Development of a Fast and Simple Method for Determination of β-Agonists in Urine by Extraction on Empore Membranes and Detection by a Test Strip Immunoassay

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A rapid screening method for β-agonists in cattle urine was developed that offers speed and protocol simplicity and that could be performed on the spot. The method consists of an extraction on Empore membranes with strong cation exchange properties and a test strip immunoassay. The Empore extraction provides a fast and simple cleanup of the urines and a concentration step with recoveries ranging from 50−80% for the β-agonists of the aniline type and around 10% for salbutamol and terbutaline. A test strip immunoassay is performed on the diluted extract. With the use of an antiperoxidase antibody, an internal negative control was included, to which all color reductions are compared. Although a portable colorimeter increased the sensitivity of the test, a visual interpretation was sufficient to give a correct yes/no answer. The whole test takes about 30 min and has a visual detection limit for clenbuterol of 1 ng/mL of urine. The test also shows good sensitivity for salbutamol, despite the low recovery with the Empore extraction.

Keywords: *Clenbuterol; β-agonists; Empore extraction membranes; test strip immunoassay*

INTRODUCTION

In human and veterinary medicine, β_2-agonistic drugs, like clenbuterol and salbutamol, are therapeutically used as broncho-dilating agents. In addition to this therapeutic use, these compounds, particularly clenbuterol, have been found to be illegally used in meat producing animals like cattle. In these animals, the β-agonists increase the protein to fat ratio, resulting in a higher production efficiency (Hanrahan, 1987). For this reason, they are also known as repartitioners. In the European community (EC), the use of β-agonists as growth promotors is forbidden (EC Directive 96/22/EC). Despite this ban and according to Hahnau and Jülicher (1996), clenbuterol has proven to be the dominating anabolic substance being illegally used in fattening animals in the last years. This abundant misuse raised serious concerns about a toxicological risk for the consumer. In the 1990s, several outbreaks of food poisoning were reported in Spain, France, and Italy by the intake of meat or liver contaminated with β-agonist residues (Martinez-Navarro, 1990; Pulce et al., 1991; Maistro et al., 1995). Apart from clenbuterol, other structurally related β-agonists were misused as growth promotors, though at a smaller extent. This is the case of salbutamol, cimaterol, bromobuterol, mabuterol, mapenterol, methylclenbuterol, clenproperol, and terbutaline (Figure 1).

In recent years, numerous efforts were concentrated in the field of residue analysis on the detection and quantification of clenbuterol and other β-agonists in a variety of biological matrices. Generally, attempts were made to develop a multianalyte analysis to encounter as many β-agonists as possible. This approach is hampered, however, by the very diverse structure and sometimes totally different physicochemical behaviors of the substances that can be used. In this respect, the β-agonists are often classified as substances of the aniline-type, e.g., clenbuterol, of the phenol-type, e.g., salbutamol, and of the resorcinol-type, e.g., terbutaline (Courtheyn et al., 1994). The last two have amphoteric characteristics due to the basic amine group and the acid phenolic hydroxyl group(s) and are more polar than the first sub-class. Montrade et al. (1993) described a multiresidue method for analysis of β-agonistic drugs in cattle urine by gas chromatography−mass spectrometry. They applied a solid-phase extraction based on mixed-phase cartridges with both cation exchange and lipophilic properties. The same extraction principle was also reported by other authors (Collins et al., 1994; Dumasia and Houghton, 1991; Leyssens et al., 1991). Immunoaffinity chromatography (IAC) was another approach for several researchers to develop a multianalyte extraction (Van Ginkel, 1991; Schilt et al., 1990; Haasnoot et al., 1993). The immunochemical technique is applicable not only to the extraction of β-agonists but also to their detection. Numerous enzyme-linked immunosorbent assays (ELISAs) have been developed for the determination of clenbuterol or β-agonists, and at present, several companies in Europe and the United States offer microtiter plate ELISA kits for their detection (Hahnau and Jülicher, 1996). These kits can only be applied in the laboratory, because they usually need specially designed shakers, sophisticated photometers, and software for calculating the concentration.

For a further reduction of the costs and of the assay time, it was an interesting challenge to develop a simple and fast qualitative screening test that could be performed at the place of sampling (farm or slaughterhouse). The basic requirements for these on-site tests are speed, protocol simplicity, and reliability, yielding a quick yes/no answer for the inspection services. The confirmatory analyses at the laboratory can then be restricted to those samples giving a positive on-site test. Test strip immunoassays have a number of properties

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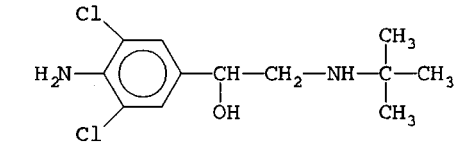
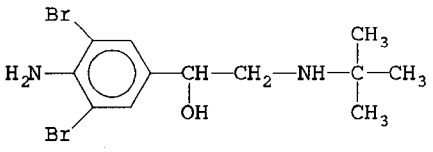
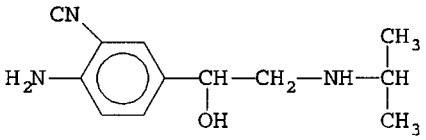


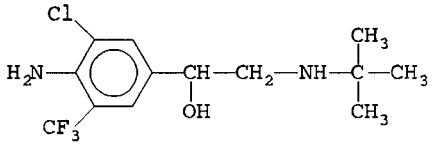
Clenbuterol



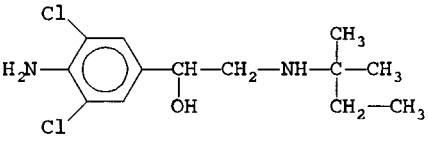
Bromobuterol



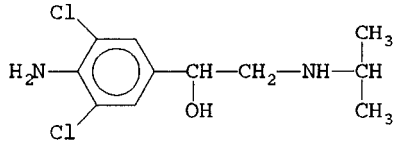
Cimaterol



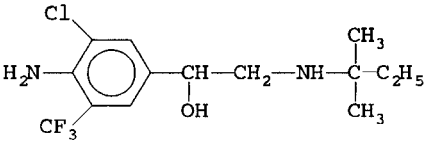
Mabuterol



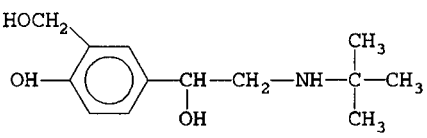
Methylclenbuterol



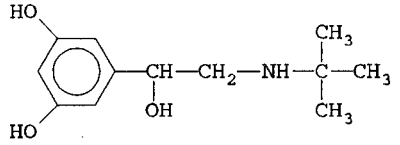
Clenproperol



Mapenterol



Salbutamol



Terbutaline

Figure 1. Structural formulas of the β-agonists involved in the study.

which make them well suited for nonlaboratory applications: the test strip is a convenient vehicle for assay reagents and enables simple separation and visualization steps (visual color interpretation). Such test strip assays, based on a competitive enzyme immunoassay have been applied with success, for instance, for the determination of mycotoxins in cereals (Schneider et al., 1991; De Saeger and Van Peteghem, 1996), antibiotics in milk (Schneider et al., 1993, 1994), and herbicides in water (Giersch, 1993). Preliminary results of the development of a test strip for clenbuterol residues in urine were reported a few years ago by Ploum et al. (1991). In the development of such a test strip immunoassay for the determination of β-agonist residues in unextracted, unpurified urine samples, it was our experience that the results were disturbed by matrix effects. The aim of this study was to develop a qualitative field test consisting of a fast and simple cleanup and extraction procedure followed by a test strip immunoassay. Extraction on the spot was not feasible with the multianalyte extractions described above, because after elution of the cartridges or IAC columns, evaporation was needed as a concentration step. For our study we used commercially available Empore extraction membranes. They contain small chromatographic particles embedded in an inert matrix of Teflon fibrils. These membranes, available in similar diameter as HPLC solvent filters and configured for a vacuum flask, were introduced a few years ago for the trace enrichment of organic compounds such as pesticides from environmental water samples (Chiron and Barcelo, 1993; Chiron et al., 1993; Durand et al., 1992). Due to the small particle size, high surface area per unit volume, and short path length, they offer rapid binding and elution characteristics and need minimal elution volumes and thus generate highly concentrated eluates. To our knowledge, until now, only one application was described of these Empore disks in veterinary drug residue analysis (Van Vyncht et al., 1994).

MATERIALS AND METHODS

Standards. Metoprolol tartrate, clenbuterol hydrochloride, salbutamol, and terbutaline hemisulfate were obtained from Sigma (Bornem, Belgium); mapenterol and methylclenbuterol were provided by the EU Reference Laboratory for Residues of Veterinary Drugs, Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin (BGVV), Berlin, Germany; bromobuterol, mabuterol, and clenproperol were provided by Dr. L. Willems-Institute (Diepenbeek, Belgium); and cimaterol was a gift from Dr. Courtheyn, Rijksontledingslaboratorium (Gentbrugge, Belgium).

Standard stock solutions were made in methanol (1 mg/mL) and stored at 4 °C.

Reagents. All solvents and reagents were of analytical-reagent grade unless stated otherwise. Ammonia (about 25%

w/w) was from UCB (Leuven, Belgium). HPLC grade methanol was from BDH Laboratory Supplies (Poole, England). HPLC grade H_2O (BDH, Poole, England) was used to prepare the solutions and buffers for the immunoassay, except for the washing solution. Goat anti-rabbit IgG, Rabbit antiperoxidase IgG, bovine serum albumin (BSA) (fraction V, Ria grade), Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), casein sodium salt (casein), and ProClin 300 were all from Sigma (Bornem, Belgium). ProClin 300 is a preservative that was added to all the solutions and buffers for the immunoassay in a concentration of 0.02%−0.05% (v/v). Rabbit anti-sheep IgG was from DAKO (Glostrup, Denmark), and H_2O_2 30% was from Merck (Darmstadt, Germany). Immunodyne ABC membrane, pore size 0.45 μm (Pall Biosupport, Portsmouth, England), was used for the dipstick preparation. This nylon Immunodyne membrane is preactivated to form covalent linkages with nucleophilic groups found on proteins and other biological macromolecules.

Urine Samples. Blank urine samples for the recovery experiments and the development of the test strip immunoassay were from untreated male veal calves and heifers that were kept at the University experimental farm (Agri-vet-Research and Development, R. U. Gent, Melle, Belgium). To test the selectivity of the test strip assay, blank urines from 10 pigs, 10 calves, and 10 steers were provided by TNO Nutrition and Food Research Institute (Zeist, The Netherlands).

Seven calf urines were kindly provided by Dr. Thomas Gude, BGVV, Berlin, and were from animal experiments. Three animals were fed the therapeutic dose (0.8 μg of clenbuterol/kg of body weight) twice daily for 10 days, and urine was sampled at 0 days (sample 1192), 4 days (sample 1195), and 7 days (sample 1197) withdrawal time. Three animals were fed the fattening dose (10 μg of clenbuterol/kg of body weight) twice daily for 21 days. Urine was sampled at 0 days (sample 8152), 2 days (sample 10025), and 4 days (sample 8280) withdrawal time. For one animal, both clenbuterol (at 5 μg/kg of body weight) and salbutamol (at 10 μg/kg of body weight) were administered simultaneously twice daily for 3 days and urine was collected at 0 days withdrawal time (urine 10402). The GC/MS analyses to check the concentrations in these urines were performed at BGVV, Berlin, with an in-house GC/MS method that was a modification of the method published by Montrade et al. (1993) and that showed a limit of identification (four ions) of 0.1 μg/L for both clenbuterol and salbutamol.

Antibody Preparation. The antibody was raised in sheep at TNO (Zeist, The Netherlands) against salbutamol−succinate−ovalbumin. The crude antiserum was aliquoted and stored at −20 °C. A stock dilution of 1:20 in a phosphate-buffered saline (PBS, 0.1 M, pH 7.2)−glycerol mixture (serum−PBS−glycerol; 1 + 9 + 10, v/v/v) was prepared and stored at −20 °C. The appropriate working solutions of the antibody were freshly prepared by diluting this stock dilution in assay buffer (0.05% BSA (w/v) in 0.04 M PBS, pH 7.2).

Synthesis of the Enzyme Label. Salbutamol−succinate−horseradish peroxidase (HRP) was synthesized following the procedure described by Degand et al. (1993).

Microtiter Plate Immunoassay. The wells of Nunc MaxiSorp flat-bottom microtiter plates (Life Technologies, Merelbeke, Belgium) were coated with rabbit anti-sheep IgG diluted in carbonate/bicarbonate coat buffer, pH 9.6, for 2 h at 37 °C. After a washing step (0.05% (w/v) Tween 20 in water), a blocking step (2% (w/v) caseine in water), and a subsequent washing step, the plates could be stored in a vacuum-sealed plastic bag at 4 °C for several weeks. Although the antibody was raised against a salbutamol conjugate, the standard curve was made with the clenbuterol standard, that is still the major β-agonist misused in cattle fattening. Hence, the concentrations that were found in the samples were clenbuterol equivalents. For the incubation step, 25 μL of clenbuterol standard (range 0.1−5 μg/L, prepared in assay buffer out of the methanolic stock solution) or five times diluted urine, 50 μL of the salbutamol−enzyme conjugate solution (1:750 000 dilution in assay buffer), and 100 μL of diluted sheep anti-salbutamol antiserum (1:250 000 in assay buffer) were added to each well. After 2 h incubation at room temperature, bound peroxidase activity was assessed by adding 150 μL of a H_2O_2/TMB substrate solution to each well. After 30 min incubation in the dark, the color reaction was stopped with 1 M sulfuric acid and the absorbance was measured at 450 nm. Concentrations were calculated using a Rodbard four-parameter logistic model. Cross-reactivities of the antiserum with β-agonists other than clenbuterol were determined at 50% displacement.

Figure 2.

Figure 3.

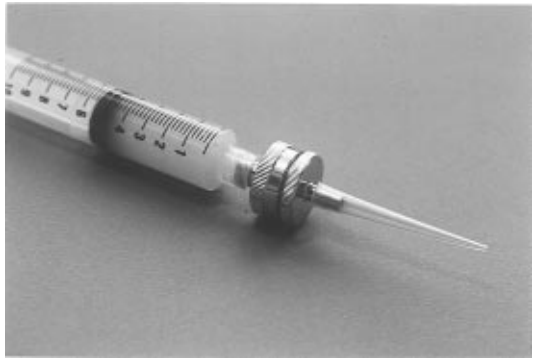


Figure 4.

Preparation of Extraction Device. With the use of a cork borer, individual 13 mm disks were punched out of 47 mm diameter Empore cation-SR ion exchange disks (catalog no. 1214-5020) from Varian Sample Preparation Products (Harbor City, CA) (Figure 2). These disks consist of a strong cation exchanger (SCX) resin that is a poly(styrene−divinylbenzene) (SDB) with sulfonic acid functions (3M, 1995). The 13 mm disk was inserted in a stainless steel filter holder (Schleicher & Schuell, Dassel, Germany) that was connected to a 10 mL plastic syringe (Becton Dickinson, NJ) at one side and a pipet tip at the other end (Figures 3 and 4).

Urine Extraction. The pH of 5 mL of urine that had been prefiltered on a 0.2 μm disposable filter (CHROMAFIL Einmalfilter, Machery-Nagel, Düren, Germany) to remove particles was adjusted to 1 with 4 N HCl. The Empore filter was preconditioned by pushing consecutively 2 mL of MeOH and 2 mL of 0.01 N HCl through the filter with the use of the syringe. Care was taken that the membrane always remained wet. The sample was then aspirated into the syringe and

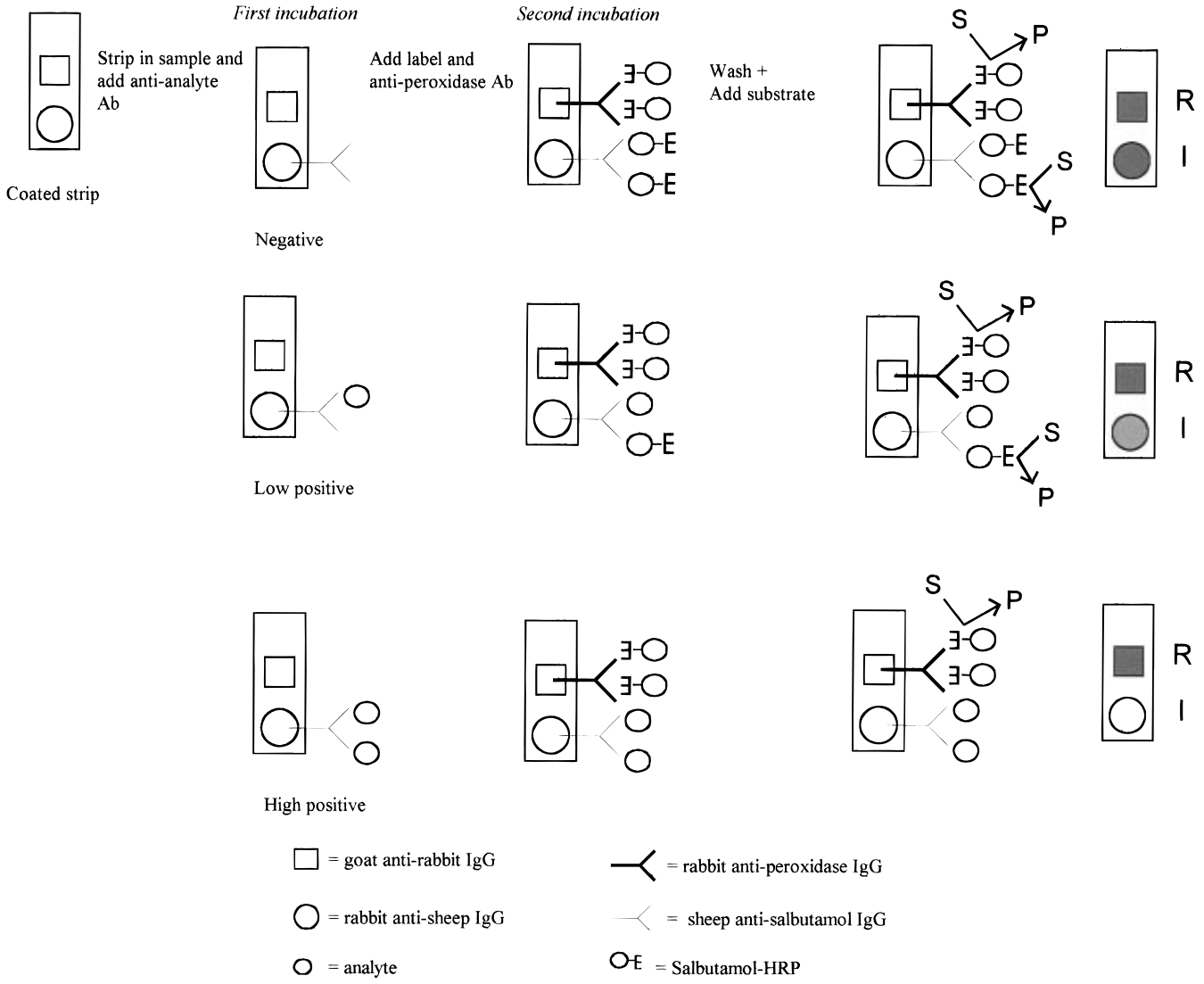


Figure 5. Schematical presentation of the test strip immunoassay.

forced through the membrane. The washing step consisted of pushing successively 1 mL of 0.01 N HCl and 1 mL of MeOH through the membrane. Finally, the β-agonists were eluted with 250 μL of MeOH containing 3% (v/v) of concentrated (25%) ammonia solution.

Preparation of the Test Strip. The Immunodyne ABC membrane was cut into strips (1 × 1.5 cm), and 1 μL of undiluted goat anti-rabbit IgG was applied as a spot at 1 cm height by means of a 0.5−10 μL Eppendorf pipet. In the same way, 1 μL of undiluted rabbit anti-sheep IgG was applied at 0.5 cm height. The strip was dried at 37 °C for 30 min and stuck on a Nunc plastic self-adhesive sealing tape. The remaining protein binding sites were blocked by immersing the strip in blocking solution for 30 min. The dipsticks were then rinsed with washing solution and soaked for 10 min in washing solution. Finally, the dipsticks were dried as described before. Strips coated in this way were ready-to-use and could be stored at 4 °C in a vacuum-sealed plastic bag for at least 6 weeks without a change in performance.

Test Strip Immunoassay (Test Strip IA) (Schematically Presented in Figure 5). The Empore extract (250 μL) was diluted by adding 2 mL of assay buffer. Five hundred microliters of this diluted extract was mixed with 250 μL of the diluted sheep anti-salbutamol antiserum (1:50 000 in assay buffer). The coated test strip was immersed in this mixture. After 10 min incubation time (first incubation), 250 μL of salbutamol−HRP solution (1:4000 in assay buffer) and 125 μL of rabbit antiperoxidase IgG solution (1:30 000 in assay buffer) were added and the incubation was continued for 2 min (second incubation). The test strip was then taken out of the solution, thoroughly rinsed with washing solution, and soaked for 5 min in the washing solution. Finally, the strip was immersed in 2 mL of substrate solution (TMB−H_2O_2) that was prepared according to Abouzied and Pestka (1994). After 3 min the color reaction was stopped by immersing the strip in washing solution and blotting the strip on absorbing paper.

The test result is visually interpreted as follows: the color of the upper spot, the reference (R) spot, was independent of the β-agonist concentration and was intense blue (black in the figure), while the color of the lower spot, the indicator (I) spot was inversely related to the β-agonist concentration in the sample. This spot had the same color intensity as the reference spot for a negative sample, while a color reduction was an indication for the presence of β-agonists. The dot color intensity of the test strips was also measured with a portable colorimeter, Minolta Chroma Meter CR-321 (Minolta, Aartselaar, Belgium), which is a compact tristimulus color analyzer for measuring reflective colors of surfaces. This color intensity was expressed as a single numerical value ΔE^*_{ab}, which is the difference between the color of a white membrane (the "target color data" that is stored in the memory of the meter) and the color of the dots of the test strip. The ratio of the color intensity of the indicator spot on the color intensity of the reference spot ($\Delta E^*_{ab}I/\Delta E^*_{ab}R$ or I/R) was 1 or slightly exceeded 1 for a negative sample and was less than 1 for a positive sample.

Recovery Testing by Liquid Scintillation Counting. This was performed using ^{3}H-clenbuterol (specific activity of 14 Ci/mmol) and ^{3}H-salbutamol (specific activity of 14 Ci/mmol) (Laboratoire d'Hormonologie, Marloie, Belgium). The recovery was determined by spiking the blank urine with a tritiated standard in the presence of cold standard (2 μg/L).

Recovery Testing by Gas Chromatography−Mass Spectrometry (GC/MS). The recovery was also determined by GC/MS, at a concentration of 10 μg/L, based on the procedure described by Montrade et al. (1993).

Recovery Testing of Salbutamol Conjugates. Salbutamol glucuronide and salbutamol sulfate standards are not commercially available. Therefore, in order to check the recovery of the salbutamol conjugates, a calf urine was used that had been found positive for salbutamol in the routine analysis carried out at TNO. The concentrations were determined by the microtiter plate ELISA and were read against a standard curve of salbutamol. The urine was taken up in the ELISA before hydrolysis and after hydrolysis without extraction, by simply diluting in assay buffer. At the same time, the hydrolyzed and unhydrolyzed urine were extracted on an Empore membrane and the concentrations were determined in the diluted Empore extracts. The hydrolysis was performed by incubating the urine for 20 h at 50 °C with Helix pomatia juice (Boehringer, Mannheim, Germany) that exhibits β-glucuronidase/aryl sulfatase activity. The experiment was carried out in duplicate and also applied to a blank urine to correct for eventual background signal.

RESULTS AND DISCUSSION

Empore Extraction. Since it was our aim to develop a rapid and simple extraction and cleanup step that could be performed out of the laboratory and that preferably gave high concentrated extracts, the multianalyte extractions that were mentioned above (Montrade et al., 1993; Collins et al., 1994; Dumasia and Houghton, 1991; Leyssens et al., 1991; Van Ginkel, 1991; Schilt et al., 1990; Haasnoot et al., 1993) were not suitable, because an evaporation of the eluate was needed. Elution with very small volumes was not possible either. Montrade et al. (1993) for instance eluted the mixed-phase cartridges with 6 mL of ethyl acetate containing 3% (v/v) concentrated (32%) ammonia solution. We measured the amount of [^{3}H]clenbuterol that eluted from these cartridges in every 1 mL portion of the 6 mL of ethyl acetate/NH_3 (97/3 v/v) by liquid scintillation counting. Most of the [^{3}H]clenbuterol eluted in the last three portions, proving that the elution volume could not be reduced. The same was observed with MeOH/NH_3 (97/3 v/v), which is a better elution solvent to combine with an immunological detection method, since it is miscible with an aqueous ELISA buffer.

The Empore disk technology was attractive due to the possibility of eluting with small volumes. Furthermore, the large commercial membranes could be downsized to small disks that, in combination with the appropriate filter holder and syringe material, formed a handy extraction device. The choice of the strong cation exchange disk was based on the different ionization behavior of the three types of β-agonists. As explained by Courtheyn et al. (1994), the three types of compounds (anilines, phenols, resorcinols) are only in the same state of positive ionization at the aliphatic amine group and uncharged at the aromatic moiety in acidic conditions.

It is generally accepted that β-agonists such as clenbuterol are not susceptible to metabolization or conjugation such as salbutamol and terbutaline. Although a large percentage (between 45% and 75%) of the excreted salbutamol has been found in calf urine as a free drug (Montrade et al., 1995), the substance was also excreted as 4-*O*-sulfate and 4-*O*-glucuronide conjugate. In order to carry out our quick assay, we excluded a hydrolysis step that would cleave these bindings and that generally takes several hours at elevated temperatures. Therefore, the pH of our sample was adjusted to 1, which is low enough to have the glucuronide or sulfate moieties in the undissociated form. Before pH adjustment and Empore extraction, the urines were first prefiltered through a disposable 0.2 μm membrane. This replaced the centrifugation step that usually precedes urine analysis and was intended to remove particles that could plug the extraction membrane.

Table 1. Recovery of β-Agonists of the Aniline Type at a Concentration of 10 μg/L in Urine, As Determined by GC/MS Analysis (n = 4), after the First and the Second Elution of the Disk with 250 μL of MeOH/NH_3 (97/3 v/v), and Total Recovery of the Two Consecutive Elution Steps

	first elution		second elution		
substance	mean recovery (%)	% CV[a]	mean recovery (%)	% CV	total recovery elution 1 + 2
clenbuterol	72	17	17	17	89
mabuterol	85	9	17	30	102
clenproperol	78	33	22	25	100
mapenterol	82	10	17	24	99
methylclenbuterol	49	19	14	13	63
bromobuterol	68	12	15	17	83
cimaterol	53	30	6	42	59

[a] % CV = % coefficient of variation.

For clenbuterol, the recovery determined with liquid scintillation counting was 69% ± 16% (n = 6) at a concentration of 2 μg/L in urine and ranged from 73 to 85% over a large concentration interval from 0.1 μg/L to 100 μg/L in urine. These values were comparable to the recovery that was found with GC/MS analysis (Table 1, first elution). The recoveries of the other β-agonists of the aniline type that were also determined with GC/MS analysis are presented in the same table.

In Table 1 the recoveries of the same substances are presented after eluting the same disk with a second portion of 250 μL of MeOH/NH_3 97/3 (v/v). For most of the substances, approximately 15% was additionally eluted by this second elution. However, eluting the disk with one portion of 500 μL instead of 250 μL did not yield this additional 15%. In Table 1, also the total recoveries of the two consecutive elution steps with 250 μL of MeOH/NH_3 97/3 (v/v) are presented. A recovery of more than 80% was obtained for all compounds, except for cimaterol and methylclenbuterol. The total recoveries for the tested aniline-type β-agonists were comparable or higher than reported by Montrade et al. (1993) or Collins et al. (1994) (except for clenproperol, mapenterol, and bromobuterol, which were not tested by either of the two groups). As our purpose was to take up the extract in an immunoassay, we preferred to elute only once with 250 μL in order to have a more concentrated extract. The wash step with 100% methanol prior to elution resulted in cleaner sample extracts, as was also reported by Collins et al. (1994) for the extraction of mixed-phase cartridges, and caused a loss of only approximately 4%, as determined with liquid scintillation counting. We also tested another type of Empore disk, namely with mixed characteristics (both cation exchange and lipophilic properties). This disk was also based on a poly(styrene−divinylbenzene) network, but with a lower loading of sulfonic acid functions. The use of this type of disk yielded similar recoveries for clenbuterol (71% recovery).

For salbutamol and terbutaline, Collins et al. (1994) reported high recoveries, namely in the same range as

Table 2. Cross-Reactivities of the Antiserum with Different β-Agonists, Relative to Clenbuterol and Determined at 50% B/B_0

substance	% cross-reactivity	substance	% cross-reactivity
clenbuterol	100	terbutaline	15
salbutamol	176	clenproperol	4
mabuterol	78	broombuterol	55
mapenterol	19	methylclenbuterol	17
cimaterol	7		

for clenbuterol and mabuterol, while Montrade et al. (1993) had the lowest recovery for these more polar substances (22% for salbutamol and 36% for terbutaline). In our study, the obtained recoveries for salbutamol and terbutaline were low as well. With liquid scintillation counting, for salbutamol at a concentration of 2 μg/L in urine, the recovery was 8% (% CV = 18, n = 5). The recoveries for salbutamol and terbutaline at a concentration of 10 μg/L in urine, as determined by GC/MS analysis were in the same range: 7% recovery for salbutamol (% CV = 27, n = 3) and 11% recovery for terbutaline (% CV = 30, n = 3). This recovery could not be enhanced by using other elution solvents such as ethanol/NH_3 97/3 (v/v) or 2-propanol/NH_3 97/3 (v/v) nor by using the mixed-phase type extraction disk (data not shown). This low recovery of about 10% does not make these compounds undetectable, however, as will further be demonstrated, taking into account that usually, for direct ELISA analysis of urines, the samples are first diluted five times, while here, a 20-fold concentration is performed.

Moreover, the recovery testing of salbutamol conjugates in a real sample showed higher recoveries. Without hydrolysis, an extraction recovery of 28% was found, as determined with the microtiter plate ELISA. After the hydrolysis step, during which the salbutamol glucuronide and salbutamol sulfate were converted into salbutamol, 37% was recovered in the Empore extract. It is almost impossible to estimate exactly which percentage of the salbutamol conjugates was recovered. Firstly, the cross-reactivity of the antiserum with the salbutamol conjugates is unknown and could not be determined, as the standards are not commercially available. Secondly, the relative proportion of unconjugated salbutamol in the urine can range between 45% and 75% (Montrade et al., 1995), depending on the withdrawal time, which was unknown for the sample that we analyzed. Finally, the difference between the extraction recovery before and after hydrolysis (28% versus 37%) was in the range of the normal variation on the extraction recovery determined for salbutamol. However, the experiment demonstrated that omitting the hydrolysis step did not reduce dramatically the extraction recovery.

Microtiter Plate ELISA. The standard curve of the microtiter plate ELISA exhibits a 50% binding value (ID_{50}) for clenbuterol of 0.58 ± 0.08 μg/L (n = 10). This ID_{50} is comparable with the most sensitive ELISAs commercially available for screening of β-agonists (Hahnau and Jülicher, 1996). Cross-reactivities of the antiserum with other β-agonists are expressed relative to clenbuterol (100% cross-reactivity) and are presented in Table 2.

Test Strip Immunoassay. In the first experiments, carried out in the development of the test strip IA, salbutamol-HRP (dilution 1:400 000) and the anti-salbutamol antibody (dilution 1:50 000) were both added to the sample simultaneously. The strip coated with the second antibody was immersed in this mixture and incubation time was 30 min. When the test was applied in this format for the detection of standards in assay buffer, a concentration of 1 μg/L produced a clear color reduction and a concentration of 5 μg/L resulted in a completely inhibited color development. The incubation time of 30 min could not be shortened, since this resulted in a weak signal. When this test strip assay was applied to standards added to diluted blank urine, the assay failed due to matrix effects: the color of the negative control was already very weak and hence a color reduction, which should indicate the presence of the analyte, was not detectable. Increasing the concentration of antibody and label to increase the "blank signal" only further reduced the sensitivity of the test. Hence, it became essential to link the test strip IA to a fast and simple extraction method. Instead of diluting the urines five times, the Empore extraction provided a 20-fold concentration (5 mL of urine was reduced to a 250 μL extract) and the urines were thoroughly cleaned up. It was obvious, however, that the test strip IA could not be carried out in the pure methanolic Empore extract. Therefore, it was necessary to determine the minimum dilution of the extract with assay buffer that did not disturb the immunological and color reaction. To test this, the strip test was carried out in assay buffer and in different dilutions of MeOH/NH_3 (97/3 v/v) (0-standards), which showed that the elution solvent should be diluted 10-fold to have no harmful effect. In practice, to the 250 μL extract was added 2 mL of assay buffer, giving a 9-fold dilution.

As explained earlier, previous attempts to further reduce the incubation time only led to a very weak color development. Therefore, the original competitive incubation step was modified to a sequential incubation step, where the analyte first had the opportunity to bind to the specific antibody in the first incubation step. In a second step, a high concentration of the enzyme label was added (1:4000 dilution) but this second incubation was kept very short (2 min). This provided an intense color development, while the short label incubation time kept the test sensitive, as was also demonstrated by Weller et al. (1992).

Simultaneously with the enzyme label, diluted anti-peroxidase IgGs were added to generate the reference spot. The principle of an internal reference system was applied by Litman et al. (1983) in the development of a homogeneous enzyme immunoassay-based test strip immunoassay for morphine. This system provided a color response on the test strip, independently of the analyte concentration, but produced from the same conjugate and developer components, namely by the antiperoxidase antibodies that bind a constant amount of hapten-enzyme conjugate. Moreover, this color response responded in parallel with the indicator color to changes in temperature, timing and the sample composition or in other words, the I/R ratio remained constant. Recently, this principle was also applied by Schneider et al. (1995) in the development of a membrane-based ELISA test for fumonisin B_1. While both research groups applied the anti-analyte and anti-peroxidase antibodies directly onto the membrane, we preferred to work with the second antibody technique. This technique provides a more economical use of the anti-analyte antibody and a higher sensitivity and reproducibility, as was also concluded by Schneider and Hammock (1992). Moreover, our attempts to coat the anti-salbutamol and anti-peroxidase antibodies directly on the membrane resulted in spots that were too small in area. Probably this was due to the glycerol present in the stock dilutions of these antisera. Therefore, the

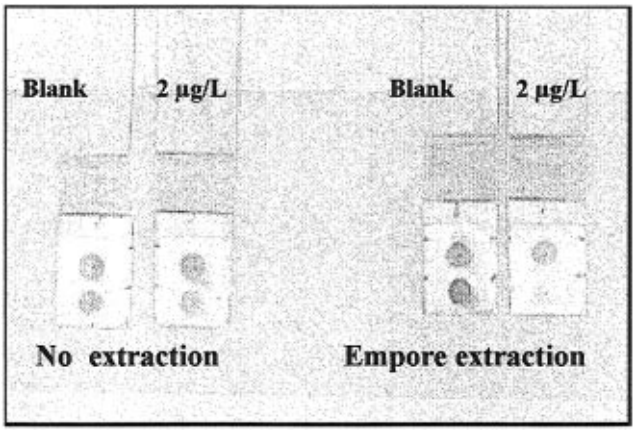


Figure 6. Comparison of a test strip immunoassay carried out on a urine blank and urine spiked at 2 µg/L, without Empore extraction (left strips) and after Empore extraction (right strips).

membrane was precoated with 1 µL of rabbit anti-sheep IgG to bind the sheep anti-salbutamol IgGs and with 1 µL of goat anti-rabbit IgG to bind the rabbit anti-peroxidase IgGs.

After optimization of the label and anti-salbutamol antibody concentrations in order to obtain a dark-colored indicator spot for a negative sample and also a clear color reduction at low antigen concentrations, the anti-peroxidase loading had to be adjusted, since this controls the reference color. With an anti-peroxidase dilution of 1:30 000 the reference spot was equally colored as the indicator spot for a blank sample. The use of the internal reference system also provides a control to the user for damage to the enzyme label or a highly disturbing matrix, since this would lead to a partial or complete reduction of the reference spot.

In Figure 6 the results are presented of the test strip IA after immersing the strip in unextracted urine (left strips). Blank urine and urine spiked with clenbuterol at 2 µg/L were diluted five times in assay buffer to reduce matrix effects, and the test strip IA was performed on 500 µL of diluted urine. At the same time, 5 mL of blank and 5 mL of spiked urine were extracted on the Empore filter and the test strip IA was performed on a 500 µL diluted extract as described before (right strips). To reach the required sensitivity, or in other words, to obtain a sufficient color reduction at the low ppb level, the Empore extraction was shown to be necessary.

Unlike the observations of Giersch (1993), we preferred the Immunodyne ABC membrane in our assay format over a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). With the latter, the spots had a faint color for the same label and antibody concentrations and showed a more irregular shape.

For the establishment of the detection limit of the dipstick assay, blank urine was spiked with clenbuterol at different concentrations, namely, 5, 2, 1, 0.5, and 0.25 µg/L. The Empore extraction and consecutive dipstick test were performed 10 times on the blank sample and each spiked sample. The result of such a test is shown in Figure 7. The detection limit was defined as the lowest concentration for which the indicator spot color was clearly lower than the reference spot in all of the 10 experiments. In 6 of the 10 tests, a concentration of 0.5 µg/L showed a clear positive result after visual interpretation. A 100% true positive result was only obtained for 1 µg/L. This is in agreement with the limit of detection of 1 µg of clenbuterol/L of urine that is required by the Standing Veterinary Committee of the

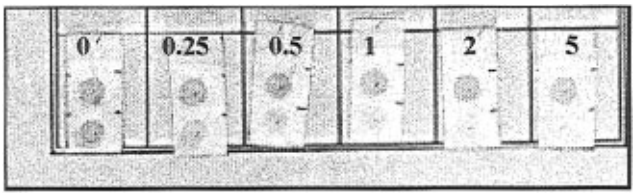


Figure 7. Results of an Empore extraction−dipstick test carried out on blank urines spiked with clenbuterol at different concentrations (5, 2, 1, 0.5, 0.25, and 0 µg/L).

Table 3. Mean Ratios of the Color Intensity of the Indicator Spot on the Color Intensity of the Reference Spot (*I/R* Ratios) As Determined by Spiking Blank Urines with Clenbuterol at Different Concentrations and Performing the Empore Extraction and Subsequent Test Strip Immunoassay

	clenbuterol conc (µg/L)					
	0	0.25	0.5	1	2	5
I/R ratio (mean)	1.10	0.91	0.67	0.52	0.49	0.43
s	0.14	0.27	0.13	0.08	0.09	0.08
n	10	5	10	10	10	10

European Commission for all detection methods used for official residue control of clenbuterol (Dr. Jülicher, BGVV, Berlin, oral communication). However, the instrumental data showed a higher sensitivity. In Table 3, the mean *I/R* ratios are presented for every concentration. At 0.5 µg/L the *I/R* ratios were significantly lower ($p < 0.05$) than for the blank samples (Student's t test). This confirmed earlier conclusions of Märtlbauer et al. (1994) that visual evaluation is less sensitive than instrumental evaluation. From the same table, the interassay reproducibility at the different concentrations could be deduced.

In order to test the selectivity of the test strip IA, the assay was applied to 30 blank samples, as was also described by Boison et al. (1996) for the evaluation of commercial rapid tests for antibiotic residues. The 30 blank samples consisted of 10 steer urines, 10 calf urines, and 10 pig urines. For all the 30 blank samples the test strip assay showed a negative result: the indicator spot was slightly darker or showed the same color as the reference spot. This is also expressed in the measured *I/R* ratios that were 1.02 ± 0.11 ($n = 10$) for the steer urines, 1.14 ± 0.13 ($n = 10$) for the calf urines, and 1.11 ± 0.09 ($n = 10$) for pig urines. However, when these urines were analyzed by the microtiter plate ELISA (without extraction but by simply diluting five times), concentrations of more than 1 ppb were found in some of these samples. Taking into account that for the test strip assay, the sample constitutes a much higher portion of the total incubation volume than for the microtiter plate ELISA, which usually results in much higher matrix interferences (Chard, 1982), it can be concluded that the Empore extraction provides a thorough cleanup of the urine matrix.

The ability of the test strip immunoassay to detect β-agonists other than clenbuterol was tested for salbutamol, bromobuterol, mapenterol, and cimaterol by spiking blank urine with different concentrations of these β-agonists and applying the Empore extraction and subsequent test strip immunoassay. The concentration was determined that caused a similar color reduction of the indicator spot as a clenbuterol spike at 1 µg/L.

This was for bromobuterol approximately 5 µg/L, for mapenterol approximately 10 µg/L, for cimaterol approximately 30 µg/L, and for salbutamol between 2 and

Table 4. Results of the Analysis of Samples from a Feeding Experiment and by GC/MS analysis, Microtiter Plate ELISA, and the Dipstick Test (Concentrations in μg/L)

	GC/MS		microtiter plate	dipstick test			
				visual result		*I*/*R* ratio	
sample no.	clenbuterol	salbutamol	ELISA	test 1	test 2	test 1	test 2
clenbuterol spike (1 μg/L)				+	+	0.57	0.55
1192	4.6		2.00	+	+	0.46	0.51
1195	0.2		0	−	−	1.09	1.04
1197	0.1		0.04	−	−	0.97	1.13
8152	127		360	+	+	0.30	0.36
8280	0.2		0.24	−	−	0.93	0.90
10025	1.7		1.03	+	+	0.70	0.70
10402	13	51	82	+	+	0.41	0.49

5 μg/L. Although bromobuterol, mapenterol, and cimaterol were comparably recovered with the Empore extraction (see Table 1), the lower cross-reactivity of these compounds with the antibody contributed to a great extent to the lower detectability. Salbutamol on the other hand is poorly recovered with the Empore extraction but cross-reacts very well with the antibody. Therefore, it could be detected at concentrations below 5 μg/L.

Finally, the Empore extraction−test strip immunoassay was also evaluated by analyzing samples from an animal experiment. The seven samples were analyzed together with a clenbuterol spike at 1 ppb. The experiment was carried out twice on different days. The strips were visually evaluated, and the color of the strips was measured with the colorimeter as well. At the same time, the urines were also analyzed with the microtiter plate ELISA, without extraction, but simply by diluting five times in assay buffer. In Table 4, the results are presented (visual result and *I*/*R* ratio) for the two dipstick tests, the microtiter plate ELISA, and the GC/MS analyses. Three urines, namely samples 1192, 8152, and 10402 were clearly positive: the color of the indicator spot was clearly reduced. The *I*/*R* ratios of these urines are below the *I*/*R* ratio of the spike (see Table 4). Both with the GC/MS analysis and with the microtiter plate ELISA, concentrations higher than 1 μg/L were found. However, there was not a great difference between the *I*/*R* ratio of highly positive urines (like samples 8152 and 10402) and the *I*/*R* ratio of a low positive urine (1192). Also from Table 3 it could be concluded that the *I*/*R* ratios were not further decreasing very much once the concentration exceeded 1 μg/L. In other words, the dipstick test yields a qualitative result (yes/no answer) rather than quantitative information. Three urines were clearly negative, namely, samples 1195, 1197, and 8280. The *I*/*R* ratios of these samples were nearly 1 or exceeded 1. Both the GC/MS analysis and the microtiter plate ELISA found concentrations below the detection limit of the dipstick test. Sample 10025 showed a positive result: the color of the indicator spot was reduced toward the reference spot, but the color reduction was not as distinct as for the clenbuterol spike at 1 μg/L. Therefore, the concentration was estimated between 0.5 and 1 ppb. This was also expressed by the *I*/*R* ratio of 0.70 that was higher than the *I*/*R* ratio of the spike (0.57). The microtiter plate ELISA also revealed a concentration around the detection limit of the dipstick assay (1.03 μg/L), while the GC/MS analysis yielded a slightly higher result (1.7 μg/L). As can be concluded from the *I*/*R* ratios, the two dipstick tests, carried out on different days, yielded reproducible results for all the samples.

Conclusions. The test described here offers a sensitive and relatively simple detection method for clenbuterol residues in urine. The whole test, including the Empore extraction could be performed in about 30 min. The Empore extraction provided a fast and simple cleanup of the urines and a concentration step with good recoveries for the β-agonists of the substituted aniline type. In connection with the subsequent test strip immunoassay, the test did not yield false positive nor false negative results on the samples that were tested. In case the Empore extraction would not take away all sample interferences, matrix effects were controlled by an internal reference spot. The internal reference system also simplified the test, since all color reductions were compared to that reference spot and the analysis of an additional negative control sample became superfluous. A portable colorimeter increased the sensitivity of the test, because it enabled us to measure small color reductions that were not detectable with the eye. However, for concentrations higher than 1 μg/L, the colorimeter did not yield much additional information, and visual interpretation was sufficient to give a correct yes/no answer. The test also showed good sensitivity for salbutamol. In the development of this test, Eppendorf pipets were used for pipeting the exact volumes. The use of dropping bottles would further simplify the test and make it performable for less trained personnel.

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