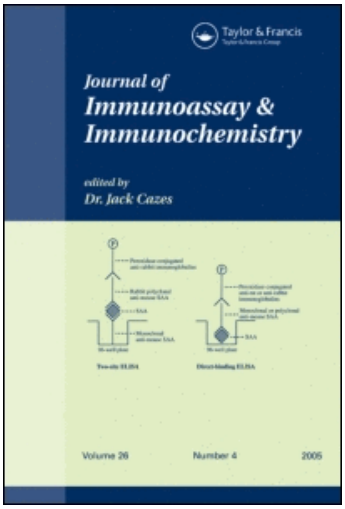

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Enzyme Immunoassays for β-Adrenoreceptor Blocking Agent, Befunolol and Its Main Metabolite, M1

Sohichiro Sato[a]; Itaru Yamamoto[a]
[a] Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan

To cite this Article Sato, Sohichiro and Yamamoto, Itaru(1983) 'Enzyme Immunoassays for β-Adrenoreceptor Blocking Agent, Befunolol and Its Main Metabolite, M1', Journal of Immunoassay and Immunochemistry, 4: 4, 351 — 371
To link to this Article: DOI: 10.1080/15321818308057015
URL: http://dx.doi.org/10.1080/15321818308057015

JOURNAL OF IMMUNOASSAY, 4(4), 351-371 (1983)

ENZYME IMMUNOASSAYS FOR β-ADRENORECEPTOR BLOCKING AGENT, BEFUNOLOL AND ITS MAIN METABOLITE, M1

Sohichiro Sato and Itaru Yamamoto

Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka 1-1-1, Okayama 700, Japan

ABSTRACT

Enzyme immunoassays for befunolol (BFE-60) and its main metabolite, M1, were established. These assays were based on the double antibody solid phase principle. Specific antisera to these agents were raised in rabbits by immunization with N-succinyl-BFE-60∿ human serum albumin (HSA) conjugate and N-succinyl-M1∿HSA conjugate, respectively. Antibody was incubated with a mixture of β-D-galactosidase (β-Gal) labelled hapten and unlabelled standard or sample drug which had been succinylated prior to assay. The antibody-bound drug was separated from free using anti-rabbit IgG immobilized on a polystyrene ball, and activity of the enzyme on the solid phase was determined fluorometrically. Succinylation of both BFE-60 and M1 resulted in 100-fold increased sensitivity with reduced cross-reactivity. The present immunoassays allow detection of 0.4 pg of BFE-60 (10 pg/ml) and 3 pg of M1 (75 pg/ml). The time courses of plasma levels of BFE-60 and M1 were examined after a single intravenous administration (1 mg/kg) or a single topical instillation (0.2, 1.0 and 5.0 % : 50 µl) to albino rabbits. BFE-60 and M1 in biological fluids required acetone-extraction before assay.

KEY WORDS: β-Blocker, Befunolol (BFE-60), a metabolite of BFE-60 (M1), Enzyme immunoassay, Polystyrene ball, β-D-Galactosidase

0197-1522/83/0404-0351$3.50/0

INTRODUCTION

Befunolol, 2-acetyl-7-(2-hydroxy-3-isopropylaminopropoxy) benzofuran hydrochloride (BFE-60), is a potent β-adrenoreceptor blocking agent (1) and has an intraocular pressure decreasing action (2,3). M1, 2-(1-hydroxyethyl)-7-(2-hydroxy-3-isopropyl-aminopropoxy)benzofuran, is the major matabolite of BFE-60 in man and animals (4-8). The pharmacokinetic behavior of BFE-60 have been investigated in several animals and man using high doses of BFE-60 (4-6) or isotope labelled BFE-60 (7,8). To determine the plasma levels of BFE-60 and M1 after topical instillation of clinical doses of this agent for glaucoma, a sensitive and specific analytical method is required. Radioimmunoassay is not suitable for this purpose, because ^{14}C-BFE-60 and ^{14}C-M1 have low specific activity (7,8). We have previously developed highly sensitive enzyme immunoassays for low molecular compounds such as cyclic nucleotides (9-11) and a β-agonist (12), and in this communication, we describe sensitive enzyme immunoassays for BFE-60 and its main metabolite, M1. By use of these assays, plasma levels of BFE-60 and M1 have been measured after a single intravenous administration or a single topical instillation to albino rabbits.

MATERIALS AND METHODS

Materials

BFE-60, its metabolites (M1: 2-(1-hydroxyethyl)-7-(2-hydroxy-3-isopropylaminopropoxy)benzofuran, M2: 2-acetyl-4-hydroxy-7-(2-

hydroxy-3-isopropylaminopropoxy)benzofuran, M1G: 2-O-glucuronide of M1, M2G: 4-O-glucuronide of M2) and ^{14}C-BFE-60 (specific activity: 1.6 Ci/mol) were donated by Kyoto Research Laboratory, Kaken Pharmaceutical Co., Ltd. (Kyoto). ^{14}C-M1 (specific activity: 1.6 Ci/mol) was synthesized by reduction of ^{14}C-BFE-60 with sodium borohydride. Chemical structures are illustrated in Figure 1. β-D-Galactosidase (β-Gal, from E. coli, grade VIII, 674 units/mg protein), human serum albumin (HSA, crystallized and lyophilized), bovine serum albumin (BSA, fraction V) and 4-methyl-umbelliferyl-β-D-galactoside (4-MUG) were purchased from Sigma Chemical Co. (St. Louis, MO). The IgG fraction of anti-rabbit IgG (lyophilized) was obtained from Miles-Yeda Ltd, (Israel). Complete and incomplete Freund's adjuvant were products of Difco Labs (Detroit, MI). Polystyrene balls (1/4 in. diameter) were purchased from Ichico

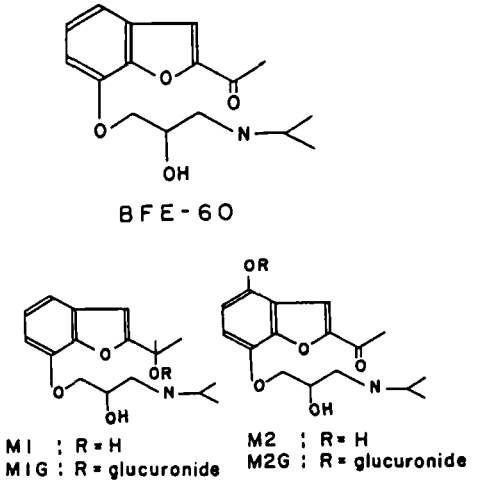

FIGURE 1. Chemical structures of BFE-60 and its metabolites (M1, M2, M1G, M2G).

Co., Ltd. (Nagoya). All other chemicals and solvents from commercial sources were of reagent grade quality.

A_1 buffer was sodium phosphate buffer (pH 6.6), 100 mmol/L , containing 100 mmol/L NaCl, 1 mmol/L $MgCl_2$ and 1 g/L BSA. A_2 buffer was sodium phosphate buffer (pH 7.0), 100 mmol/L, containing 100 mmol/L NaCl, 1 mmol/L $MgCl_2$ and 1 g/L BSA. Succinylating reagent was 20 g/L of succinic anhydride in triethylamine : acetone mixture (1 : 9, v/v).

Preparation of Immunogens

BFE-60 was conjugated to HSA by initially making a BFE-60-N-hemisuccinate (sBFE-60) and then forming an acid-amide linkage between the amino group of HSA and the carboxy group of sBFE-60. M1∿HSA conjugate was prepared in a similar fashion.

BFE-60 was succinylated with succinic anhydride in dimethylformamide : triethylamine mixture according to the method of Yamamoto et al. (12), and the hemisuccinate was purified by chloroform extraction and silica gel column chromatography. M1-N-hemisuccinate (sM1) was obtained by reduction of sBFE-60 with sodium borohydride.

sBFE-60 was conjugated to HSA by the mixed acid anhydride technique using tri-n-butylamine and isobutylchloroformate (13). sBFE-60∿HSA conjugate was purified by repeated precipitation with acetone. The final precipitate was dissolved in water, lyophilized and stored at -20°C.

The molar ratio of BFE-60 and M1 to HSA were estimated on the assumption that the molar extinction coefficient (ε) of BFE-60

and M1 are 2.4 x 10^5 at 295 nm and 1.42 x 10^4 at 246 nm, respectively. Protein concentration was estimated according to the method of Lowry et al. (14) with HSA as standard. It was found that 6.0 mol of BFE-60 or 2.8 mol of M1 was bound to 1 mol of HSA.

Immunizations

Antibody to BFE-60 or M1 was produced in albino male rabbits by repeated intradermal immunizations. The first immunization was performed with 0.5 mg of sBFE-60∿HSA or sM1∿HSA conjugate in complete Freund's adjuvant and booster doses of 0.2 mg of the immunogen in incomplete Freund's adjuvant were given at four week intervals. The titer and specificity of the antibody were determined by the method of Farr (15) using ^{14}C-BFE-60 or ^{14}C-M1. Bleedings were taken from the ear vein 7 to 10 days after the second booster, and the antisera were lyophilized and stored at -80°C until use.

Preparation of β-Gal Labelled Haptens

sBFE-60∿β-Gal or sM1∿β-Gal conjugate was synthesized with the mixed acid anhydride technique. Briefly, 2.5 µl of tri-n-butylamine was added to 5 mg of sBFE-60 or sM1 in 1 ml of acetone, held for 20 min at room temperature, and then 1.2 µl of isobutyl-chloroformate was added. After 30 min, 0.1 ml of activated sBFE-60 diluted with 4 ml of Tris acetate buffer (pH 8.5), 50 mmol/L, was added to 1 ml of β-Gal (500 µg) in the same buffer on the coupling reaction allowed to proceed for an hour at 4°C.

The conjugates were purified by Sephadex G-25 column chromatography and DEAE-Sepharose 6B column chromatography. Fractions

containing β-Gal activity were pooled and stored at -20°C after mixing with an equal volume of glycerol. β-Gal activity was stable for more than 6 months under this condition. 7.0 mol of BFE-60 was bound to 1 mol of β-Gal. The molar ratio of Ml to β-Gal was estimated to be less than 3.

Preparation of Second Antibody-bound Polystyrene Balls

Second antibody was immobilized on polystyrene balls by physical adsorption according to the method described in our previous paper (10).

Briefly, balls were soaked in a solution of the goat IgG fraction of anti-rabbit IgG (50-fold diluted with sodium phosphate buffer, pH 7.5, 50 mmol/L) containing 1 g/L NaN_3 at 4°C for 24 hours and washed three times in A_1 buffer. They were kept in the same buffer at 4°C for at least 18 hours until use.

Preparation of Assay Samples

Blood samples were taken with heparinized syringes and the separated plasma was kept at -20°C until use. 400 µl of cold acetone was added to 200 µl of plasma, mixed and precipitated in an ice bath. After centrifugation at 3000 rpm for 5 min, 300 µl of supernate was transferred to another tube and 100 µl of succinylating reagent (1 : 9 v/v mixture of 20 g/L of succinic anhydride in triethylamine and acetone) was added and the reaction allowed to proceed for an hour at room temperature. Samples were dried under vacuum, dissolved in 500 µl of A_2 buffer, and stored at -20°C until assay.

Assay procedure

The enzyme immunoassay of BFE-60 or M1 involved competition of enzyme labelled BFE-60 or M1 and standard or sample for a limited amount of antibody with separation of free and antibody bound by anti-IgG immobilized on polystyrene balls.

Succinylated standard or sample solution, 200 µl, was incubated at 4°C for 18 hours with 100 µl of β-Gal labelled hapten (2 µg/L approx.) and 100 µl of anti-hapten serum (diluted 1 : 100,000 for BFE-60 or 1 : 60,000 for M1 in A_2 buffer). A second antibody immobilized polystyrene ball was then added to each assay solution and the mixture rocked for 5 hours at room temperature. The ball was washed with A_1 buffer and transferred to tube holding 200 µl of A_2 buffer. The activity of the enzyme bound to the solid phase was determined by adding 200 µl of 4-MUG, 300 µmol/L, as substrate and incubating at 37°C for 1 hour. The enzyme reaction was terminated by addition of 2.5 ml of glycine-NaOH (pH 10.3, 100 mmol/L). The 4-MU liberated was measured by fluorescence spectrometry with excitation wavelength at 360 nm and emmission wavelength at 450 nm.

B/Bo (%) was calculated as followed.

$$B/Bo = \frac{B - Bl}{Bo - Bl} \times 100$$

B : fluorescence intensity of sample

Bo: fluorescence intensity of zero standard

Bl: fluorescence intensity of blank, with buffer replacing antibody

RESULTS

Sensitivity and Specificity of the Enzyme Immunoassays of BFE-60 and M1

Calibration curves for the enzyme immunoassay of BFE-60 and M1 show increasing inhibition of the binding of enzyme labelled hapten to the antibody by increasing amounts of unlabelled hapten (Figure 2).

In the BFE-60 assay (panel A), 30 pg of BFE-60 could be distinguished from zero. Succinylation of BFE-60 prior to the assay increased the sensitivity to 0.3 pg BFE-60. Specificity of antibody directed against against BFE-60 (lot No. 6-18-1) was assessed with some metabolites of BFE-60. Cross reactivity (B/Bo = 50 %) of M1 to BFE-60 was reduced from 1.6 % to 0.04 % by succinylation.

In the M1 assay (panel B), 100 pg of M1 could be distinguished from zero and the sensitivity was also increased to 3 pg by succinylation. Specificity of antibody directed against M1 (lot No. 6-17-6) was assessed with BFE-60 and other metabolites. Cross reactivity (B/Bo = 50 %) of BFE-60 to M1 was reduced from 1.3 % to 0.13 % by succinylation. Cross reactivities of other metabolites (M2, M1G, M2G) to both BFE-60 and M1 were less than 0.1 % in BFE-60 and M1 assays (panel A and panel B in Figure 3).

Effect of Plasma Sample on these Enzyme Immunoassays

Table 1 shows the effect of intact plasma samples from humans and rabbits on the antigen-antibody reaction. Inhibition of the Ag-Ab reaction occurred due to non-specific interfering substances and rabbit IgG. This inhibition was partly removed by boiling

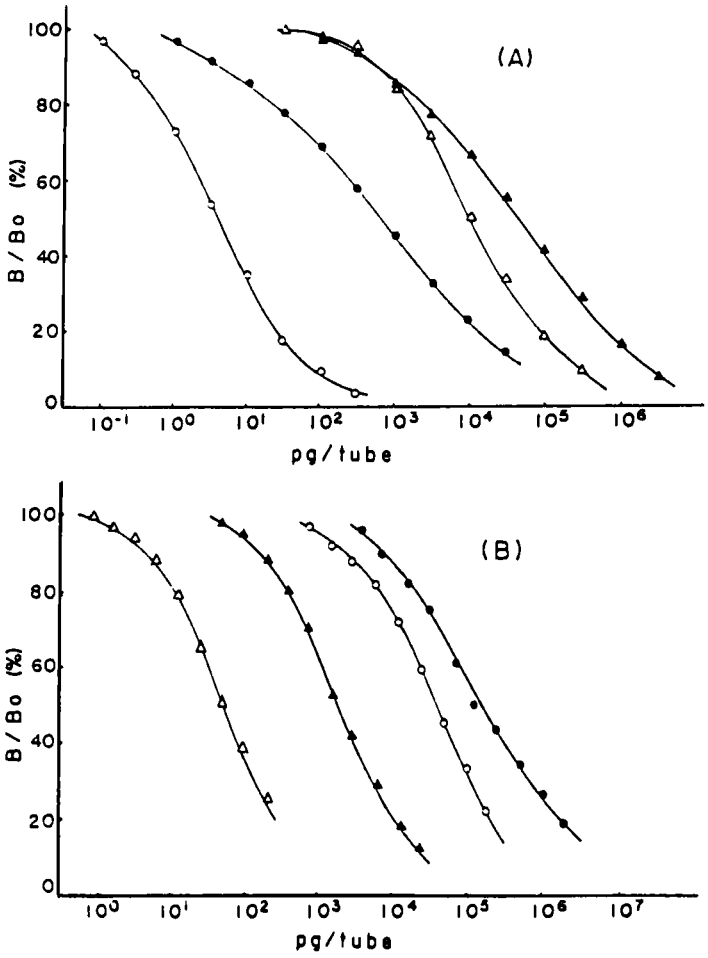

FIGURE 2. A) Typical standard curves for BFE-60 (●) and sBFE-60 (○), and cross reactivities of M1 (▲) and sM1 (△). B) Typical standard curves for M1 (▲) and sM1 (△), and cross reactivities of BFE-60 (●) and sBFE-60 (○).

for 10 min, but acetone extraction was much more effective and practically eliminated the inhibition due to individual plasmas. Calibration curves with acetone extracted and subsequently succinylated standard samples in normal plasma were compared with

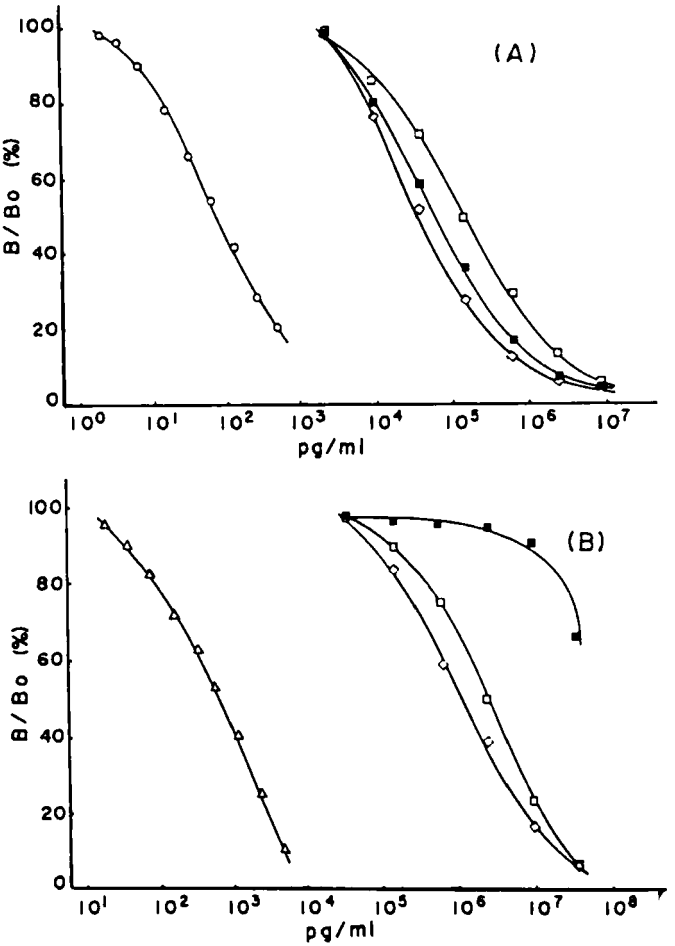

FIGURE 3. Cross reactivities of other metabolites (□: M1G, ■: M2, ◇: M2G) to BFE-60 (○) or M1 (△) on BFE-60 (panel A) or M1 (panel B) assay.

those using standards in buffer for both enzyme immunoassays (Figure 4) and showed good agreement. Recovery studies with acetone extraction and subsequent succinylation of samples using additions of 10 pg or 20 pg of authentic BFE-60 or M1 (Table 2)

TABLE 1

Effects of Human and Rabbit Plasma on the Enzyme Immunoassays [a]

Sample		Fluorescence intensity (BFE-60 assay) Intact	Boiled	Acetone extract	(M1 assay) Intact	Boiled	Acetone extract
Buffer		100	100	100	100	100	100
Human plasma	1	76.4	86.2	97.9	62.6	89.2	85.1
	2	64.7	90.7	95.8	59.0	84.4	82.6
	3	72.8	95.6	96.7	59.6	87.4	87.1
	4	67.2	82.9	99.6	50.6	83.2	83.2
	5	60.3	87.2	96.1	43.4	88.4	87.0
	6	65.7	89.6	99.8	42.8	86.6	86.0
	7	70.2	88.1	94.4	46.6	89.5	91.2
Mean		68.2	88.6	97.2	52.1	87.0	86.0
S.D.		5.39	3.98	2.01	8.25	2.40	2.87
C.V.		7.9	4.5	2.1	15.8	2.8	3.3
Rabbit plasma	1	1.4	56.3	92.9	0.9	42.2	87.1
	2	1.1	61.8	95.0	1.9	47.4	87.7
	3	0.8	71.5	95.0	0.6	55.9	91.0
Mean		1.1	63.2	94.3	1.1	48.5	88.6
S.D.		0.30	7.70	1.21	0.68	6.92	2.10
C.V.		27.3	12.2	1.3	61.9	14.3	2.4

[a] 200 µl of 10 % boiled or acetone extracted plasma was added to assay mixture containing 100 µl of diluted antiserum and 100 µl of diluted β-Gal labelled hapten solution and incubated for 18 hours at 4°C. Assays were performed as described under "MATERIALS AND METHODS".

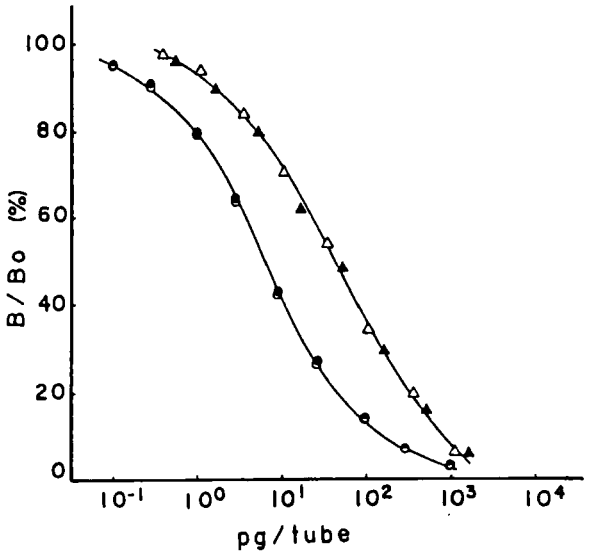

FIGURE 4. Calibration curves for BFE-60 and M1 by the proposed enzyme immunoassays with acetone extracted and succinylated standard samples in A_2 buffer (○: BFE-60, △ : M1) and normal human plasma (●: BFE-60, ▲ : M1).

TABLE 2

Recovery Studies with Human Plasma in the Proposed Enzyme Immunoassays [a]

Sample	(BFE-60 assay)		(M1 assay)	
	Measured (pg/tube)	Recovery (%)	Measured (pg/tube)	Recovery (%)
1	10.1	101	18.8	94.0
2	10.0	100	19.0	95.0
3	11.2	112	20.1	100.5
4	10.6	106	22.4	112.0
5	9.3	93	20.2	101.0
6	9.2	92	20.4	102.0
Mean ± S.D.		100.6 ± 7.63		100.8 ± 6.43

[a] Test samples were prepared by adding 10 pg of authentic BFE-60 or 20 pg of authentic M1 to normal human plasma and then assayed.

showed recovery of BFE-60 and M1 of 100.6 % and 100.8 % respectively.

Precision and Validity of the Enzyme Immunoassays

Dilution studies were carried out with rabbit plasma samples which were serially diluted 1 : 2 to 1 : 32 with A_2 buffer. These experiments resulted in a good linear relationship between assayed and measured values (Table 3).

Within and between assay reproducibility was assessed. Table 4 shows the coefficients of variation on BFE-60 and M1 assays or three concentrations with B/Bo (%) varying from about 25 to 75 %. The intra-assay coefficients of variation (CV) were 2.0 to 5.1 % in the BFE-60 assay and 3.0 to 9.7 % in the M1 assay, while inter-assay CV's were 5.3 to 6.8 % for BFE-60 and 6.8 to 14.9 % for the M1 assay. Correlationship between EIA and HPLC is shown in Figure 5. In the BFE-60 assay, the EIA correlated well with HPLC ($r = 0.994$), but in the M1 assay, EIA yielded lower values than HPLC with poorer correlation ($r = 0.727$).

Plasma Levels of BFE-60 and M1 in Rabbits After a Single Instillation or a Single Intravenous Administration

Using these enzyme immunoassays, the time course of plasma levels of BFE-60 and its main metabolite, M1, were examined.

BFE-60 ophthalmic solutions (0.2, 1.0 and 5.0 %) were instillated one drop (50 µl) into the eye and blood samples were taken at intervals and BFE-60 and M1 determined. Plasma levels of BFE-60 declined in a biphasic (0.2 and 1.0 %) or monophasic (5.0 %)

TABLE 3

Dilution Tests with Rabbit Plasma Samples in the Proposed Enzyme Immunoassays

(BFE-60 assay)

Dilution	Sample 1		Sample 2		Sample 3	
	(pg/ml)[a]	(pg/ml)[b]	(pg/ml)[a]	(pg/ml)[b]	(pg/ml)[a]	(pg/ml)[b]
1:32	18.7	598	14.3	458	16.5	528
1:16	38.5	616	27.4	438	32.7	523
1: 8	75.0	600	55.0	440	65.2	522
1: 4	154	616	107	428	131.5	526
1: 2	311	622	235	470	270.5	541
Mean ± S.D.	610.4 ± 10.7		446.8 ± 16.9		528 ± 7.65	

(M1 assay)

Dilution	Sample 1		Sample 2		Sample 3	
	(pg/ml)[a]	(pg/ml)[b]	(pg/ml)[a]	(pg/ml)[b]	(pg/ml)[a]	(pg/ml)[b]
1:32	149	4768	200	6400	172	5504
1:16	305	4880	398	6352	330	5280
1: 8	590	4720	800	6400	650	5200
1: 4	1180	4720	1450	5800	1210	4840
1: 2	2320	4640	3000	6000	2425	4850
Mean ± S.D.	4746 ± 88.1		6190 ± 275		5135 ± 287	

[a] measured value from standard curve

[b] concentration of original sample

TABLE 4

Reproducibility of BFE-60 and M1 Determinations by the Proposed Enzyme Immunoassays

(BFE-60 assay)

Sample	Intra-assay (n=8)			Inter-assay (n=5)		
	M(pg/ml)[a]	S.D.[b]	C.V.[c]	M(pg/ml)[a]	S.D.[b]	C.V.[c]
1	181.5	3.67	2.0	180.6	9.85	5.5
2	50.3	1.20	2.4	49.0	2.59	5.3
3	16.7	0.86	5.1	15.9	1.09	6.8

(M1 assay)

Sample	Intra-assay (n=8)			Inter-assay (n=5)		
	M(pg/ml)[a]	S.D.[b]	C.V.[c]	M(pg/ml)[a]	S.D.[b]	C.V.[c]
1	1651	49.9	3.0	1666	112.8	6.8
2	717.5	40.5	5.6	717.3	55.5	7.7
3	219.5	21.3	9.7	207.1	30.8	14.9

M[a] : mean of assays
S.D.[b]: standard deviation of mean
C.V.[c]: coefficient of variation

fashion, while those of M1 reached a peak at 40 min, and then declined. The ratio of BFE-60 to M1 in plasma reversed about 2 hours after instillation (Figure 6).

Plasma levels of BFE-60 after a single intravenous injection (1 mg/kg) decreased in a biphasic fashion, while those of M1 remained constant at about 10 ng/ml for an hour and then decreased (Figure 7).

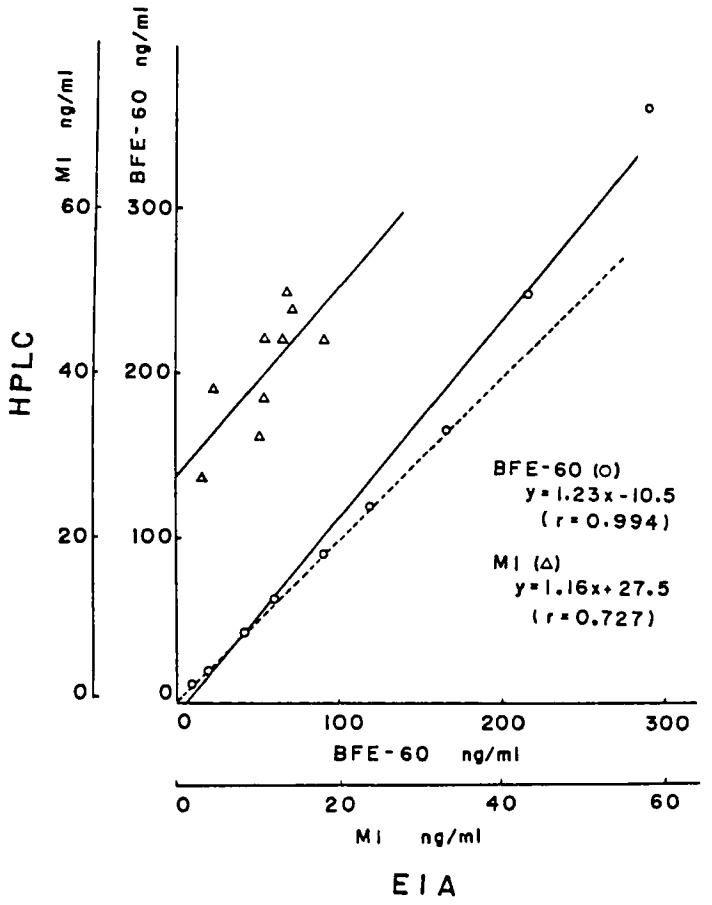

FIGURE 5. Correlation between EIA and HPLC with rabbit plasma samples (n = 9). The broken line is theoretical (y = x).

DISCUSSION

A sensitive radioimmunoassay for BFE-60 or M1 is not expected, because ^{14}C-BFE-60 and ^{14}C-M1 have low specific activity (4.99 μCi/mg, 1.6 Ci/mol). In this study, we have developed sensitive double antibody solid phase enzyme immunoassays for BFE-60 and its

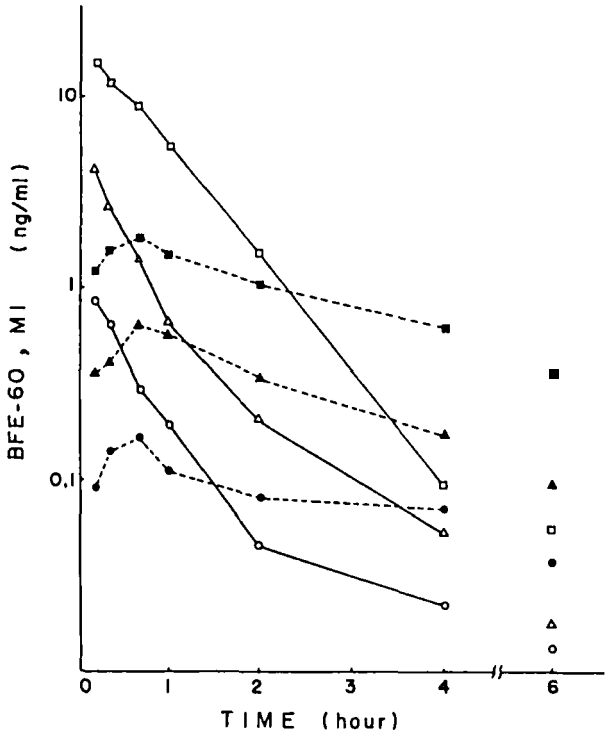

FIGURE 6. Plasma levels of BFE-60 (open marks) and M1 (closed marks) after a single instillation to rabbits with BFE-60 ophthalmic solutions (○● : 0.2 %, △▲ : 1.0 %, □■ : 5.0 %). Each point represents the mean of three animals.

main metabolite, M1. These assays allow determination of 0.4-20 pg of BFE-60 (10-500 pg/ml) and 3-200 pg of M1 (75-5000 pg/ml).

The sensitivity of these enzyme immunoassays is due to the following reasons. 1) The antisera had high specificity and affinity. BFE-60 was mainly metabolized by ring hydroxylation and 2-substituent reduction (Figure 1). Since HSA bound to the amino group of the 7-side chain of BFE-60 or M1 via a succinate bridge, the antibody might be expected to be more specific to the benzo-

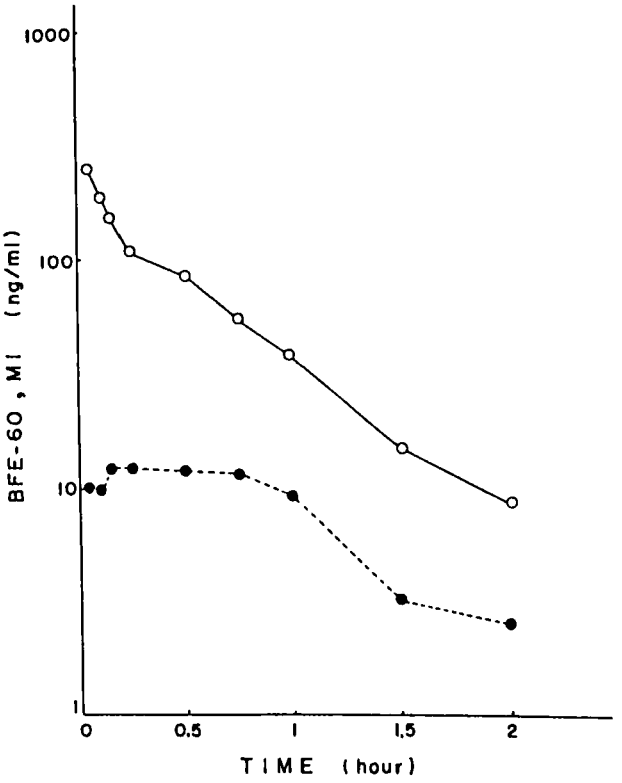

FIGURE 7. Plasma levels of BFE-60 (○) and M1 (●) after an intravenous administration (1 mg/kg) in rabbits. Each point represents the mean of three animals.

furan ring than to the 7-side chain. As a result, cross reactivity between BFE-60 and M1 was less than 0.1 % for both antisera.

2) β-Gal from E. coli used to label the hapten has a high turnover number and is readily available and stable. In addition, the enzyme activity is measured fluorometrically by using 4-MUG as substrate.

Intact plasma samples were not suitable for these enzyme immunoassays because of the presence of interfering substances,

but acetone extraction effectively eliminated these. The remaining small plasma non-specific effect did not differ significantly between individuals, allowing the use of a common normal plasma as control (Bo) instead of pre-administration plasma sample from each individual. Succinylation proceeds more effectively in acetone than in buffer, so that acetone extraction provides a convenient way of preparing samples for these enzyme immunoassays.

The dilution and recovery tests and the intra- and inter-assay precision indicate satisfactory assay performance. The enzyme immunoassays for BFE-60 and M1 described here are therefore valid and reliable. In the BFE-60 assay, EIA correlated well with HPLC, but the M1 assay correlated less well and this is now the subject of further investigation.

ACKNOWLEDGEMENTS

We wish to thank Dr. Y. Sakai, Mr. M. Tohno, and Mr. H. Kojima in Metabological Laboratory, Kaken Pharmaceutical Co., Ltd. for providing the plasma samples and measuring BFE-60 and M1 in them by HPLC.

All correspondences to : professor Itaru Yamamoto, Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka 1-1-1, Okayama 700.

REFERENCES

1. Takenaka, N., Ishihara, T., Matsuyama, Y., Matsumoto, S., Inoue, H., Okumura, M., and Honma, H. Effect of new β-adreno-receptor blocking agent, 2-acetyl-7-(2-hydroxy-3-isopropyl-aminopropoxy)benzofuran hydrochloride on heart-vascular system. Folia. Pharmacol. Jpn. 1974; 70: 385-397.

2. Tane, S., Komatsu, A., Iguchi, T., and Ooba, H. Effect of befunolol ophthalmic solution (a new beta-blocking agent developed in Japan) on eyes in normal volunteers and glaucoma subjects. Ganka-Rinsho-Iho 1979; 73: 257-262.

3. Takese, M., Araie, M., and Matsuo, T. A preliminary study of topically applied dl-befunolol hydrochloride (BFE-60), a beta-adrenergic blocking agent, for treatment of glaucoma. Folia. Ophthalmol. Jpn. 1980; 31: 266-270.

4. Ofuji, T., Kawahara, K., Matsuda, T., and Uda, K. Studies on stability and fate of befunolol in blood of mouse, rat, guinea pig, rabbit, dog, monkey, and human. Pharmacometrics 1979; 18: 1055-1062.

5. Tohno, M., Kimura, K., Nagahara, M., Sakai, Y., Ofuji, T., and Nadai, T. Identification of urinary metabolites of befunolol in dog and man principally by mass spectrometry. Yakugaku Zasshi 1979; 99: 944-957.

6. Kawahara, K., and Ofuji, T. Simultaneous determination of befunolol, a β-blocking agent, and its metabolite in human plasma by gas chromatography with electron-capture detection. J. of Chromat. 1979; 168: 266-272.

7. Tanaka, K., Ohishi, Y., Itoh, K., and Nakanishi, T. Synthesis of ^{14}C-labelled benzofuran derivative. Radioisotopes 1978; 27: 266-267.

8. Kitagawa, H., Sakai, Y., Matsumura, Y., Kojima, H., Matsuda, T., Tohno, M., and Ofuji, T. Metabolism of befunolol in rats and rabbits. Pharmacometrics 1979; 18: 889-902.

9. Yamamoto, I., and Tsuji, J. Enzyme immunoassay of cyclic adenosine 3',5'-monophosphate (AMP) using β-D-galactosidase as label. Immunopharmacology 1981; 3: 53-59.

10. Yamamoto, I., Tsuji, J., Takai, T., and Fujimoto, M. Double antibody enzyme immunoassay for the quantitation of adenosine 3',5'-cyclic monophosphate (cyclic AMP) and guanosine 3',5'-cyclic monophosphate (cyclic GMP) in tissue and plasma. J. Immunoassay 1982; 3: 173-196.

11. Yamamoto, I., Takai, T., and Tsuji, J. Enzyme immunoassay for cytidine 3',5'-cyclic monophosphate (cyclic CMP). Immuno-pharmacology 1982; 4: 331-340.

12. Yamamoto, I., and Iwata, K. Enzyme immunoassay for clenbuterol, an β_2-adrenergic stimulant. J. Immunoassay 1982; 3: 155-171.

13. Erlanger, B.F., Borek, F., Beiser, S.M., and Lieberman, S. Steroid-protein conjugate. J. Biol. Chem. 1959; 234: 1090-1094.

14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275.

15. Farr, R.S. A quantitative immunochemical measure of the primary interaction between BSA and antibody. J. Infect. Dis. 1958; 103: 239.