DETECTION OF $\beta_2$-AGONISTS IN MILK REPLACER

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ABSTRACT


$\beta_2$-Agonist drugs may be illegally used as growth promoters for feedlot calves, when mixed into milk replacer immediately before feeding. To check for the presence of clenbuterol, salbutamol and terbutaline in such food, an analytical system was established using a screening method based on two commercial qualitative competitive ELISA tests, with antibodies raised against the arylamino group and the $t$-butyl group. The extraction procedure was based on precipitation of the milk samples with acetonitrile followed by filtration. The absence of any significant interference by other substances in the filtrate allowed detection of $\beta_2$-agonist drugs in spiked samples at the lowest concentration having a repartitioning effect (50 ppb for clenbuterol, mabuterol and terbutaline, 500 ppb for salbutamol). In view of a false positive response with tetracycline in milk samples and a cross-reaction between clenbuterol and mabuterol, an HPLC-MS technique was developed which, after extraction and purification of the samples with SPE C18 Polar Plus, was able to confirm the presence of these drugs. The good recovery after extraction (ranging from 84% to 90.2%) and the low detection limit with this method (250 ng/ml for clenbuterol, mabuterol and terbutaline, and 2.5 $\mu$g/ml for salbutamol) allowed easy confirmation and simultaneous detection of the four $\beta_2$-agonists at the lowest concentrations at which they are used in adulterated milk for calves.

Keywords: adulteration, $\beta_2$-agonist, clenbuterol, contaminant, mabuterol, salbutamol, terbutaline

Abbreviations: $B$, optical density of the sample; $B_o$, maximal optical density in total absence of competition; $\%B/B_o$, percentage of inhibition; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; HPLC-MS, high-performance liquid chromatography–mass spectrometry; $m/z$, mass to charge ratio; ppb, parts per billion; ppm, parts per million; SPE, solid-phase extraction;

INTRODUCTION

Certain $\beta_2$-agonists are widely reported to be effective growth promoters (repartitioning agents) in cattle, pigs, poultry and sheep (Moser et al., 1986; Kim et al., 1987; Buyse et al., 1991; Shaoquan and Orcutt, 1991). The use of these substances in animal husbandry is potentially dangerous because their presence in the liver of treated animals can adversely affect human consumers (Martinez-Navarro, 1990; Pulce et al., 1991; Martinez et al., 1992).

To control the illegal use of $\beta_2$-agonists in animal husbandry, it is essential to have sensitive, specific and rapid analytical techniques that reveal the presence of these substances in animal tissues and animal feed. In particular, their detection in reconstituted milk is a priority, since they are often added illegally to this product during reconstitution immediately before feeding to the animals (Commission of the
European Communities, 1993). For this reason, we developed an analytical system capable of detecting the $\beta_2$-agonists most commonly used as growth promoters (clenbuterol, salbutamol, mabuterol and terbutaline) at the concentrations of 50 ppb (500 ppb for salbutamol) that have a repartitioning effect and are seven times higher than the recommended therapeutic dose (~0.8 mg/kg body weight for clenbuterol) (Meyer and Rinke, 1991; Witkamp, 1994). The method involves screening with two commercially available qualitative competitive ELISA tests for clenbuterol and terbutaline, followed by confirmatory analysis using HPLC-MS after extraction and purification of samples on a SPE column.

MATERIALS AND METHODS

Chemicals

Clenbuterol and mabuterol were obtained from Boehringer Ingelheim, Germany; terbutaline, salbutamol and tetracycline from Sigma, St Louis, MO, USA; HPLC grade reagents and solvents from J.T. Baker, Deventer, The Netherlands; ELISA plates carrying antibodies against the arylamino group present in clenbuterol and mabuterol (ELISA Technologies Clenbuterol) and the t-butyl group present in terbutaline, clenbuterol, salbutamol and mabuterol (ELISA Technologies Bronchodilator Group), intended for testing equine urine, were obtained from Neogen Corp., Lexington, MA, USA.

Milk replacers

The milk replacers for calves (both approximately 21% fat, dry weight) were obtained from Denkavit (Finish 2 Super), Voorthuizen, The Netherlands, and from Schils (Mix Ingrasso), Sittard, The Netherlands.

ELISA assay

Five 0.5 g samples of each brand of the reconstituted milk products were dispersed in water (2.5 ml) and spiked with standard aqueous solutions of the drugs to obtain concentrations of 50 ppb (500 ppb for salbutamol). Five other samples of each brand were spiked with standard aqueous solutions of tetracycline to obtain a concentration of 600 ppm. Then 1 ml aliquots of these spiked samples were diluted 1:2 with aqueous 0.2% acetic acid and centrifuged (3000g, 15 min). After removal of the upper lipid phase, the middle aqueous layer was passed through a 0.2 μm cellulose acetate filter (Sartorius, Gottingen, Germany). Then, 100 μl of the filtrate was diluted 1:2 with EIA buffer (pH 7.2, included in the ELISA test kit), used in the ELISA assay plates following the manufacturer’s instructions, and subsequently read by a Multiskan MCC/340 photometer (Titertek, Walnut Creek, CA, USA). ELISA calibration curves for the $\beta_2$-agonists were constructed for concentrations ranging from 0.25 to 10 ng/ml in the filtered milk samples. The assays were conducted in quadruplicate.