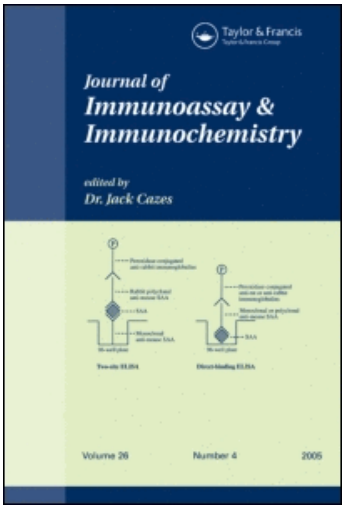

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Enzyme Immunoassay for Clenbuterol, an β_2-Adrenergic Stimulant

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ENZYME IMMUNOASSAY FOR CLENBUTEROL, AN β_2-ADRENERGIC STIMULANT

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ABSTRACT

A sensitive double antibody and heterologous enzyme immunoassay for the quantitation of clenbuterol is established. Specific antiserum to this agent was raised in rabbits by immunization with diazotized clenbuterol and human serum albumin conjugate. For competitive reactions, antibody was incubated with a mixture of diazotized clenbuterol analog (NA 1141) labelled with β-D-galactosidase and unlabelled standard or sample clenbuterol. The antibody-bound enzyme hapten was separated from free hapten by anti-rabbit IgG immobilized to a polystyrene ball. Activity of the enzyme on the solid phase was fluorometrically determined. The assay system made it possible to ascertain values as low as 0.5 pg /tube of clenbuterol. By use of this assay method, the time course of plasma levels of clenbuterol was examined after a single oral administration (20 μg) to 3 healthy volunteers. It was shown that the maximum level was achieved after 2-3 hr with approximately 10 ng clenbuterol/dl of plasma.

KEY WORDS: Plasma clenbuterol (NAB 365), NA 1141, Heterologous enzyme immunoassay, Polystyrene ball, β-D-galactosidase, β_2-stimulant.

INTRODUCTION

Clenbuterol (NAB 365), 4-amino-α-[(t-butylamino)methyl]-3,5-dichlorobenzyl alcohol hydrochloride, is a broncholytic agent with

selective activity on adrenergic β_2-receptors (1). This drug differs from the known β-mimetic drugs in its much longer duration of action. In addition, the dose of clenbuterol is extremely low because it is effective with trifling quantity (2-12). The pharmacokinetic behavior and metabolic patterns of clenbuterol were investigated in the rat, mouse, rabbit, dog, and man. In those studies, however, very high doses of clenbuterol (2-10 mg/kg) or isotope labelled clenbuterol were used (13-16). If we wish to determine the plasma levels of clenbuterol after administration of clinical doses of this agent, a sensitive and specific analytical method is required. We have successfully attempted to develop enzyme immunoassays for low molecular compounds such as cyclic nucleotides (17-19).

In this communication, we describe a sensitive enzyme immunoassay for clenbuterol. By use of this assay, plasma levels of clenbuterol after a single oral administration of 20 μg of this drug to human volunteers have been determined.

MATERIALS AND METHODS

Materials

Clenbuterol hydrochloride, its related compounds (NAB 739, NAB 821, NAB 930, NAB 933, NA 1141) and ^{3}H-clenbuterol were gifts from Teijin Institute for Bio-Medical Research (Tokyo). Chemical structures of them are illustrated in Figure 1. β-D-galactosidase (β-Gal, from *Escherichia coli*, Grade VIII, 674 units/mg protein),

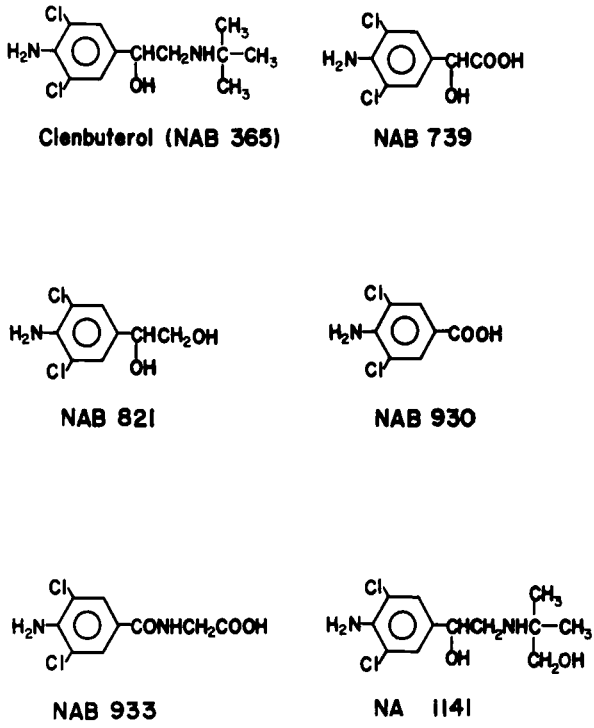

FIGURE 1. Chemical structures of clenbuterol and the related compounds.

human serum albumin (HSA, crystallized and lyophilized), bovine serum albumin (BSA, fraction V) and 4-methylumbelliferyl-β-D-galactoside (4-MUG) were obtained from Sigma Chemical Co. (St. Louis, Mo.); Immunoglobulin G (IgG) fraction of anti-rabbit IgG (prepared in goat, lyophilized), from Miles-Yeda Ltd. (Islael); Freund's complete and incomplete adjuvants, from Difco Lab. (Detroit, Mich.); Polystyrene balls (diameter, 1/4 inch), from Ichiko Co. Ltd. (Nagoya); ACS II scintillation cocktail, from RCC Amersham (London); 4-methylumbelliferone (4-MU), from Nakarai Chemicals Ltd. (Kyoto); Sodium nitrite and N,N-dimethylanilin, from

Katayama Chemicals (Osaka). All other chemicals from commercial sources were of reagent grade quality.

Animals

Male rabbits weighing approximately 1.8 kg were used for immunization.

Preparation of Immunogen

To obtain the conjugate of clenbuterol and HSA, diazotized clenbuterol was synthesized, and coupled to HSA. Briefly, 3 mg clenbuterol hydrochloride was dissolved in 400 µl of distilled water and the pH was adjusted to 1.5 by 100 µl of 1 N HCl followed by the dropwise addition of 3 mg sodium nitrite in 0.2 ml of distilled water under the dark condition at 4°C with constant stirring. The reaction mixture was allowed to stand for 30 min. The unreacted nitrous acid was removed by the addition of 140 µl of distilled water containing 7 mg of ammonium sulfamate until no more nitrogen bublles were given off. The diazotized clenbuterol was detected by reaction with N,N-dimethylanilin to form deep yellow. The diazo-clenbuterol solution was slowly added to 1 ml of 0.1 M phosphate buffer (pH 7.5) containing 65 mg HSA, the pH being adjusted to 7.5 with 1 N NaOH. The preparation was allowed to stand at 4°C overnight and then, dialyzed against 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl at 4°C for 3 days in the dark with frequent changes of the dialyzing solution. The immunogen was lyophilized and stored at -20°C until use. An experiment with ^{3}H-clenbuterol indicated a conjugate of about 7 clenbuterol residues per HSA molecule.

Immunization

Antibody to clenbuterol was produced in rabbits by repeated intradermal immunizations. The first immunization was performed with 0.5 mg of diazotized clenbuterol-HSA conjugate in complete Freund's adjuvant and the booster doses of 0.2 mg of the immunogen in incomplete Freund's adjuvant were given at two week intervals. After the resting period of two months, the fourth booster was performed again. The titre and specificity of the antibody was often inspected employing ligand binding affinity test using ^{3}H-clenbuterol as ligand as described for that of cyclic nucleotides by Yamamoto et al. (17). On the eighth day after the fifth booster, blood was taken from the ear vein. The antiserum obtained was lyophilized and stored at -20°C until use.

Preparation of Hapten-Enzyme Conjugate

NA 1141, instead of clenbuterol, was conjugated to β-Gal by diazotization. Hundred µl of distilled water containing 20 µg NA 1141 was mixed with 300 µl of distilled water and the pH was adjusted to 1.0 by adding 10 µl of 2 N HCl followed by the addition of 200 µl of $NaNO_2$ (20 µg). Ten µl of ammonium sulfamate (250 µg) was added to the mixture after incubating for 30 min. Diazotization of NA 1141 was confirmed with N,N-dimethylanilin. Then 20 µl of diazotized NA 1141 was added to a mixture of 190 µl of 0.01 M phosphate buffer (pH 7.5) containing 0.1 M NaCl and 1 mM $MgCl_2$ and 10 µl (44.5 units) of β-Gal. After standing at 4°C for 2 hr, 280 µl of the same buffer was added to the mixture and the preparation was subjected to dialyzation against the same buffer.

The enzyme activity was reduced by 70 % by the reaction procedure. The enzyme conjugate obtained was stocked 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 procedures; balls were soaked in a solution of the goat IgG fraction of anti-rabbit IgG (50-fold diluted with 0.05 M sodium phosphate buffer, pH 7.5) containing 0.1 % NaN_3 at 4°C for 24 hr and washed three times in 0.01 M phosphate buffer (pH 6.6) containing 0.1 M NaCl, 1 mM $MgCl_2$ and 0.1 % BSA (A_1 buffer). They were then kept in the same buffer at 4°C for at least 18 hr until use.

Preparation of Samples

The preliminary pharmacokinetic studies of clenbuterol in man were carried out in three male healthy volunteers (31-37 years old, 63-72 kg). A single dose of 20 μg of clenbuterol hydrochloride was orally administered to these subjects and the blood samples taken from the medial cubital vein in the bend of the elbow at the indicated times. Plasma obtained was kept at -20°C until use. The medications and samplings were carried out by Prof. Mitsuyoshi Nakajima at Hamamatsu University, School of Medicine, Hamamatsu, Shizuoka.

Preliminary Treatment of Samples

Hundred μl of human plasma was diluted in 300 μl of 0.1 M phosphate buffer (pH 6.6) containing 0.1 M NaCl and 1 mM $MgCl_2$ (A_2 buffer) and the diluted plasma was subjected to heating at

100°C for 1 min. The denatured plasma preparation was then clarified by centrifugation at 15,000 rpm for 20 min. The supernatant was stored frozen until assayed. Each assay was run in duplicate.

Assay Procedure

This enzyme immunoassay for clenbuterol was based on double antibody and heterologous solid phase method; for the first step, competition of enzyme-labelled clenbuterol analog (NA 1141) and standard or sample clenbuterol for the binding to a limited amount of antibody in aqueous phase, and for the second step, separation of free and antibody bound hapten by anti-IgG as a second antibody immobilized on polystyrene balls. For the competitive reaction assay tubes containing 50 µl of β-Gal-diazo NA 1141 conjugate (diluted 1:2,000 with A_2 buffer containing 5 % BSA), 50 µl of anti-clenbuterol serum (diluted 1:32,000 with A_2 buffer containing 5 % BSA) and 100 µl of sample or standard (clenbuterol) in A_2 buffer were incubated at 4°C for 18 hr and then, 200 µl of A_2 buffer and a second antibody immobilized polystyrene ball were added to each tube. Antibody bound β-Gal-diazo NA 1141 was bound to the polystyrene ball by rocking the ball in the mixture for 5 hr at room temperature. The ball was then washed with A_1 buffer and transferred to a tube containing 200 µl of A_1 buffer. The activity of enzyme bound to the solid phase was determined by incubating the ball in a reaction mixture of 200 µl of 0.3 mM 4-MUG and 200 µl of A_1 buffer at 37°C for 90 min and terminating the reaction by the addition of 2.5 ml of 0.1 M glycine-NaOH buffer (pH 10.3). The amount of the 4-MU liberated was determined by fluorescence spec-

trophotometry with excitation wavelength at 360 nm and emission wavelength 450 nm.

RESULTS

Calibration Curve and Cross Reactivity for Enzyme Immunoassay of Clenbuterol

A preliminary experiment revealed that NA 1141, a minor metabolite in animals but not in human showed, to lesser extent, cross-reactivity to antibody raised against diazo-clenbuterol HSA conjugate in rabbits. Therefore, we have made attempt to compare homologous and heterologous enzyme immunoassay by using clenbuterol and NA 1141, respectively as enzyme labelled hapten. Figure 2 illustrated typical calibration curves for the enzyme immunoassay of clenbuterol which shows linear displacements of enzyme labelled NA 1141 or clenbuterol by unlabelled clenbuterol, when plotted as a semilogarithmic function from 0.5 to 128 pg/tube of clenbuterol.

The sensitivity has been significantly increased by employing β-Gal-diazo-NA 1141 as enzyme-labelled hapten instead of β-Gal-diazo-clenbuterol. Therefore, β-Gal-diazo-NA 1141 was employed as enzyme labelled hapten in the present enzyme immunoassay for clenbuterol.

Specificity of anti-clenbuterol serum was assessed with some metabolites of clenbuterol such as NAB 739, 821, 930, 933 and NA 1141 in urine of human and animals such as dog and rabbit. These clenbuterol metabolites except NA 1141 were extensively less

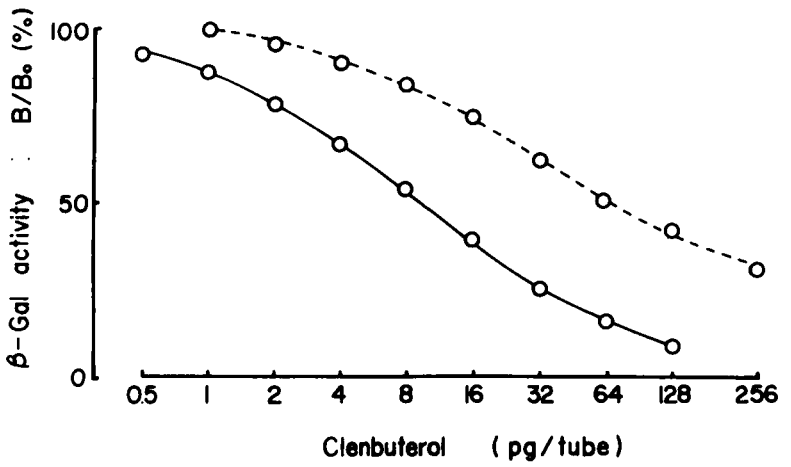

FIGURE 2. Typical calibration curves for clenbuterol by enzyme immunoassay with homologous and heterologous hapten-enzyme conjugates. Incubation tube contained anti-clenbuterol serum (diluted 1:32,000), β-Gal-diazo-clenbuterol (---O---) or β-Gal-diazo-NA 1141 (—O—) (diluted 1:2,000) and standard clenbuterol at concentrations from zero to 128 pg in a final volume 200 µl in A_2 buffer. Assays were performed as described under "MATERIALS AND METHODS". Each point is represented as mean % of duplicate assays.

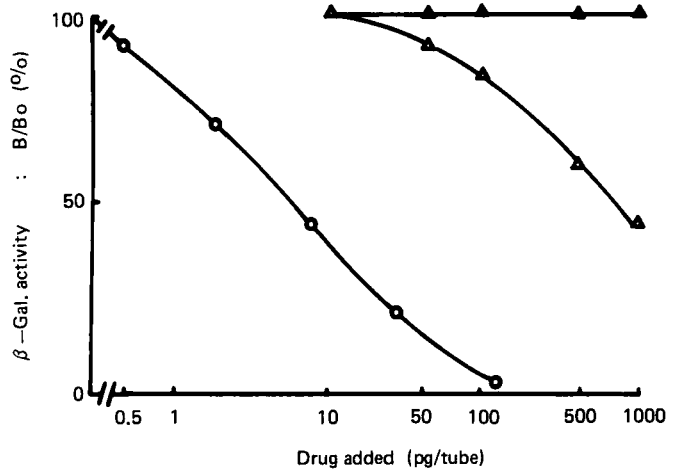

FIGURE 3. Cross-reactivity by some clenbuterol metabolites in the enzyme immunoassay. Incubation tube contained anti-clenbuterol serum (diluted 1:32,000), β-Gal-diazo NA 1141 (diluted 1:2,000) and standard clenbuterol or clenbuterol metabolites (NAB 739, NAB 821, NAB 930, NAB 933, —▲— ; NA 1141, —Δ—) in a final volume 200 µl in A_2 buffer. Assays were performed as described under "MATERIALS AND METHODS".

reactive with the antibody. NA 1141 was only one metabolite which showed cross-reactivity with this antibody. However, this compound is expected to be negligible for practical purposes, because unmetabolized clenbuterol accounts for 85-90 % of the radioactivity in the whole extract of urine in all 3 species, but all other metabolites present account for less than 4 % after radioactive clenbuterol administration, and because cross reactivity of NA 1141 in this assay is less than 1 % as shown in Figure 3. In addition, this metabolite is shown to be extremely low in human urine after an oral administration of clenbuterol. Any acetyl product is not identified in the urine (14,15).

Precision and Validity of the Enzyme Immunoassay

Plasma may contain substances which interfere with the binding reaction or the enzyme activity. Matrix problems were minimized by

TABLE 1

Dilution Test with Human Plasma in Clenbuterol Determinations by the Proposed Enzyme Immunoassay

Dilution	Measured Values (ng of Clenbuterol / dl of Plasma)		
	Sample No. 1	2	3
1 : 8	9.4	16.0	16.8
1 : 16	4.6	8.4	8.6
1 : 32	2.8	4.8	4.8
1 : 64	1.4	3.0	2.1
1 : 128	ND	1.6	1.1

ND : not determined.

Plasma samples were serially diluted with A_2 buffer and subjected to the enzyme immunoassay.

TABLE 2

Recovery Test with Human Plasma in Clenbuterol Determinations by the Proposed Enzyme Immunoassay

Number of Samples	Added (pg/tube)	Recovery (%)	S.D.
5	2	99	9.6
5	4	104	8.4
5	8	103	5.1
5	16	100	6.6
5	32	100	0.9
Mean		101	6.1

Authentic clenbuterol was added to plasma samples obtained from clenbuterol-treated volunteers. Clenbuterol was measured by the proposed enzyme immunoassay and calculated for recovery %. Each value is represented as the mean of five determinations.

TABLE 3

Coefficient of Variation in Clenbuterol Determination by the Proposed Enzyme Immunoassay

	Sample No.	Intra-assay (n=6) M[a] (ng/dl)	S.E.[b]	C.V.[c] (%)
Human Plasma	1	2.9	0.14	12.6
	2	5.4	0.08	4.1
	3	13.7	0.16	3.1
	4	19.4	0.11	1.9
	5	51.9	0.49	3.4

[a] M : mean of assays.

[b] S.E. : standard error of mean.

[c] C.V. : coefficient of variation.

incubating the assay in 0.1 M phosphate buffer (pH 6.6) containing 0.1 M NaCl, 1 mM $MgCl_2$ and 2.5 % BSA.

Dilution test was carried out with human plasma samples which were serially diluted 1:8 to 1:128 with A_2 buffer. This experiment resulted in a good linear relationship between dilution and measured values (Table 1).

Varying concentrations of authentic clenbuterol were added to a certain plasma sample obtained from a healthy volunteer who was orally administered with 20 μg clenbuterol hydrochloride. Total amounts of clenbuterol were calculated for recovery (%) by enzyme immunoassay (Table 2). The mean recovery was 101 %.

Reproducibility of the present enzyme immunoassay was evaluated on the basis of variations in assayed values for the same samples within assays.

Table 3 shows the coefficients of variation for clenbuterol levels in five human plasma samples on intra-assay. The intra-assay coefficients of variation range between 1.9 and 12.6 %.

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

By use of this enzyme immunoassay, the time course of plasma levels of clenbuterol was examined after a single oral administration of 20 μg of the drug to 3 healthy volunteers. In this experiment, 50-100 μl of plasma samples were used for the assay.

Clenbuterol level in plasma in each person was observed to rapidly increase after the oral administration. The maximum level was achieved 2-3 hr after administration with approximately 10 ng

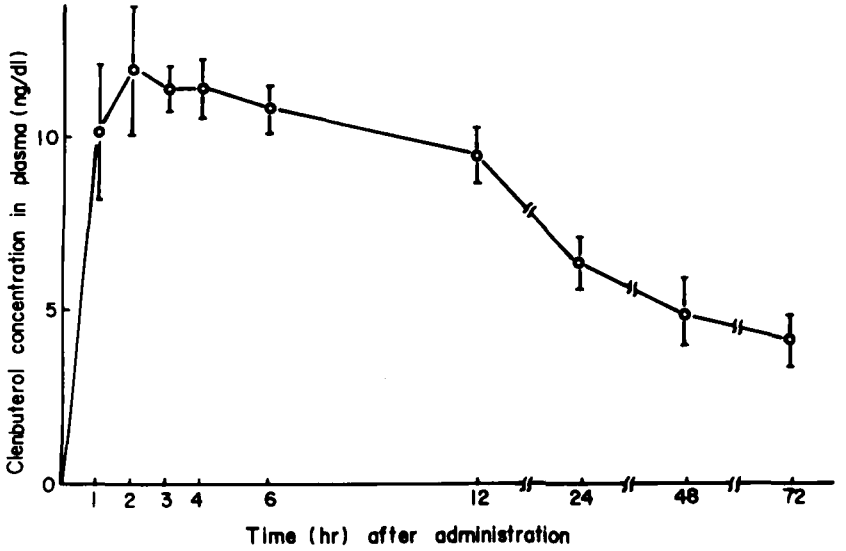

FIGURE 4. Plasma levels of clenbuterol in volunteers after a single oral administration (20 μg/body). Each point is represented as the mean value of 3 healthy volunteers.

of clenbuterol/dl of plasma. Thereafter, the levels of plasma clenbuterol were slowly decreased. Seventy two hr after administration, the concentration in the plasma was still higher than 1/3 of the maximum concentration achieved after 2 hr. These results are shown in Figure 4.

DISCUSSION

A radioimmunoassay method specific for clenbuterol was developed by Kopitar and Zimmer in 1976 (16). The sensitivity of the method of measurement was 2 to 16 ng per sample. This method could be applied for measurement of plasma levels of clenbuterol in animals treated with high dose (5 mg/kg) clenbuterol, but not

of human plasma sample, because clinical doses are 10-50 μg for treating patients with athma and other diseases (2-12).

In this study, we have developed a highly sensitive double antibody and heterologous enzyme immunoassay for clenbuterol. This assay allows to measure samples containing 0.5-100 pg clenbuterol. By use of this analytical method, plasma levels of unmetabolized clenbuterol in human volunteers after a single oral administration of only 20 μg of clenbuterol hydrochloride have been determined. Plasma levels of clenbuterol was observed to increase rapidly until 2 hr following administration. A maximum plasma level of approximately 10 ng/dl of clenbuterol is achieved 2-3 hr after the administration and the half life of clenbuterol in blood was estimated to be 30 hr. The pharmacokinetic patterns of clenbuterol in plasma are correlated well with those obtained by using isotope labelled compound (15).

Prior separation of clenbuterol from its metabolites or interfering substances are not necessary if the samples are applied to this assay system after plasma protein are removed by heating the samples at 100°C for 1 min. We have confirmed that clenbuterol is not decomposed by the heat-treatment. Assay of filtrate obtained by ultrafiltration technique indicated that about 95 % amount of clenbuterol in plasma is bound to plasma protein.

We have successfully attempted to determine plasma levels of clenbuterol in rats and humans after single or repeated administrations of 10 μg clenbuterol hydrochloride/body and in urine of volunteers after a single oral dose of 20-80 μg clenbuterol hydrochloride. Thus, those results will be published elsewhere.

High sensitivity of this enzyme immunoassay contributes largely to the following reasons. 1) hapten-heterologous technique by using clenbuterol specific antiserum and NA 1141-β-Gal instead of clenbuterol-β-Gal is introduced; the affinity of the antiserum is much higher to clenbuterol than to NA 1141, therefore specific antiserum selectively combines clenbuterol. The sensitivity, thus, raises comparing with homologous assay system. 2) β-Gal from *E. coli*. used to label hapten has high turnover, availability and stability. In addition, the enzyme activity is measured in fluorometer, by using 4-MUG as substrate. 3) Polystyrene balls used as solid phase have the broad area uniformly and excellence in physical adsorption of antibody.

The dilution and recovery tests as well as the intra-assay tests resulted satisfactory. This enzyme immunoassay is therefore valid in any routine laboratory and the application of the present assay principle will be able to expand the development of the microquantitative analysis of other drugs.

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The abbreviations used are: A_1 buffer, 0.01 M phosphate buffer, pH 6.6 containing 0.1 M NaCl, 1 mM $MgCl_2$ and 0.1 % BSA; A_2 buffer, 0.1 M phosphate buffer, pH 6.6 containing 0.1 M NaCl and 1 mM $MgCl_2$; BSA, bovine serum albumin; β-Gal, β-D-galactosidase; HSA, human serum albumin; IgG, immunoglobulin G; 4-MU, 4-methylumbelliferone; 4-MUG, 4-methylumbelliferyl-β-D-galactoside; NA 1141, 2-[2-(4-amino-3,5-dichlorophenyl)-2-hydroxyethylamino]-2-methylpropanol; NAB 365, clenbuterol, 4-amino-α-[(t-butylamino)-methyl]-3,5-dichlorobenzyl alcohol hydrochloride; NAB 739,(4-amino-3,5-dichlorophenyl)hydroxyacetic acid; NAB 821, 1-(4-amino-3,5-

dichlorophenyl)-1,2-ethandiol; NAB 930, 4-amino-3,5-dichlorobenzoic acid; NAB 933, N-(4-amino-3,5-dichlorobenzoyl)glycine.

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