ELSEVIER

Journal of Chromatography B, 724 (1999) 41–51

JOURNAL OF CHROMATOGRAPHY B

Multi-residue analysis of anabolics in calf urine using high-performance liquid chromatography with diode-array detection

Anneke Koole*, Jan-Piet Franke, Rokus A. de Zeeuw

University Centre for Pharmacy, Department of Analytical Chemistry and Toxicology, Ant. Deusinglaan 1, 9713 AV, Groningen, The Netherlands

Received 18 August 1998; received in revised form 23 November 1998; accepted 30 November 1998

Abstract

We describe the development of an HPLC method with diode-array detection (DAD) for the analysis and identification of 20 substances with anabolic properties, that are considered as potential growth promoters, to be used for the analysis of extracts of calf urine samples. The substances are separated on an RP-Select B column using a mobile phase consisting of a mixture of acetonitrile and water. Gradient elution from 43–76% acetonitrile in water with a concave curve was used to achieve a good separation of the compounds with an acceptable analysis time. For the identification, a retention parameter and the UV spectrum were used. The retention parameter was the retention time corrected with a reference mixture. The latter reduced the standard deviations to about 25% of their original values. The limits of detection of the HPLC system ranged from 0.5–5 ng injected amount for the androgens, progestagens, stilbenes and resorcylic acid lactones and to 5–10 ng injected amount for the oestrogens. After extraction from urine the limits of detection were increased by the presence of matrix components, but they were between 5 and 10 ng injected amount for most of the substances.

Keywords: Anabolic steroids

1. Introduction

Anabolic steroids and some related substances with comparable activities have been used as growth promoters during fattening of cattle for a long time [1,2]. This treatment may result in residues in the meat, which could be harmful for the consumer. The anabolic steroids are known carcinogens and prolonged ingestion of large doses disturbs the endocrine balance, leading to a large number of side effects [3]. Also, the consumption of meat that contained residues of oestrogenic compounds, has been suggested as the cause of breast enlargement in Italy [4] and precocious puberty in Puerto Rico [5]. Because of these risks, the use of anabolics as growth promoters in cattle was banned in the European Union in 1988 [6]. Yet, in the United States and several other countries the natural steroids and zeranol and trenbolone can be used as growth promoters.

For the control of the banned substances, samples taken during fattening at the farm or at the slaughterhouse are analysed for the presence of illegal growth promoters. Urine is the sample most often used for the analysis of anabolics. Analytical methods for human and equine urine [3,7] and for biological

*Corresponding author.

0378-4347/99/$ – see front matter
PII: S0378-4347(98)00556-8

samples obtained from food-producing animals [8,9] have been reviewed. Most methods employ solid-phase extraction (SPE) or immunoaffinity chromatography for clean up of the sample and GC–MS for the detection of the steroids [3,7]. However, HPLC has been used for the analysis of anabolic steroids in preparations of illegal growth promoters [10–12] and as a clean up step for biological samples [13,14]. Also, LC–MS has been used to analyse 28 anabolic steroids and metabolites [15] or conjugates of testosterone and epitestosterone [16] in extracts of human urine.

Here, the development of an HPLC–DAD method for the analysis and identification of 20 anabolics is described, which can be used for the analysis of extracts of calf urine samples in the screening for the abuse of illegal growth promoters. The aim of our research was to develop a rapid and cost-effective screening method, e.g. without MS detection, which can be used for the analysis of urine samples taken at the farm during fattening. The combination of the information provided by the retention parameter obtained with the HPLC and the UV spectrum recorded with the DAD may be sufficient for an unequivocal identification of the anabolics.

2. Experimental

2.1. Chemicals

The following chemicals were used for the experiments: For the preparation of stock solutions of the anabolics HPLC grade acetonitrile (Labscan, Dublin, Ireland) was used. In the HPLC system, gradient elution grade acetonitrile (Merck, Darmstadt, Germany) was used. Water was demineralised in house and was then purified with a Maxima ultrapure water instrument (Elga, obtained from Salm & Kipp, Breukelen, The Netherlands). Mobile phases were prepared by mixing demineralised and purified water with the modifier in the specified proportions (all v/v). Mobile phases were degassed using vacuum and sonication prior to use (vacuum was available through in-house facilities and the Bransonic ultrasonic cleaner model B2210-E-MT was from Bransonic (Bransonic Ultrasonics Corporation, Danbury, CT, USA)).

2.1.1. Anabolics

The anabolics used as reference substances were as follows. Methyltestosterone (MT) was from Serva (Serva Feinbiochemika, Heidelberg, Germany). Dienoestrol (DE), hexoestrol (HEX), and 17α-ethynyl oestradiol (EE2) were obtained from Sigma (St. Louis, MO, USA). Medroxyprogesterone (MP) was from Upjohn (Kalamazoo, MI, USA). BCR reference standards of zearalenone (Zeara), zeranol (Zer), taleranol (Tal), 19–17α-nortestosterone (αNT) and 19–17β-nortestosterone (βNT) were supplied by RIVM (Community Reference Laboratory/Laboratory for Analytical Residue Research, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; further referred to as RIVM). Zeara, β-trenbolone (Tb), 17β-oestradiol (E2), stanozolol (Stan), clostebol acetate (ClTac) and clostebol-diol (ClTdiol) standards were supplied by RIVM. Testosterone (T), βNT, progesterone (P), medrogestone (MED), trans-diethylstilbestrol (tDES) and oestrone (E1) were obtained from a local wholesaler. All stock solutions were prepared in HPLC grade acetonitrile. Calibration standards, of which 20 μl were injected, were prepared in the range of 0.25–10 μg/ml by dilution of the stock solutions with gradient grade acetonitrile. The standard solution of tDES contained a 72:28 mixture of tDES and cis-diethylstilbestrol (cDES).

2.2. HPLC columns

The columns tested were a Chromsep stainless steel HPLC column (150×4.6 mm I.D.) packed with Hypersil® ODS material (5 μm particles) (Chrompack, Bergen op Zoom, The Netherlands), a LiChroCART® 250-4 HPLC cartridge Superspher® 60 RP-select B (4 μm particles) (Merck), and a LiChroCART® 250-4 HPLC cartridge Superspher® 100 RP-18 (4 μm particles) (Merck). Guard columns were used to protect the HPLC columns. For the Hypersil column, Chromsep guard columns SS 10×3 mm reversed-phase (R3) (Chrompack) were used and for the Superspher columns, LiChroCART® 4-4

HPLC guard columns LiChrospher® 60 RP-select B (5 μm) (Merck).

2.3. HPLC-equipment

The HPLC pump was a System Gold 126 solvent module (Beckman Instruments, Mijdrecht, The Netherlands) equipped with a System Gold 168 DAD detector (Beckman). The pump and the detector were controlled with the Gold Nouveau Chromatography Data System version 1.0 (Beckman, 1996), run on an IBM personal computer 330p100 (Beckman) equipped with a HP deskjet 510 printer (Hewlett-Packard, Amsterdam, The Netherlands).

The HPLC column was a LiChroCART® 250-4 HPLC cartridge containing Superspher® 60 RP-select B material, 250×4 mm I.D. (Merck) protected by a LiChroCART® 4-4 guard column with LiChrospher® 60 RP-select B material, 4×4 mm (Merck). The injector was a Rheodyne 7725i injector equipped with a 20 μl sample loop (Rheodyne, Cotati, CA, USA).

The flow-rate was set at 0.8 ml/min. The gradient was made up from 40% acetonitrile in water (v/v) (solvent A) and acetonitrile (solvent B). For the final system, the solvent module was programmed to deliver the following gradient (see also Fig. 1):

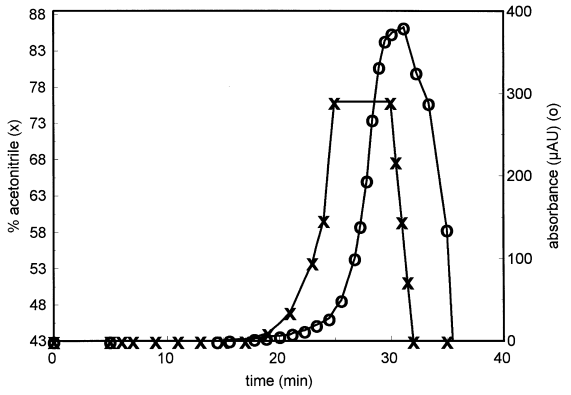

Fig. 1. Final gradient program for the separation of the anabolics on a Superspher RP-Select B column. The line with the crosses shows the pump program and the line with the dots gives the detector signal measured at 190 nm.

0–5 min: 95% A and 5% B (43% acetonitrile in water (v/v))
5–25 min: gradient from 95% A to 40% A with curve 6
25–30 min: 40% A and 60% B (76% acetonitrile in water (v/v))
30–32 min: linear gradient from 40% A to 95% A (curve 0)
32–45 min: restabilise at 95% A and 5% B (43% acetonitrile in water (v/v)).

The DAD-detector was programmed to collect data for 35 min from the start of the run. An autozero scaling was performed at the start of each new run. The scan range was 190–400 nm. Data were collected at a rate of 2 Hz. Readings were performed at 192, 230, 242, 280, or 350 nm (bandwidth 4 nm) depending on the substance studied. For routine operation, the software can be programmed to collect data at those five wavelengths (multichromatogram mode). Spectra were saved for detected peaks in this mode. Detection wavelengths for the quantitation of substances were:

192 nm: E2, EE2, E1
230 nm: DE, HEX, cDES, Tal, Zer, sometimes E2 (all substances can be detected at this wavelength)
242 nm: T, αNT, βNT, MT, tDES, P, MP, ClTac, ClTdiol, Zeara
280 nm: MED
350 nm: Tb

2.4. Experimental conditions

2.4.1. Development of the HPLC system

First, the retention times of 15 anabolics were determined on the Superspher RP-select B column with an isocratic mobile phase of 45% acetonitrile in water at a flow-rate of 0.8 ml/min. Standard solutions of the anabolics with concentrations of 0.05–0.1 mg/ml were injected. Chromatograms were monitored at 240 nm, or if necessary at a more convenient wavelength to detect peaks.

Next, the separations obtained with the seven possible gradient curves were determined with a test mixture containing Tb, E1, MT, Zeara, tDES, HEX,

cDES, P and MED at a concentration of about 10 μg/ml (for tDES and cDES the total concentration was 10 μg/ml). The gradient program used in this experiment was 5 min isocratic 40% acetonitrile in water, in 20 min to 67% acetonitrile in water using different gradient curves, 5 min isocratic 67% acetonitrile in water, in 2 min linear gradient back to 40% acetonitrile in water, and finally, 13 min restabilisation. Chromatograms were monitored at 230 nm to detect peaks. Data collection was stopped at 35 min. Gradient curve 6 was selected as the one best suitable for the separation of the anabolics. The percentages of acetonitrile were changed to 43% acetonitrile in water as initial composition and 76% acetonitrile in water as final composition to optimise the retention times of the substances. Other HPLC conditions and detector parameters are described in Section 2.3.

With the final system calibration standards in acetonitrile in the range of 0.25–10 μg/ml or 5–200 ng injected amount were analysed. The peak heights obtained at the specific wavelengths were used to construct calibration curves. The LODs were calculated at three times the noise at the specific wavelength used for those calibration curves.

2.4.2. Sample work-up procedure

The pH of a 5-ml urine sample was adjusted to 5.2 with 4 *M* hydrochloric acid. Then 1 ml 2 *M* acetate buffer pH 5.2 and 20 μl Suc d'Helix Pomatia (Sepracor/Biosepra SA, Villeneuve-la-Garenne, France) were added and the mixture was incubated overnight at 37°C. Thereafter, the sample was centrifuged for 12 min at 4000 rpm to remove particles that could block the SPE column. The C18 column (Merck) was conditioned consecutively with 2 ml methanol and 2 ml water using slight vacuum (<5 in Hg), followed by the application of the hydrolysed and centrifuged sample. Then the column was washed with 2 ml 55% methanol in water under slight vacuum. After drying for 5 min under full vacuum the steroids were eluted with 3 ml 95% acetone in water under slight vacuum. The neutral alumina column (Merck) was conditioned consecutively with 5 ml hexane and 5 ml acetone. The extract of the C18 column was then applied to the column and the run-through was collected. The column was dried briefly under vacuum and was then eluted with 2 ml 95% acetone in water. For this part of the procedure no vacuum was needed except for drying. The run-through and the extract were combined and were evaporated to dryness under nitrogen at 37°C. The residue was redissolved in 200 μl acetonitrile and 20 μl was injected into the HPLC system described above. The time needed to carry out the dual extraction is about 1 h and 10 samples can be handled at the same time. Further details on the procedure are described elsewhere [17].

2.4.3. Calculations

Chromatographic characteristics were calculated by the Gold Nouveau Chromatography Data System. Plate numbers (N) and resolutions (Rs) to the previous peak were calculated according to the German Pharmacopoeia (DAB) method of the software, which uses the Eqs. (1) and (2) [18]:

$$N = 5.54*(t_R/w_{0.5})^2 \quad (1)$$

$$Rs = 1.18*(t_{R2} - t_{R1})/(w_{0.5,1} + w_{0.5,2}) \quad (2)$$

where t_R is the retention time and $w_{0.5}$ is the peak width at half maximum for two neighbouring peaks, 1 and 2, respectively.

The selectivity factor (α) for two neighbouring peaks, 1 and 2, respectively was calculated according to Eq. (3) [18]:

$$\alpha = k'_2/k'_1 \quad (3)$$

3. Results and discussion

3.1. Development of the HPLC system

Preliminary experiments with a limited number of compounds and isocratic elution were performed to select the column and the modifier that were most suitable for our purpose. Three columns were tried: Hypersil ODS, Superspher RP-Select B and Superspher RP-18. As modifier, acetonitrile and methanol were investigated. When methanol was used with the Superspher columns very high backpressures were observed. Also, acetonitrile gave a slightly better separation on the Hypersil column. Therefore, further experiments were performed with acetonitrile as modifier. As expected, retention times

(t_Rs) on the Superspher columns were longer than on the Hypersil column, but the peaks remained sharp and high theoretical plate number were obtained. The separation of the test compounds was better on the RP-Select B column than on the RP-18 column. The optimum system in these preliminary experiments was found to be a Superspher RP-Select B column with an isocratic mobile phase of 45% acetonitrile in water.

Next, the t_Rs of 15 anabolics were determined with the optimum isocratic HPLC system described above. Only those 15 could be tested as the other compounds became available at a later stage. The results are summarised in Table 1. The first compound, Tb, eluted at a t_R of 10.7 min, which would avoid interference by polar matrix components likely to be found in extracts of urine samples. Peak shapes usually remained acceptable for the later eluting peaks, which is reflected in the theoretical plate numbers. Yet, the Stan peak was rather broad, resulting in a low plate number. This experiment was done to select compounds for a test set for the development of the gradient, which should include the various structures among the anabolics. Also, the earliest and latest eluting substances (Tb and MED) were selected. tDES and HEX were included, because they eluted very near to each other. In view of the above considerations, the test set consisted of Tb, E1, MT, Zeara, tDES, HEX, cDES, P and MED. The criteria used for the development of the gradient were:

- The first compound should elute around 10 min from the start to minimise interference by polar components in extracts of urine samples.
- The anabolics should all elute within 35 min to keep the analysis time acceptable.
- The compounds should be spread as evenly as possible over the available range to minimise interference among analytes.

A test gradient from 40 to 67% acetonitrile in water was used and the seven gradient curves that can be produced by the Beckman software were all tested. A concave curve (curve 6 of the software) resulted in a nice spread of the test compounds and left room for the substances that were not included in the test set. Yet, tDES and HEX were not separated with any of the gradients used. As MED did not elute within 35 min with the test gradient, the percentage of acetonitrile at the end of the gradient had to be increased to 76%. Also, the percentage at the start was increased slightly to 43% to make the earliest compound elute around 10 min. This resulted in the final gradient depicted in Fig. 1. The t_Rs and chromatographic characteristics found for the anabolics tested are given in Table 2 and a representative chromatogram is shown in Fig. 2. The t_Rs of the anabolics are spread quite evenly over the range of 10–35 min, but some peaks show overlap. In these cases the UV spectra of the compounds can be used to differentiate between substances. Only in the case of αNT and βNT the peaks coincide and the UV spectra are identical. However, this is not a problem as αNT is a metabolite of βNT [19].

The linear range (Table 2) was tested with calibration standards between 0.25 and 10 μg/ml or 5–200 ng injected amount. For E2, EE2, E1 and ClTac, no peak was observed with the 0.25 μg/ml standard. Stan was found to have only weak UV absorption and was very difficult to detect. LODs (Table 2) were calculated for the anabolics at their specific detection wavelengths. For all anabolics

Table 1
Retention times and chromatographic characteristics of 15 anabolics in the isocratic HPLC system[a]

Anabolic	t_R (min)	k	N	Rs	α
t_0	1.4				
Tb	10.7	6.5	7000		
βNT	11.7	7.3	6000	1.84	1.11
T	14.2	9.0	6000	3.66	1.24
EE2	15.3	9.8	5000	1.43	1.09
E1	16.3	10.5	5000	1.04	1.07
MT	17.4	11.3	6000	1.32	1.08
Zeara	19.2	12.5	5000	1.77	1.11
DE	21.6	14.3	4000	1.97	1.14
tDES	22.9	15.2	4000	0.92	1.06
HEX	23.5	15.6	5000	0.42	1.03
cDES	30.1	20.2	4000	4.10	1.30
P	37.6	25.5	8000	4.27	1.26
MP	44.9	30.7	7000	3.71	1.20
Stan	50.8	34.9	2000	1.81	1.14
MED	66.8	46.2	9000	4.48	1.32

[a] The substances were separated on a Superspher RP-Select B column with an mobile phase of 45% acetonitrile in water at a flow-rate of 0.8 ml/min.

Table 2
Retention times and chromatographic characteristics of 21 anabolics in the final gradient HPLC system[a,b]

Anabolic	No	t_R (min)	k	Rs	α	Linear range (μg/ml)	LOD (ng)
t_0		1.4					
Tal	1	10.0	6.0				2
Tb	2	12.9	8.1	5.73	1.33	0.25–10	4
Zer	3	13.8	8.7	1.64	1.08		2
βNT	4	14.8	9.4	1.63	1.08	0.25–10	1
αNT	5	14.9	9.5	0.26	1.01		2
E2	6	15.7	10.0	1.28	1.05	0.5–10	8
EE2	7	18.1	11.7	3.48	1.17	0.5–10	9
T	8	18.2	11.8	0.11	1.01	0.25–10	1
E1	9	19.5	12.7	1.79	1.07	0.5–10	7
Zeara	10	22.8	15.0	3.96	1.19	0.25–10	1
MT	11	23.2	15.3	0.49	1.02	0.25–10	2
DE	12	26.4	17.5	4.83	1.15	0.25–10	3
tDES	13	27.2	18.1	1.53	1.03	0.2–7.5	0.5
HEX	14	27.3	18.2	0.43	1.01	0.25–10	0.5
ClTdiol	15	28.0	18.7	2.16	1.03	0.25–10	1
cDES	16	28.8	19.2	2.94	1.03	0.15–3	0.5
P	17	30.6	20.5	8.45	1.07	0.25–10	0.5
MP	18	31.2	20.9	2.35	1.02	0.25–10	1
Stan		31.8	21.3	2.04	1.02		>600
MED	19	33.5	22.5	4.81	1.06	0.25–10	0.5
ClTac	20	33.7	22.7	0.80	1.01	0.5–10	3

[a] The numbers in column 2 correspond with the peak numbers in Fig. 2.
[b] Conditions were as described under Section 2.3.

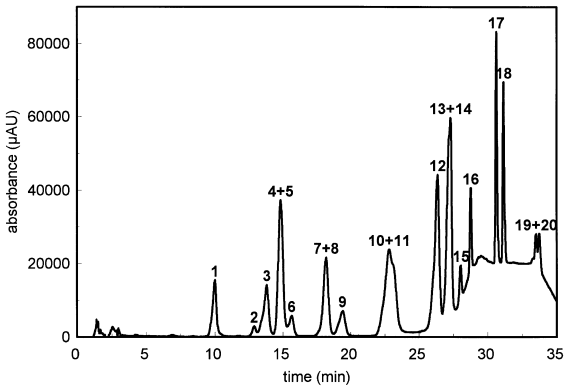

Fig. 2. Chromatogram of a standard solution containing 20 anabolics (200 ng injected amount for each substance) using the final gradient elution program and recorded at 230 nm. The numbers refer to the anabolics as given in Table 2. Conditions were as described under 'HPLC-equipment'.

except the oestrogens, the LODs were below 5 ng injected amount. The oestrogens had slightly higher LODs of 5–10 ng injected amount.

From Tables 1 and 2 it can be seen that EE2 and T, as well as Zeara and MT switched positions in the final gradient as compared to the results in the isocratic system. The t_Rs of the oestrogens, stilbenes and resorcylic acid lactones were found to vary more than the t_Rs of the other substances. This is most likely due to the more polar character of the former compounds caused by the presence of a phenolic hydroxy group. Their retention behaviour will be more influenced by slight changes in the composition of the mobile phase. A possible solution for this problem would be to add a buffer to the mobile phase. However, the use of corrected retention times (t_R^cs) made the use of a buffer unnecessary (see below). As the addition of a buffer to the mobile phase presents other problems, like crystallisation with increasing amounts of acetonitrile and the

necessity of thorough washing, the use of t_R^cs was preferred.

3.2. Identification of compounds

The retention behaviour and the UV spectrum are the primary tools in the identification of the anabolics. Although neither parameter on its own provides adequate selectivity, the combined information from the retention behaviour and the UV spectrum may provide enough power to allow unambiguous identification [20,21].

The t_Rs observed during our experiments were fairly stable, but variations occur due to small changes in mobile phase composition and room temperature. Therefore, when the retention behaviour is to be used for identification of compounds, a correction must be made to minimise variation [20].

It was decided to use a corrected retention time (t_R^c) for this purpose. This correction is made similar to the corrected h*R*f values used in the standard TLC systems for toxicological samples [22]. A calibration is made using a mixture of six reference substances, of which the mean t_R^c is accurately known from repeated analyses. The substances selected as reference substances were Tal, T, MT, tDES, cDES and MED. The t_Rs found for these references in an experiment just before or just after the analysis of an unknown specimen are plotted against their mean t_R^c to obtain a calibration graph (see Fig. 3), which can be used for the correction of peaks observed in samples by interpolation. The t_R^c of an unknown peak can also be calculated using the following Eq. (4)

$$t_R^c(X) = t_R^c(A) + (t_R^c(B) - t_R^c(A))/(t_R(B) - t_R(A))*(t_R(X) - t_R(A)) \quad (4)$$

where *A* and *B* are the bracketing reference substances around unknown substance *X*. This procedure reduced the standard deviations of the retention parameter to 7–60% (average 26%) of its original value (see Table 3). It can also be seen from Table 3 that the t_Rs of the oestrogens, stilbenes and resorcylic acid lactones varied more between experiments than the t_Rs of the androgens and the progestagens, as was mentioned above. The use of t_R^cs reduced the standard deviations of all compounds to 0.10 min or less.

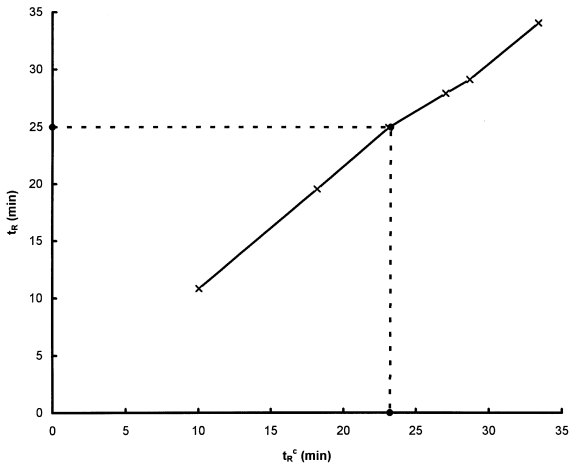

Fig. 3. Example of a calibration graph for the correction of retention times obtained with the HPLC system. t_R stands for the retention time actually measured in the experiment. t_R^c stands for the corrected retention time, which is the mean of a great number of observations done in the past. The dashed line indicates how the t_R^c for an unknown peak with an t_R of 25 is being calculated.

Although they are still slightly higher for the oestrogenic compounds than for the androgens and progestagens, the value of 0.10 min is acceptable for calculations of similarity indices. Also, by using t_R^cs the standard deviation has become more or less constant over the whole t_R range studied.

The Beckman Gold Nouveau software provided the possibility to build a library of UV spectra and an algorithm is build into it to retrieve spectra and to calculate a similarity index (SI). However, the software cannot subtract a baseline file containing DAD data to correct for absorbances caused by the solvent used to make the gradient. As the acetonitrile used to make the gradient had a significant UV absorbance between 190 and 230 nm, this seriously hampered the recognition of UV spectra of compounds that eluted in the gradient part of the chromatogram (especially after 25 min). A procedure was developed to overcome this problem. The spectrum was exported as ASCII files and baseline subtraction could be performed manually in a spreadsheet program or by a home-made software program. A standard blank spectrum taken at a t_R of 31 min can be used for subtraction, which can be corrected for the maximum absorption observed in the data-file

Table 3
Retention times and corrected retention times of 20 anabolics[b,c,d]

Anabolic	t_R (min)		t_R^c (min)	
	Avg	SD	Avg	SD
Tal[a]	10.04	0.11	10.06	0.02
Tb	12.86	0.14	12.88	0.02
Zer	13.85	0.20	13.87	0.04
βNT	14.75	0.15	14.76	0.03
αNT	14.97	0.16	14.99	0.02
E2	15.57	0.21	15.59	0.06
EE2	18.02	0.27	18.04	0.10
T[a]	18.17	0.20	18.19	0.01
E1	19.33	0.30	19.35	0.08
Zeara	22.67	0.35	22.69	0.09
MT[a]	23.13	0.26	23.17	0.07
DE	26.24	0.28	26.27	0.08
tDES[a]	27.06	0.19	27.08	0.03
HEX	27.24	0.18	27.26	0.04
ClTdiol	27.98	0.12	27.98	0.03
cDES[a]	28.73	0.08	28.72	0.05
P	30.58	0.08	30.58	0.02
MP	31.09	0.08	31.10	0.02
MED[a]	33.42	0.10	33.42	0.03
ClTac	33.64	0.09	33.66	0.04

[a] Indicates the substances used as references.
[b] Averages (Avg) and standard deviations (SD) were calculated for both retention time (t_R) and corrected retention time (t_R^c) ($n=4$).
[c] Conditions were as described under Section 2.3.
[d] The reference mixture and the analytes were analysed separately on the same day.

at the same t_R. For compounds eluting in the steep part of the gradient a percentage of the standard blank spectrum should be subtracted. The percentage to be used was calculated from the t_R of the substance (see Fig. 1). The resulting corrected spectra could be compared to corrected reference spectra, thus allowing a SI to be calculated. A more detailed discussion on the calculation of the SI for both UV spectrum and t_R can be found elsewhere [20,23,24].

The SI calculated from the UV spectrum was then combined with the SI calculated from the t_R. The program used for these calculations has been designed in such a way that it produces a list of candidates for the unknown substance encountered, in decreasing order of similarity. The substance with the highest combined SI is the most likely candidate. In the event that two or more substances are listed at the top with little difference between their SIs, data obtained from other analytical methods, e.g. TLC, GC or MS may be introduced to provide further differentiation between the top candidates to the extent that only a single candidate remains. On the other hand, if the computer finds only one candidate for the t_R and UV spectrum of the unknown and the SI for the combined findings is sufficiently high, the unknown can be considered unequivocally identified, since no other substance than the one candidate found matches the data for the unknown [20].

Comparison of the full spectrum of the unknown with the full spectrum in the library is to be preferred as this provides better identification power. However, at low analyte concentrations, the spectra obtained may become less suitable for identifications due to noise. As an alternative, peak percentages can be calculated at the five detection wavelengths. This is calculated as the percentage of the height at that wavelength to the height at the specific detection wavelength. Reference values obtained in our experiments are given in Table 4. The peak ratios can be used instead of the whole UV spectrum for the calculation of the SI. As in this case the identification power of this SI is lower than that of the SI obtained with the whole UV spectrum, it should be given less weight in the calculation of the final SI.

3.3. Potentials of the developed HPLC–DAD system

The HPLC–DAD system described here can be used for the analysis of extracts of urine samples from calf taken during fattening of the animals for the presence of residues of illegal anabolics. An example of a chromatogram obtained after extraction of a blank and spiked calf urine sample is given in Fig. 4. The samples were extracted according to a combined C18-alumina SPE procedure which is described elsewhere [17]. Detected substances can be identified on the basis of their corrected retention parameter and their UV spectrum. If unequivocal identification cannot be obtained on the basis of the observed retention parameter and the UV spectrum the latter may be combined with data from other analytical methods, e.g. TLC or GC–MS [20].

Other possible uses include the analysis of seized preparations of illegal growth promoters. For the analysis of dosage forms simple extraction methods are sufficient. Tablets can be pulverised and ex-

Table 4
Peak percentages for 20 anabolics at different wavelengths[a,b,c]

Anabolic	Percentage					LOD				
	192	230	242	280	350	192	230	242	280	350
Tal[d]	190	100	42	33	0	[d]	[d]	[d]	[d]	[d]
Tb	37[e]	17[e]	20	13	100		12	12	12	5
Zer[d]	160	100	37	36	0	[d]	[d]	[d]	[d]	[d]
βNT	18[e]	63	100	2	0	95	5	5	48	
αNT[d]	19	63	100	2	0	[d]	[d]	[d]	[d]	[d]
E2	830	100	8	48[e]	0	9	5		46	
EE2	100	13	2	6	0	11	22		22	
T	18[e]	63	100	2[e]	0	51	13	13		
E1	100	13	1	6	0	5			11	
Zeara	66[e]	103	100	47	0	12	10	10	10	
MT	19	64	100	2	0	94	5	5	94	
DE	220	100	75	15[e]	0	27	5	5	5	
tDES	380	91	100	33	0	19	4	4	4	
HEX	390	100	7	18	0	13	5	26	5	
ClTdiol	41	36[e]	100	25	0		12	5	12	
cDES	407	100	82	61	0	15	2	4	4	
P	32	71	100	1	0		6	6		
MP	36[e]	74	100	2	0	13	5	5	20	
MED[f]	29	13	16	100	1				5	
ClTac[f]	39	38	100	22	0					

[a] Empty boxes mean that the practical detection limit is larger than 200 ng or that there is interference by urine components.
[b] 'LOD' represents the practical detection limits (ng) in extracts from calf urine at the different wavelengths.
[c] Under 'percentage' the percentage of the height at that wavelength to the height at the specific detection wavelength of the anabolic is given.
[d] Not performed.
[e] Ratio may be affected in urine samples due to interference.
[f] Detection at 192, 230 and 242 nm in urine samples is hampered by a co-eluting interference [21].

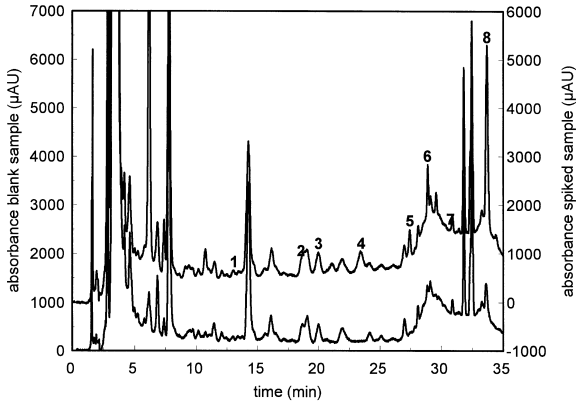

Fig. 4. Chromatograms obtained with blank (lower case) and spiked (upper case) calf urine recorded at 280 nm. The sample was spiked at the 25 ng/ml level with Tb (1), T (2), E1 (3), Zeara (4), tDES (5), cDES (6), P (7) and MED (8). Not all peaks are visible at this wavelength. Conditions were as described in Section 2.4.

tracted with chloroform [25] or methanol [10]. Oily preparations for injection can be extracted with methanol [10,11] followed by alkaline hydrolysis of the esters with potassium hydroxide [25]. Aqueous suspension and emulsions can be diluted with methanol [10,11] and implants can be extracted with methanol [11] before analysis. Hydrolysis of esters of the steroids is necessary because some may not elute from the HPLC column within 35 min using the present gradient. Alternatively, the gradient may be adapted to allow analysis of intact esters by increasing the final percentage of acetonitrile to 100% [10,11].

Several other multi-residue HPLC systems have been developed for the analysis of anabolics. Most of these used reversed-phase columns [10–12,26,27], but separations on two normal-phase columns have been reported as well [13]. In some of those studies only a limited number of compounds were used

[13,26,27]. However, these methods were not intended for the analysis of urine samples [10–12,27] or they were intended as sample pre-treatment methods prior to immunological detection [26]. Three methods have been reported for the analysis of multiple anabolics in illegal preparations [10–12]. Of those three studies only the last one may potentially be useful for the analysis of urine samples, as the first analyte elutes at 7 min. With the other two systems, the first compound elutes at a retention time of 1–2 min where polar matrix components will interfere with detection.

Two LC–MS methods have been reported for the analysis of anabolic steroids in extracts of human urine [15,16]. The first method consisted of on-line SPE coupled to a narrow-bore, isocratic HPLC–MS system. It was found to be suitable for the detection of up to 28 anabolic steroids and metabolites [15]. The use of an isocratic HPLC system will, however, result in very long retention times of more apolar anabolic steroids. Also, only 0.2–0.3 ml urine is injected into the system, whereas in our method the equivalent of 0.5 ml urine is injected into the HPLC [17]. The second method was specifically developed for the analysis of the conjugates of testosterone and epitestosterone [16] and is, therefore, not suitable as multi-residue method.

In conclusion, we have reported a method for the analysis and identification of 20 anabolics. Identification in urine is based on the corrected retention parameter plus the UV spectrum of the substance. Unambiguous identification of the 20 anabolics tested is possible, when the amounts present are sufficiently high. The detection limits of the HPLC–DAD system are all below 10 ng injected.

Acknowledgements

This research was sponsored by the European Union with AIR-grant no AIR3-CT94-1511. We want to thank Dr R.W. Stephany and Dr L.A. van Ginkel from RIVM, Bilthoven for providing us with reference standards of several anabolics. We also acknowledge G. Wieland from Merck KGaA, Darmstadt, for providing us with Superspher columns.

References

[1] P.J. Buttery, J.M. Dawson, Proc. Nutr. Soc. 49 (1990) 459–466.
[2] P. Schmidely, Ann. Zootech. 42 (1993) 333–359.
[3] D.B. Gower, E. Houghton, A.T. Kicman, Anabolic Steroids: Metabolism, Doping and Detection in Equestrian and Human Sports, in: H.L.J. Makin, D.B. Gower, D.N. Kirk (Eds.), Steroid Analysis, 1st edition, Blackie Academic & Professional, Glasgow, 1995, pp. 468–526.
[4] G.M. Fara, G. Del Corzo, S. Bernuzzi, A. Bigatello, C. Di Pietro, S. Scaglioni, G. Chiumello, Lancet ii (1979) 295–297.
[5] C.A. Saenz de Rodriguez, A.M. Bongiovanni, L. Conde de Borrego, J. Pediatr. 107 (1985) 393–396.
[6] Council directive 88/146/EEC, Off. J. Eur. Commun. L70 (1988) 16–18
[7] C. Ayotte, D. Gouderault, A. Charlebois, J. Chromatogr. B 687 (1996) 3–25.
[8] F. André, in: N. Haagsma, A. Ruiter (Eds.), EuroResidue III Conference on Residues of Veterinary Drugs in Food, Veldhoven, The Netherlands, May 6–8, 1996, University of Utrecht, Faculty of Veterinary Medicine, Utrecht, The Netherlands, pp. 53–61
[9] M. O'Keeffe, Strategies for the detection of veterinary drug residues, in: G. Enne, H.A. Kuipers, A. Valentini (Eds.), Proceedings of the Teleconference held on Internet from April 15–August 31, 1994, Residues of Veterinary Drugs and Mycotoxins in Animal Products – New Methods for Risk Assessment and Quality Control, Wageningen Press, Wageningen, 1996, pp. 31–40.
[10] M.J. Walters, R.J. Ayers, D.J. Brown, J. AOAC 73 (1990) 904–926.
[11] J.O. de Beer, J. Chromatogr. 489 (1989) 139–155.
[12] I.S. Lurie, A.R. Sperling, R.P. Meyers, J. Forensic Sci. 39 (1994) 74–85.
[13] E.H.J.M. Jansen, L.A. van Ginkel, R.H. van den Berg, R.W. Stephany, J. Chromatogr. 580 (1992) 111–124.
[14] L.A. van Ginkel, E.H.J.M. Jansen, R.W. Stephany, P.W. Zoontjes, P.L.W.J. Schwillens, H.J. van Rossum, T. Visser, J. Chromatogr. 624 (1992) 389–401.
[15] D. Barrón, J. Barbosa, J.A. Pascual, J. Segura, J. Mass Spectrom. 31 (1996) 309–319.
[16] K.A. Bean, J.D. Henion, J. Chromatogr. B 690 (1997) 65–75.
[17] A. Koole, J.P. Franke, R.A. de Zeeuw, J. Liq. Chromatogr., submitted.
[18] Gold Nouveau Software Reference Manual, Beckman Instruments, Fullerton, CA, USA, March 1996.
[19] L.A. van Ginkel, R.W. Stephany, H.J. van Rossum, H. van Blitterswijk, P.W. Zoontjes, R.C.M. Hooijschuur, J. Zuydendorp, J. Chromatogr. 489 (1989) 95–104.
[20] J. Hartstra, Computer Aided Identification of Toxicologically relevant Substances by Means of Multiple Analytical Methods, PhD Thesis, State University of Groningen, 1997.

[21] R.A. de Zeeuw, J. Hartstra, J.P. Franke, J. Chromatogr. A 674 (1994) 3–13.
[22] R.A. de Zeeuw, J.P. Franke, F. Degel, G. Machbert, H. Schütz, J. Wijsbeek (Eds.), Thin-Layer Chromatographic Rf Values of Toxicologically Relevant Substances on Standardized Systems, 2nd edition, VCH, Weinheim, Germany, 1992, pp. 19–20.
[23] P.G.A.M. Schepers, J.P. Franke, R.A. de Zeeuw, J. Anal. Toxicol. 7 (1983) 272–278.
[24] J. Hartra, J.P. Franke, R.A. de Zeeuw, GIT Labor. Medizin. 18 (1995) 272–279.
[25] P.D. Colman, E. A'Hearn, R.W. Taylor, S.D. Le, J. Forensic Sci. 36 (1991) 1079–1088.
[26] E.H.J.M. Jansen, R. Both-Miedema, R.H. van den Berg, J. Chromatogr. 489 (1989) 57–64.
[27] Y.S. Gau, S.W. Sun, J. Liq. Chromatogr. 18 (1995) 2373–2382.