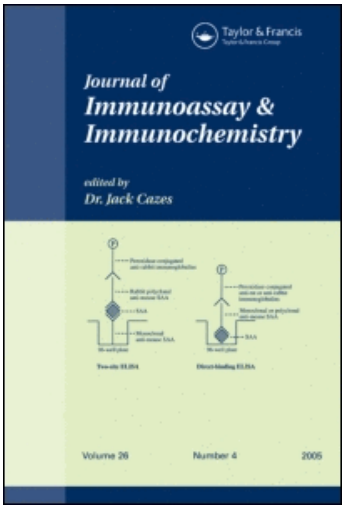

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Enzyme Immunoassay for Mabuterol, A Selective β_2-Adrenergic Stimulant in the Trachea

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ENZYME IMMUNOASSAY FOR MABUTEROL, A SELECTIVE β_2-ADRENERGIC STIMULANT IN THE TRACHEA

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KEY WORDS: enzyme immunoassay, polystyrene ball, mabuterol (KF-868), β_2-adrenergic stimulant

ABSTRACT

A sensitive double antibody and heterologous enzyme immunoassay for mabuterol was established. For competitive reactions, antibody raised against diazotized mabuterol-human serum albumin was incubated with a mixture of diazotized mabuterol analog (RC-1) labeled with β-D-galactosidase and standard or sample. Free and antibody-bound enzyme hapten were separated using anti-rabbit IgG immobilized on polystyrene balls. Activity of the enzyme on the solid phase was fluorometrically determined. The present immunoassay allows detection of 0.5 to 100 pg/tube of mabuterol.

Pharmacokinetic behavior of this agent in human plasma and urine was studied after a single oral administration (50 μg). The maximum level was achieved after 2-3 hrs with approximately 280 pg mabuterol /ml of plasma and the half life of mabuterol was estimated to be 19.5 hrs. Cumulative amount of mabuterol in the first 72 hrs urine was 64.3 ± 13.2% of the administered dose.

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INTRODUCTION

Mabuterol HCl, (±)-1-(4-amino-3-chloro-5-trifluoromethyl-phenyl)-2-(tert-butylamino) ethanol HCl, exhibits a potent and durative sympathomimetic activity at a very low dose and posseses a high β_2-receptor selectivity in the trachea. Based on these characteristics, mabuterol HCl is being developed as a bronchitis and pulmonary emphysema.

The pharmacokinetic behavior of mabuterol has been already studied in rats (1,2) following administration of high doses of ^{14}C-labeled mabuterol, and the metabolic pathway defined. Furthermore, Guentart et al.(3) performed pharmacokinetic studies in six male volunteers following a single oral administration of 40 μg of ^{3}H-mabuterol. If we wish to determine the precise plasma levels of mabuterol after administration of a clinical dose of this agent, a highly sensitive and specific analytical method is required. Recently, Yamamoto and Iwata (4) have succeeded in assaying the β_2-stimulant, clenbuterol which has a similar structure to mabuterol, by enzyme immunoassay.

In this paper, we describe a development of enzyme immunoassay and pharmacokinetic behavior of mabuterol in man.

MATERIALS AND METHODS

Materials

Mabuterol HCl (RC-0) and following metabolites found in rats and dogs were supplied by Karl Thomae GmbH (West Germany).

That is; 1-(4-amino-3-chloro-5-trifluoromethylphenyl)-2-(1,1-dimethyl-2-hydroxyethylamino) ethanol hydrochloride (RC-1), 1-(4-amino-3-chloro-5-trifluoromethylphenyl) glycol (RC-2), 4-amino-3-chloro-5-trifluoromethyl mandelic acid (RC-3), 4-amino-3-chloro-5-trifluoromethyl benzaldehyde (RC-4), 4-amino-3-chloro-5-trifluoromethyl benzoic acid (RC-5), and 4-amino-3-chloro-5-trifluoromethyl hippuric acid (RC-6). ^{14}C-mabuterol was obtained from New England Nuclear (U.S.A.). The radiochemical purity was 94.8% and the specific activity was 62 μCi/mg. β-D-Galactosidase (β-Gal, from Escherichia coli, Grade VIII, 940 units/mg protein), human serum albumin (HSA, Crystalized and lyophilized, essentially globulin free), 4-methylumbelliferone (4-MU) and 4-methylumbelliferyl-β-D-galactoside (4-MUG) were obtained from Sigma Co. (U.S.A.); anti-rabbit IgG serum (goat, heavy & light chains specific, antibody protein 12.0 mg/ml), from Difco Lab. (U.K.); Polystyrene balls (diameter, 1/4 inch), from Ichiko Co. Ltd. (Nagoya). All other chemicals from commercial sources were of analytical grade quality.

Animals for Immunization

Male albino rabbits weighing approximately 2 kg were used (Labo Animal,Tokyo).

Volunteers

Fourteen healthy male adults (age: 25-42 years old, body weight: 54-82 kg) were subjected to this study.

Administration of Drug and Collections of Blood and Urine

The tablets each containing 50 μg mabuterol HCl were administered after an overnight fast with 150 ml of water.

Blood samples were taken from the antecubita vein into vacuum blood collection tubes treated with heparin before and 1, 2, 4, 6, 8, 10, 24, 30, 48 and 72 hrs after administration. After the blood collection, plasma were prepared by an usual method and stored at -40°C before measurement.

Urine samples were collected before administration and at intervals of 0-3, 3-6, 6-10, 10-24, 24-48 and 48-72 hrs. Each aliquot of urine was stored at -40°C before measurement.

Preparation of Immunogen

Mabuterol HCl-HSA conjugate was prepared by a diazo-coupling method at 4°C according to the method of Yamamoto et al. (4) Sodium nitrite (13.3 mg) in 0.9 ml of water was added dropwise to a solution of mabuterol HCl (15.5 mg) in 2.5 ml of 0.2 N HCl with constant stirring. The reaction mixture was allowed to stand for 30 min. To remove the excess nitrous acid, ammonium sulfamate (31.1 mg) in 0.6 ml of water was added to the reaction mixture. The formation of diazonium salt was confirmed using N,N-dimethyl aniline. The diazonium solution was added dropwisely to 5 ml of 0.1 M phosphate buffer (pH 7.5) containing of 289.0 mg of HSA and the pH was adjusted to 8.0 with 1 N NaOH. The mixture was allowed to stand overnight and then, dialyzed against 0.01 M

phosphate buffer (pH 7.4) containing 0.15 M NaCl for 3 days. The number of haptenic molecule per HSA was estimated as seven on the bases of its absorbance (5,6) and of the experiment with ^{14}C-mabuterol.

Immunization

Male albino rabbits were immunized with 1 mg of immunogen emulsified in complete Freund's adjuvant and injected into the back. Booster injections at doses of 0.5 mg of the immunogen in incomplete Freund's adjuvant were given subcutaneously into the back at 4 week intervals. The titer and specificity of the antibody was determined at intervals by Farr's method (7) using ^{14}C-mabuterol (11 p mole, 500 dpm). Blood was taken from the ear vein 6 to 7 days after the second booster. Sera were lyophylized and stored at -40°C.

Preparation of Hapten-Enzyme Conjugate

The procedures described below were performed at 4°C.

RC-1, instead of mabuterol, was conjugated to β-Gal, by diazotization, because RC-1 has a slight cross-reactivity to the antibody raised against diazomabuterol-HSA and heterologous assays are generally known to improve the sensitivity of the EIAs. RC-1 solution (200 μg in 400 μl of water) was adjusted pH to 1.5 by 10 μl of 2 N HCl, followed by the dropwise addition of sodium nitrate solution (200 μg in 200 μl of water) with constant

stirring. The reaction mixture was allowed to stand for 30 min. Excess nitrous acid was removed by adding ammonium sulfamate (2.5 mg in 10 µl of water). Then 10 µl of the diazonium salt solution of RC-1 was added dropwise to a mixture of 100 µl of 0.01 M phosphate buffer (pH 7.5) containing 0.1 M NaCl and 1 mM $MgCl_2$ and 100 µl (445 units) of β-Gal. After standing for 2 hrs, the total volume was adjusted to 500 µl with the same buffer and the preparation was subjected to dialysis against the same buffer. The enzyme activity was kept 88% of original source, and the number of haptenic molecule per β-Gal was estimated as two by the experiment with ^{14}C-mabuterol. The enzyme conjugate obtained was stored at -20°C after mixing with equivalent volume of glycerin.

Preparation of Second Antibody-Bound Polystyrene Balls

Second antibody was immobilized on polystyrene balls by physical adsorption according to the following procedure: balls were soaked in a solution of the goat IgG fraction of anti-rabbit IgG (50-fold diluted with 50 mM phosphate buffer, pH 7.4, containing 0.1% NaN_3) at 4°C for 24 hrs and washed with in P-M buffer (100 mM phosphate buffer, pH 7.4, containing 0.1 M NaCl, 2 mM $MaCl_2$ and 0.1% BSA). They were then kept in the same buffer at 4°C for 18 hrs. The buffer was changed with new P-M buffer containing 1% BSA and kept another one hour until used to prevent artificial interactions of samples with the coated balls.

Assay Procedure

The enzyme immunoassay of mabuterol involved competition of β-Gal labeled RC-1 and standard or sample for a limited amount of antibody to mabuterol with separation of free and antibody-bound by anti-IgG immobilized on polystyrene balls. Fifty μl of standard human plasma (containing various concentrations of mabuterol) or sample solution was incubated at 4 C for 24 hrs with 50 μl of RC-1: β-Gal solution (diluted 1:40,000 with P-M buffer containing 5% of BSA) and 50 μl of anti-mabuterol serum (diluted 1:50,000 with P-M buffer containing 5% of BSA). Then, 50 μl of P-M buffer and a second antibody immobilized polystyrene ball were added to each assay solution. After mixing for 5 hrs at room temperature, the balls were washed with P-M buffer and transfered to a tube holding 400 μl of 0.3 mM 4-MUG. After incubation for 1 hr at 37 °C, 2.5 ml of 0.1 M Na_2CO_3 solution was added. The amount of the 4-MU liberated was determined by fluorescence spectrophotometry with an excitation wavelength at 360 nm and emission wavelength at 450 nm.

RESULTS

Calibration Curve and Cross Reactivities for Enzyme Immunoassay of Mabuterol

Typical calibration curves for the enzyme immunoassay of mabuterol show a linear displacement of enzyme labeled RC-1 by

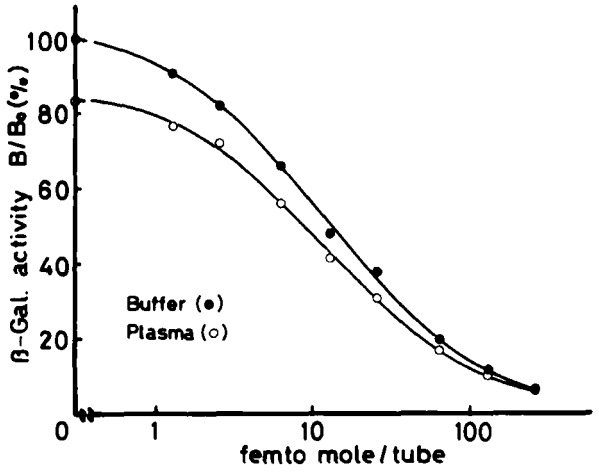

FIGURE 1. Calibration curves for mabuterol. Various amounts of mabuterol HCl were added to the pool plasma or P-M buffer and each point was determined in triplicate according to the assay procedure.

unlabeled mabuterol, when plotted as a semilogarithmic function from 1 to 200 f mole/tube of mabuterol (Fig. 1). As little as 0.5 pg of mabuterol could be distinguished from zero. Fig. 1 also shows the effect of plasma samples on this enzyme immunoassay system. Inhibition of Ag-Ab reaction occurred due to nonspecific interfering substances and rabbit IgG. To exclude nonspecific interfering substances from human plasma samples, an aliquot of normal (standard) plasma was included in each tube for drawing standard curve for mabuterol. Inhibitions by plasma were kept at a fixed rate in every assays.

The specificity of antibody directed against mabuterol was assessed with six known major metabolites (RC-2 ~ 6) of mabuterol

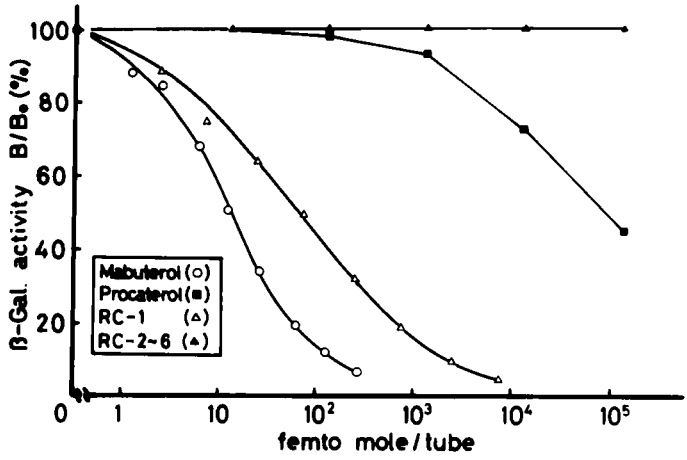

FIGURE 2. Specificity of anti-mabuterol serum, determined by EIA

found in rat urine (their chemical names listed in Materials and Methods) and procaterol which has the same phoarmacological action as mabuterol. The cross-reactivity of RC-2 ~ 6 and procaterol with anti-mabuterol serum was found to be no more than 0.02% (Fig. 2). RC-1 was the only metabolite that showed significant cross-reactivity (about 20%) with the antiserum. These results suggest that the present heterologous EIA with RC-1-β-Gal conjugate as an enzyme hapten is specific to mabuterol among mabuterol metabolites except RC-1.

Intra- and Inter-assay Coefficient of Variation for the EIAs

The coefficients of variation for mabuterol measurement were examined on seven human plasma samples. The intra-assay coeffi-

cients of variation range between 5.9 and 7.3%, while that of inter-assay between 6.7 and 11.2%. (data not shown)

Recovery and Dilution Test

Dilution tests were carried out with plasma samples which were serially diluted 1:2 to 1:64 with mabuterol free human plasma. This experiment resulted in a good linear relationship between dilution and measured values (data not shown).

Recovery studies with mean recoveries of 105.4% are shown in Table 1.

Plasma Levels of Mabuterol in Healthy Volunteers after a Single Oral Administration

The proposed assay was applied to the determination of mabuterol in plasma samples from fourteen men who were treated with a single oral doses of 50 μg mabuterol HCl. In each assay for plasma samples, mabuterol free human plasma was added to every tubes for standard curve in order to exclude the artificial interfererance from the plasma sample. Fig. 3 shows the time course of plasma concentrations of mabuterol. The levels were observed to rapidly increase in the cases after the oral administration. The maximum concentration (approximately 280 pg/ml of plasma) was observed 2-3 hrs after administration. Thereafter, the levels slowly decreased. Seventy-two hrs after administration, the plasma levels were lower than 1/10 of the

TABLE 1

Recovery Test

Added (pg/ml)	Measured (pg/ml)	Obtained (pg/ml)	Recovery Mean(%)
0	168	---	---
26	204	36	138
58	230	62	107
106	274	106	100
200	338	170	85
274	434	266	97
		Mean	105.4%

The concentrations of mabuterol were calculated using the calibration curve which was prepared by adding known amounts of mabuterol HCl to mabuterol free plasma.

maximum concentration. The half life of mabuterol in blood was estimated to be 19.5 hrs.

Cumulative Amount of Mabuterol in Urine after a Single Oral Administration

This assay was applied to the measurement of urine mabuterol from the same volunteers studied above. Fig. 4 shows cumulative amount of mabuterol (% of dose). Cumulative amount in each

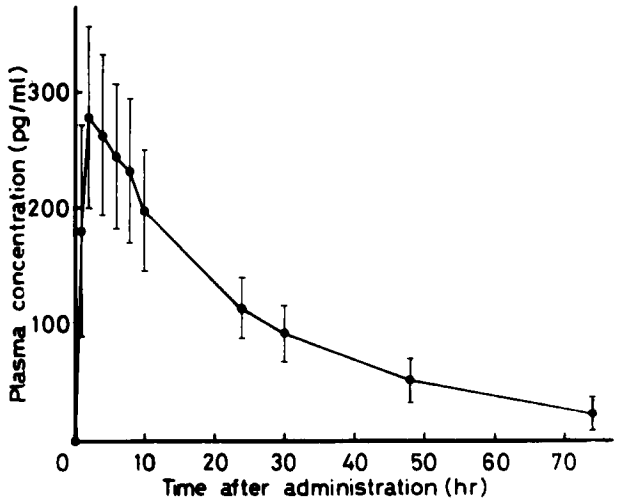

FIGURE 3. Plasma levels of mabuterol in volunteers after a single oral administration (50 μg/man). Each point is represented as the mean value of 14 healthy volunteers. The concentration of mabuterol were calculated using the calibration curve which was prepared by adding known amounts of mabuterol HCl to mabuterol free plasma.

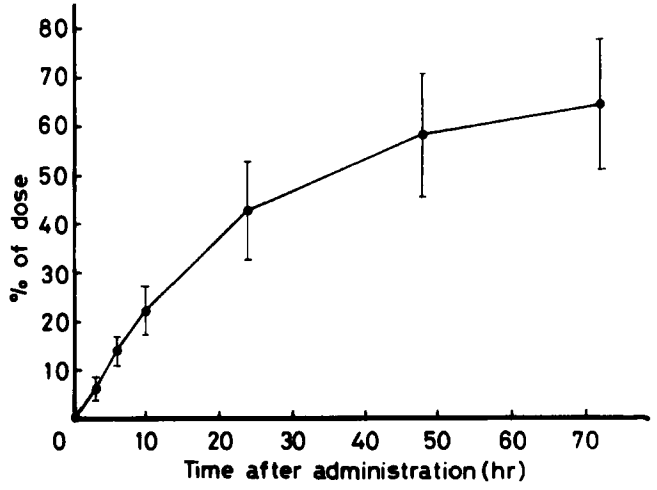

FIGURE 4. Cumulative amount of mabuterol in human urine. Urinary samples were collected before administration and at following intervals of 0-3, 3-6, 6-10, 10-24, 24-48 and 48-72 hrs. The urinary concentrations of mabuterol were measured after urinary samples were 50 times diluted with P-M buffer.

person was observed to slowly increase and at 72 hrs after the oral administration, that was 64.3 ± 13.2 %. Especially, 10 hrs after administration, the velocity of excreta in urine was in proportion to the plasma concentration.

DISCUSSION

To date, no useful method has been available for monitoring the plasma concentrations of mabuterol following clinical dose (50 μg). For example, the sensitivities of the radioimmunoassay and mass fragmentography were found to be 2 and 0.5 ng/ml plasma, respectively.

In this study, we have developed a highly sensitive double antibody and heterologous enzyme immunoassay for the quantitation of mabuterol. This analytical method made it possible to determine the mabuterol level in human plasma after a single oral dosage of 50 μg mabuterol HCl.

The calibration curve was linear in the range of 0.02 to 2 ng/ml when 50 μl of plasma sample was used and the maximum sensitivity was 1.29 f mole/tube (0.5 pg/tube).

In the assay of mabuterol with mabuterol-β-Gal, instead of RC-1-β-Gal, the expected sensitivity was not obtained (data not shown). Therefore, the heterologous enzyme immunoassay was to developed. The high sensitivity of this enzyme immunoassay is thought to be due to: 1) The anti-mabuterol antibody has both a

high titre and excellent specificity; 2) The combination of a hapten-heterologous assay having a high sensitivity with a double antibody solid phase method of their separation efficiency; 3) The enzyme activity in the conjugate remained at 88% of prereaction level in the reaction procedure, and β-Gal was uniformly conjugated to about two RC-1 molecules; 4) By soaking the second antibody balls in P-M buffer containing 1% BSA, nonspecific adsorption to the ball was reduced (the blank value was below 2% of total activity); and 5) The use of a system in which an excess amounts of second antibody were uniformly coated on polystyrene balls allowed a complete separation of antibody bound and free materials.

The cross-reactivity of RC-1 with the antiserum was found to be about 20%. The main reason for this was due to the use of RC-1-β-Gal as enzyme-hapten, instead of mabuterol-β-Gal in this enzyme immunoassay. Fortunately, the levels of RC-1 are thought to be negligible or very low in human body fluids after the administration of RC-1 at a dose of 40 μg. Following of administration of ^{3}H-labeled compound, levels of less than 10 pg/ml were reported (4).

Plasma levels of mabuterol in human volunteers after a single oral administration of 50 μg mabuterol have been determined by this method. The maximum plasma level of approximately 280 pg/ml was achieved at 2-3 hrs and the level was maintained over 200 pg/ml until 10 hrs after the administration. The half

life of mabuterol in plasma was estimated to be 19.5 hrs. These results suggest that mabuterol should be a long acting agent (8,9). One of the present authors has reported the kinetics of plasma levels of clenbuterol, an analog of mabuterol measured by using an enzyme immunoassay. The duration of clenbuterol is suggested to be much shorter than mabuterol. Clenbuterol has two chloric groups on benzene ring. One of these is displaced by CF_3 on mabuterol molecule.

The cumulative excretion of mabuterol in human urine was estimated to be 64.3 ± 13.2 % of the dose. The high excretion rate of unchanged mabuterol in human urine suggests a species difference between man and the rat. This is probably due to the fact that relatively large amounts of oxidative N-dealkylated metabolites were detected in rat urine after the administration of ^{14}C-labeled mabuterol, suggesting increased drug oxidizing activity in the liver of rats compared with man.

REFERENCES

1. Yuge, T., Hase, T., Takayanagi, T., Kamasuka, K., Amemiya, K. and Horiba, M., Pharmacokinetic studies of mabuterol, a new selective β_2-stimulant. I: Studies on the absorption, distribution and excretion in rats. Arzneim. Forsch./Drug Res. 1984; 34: 1659-1667

2. Horiba, M., Murai, T., Nomura, K., Yuge, T., Sanai, K. and Osada, E., Pharmacokinetic studies of mabuterol, a new selective β_2-stimulant. II. Urinary metabolites of mabuterol in rats and their pharmacological effect. Arzneim. Forsch. /Drug Res. 1984; 34: 1668-1679

3. Guentert, T.W., Buskin, J.N. and Galeazzi, R.L., Single dose pharmacokinetics of mabuterol in man. Arzneim. Forsch./Drug Res. 1984; 34: 1691-1696

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

5. Farr, R.S., A quantitative immunochemical measure of the primary interaction between BSA and antibody. J. Infect. Dis. 1958; 103: 239-962.

6. Erlanger, B.F., Borek, F., Beiser, S.M. and Liberman, S., Steroid protein conjugates. I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortison. J. Biol. Chem. 1959; 228: 713-727.

7. Erlanger, B.F., Borek, F., Beiser, S.M. and Liberman, S., Steroid protein conjugates. II. Preparation and characterization of conjugates of bovine serum albumin with progresterone, deoxycorticosterone and estorone. J. Biol. Chem. 1959; 234: 1090-1094.

8. Iwakura, M., Kohnoh, O. and Yamamoto, M. et al., Investigation on the optimal dose of mabuterol for bronchial asthma - Single dose study -. Sinryo to Shinyaku 1984; 22: 987-1000.

9. Kawakami, Y., Mue, S. and Miyamoto, A. et al., Clinical Investigation of mabuterol in bronchial asthma - Single dosing multicenter double blind trial -. in presentation.