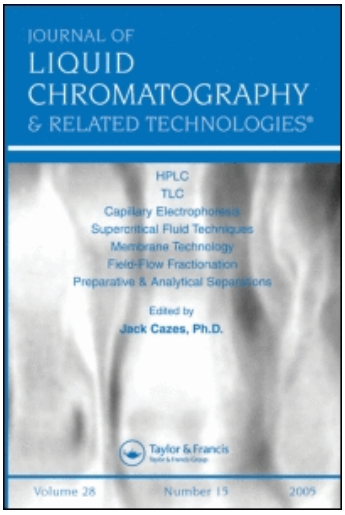

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SOLID PHASE EXTRACTION FOR MULTI-RESIDUE ANALYSIS OF ANABOLIC STEROIDS AND RELATED SUBSTANCES FROM CALF URINE USING C18 AND ALUMINA COLUMNS

A. Koole[a]; J. P. Franke[a]; R. A. de Zeeuw[a]
[a] Department of Analytical Chemistry and Toxicology, Ant. Deusinglaan 1, University Centre for Pharmacy, Groningen, The Netherlands

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A. Koole, J. P. Franke, R. A. de Zeeuw

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

ABSTRACT

A solid phase extraction method for anabolic steroids and related substances in calf urine is reported, that is suitable as a screening method for illegal growth promoters. Two types of sorbent were used: a reversed phase C18 material and a polar alumina material. After overnight enzymatic deconjugation, the 5-mL sample was first brought on the C18 column. This column was washed with 55% methanol in water and was then eluted with 95% acetone in water. The extract was directly brought on the alumina column. The run-through and an additional elution with 95% acetone in water were collected. The final extracts were analysed with an HPLC-DAD method described previously. The steroids are separated on a Superspher RP-Select B column with a gradient mobile phase consisting of acetonitrile and water. The method was found to be suitable for at least 19 illegally used anabolic steroids, having recoveries ranging from 65-110% at a spiking level of 25 ng/mL. Detection limits ranged from 0.6-80 ng injected amount or 1-160 ng/mL urine.

INTRODUCTION

Anabolic steroids and some related substances with comparable activities, all of which are here referred to as anabolic steroids, have been used as growth promoters during fattening of cattle for a long time.[1,2] This treatment may result in residues of the compound in the meat, which could be harmful for the consumer. From sports doping cases and therapeutic uses the anabolic steroids are known carcinogens and prolonged ingestion of larger doses disturb the endocrine balance, leading to a large number of side effects.[3] Although these effects are mainly expected when large doses are ingested, 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 anabolic steroids as growth promoters in cattle was banned in the European Union in 1988.[6] For the control of this ban, samples taken during fattening at the farm and after slaughter at the slaughterhouse are analysed for the presence of illegal growth promoters. Urine is the sample most often used for the analysis of the anabolic steroids.

Analytical methods for human and equine urine[3,7] and for biological samples obtained from food-producing animals[8,9] have been reviewed. Most methods employ solid phase extraction (SPE) or immuno-affinity chromatography (IAC) 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] For SPE of steroids from urine mostly reversed phase sorbents have been used, including C2[15] and C18[16-25] bonded silica. Polar sorbents, like silicagel,[20,24,26] alumina,[22,24] and aminopropyl bonded silica,[17,21,25] were used as additional clean up steps. IAC columns have been used alone[27] or in combination with C18 SPE[18] for the extraction of nortestosterone from urine. Other applications of IAC for the analysis of anabolic steroids in biological matrices have been reviewed.[28] Here, the development of a SPE method for calf urine is reported, capable of screening for a large variety of anabolic steroids used as growth promoters during fattening of cattle. Two types of sorbent were used: a reversed phase C18 material and a polar alumina material. The method was found to be suitable for at least 19 illegally used anabolic steroids belonging to the androgens, oestrogens, progestagens, resorcylic acid lactones, and stilbenes. The extracts were analysed with an HPLC-DAD method described previously.[29]

EXPERIMENTAL

Steroids and Related Substances

The anabolic steroids used as reference substances were as follows. Methyltestosterone (MT) was from Serva (Serva Feinbiochemika GmbH,

Heidelberg, Germany). Dienoestrol (DE), hexoestrol (HEX) and 17α-ethynyl oestradiol (EE2) were obtained from Sigma (Sigma Chemical Company, St Louis, USA). Medroxyprogesterone (MP) was from Upjohn (Kalamazoo, Michigan, USA). BCR reference standards of zearalenone (Zeara), zeranol (Zer), taleranol (Tal), 19-17α-nortestosterone (αNT) and 19-17 β-nortesto-sterone (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) and clostebol-diol (ClTdiol) standards were supplied by RIVM. Testosterone (T), βNT, progesterone (P), medrogestone (MED), diethylstilbestrol (tDES and cDES) and oestrone (E1) were obtained from a local wholesaler.

All stock solutions were prepared in HPLC grade acetonitrile. Calibration standards were prepared in the range of 0.25-10 μg/mL, of which 20 μL were injected, by dilution of the stock solutions with either HPLC grade or gradient grade acetonitrile. Solutions used for spiking of samples were prepared by dilution of the stock solution with gradient grade acetonitrile. The standard solution of tDES and cDES was found to contain a 72:28 mixture of trans- and cis-DES.[29] Some of the substance are sensitive to day light. The ratio of tDES to cDES changes under the influence of day light.[30,31] Standard solutions should, therefore, be kept in the dark.

Other Chemicals

Water was demineralised in house and when it was used for HPLC it was purified with a Maxima ultrapure water instrument (Elga, obtained from Salm en Kipp BV, Breukelen, The Netherlands). Methanol, acetone, and n-hexane were analytical grade (Merck KGaA, Darmstadt, Germany). Acetonitrile for HPLC (Labscan, Dublin, Ireland) was used for the preparation of stock solutions of the reference standards. Acetonitrile, gradient grade for chromatography (Merck), was used for mobile phases, for the dilution of calibrations standards and for the redissolution of samples. Ethyl acetate was analytical reagent grade (Labscan). Fuming hydrochloric acid (37%) and anhydrous sodium acetate were analytical grade from Merck. Aluminium oxide 90, active, neutral, activity I, particle size 0.063-0.200 mm (70-230 ASTM) for preparative chromatography was from Merck. Suc d'Helix Pomatia was from Sepracor/Biosepra SA (Villeneuve-la-Garenne, France)

Prepared Solutions

Concentrated hydrochloric acid (3.45 mL) was diluted with 6.55 mL demineralised water to obtain 4 M hydrochloric acid. The 2 M acetate buffer (pH 5.2) was made by dissolution of 1.64 g sodium acetate in about 7 mL

demineralised water, adjustment of the pH to 5.2 with 4 M hydrochloric acid and making up of the volume to 10 mL with demineralised water. For 55% methanol in water (v/v) and 95% acetone in water (v/v) appropriate amounts of the organic solvent and demineralised water were mixed. Solvent A of the mobile phase (40% acetonitrile in water v/v) was prepared by mixing 400 mL gradient elution grade acetonitrile with 600 mL demineralised and purified water. Solvent B was gradient grade acetonitrile. Both mobile phase solvents were degassed using vacuum and sonication prior to use.

Urine Samples

Blank calf urine samples and reference blank bovine urines (5 mL lyophilised, codes bov01-bov20) were provided by RIVM. Samples were stored at -18°C until analysis to prevent decomposition.

SPE Columns

LiChrolut® RP-18 columns for solid phase extraction with 200 mg sorbent, and Extrelut® 20 pre-packed columns for extraction of lipophilic compounds from aqueous solutions (20 mL samples) were from Merck. Neutral alumina columns were home-made using cleaned standard 3-mL polypropylene SPE columns (id 9 mm): 1.00 g of neutral alumina was dry-packed between two cleaned PTFE frits. For cleaning of the polypropylene columns the used sorbent was removed and the columns were cleaned with water and methanol. The PTFE frits were cleaned with methanol.

Equipment

A PHM 62 standard pH meter with combined pH electrode GK2501C was from Radiometer (Copenhagen, Denmark). The Megafuge 1.0 was obtained from Heraeus Sepatech GmbH (Osterode, Germany) and the vortex mixer from Wilten & Co BV (Etten-Leur, The Netherlands). The waterbath (Gebr. Haake, Berlin, Germany) was operated at 37°C. The SPE-column processing system was a Baker SPE 12-g vacuum manifold (Mallinckrodt Baker BV, Deventer, The Netherlands). A Bransonic ultrasonic cleaner model B2210-E-MT was obtained from Bransonic (Bransonic Ultrasonics Corporation, Danbury, CT, USA). Vacuum and nitrogen were available through in-house facilities.

HPLC System and Conditions

The HPLC pump was a System Gold® 126 solvent module (Beckman Instruments Inc., 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) 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, 250x4 mm (Merck), protected by a LiChroCART® 4-4 guard column with LiChrospher® 60 RP-select B material, 4x4 mm (Merck). The injector was a Rheodyne 7725i injector equipped with a 20 µL sample loop (Rheodyne, Cotati, CA, USA).

The flow was set at 0.8 mL/min. The gradient was made up from 40% acetonitrile in water (v/v) (solvent A) and gradient grade acetonitrile (solvent B). The solvent module was programmed to deliver the following gradient:

0-5 min: 95% A and 5% B (43% acetonitrile in water (v/v))
5-25 min: concave 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: restabilize at 95% A and 5% B (43% acetonitrile in water (v/v))

The DAD-detector was programmed to collect data for 35 minutes 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 steroid 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 steroids were:

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

Methods

Final Extraction Procedure

The pH of the 5-mL 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 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 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 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.

In all cases, care should be taken to prevent the columns to run dry during the conditioning, sample application and washing steps.

Validation of the Final Procedure

In the first validation experiments, drug-free calf urine samples, spiked at 5 different levels, were extracted together with a blank sample. The spiking levels were 10, 25, 50, 100, and 200 ng/mL, respectively, for which 10-50 µL of a suitable spiking solution were used. The samples were then hydrolysed, extracted and analysed according to the procedures described above. Absolute recoveries were calculated using calibration standards in the range of 0.25-10 µg/mL (5-200 ng injected amount). If necessary, a correction was made for endogenous peaks in the extract of the blank sample. Calibration curves for extracted samples were constructed from the results. These calibration curves were used for the calculation of precision and accuracy in the experiments described below.

In the second validation experiment, the repeatability was determined at a spiking level of 10 ng/mL. Four samples were spiked at the 10 ng/mL level with 20 µL of a 2.5 µg/mL solution of the respective steroid. They were then hydrolysed, extracted, and analysed according to the procedures described above, together with a blank sample. Absolute recoveries were calculated using the respective calibration standards in the range of 0.25-10 µg/mL (5-200 ng injected amount). The repeatability was calculated as the relative standard deviation of the recoveries obtained for the four spiked samples. Accuracy and precision were calculated with the calibration curves for extracted samples. The accuracy was defined as the relative difference between spiked level and level found. The precision was calculated as the relative standard deviation of the levels found for the four spiked samples.

In the third validation experiment, the repeatability and reproducibility were determined at a spiking level of 25 ng/mL. Two samples were spiked at the 25 ng/mL level with 25 μL of a 5 μg/mL solution of the respective steroid. They were then hydrolysed, extracted, and analysed according to the procedures described above, together with a blank sample. The same experiment was performed on five different days. On the last two days, a new lot of C18 SPE columns was used. Absolute recoveries were calculated using the respective calibration standards in the range of 0.25-10 μg/mL (5-200 ng injected amount). The repeatability and reproducibility were calculated from one-way ANOVAs.[32] The reproducibility was defined as the relative standard deviation of the recoveries obtained on different days. Accuracy and precision were calculated with the calibration curves for extracted samples. The accuracy was defined as the relative difference between spiked level and level found. The precision within and between days was calculated from one-way ANOVAs.[32] To assess whether the use of the new lot of SPE columns had affected the results a Mann-Whitney rank sum test was performed.[32]

Determination of False Positives and False Negatives

In the final validation experiment the number of false positives and false negatives was determined. For this experiment a reference bank of 20 blank bovine urine samples was used. For each sample in the bank, a blank and a spiked sample were analysed. Samples were spiked at the following levels using 50 μL of a suitable spiking solution: 9.2 ng/mL Tb, 4.8 ng/mL βNT, 30.4 ng/mL E1, 9.9 ng/mL Zeara, 10.7 ng/mL DE, 7.7 ng/mL tDES, 4.8 ng/mL ClTdiol, 3.1 ng/mL cDES, 3.1 ng/mL MP, 5.2 ng/mL MED. The extracts were analysed together with a standard solution, which was used to estimate the retention times of the other steroids and as an indication for the recovery of the steroids in the spiked samples. When the recovery of the spiked steroids was 100%, the peak found in the spiked sample would be equal to the peak in the standard. Chromatograms were studied for the presence of peaks at 192, 230, 242, 280, and 350 nm. The criteria for peak detection were: a) The peak had to be within a reasonable distance (± 1.5 standard deviation) from the expected retention time of the steroid, and b) The peak height had to be larger than three times the noise at that wavelength. The UV spectra of detected peaks in the spiked urine samples of calves and the young bull were exported as ASCII files and correlation coefficients between reference spectrum and spectrum in the sample were determined as a measure of the similarity between the two spectra.[33]

Calculation of Results

All results were calculated using peak heights at the specific detection wavelengths of the steroids, as given in the section 'HPLC System and Conditions' above. For all statistical tests, a significance level of 5% was used.

The limits of detection (LODs) for the 19 anabolic steroids for the HPLC-DAD system for calibration standards and for analytes extracted from calf urine were calculated using calibration standards in the range of 5-200 ng (injected amount). From the chromatograms the average noise was determined for both calibration standards and urine samples. The calculated amount at three times the noise level for calibration standards was taken as the LOD of the HPLC-DAD system. The average blank signal of four calf urine samples plus three times the noise level for urine samples or the standard deviation of the four blank signals, depending on the number of positive blanks, was used to calculate the LODs for steroids extracted from calf urine.

RESULTS AND DISCUSSION

Development of the Procedure

The development and characteristics of the HPLC-DAD system will be published elsewhere.[29] The system was originally set up for 21 anabolic steroids and related substances. However, stanozolol could not be detected at low levels due to its weak UV absorption. Also, the stanozolol peak observed was rather broad. During the first studies with urine samples it became clear that a matrix interference co-eluted with clostebol acetate. As it was not possible to get rid of this peak, clostebol acetate was not included in further studies. However, the acetate ester is not excreted as such and the metabolite ClTdiol can be determined.[34] Initial studies were performed with 16 anabolic steroids. Tal, Zer, and αNT became available in a later stadium and they were not used in all experiments.

The first step in the analytical procedure is the hydrolysis of conjugates of the steroids and their metabolites.[35,36] Suc d'Helix Pomatia was used, because it is suitable for both glucuronides and sulphates. Deconjugation for 2 hours at 50°C may be performed, but overnight hydrolysis was found to result in cleaner extracts (R.K. Vermeulen, RIVM, personal communication, 1996).

A C_{18} SPE column was used as a first clean up step. Yet, it rapidly became apparent that these columns, which are frequently used to extract drugs from human urine, had difficulties in adequately cleaning up bovine urines. The resulting extracts showed a considerable number of endogenous peaks. In order to improve the results the percentage of methanol in the wash step was optimised to get the cleanest possible extract with a good and reproducible recovery of the steroids. Also, a wash step with hexane, performed immediately after the wash with the methanol-water mixture, was evaluated. However, significant losses of the steroids were already observed with small volumes of hexane. The elution solvent and elution volume were also optimised.

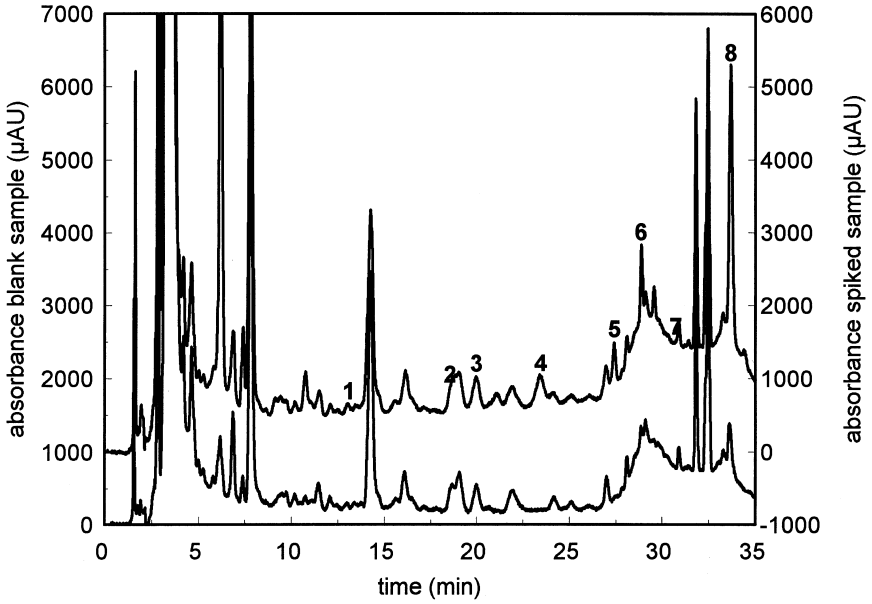

Figure 1. Chromatograms of a extracts of a representative calf urine sample obtained with the final SPE procedure recorded at 280 nm. The lower trace shows a blank and the upper trace a spiked calf urine sample. The sample was spiked at the 25 ng/mL level with Tb (1), T (2), E1 (3), Zeara (4), tDES (5), cDES (6), P (7), and MED (8). Not all spiked analytes can be detected at this level at 280 nm.

Hexane proved to be unsuitable as elution solvent, because none of the spiked substances was recovered. This can be explained from the fact, that the column is now dry and the solvent is not able to dissolve the substances. Ethyl acetate appeared to be a good elution solvent as was found before by others.[17,21] Although all the steps in the C18 SPE procedure were optimised, the extracts of the urine samples still contained a lot of interfering compounds. Therefore, an additional clean up step with an alumina SPE column was evaluated. The extract resulting from the C18 SPE step was brought on the column without evaporation. The steroids should pass through the column unretained, whereas matrix components are being retained. Neutral alumina was found to result in the cleanest extracts with a good recovery for most of the steroids in the run-through. However, Zeara was retained by the alumina when organic solvents were used for sample application and elution. In the literature a solution of 95% acetone in water was suggested as a good elution solvent for oestrogenic compounds from alumina columns.[37-39] This solvent was further evaluated. It was also found to be equally suitable as elution solvent for the C_{18} SPE column as ethyl acetate. Therefore, the ethyl acetate, which had

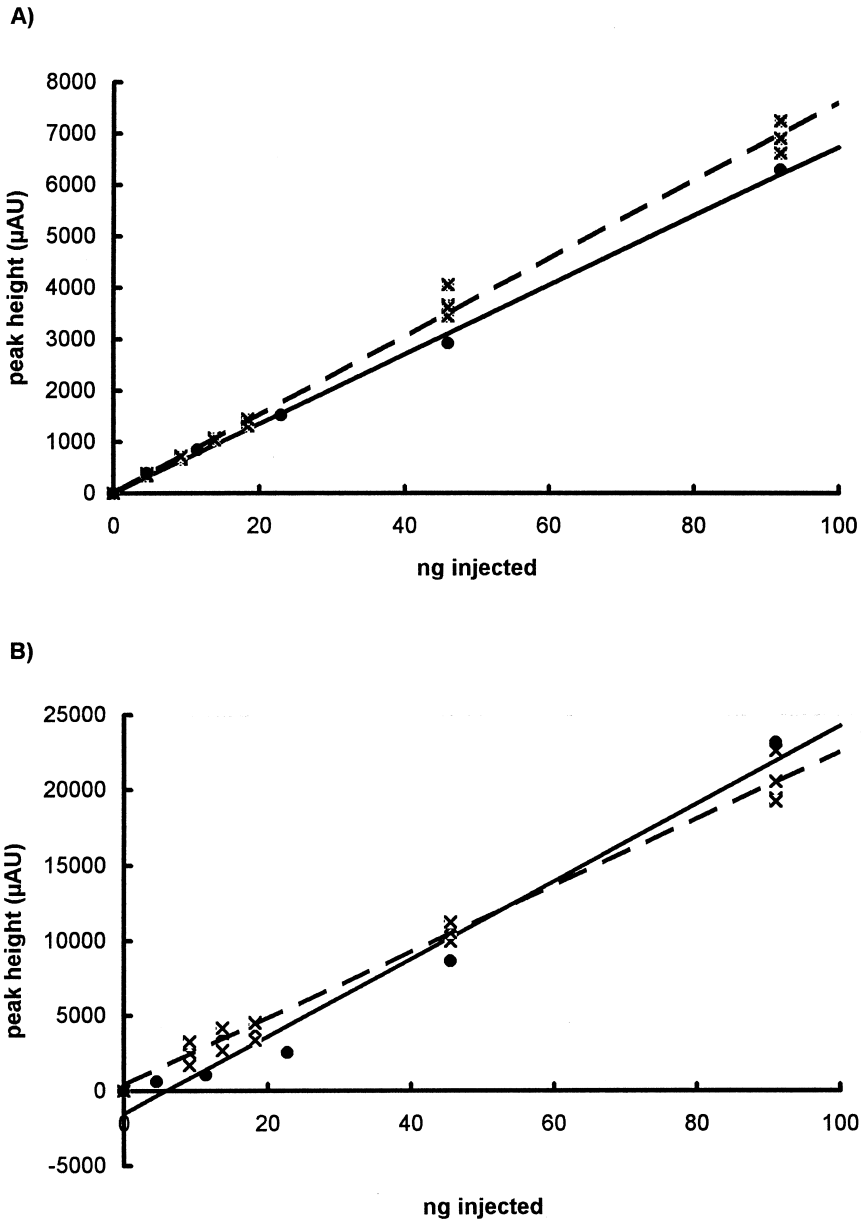

Figure 2. Chromatogram of a extracts of a representative blank cow urine sample obtained with the final SPE procedure recorded at 230 nm. Note that the scale of this sample is ten times larger than for a representative calf urine sample.

Table 1

Comparison of the Calibration Curves (y - a*x + b; y: Peak Height (μAU), x: ng Injected in Case of 100% Recovery) for the Anabolic Steroids and Related Substances Calculated for Calibration Standards and for Extracted Spiked Samples[a]

	Standards			Samples		
Steroid	**b**	**a**	**r^2**	**b**	**a**	**r^2**
Tb	37	75	0.9981	3	68	0.9981
βNT	-150	190	0.9969	-460	190	0.9906
E2	390	220	0.9871	-1500	260	0.9741
EE2	25	140	0.9983	-1320	240	0.9716
T	77	210	0.9916	-940	140	0.9781
E1	340	220	0.9961	-1700	210	0.9627
Zeara	-20	100	0.9983	-94	83	0.9897
MT	-57	120	0.9977	-160	130	0.9978
DE	460	220	0.9520	-940	160	0.9657
tDES	120	240	0.9912	-350	200	0.9878
HEX	120	290	0.9872	-880	240	0.9755
CITdiol	-31	160	0.9963	-230	160	0.9944
cDES	170	310	0.9903	-800	320	0.9780
P	110	400	0.9981	-3000	390	0.9832
MP	-230	340	0.9969	-160	330	0.9990
MED	7.5	340	0.9977	-680	300	0.9938

[a] For the calibration curve for the standards four data points per concentration were used. For the calibration curve for extracted samples one data point per spiking level was used.

previously been used as elution solvent for the C18 SPE column, was replaced by 95% acetone in water. In the end, after the collection of the run-through, the alumina column was eluted with 2 mL 95% acetone in water, which resulted in a recovery of Zeara of about 80%.

The resulting final procedure given in the methods section was further evaluated with urine samples from calves and cows. Whereas extracts of calf urine were relatively clean, extracts of cow urine were relatively dirty and many interfering peaks were observed in the chromatograms (see Figures 1 and 2).

This problem was expected as urine from adult animals is known to contain metabolites of endogenous steroids[40] and other potentially interfering substances.

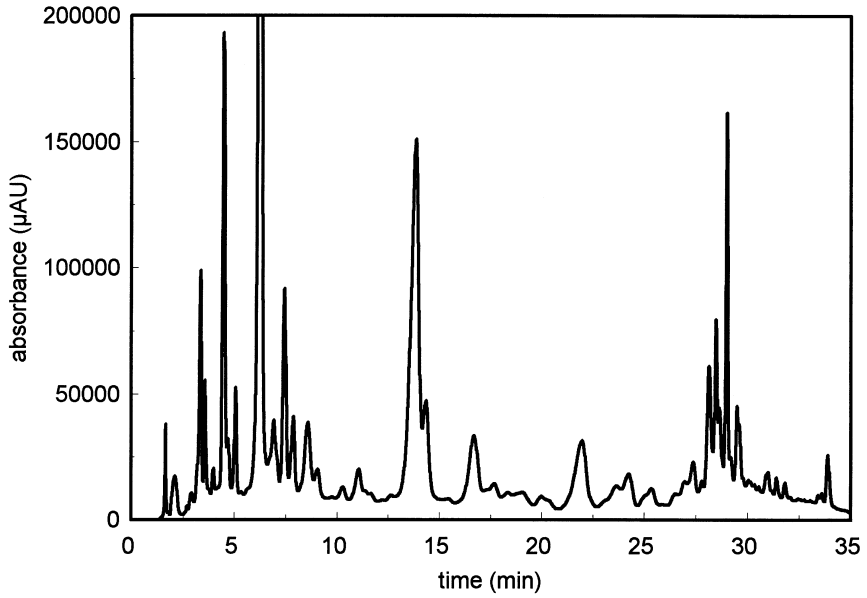

Figure 3. Calibration curves of Tb (A) and E2 (B) for calibration standards (straight line) and after extraction from urine samples (dashed line, dots indicate the signals obtained for the different samples).

Validation

Recoveries of the anabolic steroids were determined at spiking levels of 10, 25, 50, 100, and 200 ng/mL urine. Some compounds could not be detected at the 10 ng/mL spiking level either because of interference (T, cDES, P) or because this level resulted in amounts of steroid in the extract below the detection limit (EE2, E1). Interfering peaks also caused problems with the determination of the recovery of T, DE, and P at other spiking levels. For the other steroids, the recovery remained fairly constant over the concentration range studied.

The results of this experiment were used to construct calibration curves of the steroids extracted from urine samples, which were used to calculate accuracy and precision in later experiments. In Table 1 and Figure 3 the calibration curves calculated for calibration standards and for extracted spiked samples are compared. Both curves had high correlation coefficients indicating that the curves were linear. Generally, the correlation coefficient for extracted samples was somewhat lower than for the calibration standards. The constant for the calibration standards was in most cases small and was never significantly

Table 2

Repeatability of the Extraction of 16 Analbolic Steroids and Related Substances Spiked at the 10 ng/mL Level from Calf Urine*

Steroid	Recovery (%)	Repeatability (%)	Accuracy (%)	Precision (%)
Tb	117	7	20	8
βNT	105	9	41	6
E2	153	73	182	33
EE2	nd		nd	
T	42	72	61	19
E1	96	41	137	19
Zeara	91	10	-4	12
MT	130	2	46	2
DE	109	6	107	4
tDES	84	7	7	6
HEX	121	5	104	3
CITdiol	58	11	-17	7
cDES	133	51	267	20
P	128	9	158	5
MP	108	2	11	2
MED	111	12	67	10

* n = 4; nd = not detected.

different from zero. For the extracted samples, sometimes rather large, negative constants were obtained. In these cases an interference was observed in the blank samples. When the x-coefficients of both curves are compared an indication is obtained for the recovery from extracted samples. Generally, the x-values of both curves are similar, indicating near-quantitative recoveries.

Then, the repeatability was determined at the 10 ng/mL spiking level (n=4). The results are summarized in Table 2. As this spiking level is below the detection limit of the oestrogenic compounds (E2, EE2, and E2), they were either not detected or unrealistic recoveries with very large standard deviations were observed. In this urine sample, matrix compounds interfered with the detection of T, MT, cDES, and P resulting in too large or too small recoveries and/or large standard deviations. Accuracy and precision were calculated using the calibration curves for steroids extracted from urine samples. For the steroids for which interferences caused problems in one or both of the experiments large differences from the spiked amounts were obtained as reflected by large deviations under accuracy. For HEX, ClTdiol, and MED differences in the recovery obtained in the two experiments explained the large difference found under accuracy.

Table 3

Repeatability and Reproducibility of the Extraction of 16 Anabolic Steroids and Related Substances Spiked at the 25 ng/mL Level from Calf Urine[a]

Steroid	Rec	CV1	CV2	Acc	P1	P2
Tb	93	4	19	4	4	20
βNT	88	16	3	1	13	10
E2	64	98	199	8	48	105
EE2	30	132	371	0.3	32	75
T	65	35	38	5	40	71
E1[b]	94	34	17	81	21	12
Zeara	73	8	44	-3	8	34
MT	102	10	11	2	9	9
DE	70	8	21	6	5	17
tDES	73	6	32	-13	5	26
HEX	101	14	15	24	12	15
CITdiol	109	14	23	6	14	22
cDES	92	9	25	59	6	19
P	90	2	19	53	2	12
MP	93	8	13	-2	8	4
MED	79	3	15	12	3	14

[a] Duplicate analyses were performed on five different days. Rec is the absolute recovery (%), CV1 the repeatability (%), CV2 the repeatability (%), Acc is the accuracy (%), P1 the precision within-days (%), and P2 the precision between-days (%). [b] Data for day 5 were excluded because an exceptionally high value was observed in the blank sample.

Repeatability and precision were in most cases similar. However, for T, cDES, and P the precision was much better. In these cases, where interference by matrix components made determination of the recovery difficult, the use of a calibration curve for the steroid extracted from urine was very useful.

In the third experiment, the repeatability and reproducibility were determined at the 25 ng/mL spiking level (n=2 on 5 different days). The results are summarized in Table 3. This spiking level is near the detection limit of the oestrogenic compounds (E2, EE2, and E1) and an interfering peak was observed around the retention time of T and EE2. This resulted in low recoveries and large standard deviations. For E1 the data of day 5 were excluded, because a very large interference peak was observed in the blank, which was larger than the peaks observed in the spiked samples. The exact cause of this is unknown, but it was observed in some other experiments. It seemed to be related to the amount of water that was left in the extract. The repeatability was in most cases similar to that found with the samples spiked at the 10 ng/mL level. The day-to-

day reproducibility was in most cases somewhat worse. The accuracy was generally better at the 25 ng/mL level than at the 10 ng/mL. For E2, T, and EE2 the accuracy was very good, despite the fact that the recovery was low. Here again the use of a calibration curve for the steroid extracted from urine was very useful. The large value under accuracy found for E1 can be explained from the fact that the spiking level is near the detection limit. For P and cDES, also, a large deviation was found under accuracy. This can be explained by the presence of the matrix peak in the sample used for the construction of the calibration curve extracted from urine. As observed for the 10 ng/mL level, the precision values were similar to those for the repeatability and the reproducibility. The large standard deviations found for T are caused by the matrix interference.

For the reproducibility experiment at the 25 ng/mL spiking level two different lot numbers of C18 SPE columns were used. Mann-Whitney tests were performed to assess whether there were significant differences between the recoveries obtained with the two lots. This was the case for E2, Zeara, and ClTdiol, where the recoveries of day 1-3 were significantly higher, and for EE2 and MP, where the recoveries of day 4 and 5 were significantly higher. The results for E2 and EE2 can be explained by negative recoveries found for several samples due to the interfering matrix peak. For ClTdiol, the results on day 1, 2, 3, and 5 were similar and those on day 4 were worse for unknown reasons. For MP and Zeara, the difference between the two datasets appears to be real, but the effect of the different lots on the two steroids is different. No explanation for this behaviour was found. Since the above findings indicate that lot-to-lot variabilities may occur for at least some steroids, it is recommended that recoveries be checked before a new lot number of SPE columns is used for routine samples.

Determination of False Positives and False Negatives

The number of false positives and false negatives was determined with 20 reference blank bovine urine samples. This bank contained representative blank urine samples from various kinds of bovine animals. The samples were subdivided in four groups: cows (n=9), bulls (n=4), calves (n=4), young animals (n=3). The calves were all younger than 6 months. The category young animals consisted of samples of a young cow, a young bull, and a heifer, all aged between 6 and 12 months (L.A. van Ginkel, RIVM, personal communication, 1997). Although it was determined in earlier experiments that the method was not very suitable for the analysis of cow urine, it was decided to analyse all 20 blank samples to see to what extent the method may be useful in the analysis of adult animals.

False positives were determined with blank urine samples. The chromatograms were checked for the presence of peaks around the expected retention times of the steroids. The criteria for peak detection were: a) The

Table 4

Number of False Positives and False Negatives for the Anabolic Steroids and Related Substances in Calf Urine Samples[a]

Steroids	False Positives	False Negatives
Tal	3	
Tb[b]	0	4 (0)
Zer	2	
βNT	0	3
αNT	0	
E2	0	
EE2	2	
T	4	
E1	0	0
Zeara	0	2
MT	1	
DE	4	0
tDES	1	0
HEX	0	
CITdiol	0	1
cDES	3	0
P	3	
MP	0	0
MED	4	0

[a] n = 4. False positives were determined in blank urine samples and false negatives for 10 steroids were determined with blank urine samples spiked around their respective detection limits. [b] Tb was spiked below the detection limit, but a peak was visible at the proper retention time in all samples as indicated between brackets.

peak had to be within a reasonable (within 1.5 standard deviation) distance from the expected retention time of the steroid, and b) The peak height had to be larger than three times the noise. False negatives were determined for 10 steroids spiked in the samples around their respective detection limits. The results of these experiments with calf urine samples are summarized in Table 4. Only the results at the specific detection wavelength of the steroids are given here. Using the criteria given above peaks of endogenous steroids detected in blank urine samples will be marked as false positives. In these cases normally an action limit is used, above which a sample is marked as positive. As it was intended to combine the information of the retention time and the UV spectrum,

we wanted to inspect all UV spectra of detected compounds and not only of the compounds that exceeded the action limits. Therefore, it was decided to use the criteria given above instead of action limits.

Calf urine resulted in clean extracts (see Figure 1). False positives were observed for several steroids, but generally the peaks were only small. The peak that co-eluted with Tal was always smaller than 2 mAU and it had a different UV spectrum with a broad maximum around 250 nm. Around the retention time of Zer two larger peaks eluted. The first had a UV spectrum that was recognized as originating from an oestrogen. The second peak had a maximum at around 255 nm, but no identity could be proposed as yet. The presence of those peaks made detection and recognition of the UV spectrum of Zer rather difficult. Around the retention times of T and EE2 a peak with maxima of 207 and 255 nm was observed. In two calf urine samples, the UV spectrum of the peak eluting at this retention time was recognized as that of T. A peak was present around the retention time of E1 with a maximum of 265-270 nm. This peak was quite large, but E1 could be detected at 192 nm although recognition of the spectrum was a problem. In one calf urine sample a large interfering peak was observed around the retention time of MT. This peak had maxima at 243 and 287 nm. The peak eluting around the retention time of DE was always smaller than 2 mAU. From 26 min on, the spectra of interferences generally showed maxima around 250 and 285 nm, which were always smaller than 3 mAU.

In calf urine samples, the number of false negatives was limited. With regard to Tb, it later became clear that this compound had been spiked below the detection limit. However, a small peak was visible at the proper retention time in all calf urine samples. Similarly, βNT and Zeara were detected in all four calf urine samples, but only in some cases was the peak larger than three times the noise level.

The number of false positives and false negatives found in adult urine samples was quite large. In cow urine, more than 5 samples had peaks at the retention times of nearly all steroids except Tb, βNT and E2. For bull urine the situation was slightly better. No false positives were found for Tb, βNT, αNT, E2, and HEX and only one sample contained a peak with the same retention time as E1 and P. In the category of young animals, the samples of the young cow and the heifer showed a peak pattern similar to that of cow urine with many false positives. Only for Tb, E2, EE2, and E1 were no false positives observed. The urine of the young bull was rather clean with false positives only for Tal, Zer, T, P, and MED. This pattern is similar to that observed for calf urine. Due to the large number of false positives in the urine samples of adult animals, the number of detected false negatives was small. However, βNT showed only a limited number of false positives, yet it was not detected in 16 of the 20 spiked samples. Only in 5 of these negative samples a peak was detected that was smaller than three times the noise level.

UV spectra were used for the recognition of steroids. For the spiked calf urine samples and the spiked urine sample of the young bull, the UV spectra were compared to library spectra and they were visually inspected. As the steroids were spiked around their respective detection limits, spectra were distorted due to noise. Therefore, the correlations between reference spectrum and spectrum in the sample were lower (in almost all cases $r < 0.8$). However, visual inspection of the spectra showed that expected peak maxima were present in most cases. In some spectra, other UV maxima were observed caused by matrix components that eluted around the retention time of the steroid. Usually, the interfering peaks were smaller than 2-3 mAU and will only give problems when low amounts of analyte (5-20 ng, depending on retention time and spectrum of the steroid) are present in the sample. In these cases recognition of the UV spectrum will always be difficult and further confirmatory analysis is necessary.

Some additional notes are to be made. The pH value of the calf urine samples was generally lower than the pH of samples from the other animals (8.3 vs 8.6, $p < 0.05$). Also, much less 4 M hydrochloric acid was needed for calf urine to adjust the pH to 5.2 (3 drops vs 14 drops for adult urines, $p < 0.001$). Therefore, calf urines are better adjusted with 0.1 M hydrochloric acid. The extracts of calf urine samples were colorless, whereas bull urine resulted in yellowish green colored extracts and cow urine in pink to purple colored extracts. There was only one exception to this rule. Sample bov11 from a cow resulted in a yellowish brown colored extract. The samples of the young cow and the heifer behaved similarly to the samples of cow urine and the extracts were colored pink. The sample of the young bull, however, was in all respects similar to the calf urine samples.

Detection Limits

The LODs for the 19 anabolic steroids and related substances for the HPLC-DAD system and for steroids extracted from calf urine were calculated using calibration standards in the range of 5-200 ng (injected amount). From the chromatograms the average noise was determined for both calibration standards and urine samples. The injected amount calculated at three times the noise level for calibration standards was taken as the LOD of the HPLC-DAD system. The average blank signal of four calf urine samples plus three times the noise level for urine samples or the standard deviation of the four blank signals depending on the number of positive blank samples was used to calculate the LODs for steroids extracted from calf urine. The results are summarized in Table 5.

For all steroids the LODs for calibration standards of the HPLC-system were below 10 ng injected amount. For 10 of the compounds this was also the case after extraction from calf urine. One of the samples contained a large interference at the retention time of MT, whereas in the other samples no matrix peak was observed. The high detection limit for E1, T, EE2, and P were also the

Table 5

Detection Limits (ng Injected) of the Anabolic Steroids and Related Substances in the HPLC-DAD System and for the Anabolic Steroids Extracted from Urine Samples

Steroid	HPLC	Urine
Tal[a]	2	20
Tb	4	10
Zer[a]	2	80
βNT	1	2
αNT[a]	2	2
E2	8	9
EE2	9	34
T	1	54
E1	7	19
Zeara	1	4
MT	2	39[b]
DE	3	9
tDES	0.5	2
HEX	0.5	0.6
CITdiol	1	2
CDES	0.5	43
P	0.5	18
MP	1	2
MED	0.5	3

[a] No extensive experiments with urine samples could be performed due to time limitations and detection limits are estimated. [b] Based on one sample with an interfering peak; other blanks were negative resulting in a detection limit of 3 ng.

result of interference. Two peaks eluting around the retention time of Zer made detection of this growth promoter difficult, but its metabolite Tal could be determined at lower levels although, here too, interference caused some problems. The LODs of the oestrogens were higher, because of the higher noise level in samples at 192 nm.

Limitations of and Potentials of the Procedure

An LOD of 10 ng injected amount is equivalent to 20 ng/mL in urine samples. Therefore, all detection limits reported here are higher than the requirement of EU legislation (0.5 ng/mL),[41] although the LODs for the HPLC-

DAD system were sufficiently low.[29] However, the method may be suitable for use during the fattening of calves, when concentrations of the substances in urine are higher. When an autosampler is used in combination with the HPLC system, the redissolution volume can be chosen somewhat smaller and the detection limits may be lower for most of the compounds. This will only be the case for the steroids, where no interference was observed. An additional IAC extraction may be included in the procedure and this could result in cleaner extracts. Due to time restraints this was not tried.

From Table 4 it becomes clear that there are no false positives for Tb in calf urine. For the urine samples of adult animals, no false positive results were obtained either. Also, only two cow urine samples gave false negatives at a spiking level just below the detection limit. The repeatability, reproducibility, accuracy and precision at a spiking level of 10 ng/mL and 25 ng/mL were good (Tables 2 and 3). Therefore, this method appears to be suitable for the quantitative analysis of Tb in all bovine urine samples. However, the detection limit, which is currently about 10-20 ng/mL depending on the noise level, is too high to meet the requirements in the EU legislation.[41] It may be possible to increase the sample volume to solve this problem. It should be noted that in adult urine samples a peak was observed at the retention time of Tb at 230 nm, but this does not interfere with the analysis at 350 nm. Yet, when UV spectra are used for identification of the substance, Tb should be separated from this interference. This may be achieved by changing the percentage of acetonitrile in the mobile phase or possibly by performing a pre-extraction with Extrelut columns, which is already included in our procedure for the analysis of the β-agonists.[42] Several preliminary experiments with Extrelut showed adequate recoveries of Tb (over 80%) and somewhat cleaner extracts of cow urine could be obtained. The pre-extraction should be performed after the deconjugation step, because conjugates of DES could not be recovered from the column. Because of time limitations, this option was not investigated further.

One method has been reported for the extraction of several androgens from human urine using on-line extraction with a C2 column followed by LC-MS analysis. This method is claimed to be suitable for 28 anabolic steroids, including several metabolites. No LODs are given, but 100 ng steroid in 0.2 mL urine could be detected.[15] Another method was described for the analysis of three sulfoconjugated androgens in equine urine using C18 SPE with HPLC-UV detection.[43] Other reported SPE methods are single residue methods[18] or use GC-MS,[16,19,20,24-26] GC-MS-MS,[21] GC-FID[17] or immuno-assays[22,23,26] for the detection of the steroids. IAC uses the more specific interaction between analyte and antibody for the extraction of the substance of interest from the matrix. However, for multi-residue analysis either a good cross reactivity of the antibody with all analytes should exist or a combination of antibodies should be used. An example of the first strategy is the use of the salbutamol antibody in a multi-residue method for the screening of the beta-agonists.

For the analysis of the anabolic steroids no single antibody is available that can extract all substances of interest. Therefore, at least six antibodies must be combined in a multi-IAC column and even then not all steroids are retained.[28]

In conclusion, the SPE approach reported here can be used as a multi-residue method for the analysis of at least 19 illegally used anabolic steroids and related substances or their metabolites in calf urine. The detection limits of these steroids are still too high to meet the requirements of the EU legislation, but the method may be suitable for use during fattening, when higher concentrations of the substances are expected. The method is suitable for the quantitative determination of Tb in urine of calves and adult animals.

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