Journal of Chromatography, 564 (1991) 551–556
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5716

Residue screening for the β-agonists clenbuterol, salbutamol and cimaterol in urine using enzyme immunoassay and high-performance liquid chromatography

H. H. D. MEYER*, L. RINKE and I. DÜRSCH

Institut für Physiologie der Süddeutschen Versuchs- und Forschungsanstalt für Milchwirtschaft, Technische Universität München, 8050 Freising-Weihenstephan (Germany)

ABSTRACT

The antibody for enzyme immunoassay was raised against clenbuterol-diazo-BSA, and salbutamol-carboxymethyl ether-biocytin was used as a label. Procedural blanks from 500 negative urine samples were always <0.2 ppb salbutamol or <0.02 ppb clenbuterol equivalents, and a residue level of 1 ppb was detected with good reliability. After treatment of veal calves with anabolic dosages, residue levels in urine amounted to 10–200 ppb clenbuterol or salbutamol. β-Agonists were separated by high-performance liquid chromatography on LiChrospher RP-Select B columns, and acidic methanol–buffer or acetonitrile–buffer mobile phases. Combinations of high-performance liquid chromatography and enzyme immunoassay were used for confirmation.

INTRODUCTION

β-Agonists improve nitrogen retention, induce a reduction of body fat and promote muscle growth. Hence, farm animals can be produced more economically, an effect that has stimulated much research in recent years [1]. On the other hand, residues may accumulate in edible tissues, as has been reported for clenbuterol [2], but there is almost no information on residues for other β-agonists, such as salbutamol or cimaterol. The residues in food of animal origin may have pharmacological acitivity in humans (antiasthmatic, tocolytic) due to greater or lesser oral bioavailability of these compounds. Hence, a risk for the consumer cannot be excluded, and there is an urgent need for efficient screening procedures. False-negative results must be avoided during screening, and sensitive non-specific enzyme immunoassays (EIA) might be a useful tool for the estimation of the three β-agonists in urine samples within one test. In the case of positive results, identification and confirmation of the reacting compounds is necessary; combined high-performance liquid chromatographic (HPLC) and EIA procedures were developed for these purposes.

0378-4347/91/$03.50

EXPERIMENTAL

Materials

Clenbuterol and cimaterol were kindly provided by Boehringer Vetmedica (Ingelheim, Germany); all further β-agonists and other chemicals were commercially available from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

Animals

To a veal calf (six weeks old, 65 kg body weight, female Brown-Swiss), salbutamol (50 μg/kg body weight daily) was given via milk replacer for seven days. After withdrawal for fourteen days, the animal was treated again with the same dosage for another fourteen days and slaughtered. Urine was collected after stimulation and stored at −24°C until analysis.

Analytical strategy

For screening purposes, urine samples were cleaned up and submitted to EIA. For the identification step, urine samples were cleaned up, then subjected to HPLC procedure 1 followed by EIA. For confirmation, samples were cleaned up then submitted to HPLC procedure 2 followed by EIA.

Clean up

Cartridges containing 100 mg of C_{18} silica gel (Baker, Gross-Gerau, Germany; product No. 7020-1) were rinsed with 2 ml of methanol and equilibrated with 2 ml of 1 m*M* NaOH. A 1-ml volume of urine was applied to the gel, which was then washed with 2 ml of 1 m*M* NaOH followed by 4 ml of methanol–water (15:85 v/v); the β-agonists were eluted with 1 ml of 100% methanol. All solvents were pumped through the gel with the aid of 10-ml syringes, and the cartridges could be reused up to five times. The eluate was concentrated to dryness under vacuum. The residue was dissolved in 200 μl of assay buffer, and two 20-μl volumes were used for EIA.

HPLC procedure 1

A 2-ml urine sample was cleaned up, the residue of the eluate was dissolved in 600 μl of water, and 500 μl were applied to an HPLC column (LiChrospher RP-select B, 125 mm × 4 mm, I.D., 5 μm, Merck) by a Beckman 112 solvent-delivery module (Beckman, Munich, Germany). Under these conditions the β-agonists were concentrated on the column top; the large volume improves precision and minimizes contamination of the sample applicator. Elution was done with 20 m*M* KH_2PO_4, 30 μ*M* EDTA (pH 3.9)–acetonitrile at 97:3 (v/v) for 5 min, followed by an 80:20 (v/v) ratio for 7 min, and then by 100% acetonitrile for 4 min; the flow-rate was 1 ml/min, at 25°C. Fractions of 0.5 ml were collected from 2 to 15 min, and 20 μl of each fraction were directly analysed in duplicate by EIA

for detection of residue levels in the picogram and nanogram range. Clenbuterol, cimaterol and salbutamol were monitored simultaneously at 260 nm if applied in the microgram range.

HPLC procedure 2

Urine was processed as for HPLC procedure 1 but using a longer column (LiChrospher RP-select B, 250 mm × 4 mm I.D., 5 μm) and a mobile phase of different selectivity (20 m*M* KH_2PO_4. 30 μ*M* EDTA (pH 3.9)–methanol (92.5:7.5, v/v) at a flow-rate of 1 ml/min at 25°C). Around the retention time of salbutamol (12.0–17.7 min), 0.3-ml fractions were collected, and 50 μl of each fraction were analysed in duplicate by EIA. For clenbuterol a more lipophilic mobile phase was required (phosphate buffer–methanol, 75:25, v/v) and fractions from 16.9 to 24.4 min were collected.

Enzyme immunoassay

Clenbuterol-diazo-bovine serum albumin (BSA) was prepared as previously described [3], and antibodies were raised in rabbits. The labelled hormone was prepared as follows. Salbutamol and monochloroacetic acid were heated under alkaline conditions [4] to produce salbutamol-4-carboxymethyl ether (CME), which was linked to biocytin via a peptide bond. This preparation of salbutamol-CME-biocytin is similar to that described for biocytinyl-estradiol, and the complete assay protocol for the estimation of β-agonists using the biotin-avidin amplification system was like the method for estradiol [5]. The antibody was diluted 1:120 000 (Code: Brun 26.04.89). Briefly, microtiter plates were coated with affinity-purified sheep immunoglobulin G (antirabbit IgG), then hormone-specific antibody plus labelled and unlabelled hormone were added and incubated until equilibrium. Streptavidin-peroxidase was added, the plates were washed, the enzyme was incubated for colour production, the colour was measured, and the unknowns were calculated.

RESULTS

The calibration curves of the assay system are shown in Fig. 1. Besides good binding for clenbuterol (100%) there was a group of substances having *ca.* ten times less affinity to the antibody (cross-reactivities as calculated at 50% relative binding: terbutaline, 13%; salbutamol, 12%; cimaterol, 10%) whereas physiological agonists or structurally only closely related compounds showed no binding (cross-reactivities: epinephrine, $<0.01\%$; norepinephrine, $<0.01\%$; bromhexine, $<<0.01\%$; ambroxol, $<<0.01\%$). The calibration curves obtained for the different compounds were almost parallel, indicating rather homogeneous binding sites. The absolute detection limits amounted to *ca.* 1 pg/well clenbuterol and *ca.* 10 pg/well salbutamol, cimaterol or terbutaline.

The EIA system was used for the estimation of β-agonists in bovine urine.

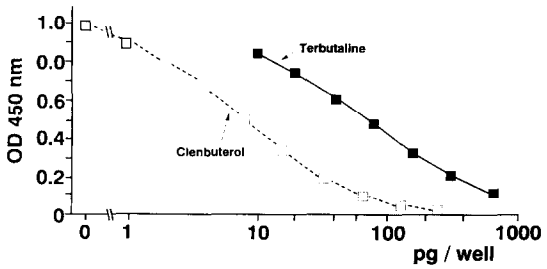

Fig. 1. EIA standard curves for clenbuterol and terbutaline.

Without clean-up, procedural blanks of negative samples ($n = 19$) were inconsistent (0.2–1.9 ng clenbuterol equivalents per ml). After clean-up, procedural blanks were always <0.02 ng clenbuterol equivalents per ml in 500 negative samples stored properly at $-24°C$. After prolonged storage (one to four weeks) at higher temperature, elevated background levels were found (up to 0.22 ng/ml). Recoveries after clean-up of urine amounted to 90–100% for clenbuterol and 65–80% for salbutamol or cimaterol; terbutaline was not extracted by this method. The coefficients of variation (C.V.) found in our laboratory were acceptable (intra-assay variation: $\bar{x} = 0.53$ ng/ml clenbuterol, $n = 10$, C.V. = 9.0%; inter-assay variation: $\bar{x} = 0.95$ ng/ml clenbuterol, $n = 9$, C.V. 9.9%). After treatment of a veal calf, levels of salbutamol in urine increased immediately (Fig. 2), but

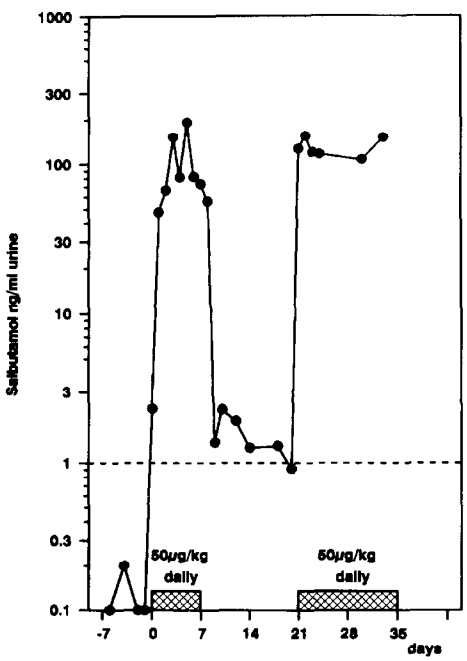

Fig. 2. Residues found in calf urine after treatment with salbutamol.

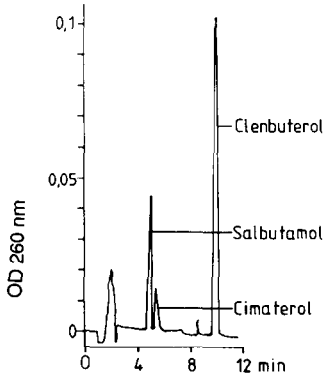

Fig. 3. Separation of β-agonists with HPLC procedure 1.

were quite variable over the whole treatment period. After withdrawal, levels decreased to <10 ng/ml within a few days, but were mostly >1 ng over two weeks of withdrawal; the second treatment induced elevation to former levels.

The rapid separation of the respective β-agonists by HPLC using a simple acetonitrile gradient is shown in Fig. 3. If field samples were found positive after screening the respective immunological activity was identified by HPLC followed by EIA. Previously activities were found only in the positions of salbutamol and clenbuterol. The findings were confirmed with HPLC procedure 2 followed by EIA of the fractions around the retention time of the relevant hormone (Fig. 4); levels of 0.5 ng/ml salbutamol cause a clear peak ten times higher than signals from negative samples. In suspected samples the same elution pattern was found, which confirmed the presence of salbutamol. In this system cimaterol is eluted *ca.* 2 min earlier, whereas clenbuterol is much more lipophilic and a higher concentration of methanol is needed to get a similar retention time.

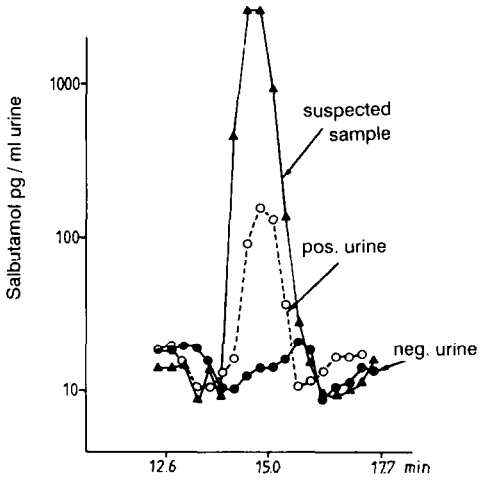

Fig. 4. Confirmation for salbutamol in urine using HPLC procedure 2 and detection by EIA.

DISCUSSION

The EIA as developed provides a tool for the simultaneous screening of β-agonists with a *tert.*-butyl or isopropyl moiety linked to the amino group. The smaller methyl group of epinephrine causes negligible affinity. On the other hand, the antibody is sufficiently non-specific for the detection of a multitude of compounds substituted on the aromatic ring only. However, a bridge heterology within the antigen and marker was needed for obtaining a good sensitivity. Clean-up of urine is useful in order to improve the reliability of the assay, but the resorcinic β-agonists do not bind to RP-silica gel in alkaline buffers, owing to deprotonation of a hydroxy group. A simultaneous screening for terbutaline, orciprenaline and other β-agonists would be very useful, but a common clean-up for these compounds still needs to be developed. On the other hand, residue levels in urine during treatment are quite high, and detection of treatment would always be possible without any clean-up. However, after withdrawal of salbutamol, levels decrease rapidly and only after prepurification can a significant elevation be monitored for at least two weeks.

The HPLC methods described provide a useful tool for differentiation of the different compounds and for a first confirmation, but positive samples become much more laborious and need more EIA reagent. It is obvious that EIA methods are generally superior to other methods with respect to simplicity and costs if they are used for screening purposes, because they have the potential for the analysis of large numbers of samples.

ACKNOWLEDGEMENTS

The authors thank the Bundesgesuntheitsamt (Berlin, Germany) and the Schaumann-Stiftung (Hamburg, Germany) for supporting the project.

REFERENCES

1 J. P. Hanrahan, *β-Agonists and their Effects on Animal Carcass Quality*, Elsevier, London, New York, 1987.
2 L. Rinke and H. H. D. Meyer, *J. Animal Sci.*, submitted for publication.
3 I. Yamamoto and K. Iwata, *J. Immunoassay*, 2 (1982) 155.
4 *Organikum, Organisch-chemisches Grundpraktikum*, Deutscher Verlag der Wissenschaften, Berlin-Ost, 1973.
5 H. H. D. Meyer, H. Sauerwein, B. M. Mutayoba, *J. Steroid Biochem.*, 35 (1990) 263.