values. On the other hand, Cu^{2+}, Fe^{2+} and Fe^{3+} caused the decrease of the B/T values (Fig. 5).

The standard curve of L-epinephrine was obtained by drawing a logit plot[12] (Fig. 6). It showed the linearity at the range of 0.1—10 pmol of L-epinephrine. The coefficients of variation of B_0 and B at 2 pmol of L-epinephrine were 2.4 and 5.2% ($N=5$) respectively. From a Scatchard plot of the data of the standard curve, the affinity constant (K) was $4.4\times10^8\,M^{-1}$.

The specificity of the RIA was examined against the analogs of epinephrine by drawing the dose-response curves (Fig. 7). The values for 50% inhibition of the analogs, together with their structures, are shown in Table I.

Catecholamines and their 3-O-methylated compounds—DL-Metanephrine (MN), having the same side chain as epinephrine, showed the highest inhibition among the other analogs (Fig. 7-A). Other metabolites and L-DOPA—These compounds showed little cross-reactivity (Fig. 7-B). Structural related compounds—DL-Synephrine, having the same side chain as epinephrine, showed the same inhibition as MN. HC-EA described in Materials showed a somewhat different curve from those of other compounds, and unexpectedly high cross-reactivity (Fig. 7-C). Adrenochrome derived from epinephrine and T-14893, a β-stimulant, showed little cross-reactivity, while adrenalone, having the similar structure as epinephrine, had a relatively high cross-reactivity (Fig. 7-D).

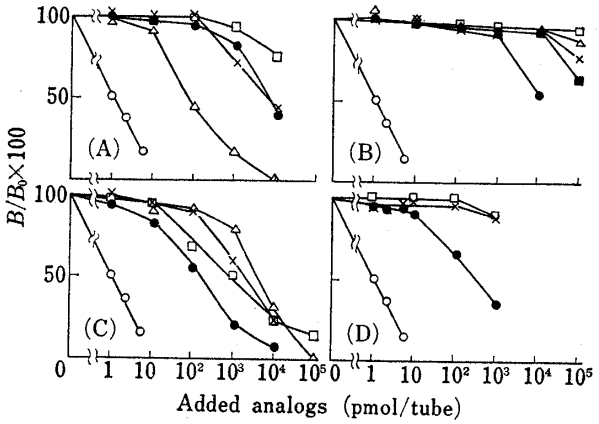


Fig. 7. Dose-response Curves of the Analogs of Epinephrine

Abbreviations are listed in Table I.

(A) —□—: NMN, —●—: NE, —×—: DA, —△—: MN, —○—: E.
(B) —□—: HVA, —△—: VMA, —×—: DOPAC, —■—: DOMA, —●—: DOPA, —○—: E.
(C) —□—: HC-EA, —△—: MK, —×—: IPN, —●—: SN, —○—: E.
(D) —□—: ADC, —×—: T_1, —●—: ADL, —○—: E.

Discussion

The antisera with high titre suitable for RIA of epinephrine were obtained by the successive immunizations at the interval for ten days.

Various techniques for separation of B and F have been available.[13] However the typical methods such as double-antibody, ammonium sulfate precipitation and dextran-coated charcoal methods would be unsuitable for RIA of epinephrine, for epinephrine has a property of non-specific binding to the protein[6] precipitated with salt or to the *anti-γ*-globulin anti-

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10) J.R. Doty, *Anal. Chem.*, **20**, 1166 (1948).
11) T.D. Sokoloski and T. Higuchi, *J. Pharm. Sci.*, **51**, 172 (1962).
12) D. Rodbard, R.L. Rayford, J.A. Cooper, and G.T. Ross, *J. Clin. Endocrinol. Metab.*, **28**, 1412 (1968).
13) D.S. Skelley, L.P. Brown, and P.K. Besch, *Clin. Chem.*, **19**, 146 (1973).

sera, and the metal ions contained in charcoal might influence on the assay system described above. On the other hand the filtration method with the membrane filter is free from these influences.

The incubation for 14 hours at 37° was so long to complete the reaction of hapten with the antibody. Further this antibody did not distinguish D- from L-epinephrine. These properties are different from those of antibodies generally described.[14]

As shown in Fig. 7, and Table I, the sensitivity and specificity of this RIA are so high that 0.1 pmol of epinephrine in biological materials can be determined in the presence of analogs. Thus this RIA would be superior to the other methods for determination of epinephrine[15] in sensitivity and convenience and may be one of the highest sensitive RIA's using ^{3}H-labeled compounds.[13]

The increase of 50% inhibition value (Table I) occurred by modifying the catechol moiety of epinephrine (DL-metanephrine and DL-synephrine) was not so large comparing with that by changing the side chain (L-norepinephrine and dopamine). These results indicate that the antibody obtained here recognizes more strictly the side chain than the catechol moiety of the compound. The unusual response of HC–EA suggests that the antibody might recognize the methylene bridge in the conjugate[1,3b] or the length of the side chain of epinephrine. The latter possibility occurs from the side chain structures C–C–N–CH_3 for epinephrine and C–N–C–CH_3 for HC–EA.

Acknowledgement This work was supported by a grant from the Ministry of Health and Welfare and a Grant-in-Aid for Scientific Research 248353 from the Ministry of Education, Science and Culture.

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