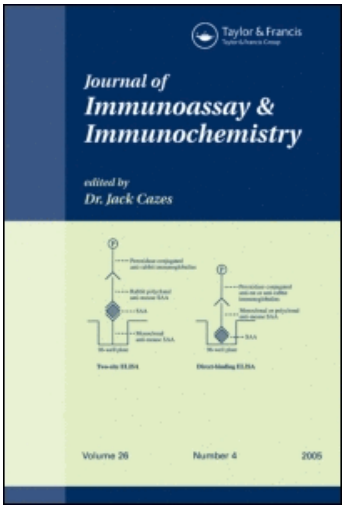

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A Competitive Enzyme Immunoassay for Albuterol: Its Application for the Drug Screening in Urine

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A COMPETITIVE ENZYME IMMUNOASSAY FOR ALBUTEROL: ITS APPLICATION FOR THE DRUG SCREENING IN URINE.

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ABSTRACT

A competitive enzyme immunoassay using purified monoclonal IgG1 and an alkaline phosphatase-albuterol derivative has been developed for the quantification of albuterol in urine. The calibration curve obtained in optimal incubation conditions is characterized by a minimum detectable level of 26 fmol/well and a working range from 52 fmol to 4,2 pmol/well. This method allows the precise and accurate quantification of albuterol in horse urine without any clean up or extraction step. Moreover the definition of its specificity shows a cross-reactivity of the antibody with the glucurono-/sulfo-conjugates of albuterol. This property is particularly interesting for the screening of urinary albuterol residues in meat producing animals. (KEYWORDS: albuterol, clenbuterol, enzyme immunoassay, meat production, drug residues).

INTRODUCTION

Albuterol (salbutamol), a β2-adrenergic agent, used for the treatment of lung diseases associated with air flow obstruction in humans (1), has also been applied in veterinary medicine for illegal purposes: its relaxing capacity on the bronchial smooth muscle has been exploited for race horse doping (2). Moreover, albuterol, as other β2-adrenergic agents, is known as a potent growth factor. This property has been used to produce an increase in skeletal muscle and to reduce the body fat content in sheep, pig and cattle (3). Residues of these production enhancing drugs in meat products may represent a threat to the consumers health. Facing this potential important public health problem (4), the Veterinary Branches of the European Communities recommend testing of animals for the detection of these drug residues, in the slaughter houses (5-6). The physicochemical methods available now (7-11) for the albuterol determination are time consuming and cannot be processed in a large scale for screening purpose. However, the detection of these drugs in urine has recently been recommended (5).

We describe here the development and the analytical validation of a competitive enzyme immunoassay for albuterol and its application for the screening of this drug in urine samples.

MATERIAL AND METHODS

Material

The following materials were purchased from the suppliers indicated. Albuterol, albuterol hemisulfate, β-glucuronidase (Sigma Chemical Co, St-Louis, MO). Succinic anhydride (Fisher Scientific, Muskegon, MI). Intestine alkaline phosphatase, paranitrophenylphosphate (Boehringer Mannheim, West Germany). Protein G Sepharose (Mab trap G), Sepharose CNBr (Pharmacia Uppsala, Sweden). 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Aldrich, Wilwaukee, WI). [^{3}H] albuterol (17 Ci/mmol) (Negev Nuclear Research Center, Beer Sheva, Israel). [^{14}C] succinic anhydride (111 mCi/mmol) (Amersham, Oakville, Ont.). Immunoplates A/C Nunc were obtained from Roskilde, Denmark. Behring ELISA processor (BEP) (Behring Institute, Marburg, West Germany) was used for the ELISA development. All other reagents were of analytical grade and obtained from Fisher Scientific.

Methods

Production and Purification of Monoclonal IgG Against Albuterol

The production of a monoclonal antibody against albuterol has been described previously (12). The characterization of this material revealed the monoclonal nature of this antibody belonging to the IgG1 isotype with an affinity constant of 1.03 nM^{-1}. Its specifity has been assessed by testing its cross-

reactivity towards to albuterol analogs. The antibody displays only significant cross-reactivity to clenbuterol (75%) (12).

Ascitic fluids have been obtained after 18 days in Balb/c mice by intraperitoneal injection of 1.6×10^6 cells/mouse. These animals were pretreated with pristane and irradiated at 200 rad, the day before injection (13).

IgG1 in the ascitic fluid was purified using the Mab trap G kit according the instructions of the manufacturer. The purity of the material was checked using the 278 nm/251 nm absorbance ratio (> 1.5), and its recovery by absorbance at 278 nm (14).

Preparation of the Alkaline Phosphatase-Albuterol Derivative

The alkaline phosphatase-albuterol derivative was obtained in a two steps reaction.

Albuterol hemisuccinate was first synthesized according Beaulieu et al. (15), with minor modifications. [^{3}H] albuterol and [^{14}C] succinic anhydride were added for the monitoring of the derivatization procedure. The synthesized material was purified by dialysis to constant [^{3}H]/[^{14}C] ratio.

Albuterol hemisuccinate was coupled to calf intestine alkaline phosphatase using EEDQ, as described by Belleau and Malek (16). 0.075 mmol of the albuterol hemisuccinate (25 mg) were first activated with 0.121 mmol EEDQ (30 mg) in 2 mL ethanol for 16 hours at 20°C. The activated derivative was then added to 17.8 nmol (2.5 mg/1mL) of alkaline phosphatase

solution in 0.1 M NaHCO3 at pH 9.5. The coupling procedure was performed for 16 hours at room temperature. The solution was then dialysed against Tris-HCl buffer 100 mM, pH 7.4, containing ZnSO4 (10 mg/L). The efficiency of the coupling procedure was estimated by counting the radioactivity incorporated and measuring the enzyme activity.

Enzyme Immunoassay

Nunc immunoplates were coated (1 hr at 37°C) with affinity purified monoclonal IgG (100 µl containing 250 ng/well) diluted in the coating buffer (Tris-HCl 50 mM, pH 8,5, NaCl 100 mM). The plates were then washed 5 times with the washing buffer (Tris-HCl 50mM, pH 7.4, NaCl 100 mM, Tween 20 0.5 mL/L) and incubated for 1 hour at 37°C with 200 µl of the saturation buffer (Tris-HCl 50 mM, pH 7.4, NaCl 100 mM, bovine serum albumine 2 g/L). The plates were then rinsed 3 times with the washing solution and incubated with the reaction medium which consists of alkaline phosphatase labelled albuterol (50 µL), albuterol standard solutions (50 µL) or biological sample (10 µL) completed to 50 µl with the incubation buffer (40 µL), giving a final volume of 100 µL in each well. The albuterol alkaline phosphatase conjugate was used at final dilution of 1/2000 corresponding to 89.48 fmoles of alkaline phosphatase linked to 20.94 pmoles of albuterol.

The plates were incubated for a period of 4 hours at 37°C. After 3 washing steps, alkaline phosphatase enzyme activity was developed using 100 µL of substrate solution (100 mM diethanolamine buffer pH 9,8, paranitrophenylphosphate 4mM and MgSO4, 100 mg/L) per well. The enzyme reaction was stopped after half an hour by adding 100 µl of NaOH 2 N. Absorbances were then measured with the BEP System at 405 nm using 490 nm as a reference wavelength.

Specificity of the Enzyme Immunoassay

To evaluate the specificity of the immunoreactive signal detected by enzyme immunoassay, urine samples spiked with albuterol and horse urine after a 10 mg IV dose (12) were submitted to high performance liquid chromatography (HPLC). The chromatograms obtained following fluorimetric detection were compared to the immunoreactivity profiles set on the eluted fractions by enzyme immunoassay procedure. Aliquots (1 mL) of the urine samples were first submitted to a clean-up extraction by immunoaffinity chromatography followed by an HPLC step as previously described (17) using fluorimetric detection. Eluted fractions (1 ml) were collected and pH adjusted at 7.4 with Tris 500 mM (50 µl) for the enzyme immunoassay detection.

In order to identify glucurono-/ sulfo-conjugates of albuterol, urine samples were submitted to HPLC before and after enzymatic

hydrolysis according to the procedure of Koster et al. (18). Chromatograms obtained by fluorimetric detection and immunoreactivity profiles were compared in both conditions.

Quantification of Albuterol in Urine by Enzyme Immunoassay

Quantification of albuterol was performed directly, without any extraction step, on the same samples than those used for the study of the specificity. The concentrations of immunoreactive albuterol were correlated with those measured by direct radioimmunoassay (12) and by HPLC/fluorescence after an immunoaffinity clean-up (17).

Data analysis.

The results were analyzed using a fitted calibration curve (19).

RESULTS

Characteristics of the ELISA System

Density of alkaline phosphatase labelling with albuterol.

The efficiency of conjugation of albuterol to alkaline phosphatase was estimated by determining the incorporation of [^{3}H] albuterol giving a yield of 234 moles of hapten per mole of enzyme. This labelling step did not result in any loss of enzyme activity.

ELISA Calibration Curve

Figure 1 shows a typical calibration curve obtained in the conditions selected above, in presence of fixed amounts of

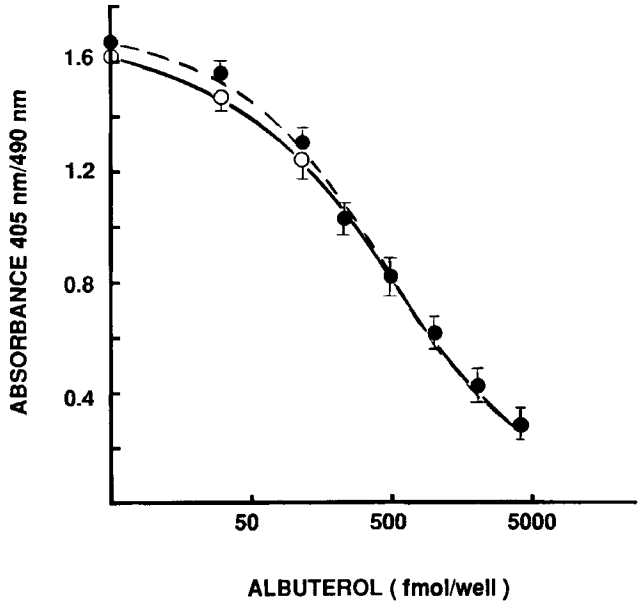

Figure 1: Calibration curve for the enzyme immunoassay of albuterol in incubation buffer (-O-) or in presence of 10 µl of horse urine (-●-).

[albuterol-enzyme] complex with increasing amounts of albuterol, ranging from 50 fmol to 5 pmol/well. The minimal detectable concentration of albuterol determined as the absorbance of the zero mean concentration minus 2 SD equals 26 fmol/well. The working concentration range is from 52 fmol to 4.2 pmol/well. The absorbance of this upper limit being statistically different (mean plus 2 SD) that this measured for a blank value. The non interference of horse urine in this direct assay has been demonstrated by statistical analysis of

the calibration curves in buffer with and without horse urine (10 µl/well) by All Fit program (20). No significant difference could be detected ($F(4.8) = 0.49$, $P = 0.05$).

Analytical Validation

Intra and inter assay precision was assessed on 15 replicates of pooled urine samples spiked at 3 different albuterol levels: 160, 80, 40 pmol/mL in five replicates. The samples were analyzed on three differents plates. The intra-assay variation CV were 2.4, 7.8 and 7.9%, respectively. The values for inter-assay CV were 7.4, 10.7 and 7.6%. The accuracy of the assay was tested using a blank urine spiked with albuterol to concentrations of 20, 40, 80, 160 and 320 pmol/mL. The linear regression curve for the correlation between albuterol concentrations expected and those measured is described by the following equation: $\hat{Y} = 1.099X + 0.45$ ($r^2 = 0.983$).

Similarly, a horse urine after an IV dose of 10 mg of albuterol was serially diluted and assayed by enzyme immunoassay. The results are shown in figure 2 ($r^2 = 0.964$, $\hat{Y} = 0.014X + 3.63$).

Specificity of the ELISA Method

Typical chromatograms set by fluorescence and immunoenzymatic detection on eluted fractions after HPLC of a blank urine extract, and an extract of an horse urine sample

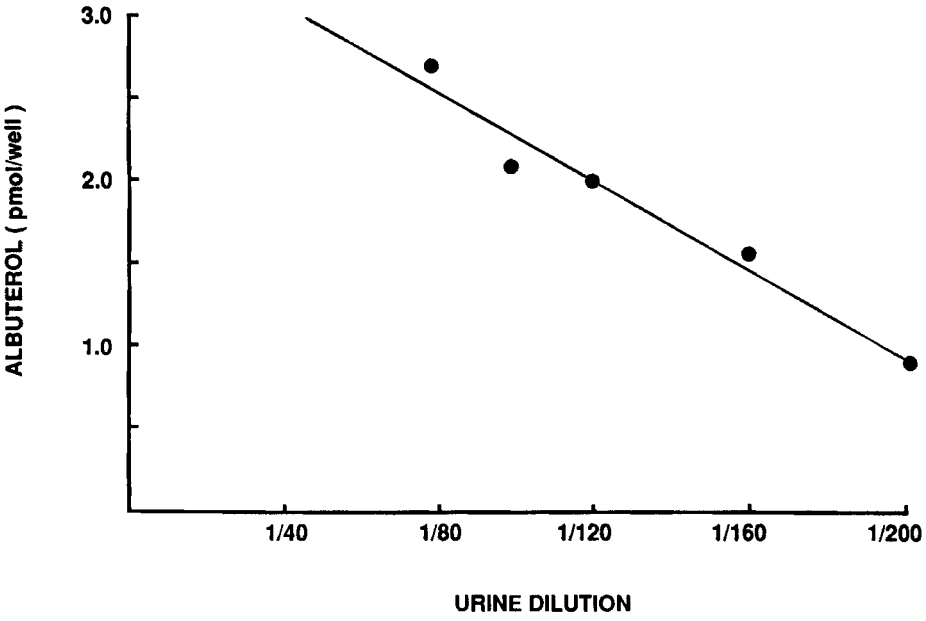

Figure 2: Correlation (r^2 = 0.964, $\hat{Y}$ = 0.014X + 3.63) between successive dilutions of a horse urine containing albuterol and the concentrations measured by enzyme immunoassay. Each value represents the mean of two concentrations.

containing albuterol are shown in figure 3. The immunoreactive signal detected by ELISA corresponds to that of albuterol standard following fluorimetric detection, with a retention time of 10 min. However, the chromatographic profiles of extracts of horse urine samples after an IV dose of albuterol display 2 peaks eluted at 6.4 and 10.18 mins detected both by fluorimetric and immunological methods.

To confirm the nature of the peak eluted at 6.4 mins, the urinary sample has been submitted to β-glucuronidase digestion

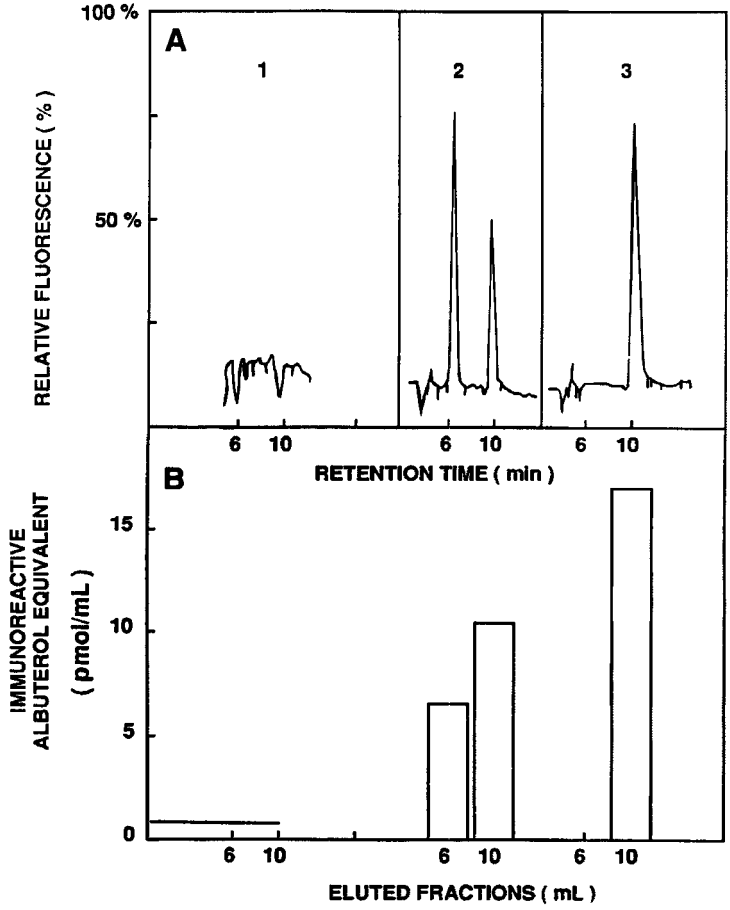

Figure 3: Typical chromatograms (A) and immunograms (B) of a blank urine (1), an horse urine extract after immunochromatography clean up (2) and β-glucuronidase hydrolysis (3).

prior the chromatography step. Indeed, the hydrolysis of glucurono-/sulfo-derivatives of albuterol is demonstrated by the disappearance of the immunoreactive peak eluted at 6.4 mins, with a shift of the signal to the elution position of the albuterol standard confirming therefore the presence of the conjugate derivative of albuterol both in the chromatogram and immunogram of these urine extracts.

The cross-reactivity of the antibody used with the metabolites has been estimated by determining the cumulative amount of drug excreted in urine versus time by both specific HPLC method and by ELISA. The cumulative amount of albuterol excreted estimated by ELISA is found to be the double of that determined by HPLC procedure which allows the separation of unchanged albuterol from its metabolites. We could conclude therefore a full cross-reactivity of the antibody with the metabolites particularly the glurucono- and sulfo- derivatives which are found the major ones excreted in urine.

Urinary Concentrations of Albuterol Quantified by ELISA

Table 1 represents the values of albuterol quantified by direct ELISA and RIA (12) methods and those measured by fluorescence after HPLC chromatography. The correlation between albuterol concentrations measured by RIA/ELISA and HPLC/ELISA methods is respectively described by the following equation: $\hat{Y} = 1.168X - 51$ ($r^2 = 0.998$), $\hat{Y} = 0.690X - 176$ ($r^2 =$

TABLE 1

URINARY LEVELS OF ALBUTEROL (pmol/mL)
AFTER AN IV DOSE OF ALBUTEROL
QUANTIFIED COMPARATIVELY BY IMMUNOLOGICAL
AND INSTRUMENTAL METHODS.

Sample	ELISA	RIA	HPLC
1	0	0	0
2	5,333	6,208	3,562
3	1,220	1,237	365
4	792	862	370
5	525	562	178
6	458	471	150
7	346	358	92
8	225	196	44
9	217	187	36
10	187	233	36

0.989). These lower levels obtained by HPLC confirm the cross-reactivity of the antibody used with the albuterol metabolites which are separated from the parent compound in the chromatographic conditions used.

DISCUSSION

In the recent years, misuse of drugs has been a major incentive for the development of analytical methodologies, particularly non radioisotopic immunoassays for the quantification of these xenobiotics. Among these drugs, β2 adrenergic agents have been suspected to be widely used for

illegal purposes (5-6, 21). Although an ELISA method for clenbuterol, using a rabbit polyclonal antiserum has been proposed for the screening of this adrenergic agent (22-23), an immunoassay with sufficient sensitivity and specificity is still lacking. We report here the set up of a competitive enzyme immunoassay using a monoclonal antibody and its application for the determination of albuterol in biological fluids. The availability of such monoclonal antibody (12) would allow the standardization of the assay of this drug for large scale studies. The immunoassay reported here is convenient since it does not require a clean up extraction in the procedure. The high sensitivity of the present assay in the fmole range is sufficient for the screening of albuterol for which ppb-levels limits were fixed by official agencies (5-6). Moreover, the cross-reactivity of the antibody with glucurono-/sulfo-conjugates of albuterol renders this assay particularly attractive for the detection of this drug and its metabolites found at high levels in urine. The application of the present enzyme immunoassay is not limited for the screening of this drug in veterinary medicine but would be suitable for the clinical monitoring of this β2 adrenergic agonist in therapeutic adjustments in human medicine.

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