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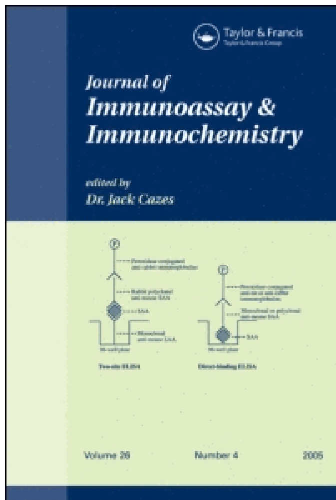

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Radioimmunoassay for Albuterol Using a Monoclonal Antibody: Application for Direct Quantification in Horse Urine

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RADIOIMMUNOASSAY FOR ALBUTEROL USING A MONOCLONAL ANTIBODY: APPLICATION FOR DIRECT QUANTIFICATION IN HORSE URINE.

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ABSTRACT

A monoclonal antibody was synthesized in mouse against the O-(3-carboxypropionyl) derivative of albuterol linked to bovine serum albumin. Isotyping of this material revealed the IgG1 class characterized by an affinity constant of 1.03 nM^{-1} and a density of sites of 0.55 nM. This antibody was found specific as its cross-reactivity to structurally related molecules was less than 1% except for clenbuterol (75%). A radioimmunoassay was set up with culture supernatant (final dilution 1/1000) and [^{3}H] albuterol. The calibration curve was characterized by a maximum binding of 28%, an ED50 of 1.15 pmol per tube, the detection limit was 28.8 fmol/tube and the linearity of the response was up to 39.8 pmol/tube. This RIA method has been used for direct quantitation of albuterol in horse urine without any clean-up or extration step. (KEY WORDS: albuterol, clentuberol, radioimmunoassay, monoclonal antibody).

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INTRODUCTION

Beta-2 agonists have been extensively used for the treatment of constrictive and obstructive pulmonary diseases, in human and veterinary medicine. Among these drugs, albuterol has been used for the improvement of the pulmonary function in race doping and for the increase of the muscular mass of oxen and calves. For these reasons, the monitoring of albuterol is required for the quality control of drug residues in meat as well as for the detection of this substance in anti-doping purposes.

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The methodologies available for the determination of albuterol in biological samples include gas chromatography-mass spectrometry (1), high pressure liquid chromatography with fluorometric (2) and electrochemical (3-4) detection, high performance thin layer chromatography (5) and radioimmunoassay using a polyclonal antiserum (6). Most of these procedures require laborious extraction steps and this is a limiting factor for the processing of a large number of samples.

The present study describes a sensitive radioimmunoassay to quantitate albuterol using a monoclonal antibody. This method has been validated for the direct quantitation of albuterol in horse urine samples.

MATERIAL and METHODS

Material

The following materials were purchased from the suppliers indicated: albuterol hemisulfate, terbutaline, bovine serum

albumin and bovine gamma globulins Cohn fraction II, III (Sigma Chemical Co., St.Louis, MO), tris-hydroxymethylaminoethane, charcoal Norit, polyethylene glycol 8000, Scintiverse BD (Fisher Sci, Muskegon, MI), dextran T70 (Pharmacia, Uppsala, Sweden), Freund complete adjuvant (CFA) and cell culture media: Hypoxanthine aminopterine thymidine (HAT), Rosewell Park Memorial Institute (RPMI) (Difco, DETROIT, MI). Microplates (Nunc, Roskilde, Denmark), Ultrasphere ODS column (Beckman, Palo Alto, CA). Ouchterlony immunodiffusion kit (ICN Biochemicals, Costa Mesa, CA). Mouse Immunoglobulin Subtype Identification kit (Boehringer Mannheim, Indianapolis, IN).

Fenoterol and clenbuterol were a generous gift of Boehringer Ingelheim (GFR). Tritiated [^{3}H] albuterol was custom labelled by the Negev Nuclear Research Center (Beer Sheva, Israel) with a specific acitvity of 17Ci/mmol.

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Methods

Synthesis of salbutamol succinate derivative and its coupling to bovine serum albumin (BSA).

The synthesis of the O-(3-carboxypropionyl) derivative of salbutamol has been performed according to Beaulieu et al (7). Briefly, 200 mg of salbutamol base were dissolved in 25 ml of absolute ethanol in presence of 10^7 dpm of [^{3}H] salbutamol. While stiring, both succinic anhydride (90 mg) and [^{14}C]

labelled succinic anhydride (2.7 x 10^6 dpm) were added. The harvested cristals were washed with ethanol until a constant ratio $^3H/^{14}C$ was obtained. The melty point (142°C) of the derivative confirmed the salbutamol benzylic succinate derivative was obtained.

Forty mg of this derivative were dissolved in 25 ml of dioxane, 3 ml water and 300 µl triethanolamine were coupled to BSA (86 mg) previously dissolved in 25 ml of distilled water in resence of 300 µl triethanolamine. The solution was finally dialyzed against distilled water giving a yield of 3.87 moles of hapten/mole of BSA.

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Production of the monoclonal antibody

Immunization protocol

Three Biozzi mice (IFFA Credo, France) were immunized intraperitoneally with 20 µg of the O-(3-carboxy propionyl) derivative of albuterol covalently linked to BSA, emulsified in 0.25 mL of the CFA. Three weeks later, the blood of mice withdrawn from the tail was screened for the presence of anti-albuterol antibodies. The mouse exhibiting the highest titer was boosted intraperitoneally with 40 µg of albuterol conjugate emulsified in 0.25 mL CFA, 3 to 4 days prior to the fusion.

Fusion

Spleen cells (1.4 x 10^8 cells) were fused with SP2/0 mouse myeloma cell line (2.8 x 10^7 cells) using the polyethylene glycol method described by Fazekas de Saint Groth (8,9). The spleen cell suspension (10^6 cells per well) was seeded in 96 well microplates with mouse peritoneal macrophages as feeder layer. Fusion hybrids were microscopically observed, 10 days after. On days 13 and 19, the supernatants of growing hybrids were screened for the presence of anti albuterol IgGs, using the radioimmunoassay procedure described below.

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Cloning

After the screening of the cell supernatants, the cloning was performed twice by limiting dilution at 0.5 and 1 cell per well in 96 wells microplates using human umbilical cord serum as feeder.

Screening for the production of anti-albuterol IgG antibodies

Anti-albuterol IgG antibodies in the sera of immunized mice and the culture supernatant were screened by RIA. Briefly, 100 µL of the test sample diluted to 1:4 with 200 mM phosphate buffer, pH 7.4, were incubated with 100 µL of [^{3}H] albuterol in a final volume of 0.5 mL for 4 hr at 4°C. Polyclonal rabbit antiserum (final dilution 1/2500) (10) and culture medium were used as positive and negative controls

respectively. The separation of bound from free [^{3}H] albuterol was obtained by adding 0.5 mL charcoal mixture consisting of charcoal Norit 10 g, dextran T70 1.0 g, bovine gammaglobulins fraction II and III 2.8 g/L in 200 mM phosphate buffer, pH 7.4. The mixture was incubated for 15 min at 4°C, followed by centrifugion at 3,000 g, for 15 min at 10°C.

The supernatants were then decanted and their radioactivity was determined in the external standard mode (Beta counter, LKB, Bromma, Sweden).

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Radioimmunoassay for quantitation of albuterol using the monoclonal antibody

The assay buffer was a 50mM Tris/HCl, pH 7.4. The incubation medium (0.5mL) consisted of standards (1.05 to 266 pmol/mL) or urine samples (100 µL), [^{3}H] albuterol (100 µL, 10000 dpm), culture supernatant containing the monoclonal antibody at the final dilution of 1/1000 (100 µL) and assay buffer (200 µL). Calibration curves were constructed by serial dilutions of a stock solution from 1.05 to 266 pmol/mL. The non-specific binding was defined in the absence of the monoclonal antibody and a quality-control sample which consisted of blank horse urine spiked with albuterol at 20 pmol/mL was included in each assay batch. After incubation for 2 hr at room temperature, the separation of bound from free fraction was

carried out by adding 40 µL of a rabbit anti-mouse IgG antiserum and 0.5 mL of PEG 8000 (15% w/w) and 500 µg of microcrystalline cellulose to each tube. The tubes were then vortexed, allowed to stand for 15 min at 10°C and were centrifuged at 3,000 g, for 15 min at 4°C. The supernatants were carefully aspirated out and the pellet corresponding to the bound fraction was resuspended in 0.5mL of the incubation buffer, and the radioactivity was counted in a beta counter.

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In order to assess the specificity of the monoclonal antibody, the cross-reactivity of the structurally related molecules and those used in horse doping were examined. The cross-reactivity (%) was defined as the ratio dose at 50% displacement of the tracer (ED50) for albuterol relative to ED50 for each of the following substances: terbutaline, isoproterenol, fenoterol, clenbuterol, dobutamine, amphetamine, ephedrine, and phenylephrine.

Urinary excretion profile of albuterol following an IV dose in horse

One quarter horse, 6 years old female (465 kg), without any medication during the week preceding the experience was used. During the experiment, the horse was restrained but had access to water ad libitum. A bolus of albuterol (10 µg/kg body weight) in 100 mL NaCl 0.9% was injected intravenously via a

catheter inserted in the jugular vein. Urine was sampled via a Foley catheter aseptically installed in the bladder, before administration of the drug and at each hour for a 12 hr period. The volume of each sample was measured and the sample stored at -30°C. The pH of urine samples was adjusted to pH 7.0-7,.5 and the samples were then filtered or centrifuged (2 500 g, 10 min) to eliminate precipitated phosphate or urate before albuterol analysis.

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Immunogram of urinary albuterol

To evaluate the specificity of the radioimmunoassay procedure, aliquots of urinary samples from a horse receiving a 10 µg/kg body weight were submitted to a clean-up extraction step using PRP-1 cartridge (Chromatographic spec., Milford, Mass) preactivated with 5 mL of methanol and 5 mL of distilled water. Following the application of the urine samples (1 mL), the cartridge was washed with 5 mL distilled water; albuterol was finally eluted with 2 mL methanol. The eluate was then evaporated to dryness in a speed vac concentrator (Savant, Farmingdale, NJ). The residue was reconstituted in 150 µL of mobile phase and 100 µL were injected in the HPLC system.

The chromatographic system consisted of a pump (Model 510, Waters Ass, Milford, Mass) an injection valve (Rheodyne, Model 7125, Berkeley, CA) with a 100 µL loop. The reverse phase HPLC column used was Ultrasphere ODS, 5 µm, 25 cm x 4.6 mm ID

(Beckman, Palo ALto, CA). The mobile phase consisted in a mixture of 50 mM ammonium acetate buffer pH 6, methanol, acetonitrile (80:8:12 v/v). The flow rate was at 0.8 ml/min. Fractions of 1 mL were collected (FRAC 100 C collector, Pharmacia, Uppsala, Sweden). The fractions were then evaporated to dryness as described earlier to determine albuterol by RIA.

Data analysis

The curves were analysed by non linear regression using a four parameter logistic equation providing estimates for asymptotic maximal and minimal binding, the slope factor, and ED50 of the curve (11).

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RESULTS

Monoclonal antibody characteristics

Isotyping of the monoclonal antibody

Isotyping of the monoclonal antibody was carried out by Ouchterlony immunodiffusion using the commercial kit from ICN. A precipitation band was observed only when the antibody reacted with rabbit anti IgG1. No reaction was detected with antiserum against other mouse IgG subclasses, IgA and IgM (figure 1). Identical results were confirmed by an ELISA method using the Mouse Immunoglobulin Subtype Identification kit and by immunodot (12-13).

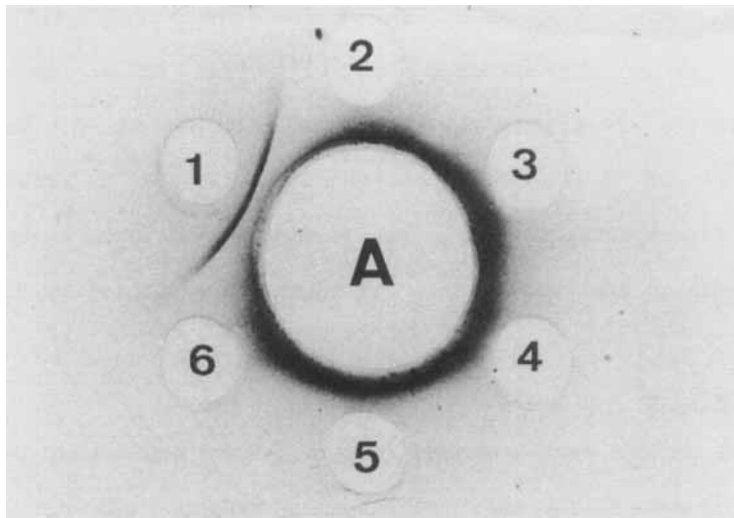

Figure 1: Isotyping of monoclonal antibody by Ouchterlony immunodiffusion. Culture supernatant: A. Rabbit antiserum against mouse immunoglobulin: 1(IgG1), 2(IgG2a), 3(IgG3), 4(IgG4), 5(IgA), 6(IgM).

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Specificity of the monoclonal antibody

The comparative cross-reactivity of albuterol analogs and other drugs used in horse doping is shown in Table 1. It is interesting to note that only clenbuterol displays a high cross-reactivity (75%) followed by terbutaline (7%). Cross-reactivity less than 1% could be detected for other tested molecules.

TABLE 1

COMPARATIVE CROSS REACTIVITY OF ALBUTEROL ANALOGS IN THE RADIOIMMUNOASSAY

Drug	ED50 (pmol/mL)	Cross-reactivity (%)
Albuterol	12	100
Clenbuterol	16	75
Terbutaline	171	7
Fenoterol	< 2 500	< 1%
Isoproterenol	< 2 500	< 1%
Dobutamine	< 2 500	< 1%
Amphetamine	< 2 500	< 1%
Ephedrine	< 2 500	< 1%
Phenylephrine	< 2 500	< 1%

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Affinity constant of the monoclonal antibody

The Scatchard analysis revealed the presence of a single homogeneous antibody population characterized by an affinity constant of 1.03 nM^{-1} with a density of sites of 0.55 nM (14).

RIA characteristics

Figure 2 shows a typical calibration curve which is characterized by a maximal binding of 27.7% (SD = 0.1%) of the total labelled albuterol added, a ED50 of 1.15 (SD = 0.032) pmol per tube and a slope factor of 1.01 (SD = 0.023). The minimum detectable amount (Bo mean minus 2 SD) was 28.8 fmol/tube and

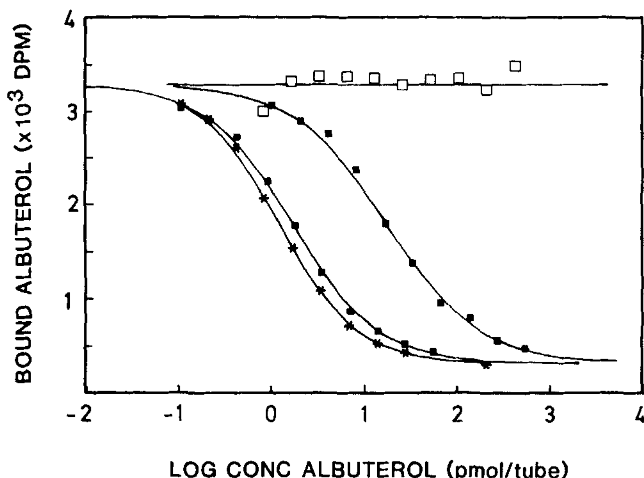

Figure 2: A typical calibration curve for albuterol radioimmunoassay. Competition curve between albuterol and its tritiated derivative using the monoclonal antibody (●). The parallel inhibition of radioligand binding with serial dilutions of urinary excreted albuterol (★) and blank urine spiked with albuterol standard (□) was reported.

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the linearity of the response was up to 39.8 pmol/tube. The non-specific binding of [^{3}H] albuterol ranged between 1-1.5%.

Similarly, excreted urinary albuterol was tested by analysing the parallelism between the calibration curve and a dilution curve of horse urine.

The mean slope factor of these calibration and dilution curves (0.96 $\pm$ 0.027 and 1.01 $\pm$ 0.023 respectively) were not statistically different ($F = 3.87$, $P < 0.01$). Similar results were obtained with successive dilutions of a calibration curve spiked in urine.

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Validation of the assay

The precision of the assay was assessed by testing blank urine spiked with albuterol concentrations of 4, 16, 32 pmol/mL. These samples were analysed in five replicates over five consecutive days. The intra-assay coefficients of variation were 5.9, 5.3 and 6.8%, respectively. The inter-assay coefficients of variation were 12.4, 7.8 and 8%, respectively.

The accuracy of the assay was tested using a blank urine to which albuterol was added to give a final concentration of 1.05 to 133 pmol/mL. The linear regression curve for the correlation between albuterol concentrations expected and those measured is described by the following equation: $Y = 0.898X + 0.75$ ($r = 0.995$, $P < 0.01$). The absolute analytical recovery of albuterol was over 95% for the range of concentrations studied.

Specificity of the radioimmunoassay

In order to assess the specificity of the radioimmunoassay, urinary sample collected following an intravenous dose of 10 µg/kg in horse was diluted at 1/80, 1/160, 1/320 and 1/640 with blank urine. Aliquots of the diluted samples were subjected to direct radioimmunoassay before and after HPLC chromatography. The peak was measured at the elution volume (5 ml) of the authentic albuterol standard (Figure 3). These values were correlated to that measured by direct radioimmunoassay. The linear regression curve is given by the equation: $Y = 0.849X - 0.45$ ($r = 0.995$, $P < 0.01$).

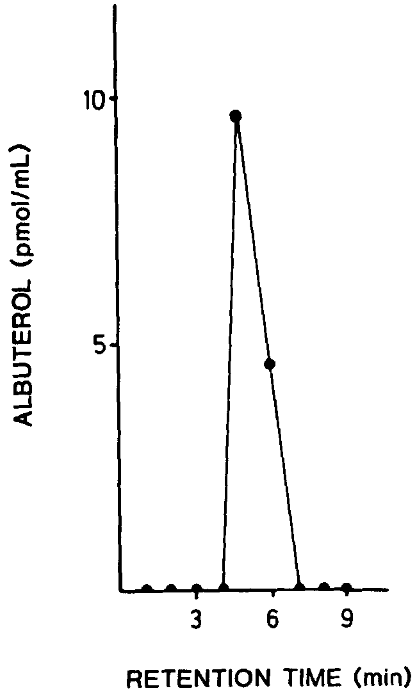

Figure 3: Immunoreactivity profile of an urine sample after HPLC separation. The specificity of the assay is demonstrated by the presence of a unique immunoreactivity peak which co-elute with albuterolstandard.

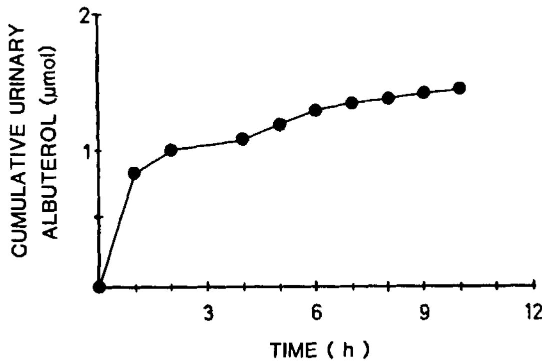

Figure 4: Urinary excretion profile of albuterol in a horse after an i.v. administration of 10 μg/kg of this drug.

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Urinary excretion profile of albuterol

The cumulative urinary excretion profile of albuterol represented by the amount of albuterol excreted in function of time is presented in Figure 4.

DISCUSSION

The present study describes the development of a monoclonal antibody against albuterol and its application in a direct radioimmunoassay of this drug in horse urine.

The monoclonalicity of the antibody has been assessed by limiting dilution method and confirmed by the IgG isotype. The high affinity constant of this antibody could be useful for the detection of albuterol in nanomolar range. It is interesting to note that this antibody displays a high cross reactivity only for clenbuterol among the beta-2-agonists tested which would facilitate the detection of this compound with the present assay. The HPLC-RIA immunogram on the urine extracts depicts an unique immunoreactivity peak corresponding that of albuterol, confirming the specificity of the assay. As no clean up procedure is required, the present assay is particularly useful for the handling of a large number of samples.

In conclusion, the present method gives adequate reliability and sensitivity for the detection and quantitative determination of the albuterol in urine at subnanomolar levels. This could be exploited for the monitoring of both albuterol and clenbuterol in veterinary medicine.

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