Journal of Chromatography, 564 (1991) 413–427
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5706

Test strip enzyme immunoassays and the fast screening of nortestosterone and clenbuterol residues in urine samples at the parts per billion level

M. E. PLOUM, W. HAASNOOT*, R. J. A. PAULUSSEN, G. D. VAN BRUCHEM, A. R. M. HAMERS, R. SCHILT and F. A. HUF

Department of Biopharmaceutical Analysis, State Institute for Quality Control of Agricultural Products (RIKILT), Bornsesteeg 45, 6708 PD Wageningen (The Netherlands)

ABSTRACT

The preliminary results of an investigation into the development of "on-site" test strip enzyme immunoassays for the screening of urine samples for the presence of growth promoters, such as 17β,19-nortestosterone and clenbuterol at the parts per billion level are described. Urine samples, enzyme-labelled analyte and a nitrocellulose test strip, containing immobilized antibodies, are incubated together, after which the strip is placed in a chromogen-containing substrate solution for colour reaction. Using prefabricated strips, the tests can be performed in 45–60 min. A similar assay was worked out using a dot-blotting device, allowing the test to be performed in 20–50 min. The tests are simple and easy to perform outside the laboratory. Urine samples identified positive by gas chromatography–mass spectrometry were also found to be positive with these test strips and, so far, no false-positive results have been encountered. With standard additions to blank urine samples, positive samples could be distinguished above the 5 ng/ml level. However, samples from treated calves contain one or more metabolites of the parent compound, which increase the sensitivity of the assays. Although the tests described can be improved and still have to be evaluated further by analysing more urine samples, the preliminary results are very promising and give a lead to further research into the applicability of such "on-site" tests in residue analysis.

INTRODUCTION

The use of growth promotors, such as anabolic steroids or β_2-agonistic drugs, in animal husbandry is prohibited within the EC [1]. In The Netherlands, urine is chosen as the matrix to screen for the presence of such compounds, using gas chromatography combined with mass spectrometry (GC–MS). With GC–MS, several anabolics can be determined simultaneously in urine, after an extensive sample clean-up, above a level of 1–5 ng/ml [2]. Using immunoaffinity chromatography for sample clean-up, at least four of the β_2-agonistic drugs can be determined in urine above the 3 ng/ml level [3]. The GC–MS procedures, in combination with the extensive sample clean-up, make such analyses slow and expensive. A substantial reduction in the control costs may be achieved by a two-stage cc ntrol programme, in which the GC–MS procedures are only employed for

0378-4347/91/$03.50

those samples found positive in a first stage involving rapid and inexpensive screening methods.

Fast and relatively cheap methods for the compounds of interest at the required low parts per billion (ppb) level are mostly based on immunoassays, such as radioimmunoassays (RIAs) or enzyme immunoassays (EIA) [4–8]. RIAs are restricted to laboratory use only, and to achieve a further cost reduction, a screening test at the place of sampling, *i.e.*, the farm or slaughter-house, is preferred. For example, for the determination of the (naturally occurring) steroid hormone progesterone in milk, several "on-site" tests are commercially available [9]. These assays are designed primarily for use by dairy producers and veterinarians for assessment of the stage of the oestrus cycle and the determination of pregnancy or non-pregnancy. Most of them are aimed at determining the relative progesterone concentration ("high" or "low"), making a distinction at the low ppb level (5–10 ng/ml), rather than obtaining a precise concentration. These tests are all based on enzyme immunoassays, in which progesterone is bound by antibodies coated to a solid phase (plastic test-tubes, microtitre plates or strips, dip-sticks or fibre-discs). A progesterone–enzyme conjugate added to the test attaches to the non-occupied antigen binding sites and produces a colour reaction with a chromogen-containing substrate. The amount of colour development is inversely proportional to the concentration of the hormone in the sample. The tests require times varying between 2 and 40 min.

In our laboratory, microtitre plate enzyme immunoassays have been developed for clenbuterol and nortestosterone (NT) determination in urine samples. In this paper, the preliminary results of a conversion of these assays into "on-site" strip tests are described.

EXPERIMENTAL

Chemicals

Horseradish peroxidase (HRP), *Helix pomatia* digestive juice (containing a minimum of 40 U/ml β-glucuronidase and 20 U/ml arylsulphatase), estrone and 5α-dihydrotestosterone were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), ovalbumine, 17β,19-nortestosterone (β-NT), norgestrel, progesterone, 17α-testosterone, 17β-testosterone, methyltestosterone, 17α-estradiol, 17β-estradiol, ethinylestradiol, norethindrone, estriol, 5α-androstane-3α,17β-diol and fenoterol were obtained from Sigma (St. Louis, MO, U.S.A.). 19-Nor-4-androsten-6β-ol-3,17-dione, 5α-estrane-3β,17α-diol and nortestosterone sulphate were obtained from Steraloids (Wilton, NH, U.S.A.). Clenbuterol hydrochloride was a gift from Boehringer (Ingelheim, Germany). Hexadeuterated clenbuterol was synthesized at the Department of Organic Chemistry, Agricultural University (Wageningen, The Netherlands). Reinforced nitrocellulose was obtained from Schleicher & Schüll (Dassel, Germany) and tetramethylbenzidine (TMB) membrane peroxidase substrate system from Kirkegaard and

Perry Labs. (Gaithersburg, MD, U.S.A.). BCA Protein Assay Reagent, N,O-bis-(trimethylsilyl)trifluoroacetamide(BSTFA) and trimethylchlorosilane (TMCS) were obtained from Pierce (Rockford, IL, U.S.A.). 4-Chloro-1-naphthol was obtained from Aldrich Chemie Benelux (Brussels, Belgium). 17β-Trenbolone was a gift from Roussel-Uclaf (Romainville, France). Salbutamol sulphate and terbutaline sulphate were obtained from Bufachemie (Castricum, The Netherlands). Carbuterol hydrochloride was a gift from Warner-Lambert (Bornem, Belgium) and cimaterol from D.G. Mann Testing Labs. (Mississauge, Canada). 19-Nor-4-androstene-3,17-dione and trideutero-17β,19-nortestosterone were kindly donated by the National Institute of Public Health and Environmental Hygiene (RIVM, Bilthoven, The Netherlands) and 17α,19-nortestosterone (α-NT) and 19-nor-4-androsten-15β-ol-3,17-dione were gifts from Organon (Oss, The Netherlands). Amberlite XAD-2 was purchased from Serva (Heidelberg, Germany) and was purified by washing with 2 *M* sodium hydroxide solution, 2 *M* hydrochloric acid, acetone, ethanol and water.

All other chemicals were purchased from Merck or Sigma and were of the highest purity available.

ANTIBODY PREPARATION

Steroid hormones and β_2-agonists are too small to be immunogenic, and must therefore be conjugated to a molecule large enough to elicit an immune response. 19-Nortestosterone-17β-hemisuccinate–bovine serum albumin (ratio 18 mol/mol) was prepared according to Kyrein [10]. Clenbuterol was conjugated to BSA (molar ratio 14) after diazotization, as described by Yamamoto and Iwata [11]. These conjugates were used for the immunization of New Zealand White rabbits. The antisera were collected and stored at -20°C.

Preparation of enzyme conjugates

17β,19-Nortestosterone and clenbuterol were conjugated to horseradish peroxidase in the same way as described above for the conjugation to BSA. The molar ratios, determined according to Erlanger *et al.* [12], were 1.3 mol of nortestosterone and 11 mol of clenbuterol per mole of HRP.

Preparation of the strip

Nitrocellulose was cut into strips (0.5 x 1 cm) and 1 μl of antiserum was applied to the strip as a spot by means of a 0.5–10 μl Eppendorf pipette. The nitrocellulose strip was stuck on a plastic self-adhesive plate sealer (for microtitre plates; Costar, Cambridge, U.K.). The strip was preincubated with 2% BSA solution for 30 min at 37°C, air-dried and stored at 4°C.

Sample materials

Nortestosterone-containing urine samples were obtained from four animal experiments. In *experiment 1*, a male veal calf (calf No. 13), aged 26 weeks and weighing 183 kg, was injected intramuscularly in the neck with 200 mg of 19-nortestosterone-17β-laurate and 20 mg of estradiol-17β-benzoate (dissolved in 4 ml of arachis oil containing 10% benzyl alcohol). Another male veal calf (calf No. 12) of the same age and weight served as a control. From both calves urine samples were collected from 2 days before to 19 days after administration of the steroids. This experiment was performed in March 1987 at the Institute for Animal Nutrition Research (ILOB) at Wageningen, The Netherlands.

In *experiment II*, one male veal calf (calf No. 77), aged 18 weeks, was injected intramuscularly in the neck with 200 mg of 19-nortestosterone-17β-laurate, another male veal calf (calf No. 78), of the same age, was treated in the same way with 400 mg of 19-nortestosterone-17β-laurate and a third male veal calf (calf No. 79), of the same age, served as a control. From the three calves urine samples were collected twice a day up until 5 days after administration of the steroid. This experiment was performed in July 1988 at the Institute for Livestock Feeding and Nutrition Research (IVVO, Lelystad, The Netherlands).

In *experiment III*, two male veal calves (calves Nos. 1 and 3), aged about 6 weeks, were injected intramuscularly in the neck with 400 mg of 19-nortestosterone-17β-laurate weekly for a period of 4 weeks. A third male veal calf (calf No. 2), of the same age, served as a control. Urine samples were collected daily from the day of the first application until 5 days after the final application. This experiment was performed in February 1989 at the IVVO.

In *experiment IV*, a male veal calf (calf No. 1), aged about 10 weeks, was injected intramuscularly in the neck with 250 mg of 19-nortestosterone-17β-laurate. Urine samples were collected daily from 1 to 16 days after application. This experiment was performed in July 1986 at the IVVO.

Blank urine samples from calves of different age were obtained from the IVVO. Clenbuterol-containing urine samples were obtained from the General Inspection Service (Kerkrade, The Netherlands). All samples were stored at -20°C until used.

Enzyme immunoassay

For cross-reactivity determinations, a competitive EIA was developed, applying clenbuterol–HRP [for β_2-agonists] or nortestosterone–HRP (for steroids) as a label.

Microtitre plates were coated overnight with 100-μl aliquots of sheep-anti rabbit IgG [10 μg/ml in 50 m*M* sodium carbonate (pH 9.6)] at 4°C. The plates were washed four times with phosphate-buffered saline (PBS)–Tween [5.4 m*M* Na_2HPO_4–1.3 m*M* KH_2PO_4–150 m*M* NaCl (pH 7.4)–0.05% Tween-20] with a Wellwash 4 microplate washer (Denley Instruments, Billinghurst, U.K.) Aliquots of 50 μl of serially diluted β_2-agonists or hormones were added to the wells,

followed by 25 μl of appropriately diluted clenbuterol–HRP or nortestosterone–HRP and finally 25 μl of antiserum (all in PBS–Tween) were added. The plates were incubated for 2 h at 37°C. After washing (as described above), the bound peroxidase was assessed with 100 μl of a freshly prepared solution of 2.2 m*M* *o*-phenylenediamine–0.012% hydrogen peroxide in 100 m*M* citrate–200 m*M* K_2HPO_4 (pH 5.0). After incubation in the dark for 30 min at 20°C the reaction was stopped by addition of 50 μl of 12.5% sulphuric acid. The product of the peroxidase reaction was determined at 490 nm with an Argus 400 Microplate reader (Canberra Packard, Downers Grove, IL, U.S.A.). Cross-reactivities were determined at 50% displacement.

Strip tests

To a strip, placed in a glass test-tube (6 × 0.9 cm I.D.), 1 ml of sample and 15 μl of diluted nortestosterone–horseradish peroxidase (NT–HRP) or diluted clenbuterol–HRP (CL–HRP) were added. After incubation at room temperature, the strip was rinsed with water and placed in another test-tube containing 0.8 ml of a substrate solution. During the experiments two different substrates were used. Substrate I consisted of a filtered 4-chloro-1-napthol solution [3 mg in 16 ml of ethanol–water (2:98)] to which, immediately before use, 11 μl of hydrogen peroxide (30%) were added. Substrate II was a TMB membrane peroxidase substrate system, which was used as described by the manufacturer.

After 1 min, the colour reaction was stopped by washing the test strips with water. The strips were reviewed for colour production, *i.e.*, positive samples reduce the colour production.

Dot-blot assay

A sheet of nitrocellulose was placed on top of three sheets of filter-paper (Whatman, Maidstone, U.K.) and the group of sheets were brought into a Milliblot-D system (Millipore, Bedford, MA, U.S.A.). Successively, 10 μl of a ten-fold diluted antibody solution and 100 μl of a BSA solution (2% in PBS) were added to the wells and after incubation for 30 min at 37°C the solution was sucked through the nitrocellulose by applying reduced pressure to the Milliblot system, using a water-jet pump. Next, 0.8 ml of sample, containing 12 μl of appropriately diluted antigen-HRP conjugate, was sucked slowly through the nitrocellulose. To remove non-bound antigen-HRP, the wells were washed twice with 200 μl of water. Next, 100 μl of the TMB substrate (substrate II) were added to the wells and after 1 min the wells were emptied and the nictrocellulose was taken out of the device, rinsed with water to stop the colour reaction and evaluated for colour production.

GC–MS procedures

For confirmation of the presence and quantification of the analytes, the following GC–MS procedures were used.

Apparatus. The GC–MS system (Hewlett-Packard, Rockville, MD, U.S.A.) consisted of a Model 5890 gas chromatograph, a Model 5970 mass-selective detector, a Model 7673-A autoinjector and a Model 59970-C data system. The column was CP-Sil-5 CB (25 m x 0.25 mm I.D.) (Chrompack, Middelburg, The Netherlands) with a film thickness of 0.12 μm, with helium as the carrier gas at a linear flow-rate of 30 cm/s.

Procedure for clenbuterol. Prior to the sample preparation, hexadeuterated clenbuterol was added to the urine samples at 10 ng/ml. To 10 ml of the sample, a few drops of 10 *M* sodium hydroxide solution were added to obtain a pH between 10 and 11. After centrifuging for 5 min at 200 *g*, the supernatant was transferred to a C_{18} solid-phase extraction column (3 ml, Analytichem International, Harbor City, CA, U.S.A.). This column was previously activated with one column volume of methanol and subsequently with two column volumes of water. Next, the column was washed twice with 2.5 ml of methanol–water (50:50), dried by sucking air through it for 5 min and subsequently eluted with two 1-ml volumes of methanol. The eluates were combined, dried by adding sodium sulphate and the methanol was evaporated to dryness at 45°C under a stream of nitrogen. To the residue, 100 μl of BSTFA–ethyl acetate (50:50) were added and the mixture was incubated at 70°C for 30 min. After cooling to room temperature, the solution was evaporated and the residue dissolved in 25 μl of ethyl acetate, of which 5 μl were injected in the splitless mode into the GC–MS system utilizing selected-ion monitoring (SIM).

The amount of clenbuterol in the sample was calculated by comparing the ratio of the peak area of clenbuterol (*m/z* 86) to that of hexa-deuterated clenbuterol (*m/z* 92) with the ratios obtained with a calibration graph. For identification of clenbuterol, detection was performed with a combination of electron-impact and (positive-ion) chemical ionization mass spectrometry as described previously [3].

Procedure for nortestosterone. Prior to the sample preparation, trideutero-17β,19-nortestosterone was added to the samples at 10 ng/ml. The urine sample (25 ml) was mixed with 6 g of water-saturated Amberlite XAD-2 for 15 min and the mixture was transferred into a glass column (100 mm x 9 mm I.D.). The settled XAD-2 was washed with three portions of 10 ml of water and dried under a stream of nitrogen. The conjugated analytes (α- and β-NT are mainly present in urine as conjugates of glucuronic and sulphuric acids) were eluted with 25 ml of methanol–ethyl acetate (50:50) and the eluate was evaporated to dryness at 50°C. To the residue, 2 ml of 0.25 *M* acetate buffer (pH 4.8) and 50 μl of *Helix pomatia* juice were added and the mixture was incubated at 37°C for 2 h. Next, four drops of 6 *M* hydrochloric acid and 20 ml of ethyl acetate were added. After mixing for 15 min, the water layer was removed and the ethyl acetate layer was incubated for 1 h at 37°C and subsequently washed with two 3-ml portions of 10% (w/w) sodium hydrogencarbonate and 3 ml of water. The ethyl acetate layer was dried by adding sodium sulphate and evaporated to dryness. The residue was dissolved

in 250 μl of toluene–ethyl acetate (85:15) and transferred to a 1-ml silica gel column (Bond Elut; Analytichem International). This column was previously activated with one column volume of toluene–ethyl acetate (85:15). The column was eluted with two 1-ml portions of toluene–ethyl acetate (85:15) and the eluates were combined and evaporated to dryness at 45°C with a stream of nitrogen.

The analytes were converted into monotrimethylsilyl derivatives by adding 100 μl of a freshly prepared mixture of 100 μl of trimethylchlorosilane (TMCS), 1 ml of BSTFA and 9 ml of pyridine and heating for 1 h at 60°C. After evaporation under a stream of nitrogen (40°C), the residue was dissolved in 25 μl of isooctane–*n*-decane (4:1). For analysis, 5 μl were injected in the splitless mode into the GC–MS system utilizing SIM. The analytes were considered to be positively identified if the GC retention times of the derivatives and the ratios of the intensities of the fragments with *m/z* values of 215, 256, 331 and 346 (base peak) agreed with those of the standards to within ±5 s and ±10%, respectively. For quantitative purposes, the ratio of the peak area of the analytes (*m/z* 346) to the trideuterated internal standard (*m/z* 349) were compared with the ratios obtained with a calibration graph.

RESULTS AND DISCUSSION

Development of a strip test for nortestosterone

17β,19-Nortestosterone was coupled to BSA through a hemisuccinate bridge at the 17-position. This conjugate was used to raise polyclonal antibodies, which will also react with steroids with a comparable structure, especially those with

TABLE I

CROSS-REACTIVITIES OF ANTIBODIES AGAINST 17β,19-NORTESTOSTERONE TOWARDS SOME STEROID HORMONES

Steroid hormone	Cross-reactivity[a] (%)	Steroid hormone	Cross-reactivity[a] (%)
17β,19-Nortestosterone	100	5α-Dihydrotestosterone	0.2
17α,19-Nortestosterone	69	17α-Testosterone	0.08
Nortestosterone sulphate	100	17β-Testosterone	<0.05
Norgestel	26	Methyltestosterone	<0.05
Norethindrone	20	5α-Androstane-3α,17β-diol	<0.05
19-Nor-4-androstene-3,17-dione	242	17α-Estradiol	<0.05
19-Nor-4-androsten-6β-ol-3,17-dione	7.8	17β-Estradiol	<0.05
19-Nor-4-androsten-15α-ol-3,17-dione	6.2	Estrone	<0.05
17β-Trenbolone	7.9	Estriol	<0.05
Progesterone	0.3	Ethinylestradiol	<0.05
5α-Estrane-3β,17α-diol	0.2		

[a] Cross-reactivities were determined in a competitive EIA, and calculated at 50% inhibition of the binding of 35 ng of nortestosterone–horseradish peroxidase. Values are means of three individual experiments.

similar A and B rings. Changes in the D ring will have only a minor influence on the reactivity. The conjugate was chosen to obtain antibodies that will react strongly with 17α,19-nortestosterone, a major metabolite found in the urine of β-nortestosterone-treated calves [13], and with 19-norandrostene-3,17-dione, which can be formed by oxidation of α- and β-NT in urine samples [14]. Further, such antibodies can be used to directly determine glucuronic and sulphuric acid conjugates (at the 17-position of the steroid), in which forms most of the nortestosterone is excreted in urine [15–17].

The cross-reactivities of the antibodies, expressed at the 50% displacement level (Table I), for some naturally occurring steroids, estradiol, estrone, progesterone and testosterone, and some synthetic steroids were determined in a competitive EIA. As predicted, high cross-reactivities were found for the major metabolite α-NT, the sulphate ester and the oxidation product of the parent compound, whereas only low reactivity occurred with the natural steroids. Trenbolone, another synthetic steroid that can be (illegally) used as growth promoter in cattle breeding, also showed a cross-reaction. Thus, when these antibodies are applied in an EIA, this hormonal substance also produces a positive result.

To permit the screening of urine samples for the presence of nortestosterone outside the laboratory, the microtitre plate EIA was converted into a strip test, which can be performed without the need for laboratory equipment. The antibodies were immobilized on (reinforced) nitrocellulose strips (0.5 x 1 cm), applied as a concentrated spot by means of a micropipette. To a nitrocellulose membrane, both negatively and postively charged proteins can be immobilized. Although the mechanism of this interaction, implying hydrophobic forces, is not fully understood, this material is most commonly used for protein blotting, *i.e.*, the transfer of proteins, separated by electrophoresis, from a gel to a membrane.

The optimum amount of antibody, *i.e.*, the minimum amount of antiserum necessary to produce a clear spot with the substrate, was determined with one- to forty-fold dilutions of the antiserum (1–44 μg of protein). Using 75 ng of peroxidase conjugate as a tracer, 11 μg of antiserum protein (in 1 μl) were chosen as the optimum. The vulnerable nitrocellulose was then protected by fixing it to a plastic plate sealer (for microtitre plates). Subsequently, the remaining binding sites on the strip were blocked with 2% BSA solution, to prevent binding of the HRP conjugate.

The optimum amount of nortestosterone–peroxidase conjugate to produce a clearly coloured spot within an acceptable time was determined in the range 1–100 ng and with incubination times between 0 and 60 min. Optimum conditions were set at 45–60 min incubation in the presence of 9 ng of HRP conjugate. The colour reaction with 4-chloronaphthol took *ca.* 1 min.

When the test strip was applied under these conditions, negative samples resulted in coloured spots, whereas no colour was observed with 10 ng of the analyte in 1 ml of PBS (Fig. 1). An amount of 5 ng/ml produced a clearly distinguishable colour reduction.

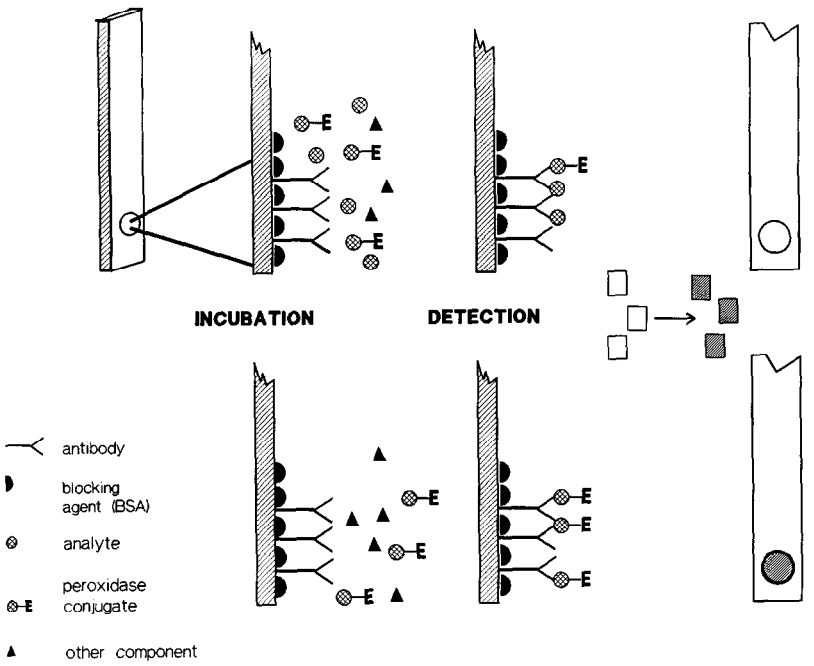

Fig. 1. Schematic representation of the strip test. A positive sample (top), giving no colour, and a negative sample (bottom), producing a coloured spot, are shown.

Determination of nortestosterone in urine samples

Nortestosterone-containing urine samples were obtained from six different calves from four individual animal experiments. Blank samples were obtained from the same animals, prior to treatment with nortestosterone laurate, and from untreated control calves from the same experiments. Most samples were taken earlier and, although kept at −20°C, were basic (pH up to 10). The pH of all samples was adjusted to 7–8 by adding a few drops of 1 *M* hydrochloric acid. Solids were filtered from the samples within a 5-μm filter, and 1-ml aliquots were placed in glass test-tubes. The nortestosterone-peroxidase tracer was added, followed by the strips, and the test was carried out as described above.

The detection limit was determined by adding different amounts of a β-NT standard solution to blank urine samples. Positive results (no colour) were observed at 20 ng/ml, which is twice that determined with β-NT solutions in buffer.

To explore the possible effects of the chloronaphthol substrate on the sensitivity of the assays, the TMB membrane peroxidase substrate system of Kirkegaard & Perry Labs. was tested; this is a precipitating substrate for membrane enzyme immunoassays consisting of a TMB solution, a hydrogen peroxide solution and a TMB membrane enhancer to precipitate the coloured reaction product. With this substrate, a two-fold reduction in the peroxidase conjugate concentration could

be applied, resulting in detection limits (non-coloured spots) of *ca.* 2 ng/ml for β-NT. With buffer solutions, blanks and positives could be discriminated at ±0.5 ng/ml. Compared with chloronaphthol, the TMB system gave a higher background intensity, but this could be effectively reduced by a slight increase in the TMB membrane enhancer concentration in the substrate solution.

The strip test was then applied to urine samples from the six treated animals, using the TMB substrate. To determine the actual α- and β-NT concentrations, the urine samples were also analysed by GC–MS (see Experimental). All samples were found positive with the strip test, even the urine sample with a 17β,19-nortestosterone concentration of only 1.3 ng/ml (Table II). The detection limit for β-NT was clearly lower than that with β-NT added to blank urine, which may be due to the presence of metabolites of the parent compound (as α-NT) in samples from actually treated animals that also bind to the antibodies, resulting in a lower detection limit for β-NT.

To determine the lowest detectable concentration, two of the positive samples were serially diluted with blank urine. The test strips were found positive above

TABLE II

COMPARISON OF THE NORTESTOSTERONE TEST STRIP ASSAY WITH GC–MS DETERMINATIONS OF THE 17α- AND 17β-NORTESTOSTERONE CONTENTS OF URINE SAMPLES FROM TREATED CALVES

The strip test was performed with TMB as substrate.

Experiment No.	Animal No.	Days after start of treatment	GC–MS determination[a]		Test strip	
			17α-NT (ng/ml)	17β-NT (ng/ml)	Result	Spot visible
I	12	Blank[b]	–[d]	–	Negative	Yes
I	13	Blank[c]	–	–	Negative	Yes
I	13	5	6.2	5.9	Positive	No
II	77	Blank[c]	–	–	Negative	Yes
II	77	4	80	5.3	Positive	No
II	78	Blank[c]	–	–	Negative	Yes
II	78	4	16.4	5.7	Positive	No
II	79	Blank[b]	–	–	Negative	Faint
III	1	Blank[c]	–	–	Negative	Yes
III	1	5	41	7.8	Positive	No
III	2	Blank[b]	–	–	Negative	Yes
III	3	Blank[c]	–	–	Negative	Yes
III	3	5	60	4.3	Positive	No
IV	1	5	8.6	1.3	Positive	No

[a] See Experimental.

[b] Untreated calf (control).

[c] Sample taken before application of nortestosterone.

[d] Nortestosterone not detectable (limit of determination 0.5 ng/ml).

TABLE III

DETERMINATION OF THE DETECTION LIMIT IN THE NORTESTOSTERONE STRIP TEST

Samples from calves 1 and 3 from animal experiment III (see *Sample materials*) were diluted with blank urine from the same animals, to determine the lowest detectable amount of nortestosterone with the test strip. The strip test was performed with TMB as substrate.

Animal No.	Dilution of sample with negative urine (v/v)	GC–MS determination		Test strip	
		α-NT (ng/ml)	β-NT (ng/ml)	Result	Spot visible
1	100:0	41	7.8	Positive	No
	75:25	30.7	5.9	Positive	No
	50:50	20.5	3.9	Positive	No
	25:75	10.2	2.0	Positive	No
	10:90	4.0	0.8	Negative	Faint
	1:99	0.4	0.0	Negative	Yes
	0:100	0	0	Negative	Yes
3	100:0	50	4.6	Positive	No
	75:25	37.5	3.4	Positive	No
	50:50	25	2.3	Positive	No
	25:75	12.5	1.2	Positive	No
	10:90	5	0.5	Negative	Faint
	1:99	0	0.5	Negative	Yes
	0:100	0	0	Negative	Yes

1–2 ng/ml β-NT (Table III). Blank samples from the same animals and from the control calves were also tested, and all produced clearly coloured spots. When compared with a buffer control, the intensity of the spots was lower, owing to matrix effects. Also, some variation in the matrix effect was found among the

TABLE IV

CROSS-REACTIVITIES OF ANTIBODIES AGAINST CLENBUTEROL TOWARDS SOME β_2-AGONISTS

β_2-Agonist	Cross-reactivity[a] (%)	β_2-Agonist	Cross-reactivity[a] (%)
Clenbuterol	100	Salbutamol	3.3
Carbuterol	7.9	Terbutaline	2.8
Cimaterol	3.3	Fenoterol	<0.01

[a] Cross-reactivities were determined in a competitive EIA, and calculated at 50% inhibition of the binding of 150 ng of clenbuterol–horseradish peroxidase. Values are means of three individual experiments.

various blank samples, and therefore samples were only judged positive when no coloured spot was observed.

Strip assay for clenbuterol

Clenbuterol was coupled to BSA through the aromatic amine, after diazotization of the amine group, as described by Yamamoto and Iwata [11]. The polyclonal antibodies raised against this BSA conjugate will react with the other side of the molecule, which contains a tertiary methyl function that is also present in some related β_2-agonists (*e.g.*, salbutamol, terbutaline and carbuterol). The cross-reactivities of the antibodies for these compounds were determined in a competitive EIA (Table IV), and appeared to be low (<8%), indicating an important role of the other side-groups of the aromatic ring. Still, these β_2-agonists may influence the determination of clenbuterol in an EIA, when simultaneously present.

A strip assay was developed with these antibodies as decribed above for nortestosterone, using TMB as substrate. A 12.5-μg amount of antiserum protein and 36 ng of peroxidase conjugate were chosen as optimum.

Measurement of clenbuterol in urine samples

Urine samples from the nortestosterone animal experiments (II and III) and

TABLE V

COMPARISON OF THE CLENBUTEROL TEST STRIP ASSAY WITH GC–MS DETERMINATIONS OF THE CLENBUTEROL CONTENTS OF URINE SAMPLES

Urine samples were obtained from the General Inspection Service (Kerkrade, The Netherlands). The strip test was performed with TMB as substrate.

Sample No.	GC–MS determination (ng/ml)	Test strip	
		Result	Spot visible
1	9.9	Positive	No
2	13.6	Positive	No
3	13.3	Positive	No
4	18.9	Positive	No
5	16.3	Positive	No
6	16.2	Positive	No
7	11.7	Positive	No
8	86.9	Positive	No
9	0.2[a]	Negative	Faint
10	0.1[a]	Negative	Faint
11	0.3[a]	Negative	Faint
12	0.2[a]	Negative	Faint
13	0.2[a]	Negative	Faint
14	<0.1[a]	Negative	Yes

[a] GC–MS results below 1 ng/ml are normally reported negative.

blank control samples were used initially to test the clenbuterol strips in urine. With all samples, coloured spots were observed with approximately similar intensities and, compared with the nortestosterone-strips, matrix effects were less obvious. The detection limit (not producing a coloured spot) with clenbuterol standard added to blank urine samples was *ca.* 5 ng/ml.

The strip test was then applied to urine samples obtained from the General Inspection Service of the Dutch Ministry of Agriculture, which were also analysed by GC–MS (see Experimental) (Table V). All samples identified positive with the GC–MS procedure were found positive with the test strips. Some samples with very low clenbuterol concentrations (0.1–0.3 ng/ml) still resulted in a reduced colour of the spots, which could be produced by metabolites of the parent compound present in the samples.

Residue analysis with a dot-blotting device

Experiments were also performed with a 96-well dot-blotting device (Fig. 2). An advantage of this system over the above-described strip tests is that the samples are sucked through the nitrocellulose filter by a decreased pressure applied to the apparatus, creating an optimum interaction between the analytes and the immobilized antibodies. After binding of the antibodies and blocking the filter with BSA (as described for the strips), which takes less than 30 min, the actual

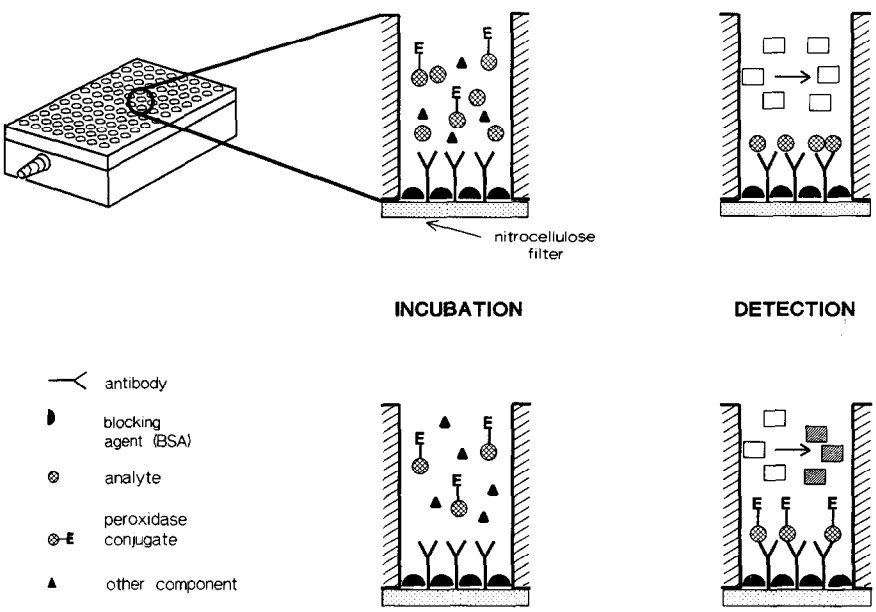

Fig. 2. Schematic representation of the dot-blot assay for residue determination, showing a positive sample, giving no colour production (top), and a negative sample, giving a colour reaction of the substrate (bottom).

analysis time is 20–30 min (depending on the number of replicates of each sample). The detection limits achieved with this system were comparable to those of the strip tests.

CONCLUSIONS

Using nitrocellulose-immobilized antibodies together with peroxidase-labelled analytes as tracer and a colour-producing substrate, strip tests can be created for fast residue analysis of growth-promoting agents, such as the steroid nortestosterone and the β_2-agonist clenbuterol, in urine at the low ppb level without any sample preparation. Using prefabricated strips or a dot-blotting device with precoated sheets, the test can be performed within 15–25 min, and can easily be executed outside the laboratory.

So far, with urine samples positively identified by GC–MS, positive results were also found with these strip tests, and no false-positive results were obtained with the blank urine samples tested. The distinction of positive and negative urine samples can be made at *ca.* 5 ng/ml with standard solutions added to blank urine, but with samples from treated animals lower concentration can be detected, owing to the effects of metabolites present in the samples that also react with the antibodies. Larger series of samples (both positive and negative) have to be analysed with the assays for β-nortestostrerone and clenbuterol to evaluate their characteristics fully, but the data presented here are very promising. Future research will be focused on (i) the optimum configuration of the tests strips for on-site application, (ii) reduction of the detection limits and (iii) a further extension of the number of analytes that can be determined with these strips by changing the binding components and/or enzyme-labelled tracers.

ACKNOWLEDGEMENTS

The authors thank H. Hooijerink, E.O. van Bennekom and P. Stouten for performing the GC–MS analyses. Further, they are indebted to P.L.M. Berende for his work on the animal experiments and the supply of sample materials.

REFERENCES

1 Council of the European Communities, Council Directive 86/469/EEC of 16 September 1986, *Off. J.Eur. Commum.*, L275 (1986) 36.
2 J. M. Weseman, H. Hooijerink, E. O. van Bennekom and R. Schilt, *Standard Operating Procedure A0511*, RIKILT, Wageningen.
3 R. Schilt, W. Haasnoot, M. A. Jonker, H. Hooijerink and R. J. A. Paulussen, in N. Haagsma, A. Ruiter and P. B. Czedik-Eysenberg (Editors), *Proceedings of the EuroResidue Symposium, Noorderwijkerhout, May 21–23, 1990*, Rijksuniversiteit Utrecht, Utrecht, 1990, p. 320.
4 K. Vogt, *Arch. Lebensmittelhyg.*, 38 (1987) 71.
5 E. Rattenberger, P. Matze, P. Funke and H. H. D. Meyer, *Arch. Lebensmittelhyg.*, 38 (1987) 73.

6 E. H. J. M. Jansen, R. H. van den Berg, G. Zomer and R. W. Stephany, *J. Clin. Chem. Clin. Biochem.*, 23 (1985) 145.
7 H. H. D. Meyer and P. Funcke, in S. Görög (Editor), *Proceedings of the 3rd Symposium on the Analysis of Steroids, Sopron, October 20–22, 1987*, Akadémiai Kidaó, Budapest, 1988, p. 225.
8 G. Degand, P. Schmitz and G. Maghuin-Rogister, *J. Chromatogr.*, 489 (1989) 235.
9 R. L. Nebel *J. Dairy Sci.*, 71 (1988) 1682.
10 H. J. Kyrein, *Z. Lebensm.-Unters.-Forsch.*, 177 (1983) 426.
11 I. Yamamoto and K. Iwata, *J. Immunoassay*, 3 (1982) 155.
12 B. F. Erlanger, F. Borek, S. M. Beiser and S. Lieberman, *J. Biol. Chem.*, 288 (1957) 713.
13 W. Haasnoot, R. Schilt, A. R. M. Hamers, F. A. Huf, A. Farjam, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 489 (1989) 157.
14 L. A. van Ginkel, H. van Blitterswijk, J. Zuydendorp, P. W. Zoontjes, H. J. van Rossum and R. W. Stephany, *Report No. 368301 009*, RIVM, Bilthoven, 1986.
15 C. H. L. Shackleton, *J. Chromatogr.*, 379 (1986) 91.
16 M. C. Dumasia, E. Houghton and S. Sinkins, *J. Chromatogr.*, 377 (1986) 23.
17 E. Houghton, M. C. Dumasia, P. Teale, M. S. Moss and S. Sinkins, *J. Chromatogr.*, 383 (1986) 1.