Comparison of purification procedures for the isolation and detection of anabolic residues in faeces using gas chromatography-mass spectrometry†

The Analyst

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Received 2nd July 1998, Accepted 28th September 1998

Within several regional field laboratories and the national reference laboratory a harmonised methodology for the analysis of anabolic residues in faecal samples was developed. The method consists of a liquid–liquid and a solid-phase extraction step, followed by a high-performance liquid chromatography purification step. Using gas chromatography-mass spectrometry, currently illegally used anabolic steroids can be detected in faeces at the ppb level. Within this context acidification, followed by centrifugation under cooling, allows efficient, practical and rapid defatting of faecal samples. Furthermore, a combination of a silica and an aminopropyl solid-phase extraction column was found to give the best results as regards the sample purification process.

For regulatory control of the illegal use of anabolic steroids, various biological specimens such as edible tissues, kidney fat and urine can be used. In Belgium, faecal samples are of increasing importance, because of their ease of collection from living animals.

Within a working group consisting of several regional field laboratories (RFL) and the national reference laboratory (NRL), experiments were performed for comparison of their methods for faecal analysis, with the aim to derive a harmonised methodology for the tracing of anabolic steroids in faecal samples, which consequently will improve the inter- and intra-laboratory reproducibility. The different methods consist of the following main steps: liquid–liquid extraction (LLE), solid-phase extraction (SPE), high-performance liquid chromatography (HPLC) purification and gas chromatography-mass spectrometry (GC-MS) detection as presented in Fig. 1. The methods have partly been described elsewhere.[1–5] The defatting step and SPE clean-up differ mainly within the different laboratories. Considering the importance of these steps, the occurrence of contradictory results can be expected. For this reason, the different defatting techniques and SPE methods were compared, finalising with the determination of limit of detection values (LODs) as well as recovery experiments. In the near future, a complete study including a collaborative study will be carried out.

Experimental

Samples

As samples, fortified blank faecal material obtained from dairy cows in active lactation was used. For spiking purposes, a mixture of currently used anabolic steroids was used at concentrations ranging from 0.5 to 10 ng g^{-1}.

Reference compounds and standard solutions

The steroids as listed in Table 1 were obtained from Steraloids (Wilton, NY, USA) or Sigma (St. Louis, MO, USA). The following compounds were selected: β-boldenone (Bol) (1,4-androstadien-17β-ol-3-one), chlorandrostenedione (ClAD) (4-chloroandrost-4-ene-3,7-dione), chlormadinone acetate (CMA) (4,6-pregnadien-6α-chlor-17α-ol-3,20-dione acetate), chlorotestosterone acetate (ClTA) (4-androsten-4-chloro-17β-ol-3-one acetate), ethinylestradiol (EE2) [1,3,5(10)-estratrien-17α-ethinyl-3,17β-diol], fluoxymesterone (FMT) (4-androsten-

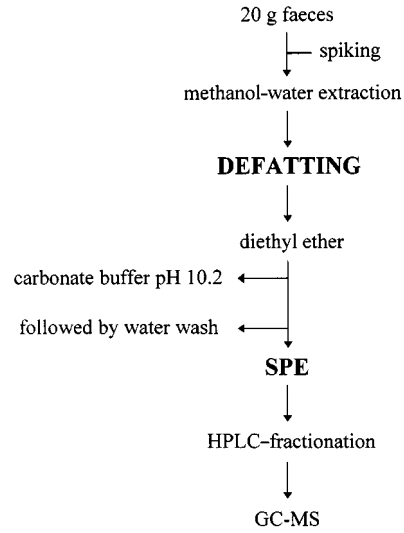

Fig. 1 Summary of the methods used for the tracing of anabolic steroids in faeces.

† Presented at the Third International Symposium on Hormone and Veterinary Drug Residue Analysis, Bruges, Belgium, June 2–5, 1998.

9α-fluoro-17α-methyl-11β,17β-diol-3-one), levonorgestrel (NG) (13β-ethyl-17β-hydroxy-18,19-dione-17α-pregn-4-en-20-yn-3-one), medroxyprogesterone acetate (MPA) (4-pregnen-6α-methyl-17α-ol-3,20-dione acetate), methandriol (MAD) (5-androsten-17α-methyl-3β,17β-diol), methylboldenone (MeBol) (1,4-androstadien-17α-methyl-17β-ol-3-one), methyltestosterone (MT) (4-androsten-17α-methyl-17β-ol-3-one), norethandrolone (NE) (4-estren-17α-ethyl-17β-ol-3-one), α-nortestosterone (aNT) (4-estren-17α-ol-3-one), stanozolol (Stan) (5α-androstan-17α-methyl-17β-ol[3,2-c]-pyrazole), trenbolone (TB) (4,9,11-estratrien-17α-ol-3-one) and zeranol (Z) (3,4,5,6,7,8,9,10,11,12-decahydro-7,14,16-trihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1-one). For each steroid a stock solution was prepared at a concentration of 1 mg ml^{-1} in methanol, and from these a working solution at a concentration of 1 ng μl^{-1} in methanol. A separate working solution was made for the internal standards equilenin (3-hydroxyestra-1,3,5,7,9-pentaen-17-one) and methylprogesterone (16β-methyl-pregn-4-ene-3,20-dione). All solutions were stored at 4 °C.

The following tritiated steroids were available: ^{3}H-labelled ethinylestradiol, ^{3}H-labelled β-nortestosterone and ^{3}H-labelled methyltestosterone. These compounds were kindly donated by 'CER' (Marloie, Belgium).

Apparatus

The HPLC purification system was composed of a Varian (Palo Alto, CA, USA) gradient pump, diode-array detector and autosampler, a FRAC-100 fraction collector (Pharmacia, Uppsala, Sweden) and a ten-port switching valve (Valco, Houston, TX, USA). GC-MS was performed on an ITS40 (Finnigan-MAT, San Jose, CA, USA) including a CTC-A200S autosampler, a Varian 3400 GC with 1077 capillary split/splitless injector and a Finnigan-MAT ion trap mass spectrometer. As liquid scintillation system, an LS 1801 beta counter from Beckman (Fullerton, CA, USA) was used.

Table 1 Limits of detection (LODs) for various anabolic steroids in faeces

HPLC fraction	Compound (code)	Diagnostic ions[a]	LOD/ ng g^{-1}
1	Trenbolone (TB)	414/283/309/399	5
	Fluoxymesterone (FMT)	552/462/407/319	2
	Boldenone (Bol)	206/430/325/415	1
	Methylboldenone (MeBol)	206/444/339/429	1
	Zeranol (Z)	433/307/335/389	1
	Ethinylestradiol (EE2)	425/285/300/440	1
	α-Nortestosterone (aNT)	418/403/313/328	1
	Levonorgestrel (NG)	456/316/301/441	1
	Chlorandrostenedione (ClAD)	464/466/429/449	2
2	Methyltestosterone (MT)	301/446/356/341	0.5
	Methandriol (MAD)	253/268/343/358	2
	Chlormadinone acetate (CMA)	488/490/453/473	2
	Medroxyprogesterone acetate (MPA)	470/455/441	2
	Norethandrolone (NE)	287/446/356/300	0.5
3	Stanozolol (Stan)	143/472/168/457	> 10
4	Chlortestosterone acetate (ClTA)	436/401/438/421	2

[a] Diagnostic ions according to their abundances.

Solvents/chemicals/material

All solvents were of analytical-reagent grade. Methanol, hexane and isooctane were obtained from Merck (Darmstadt, Germany), light petroleum (bp 30–60 °C) from J. T. Baker (Phillipsburg, NY, USA) and diethyl ether (free from peroxides) from Gifrer & Barbezat (Décines, France). The scintillation liquid pico-fluor was purchased from Packard (Downers Grove, IL, USA).

Sodium carbonate and hydrogencarbonate were from Merck. Phosphoric acid (minimum 85%) was obtained from UCB (Brussels, Belgium).

MSTFA [*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide] was purchased from Macherey-Nagel (Düren, Germany), ITMS (iodotrimethylsilane) from Janssen Chimica (Geel, Belgium) and DTE (dithiothreitol) from Sigma-Chemie (Brussels, Belgium).

The Bond-Elut Si (500 mg/3 ml) and NH_2 (100 mg/1 ml) SPE columns were from Varian, and the Extract-Clean NH_2 (500 mg/3 ml) and C_{18} (500 mg/6 ml) SPE columns from Alltech (Arlkington Heights, IL, USA).

Results and discussion

An important aspect within the development of a method is its validation. Considering the fact that the use of growth promoters in animal production is forbidden, the method proposed in this paper is a qualitative method.[6] Validation parameters such as specificity and selectivity have to be verified, *i.e.*, the identity of the analyte has to be confirmed. For this purpose, the criteria laid down in the Commission Decision 93/256/EEC have to be applied.[7] These criteria, based on retention time and mass spectral data, were applied for the evaluation of the effect of the defatting and SPE procedures on the identification of the various anabolic steroids listed in Table 1.

Comparison of defatting techniques

Based on literature information, faecal samples can be expected to contain about 20% fat.[8] Its elimination has to be considered, since otherwise injection problems can occur during the HPLC

Table 2 Overview of the different experimental SPE conditions

Step	Si	Si + NH_2	NH_2	C_{18}
Conditioning	Hexane, 2 × 2.5 ml	Hexane, 2 × 2.5 ml	Ethyl acetate, 3 × 2 ml	Methanol, 2 × 3 ml; water, 2 × 3 ml
Residue uptake and sample deposit	Chloroform, 500 μl; hexane, 5 ml	Chloroform, 500 μl; hexane, 5 ml	Ethyl acetate, 2 ml	Methanol, 1.25 ml; water, 3.75 ml
Wash	Hexane, 2 × 2.5 ml	Hexane, 2 × 2.5 ml ↓ Connection of NH_2 column ↓ Hexane, 2 × 2.5 ml	—	Methanol–water (1 + 4), 3 ml
Elution	Chloroform–acetone (4 + 1), 2 × 2.5 ml	Chloroform–acetone (4 + 1), 2 × 2.5 ml	Ethyl acetate, 3 × 1 ml	Acetonitrile, 3 × 1 ml

purification step. For defatting purposes, hexane and light petroleum are commonly used solvents. A major drawback of these solvents is the significant loss of gestagenic-type compounds. For ClTA, for instance, more than 50% loss has been observed.[9] Recently, the usefulness of isooctane was investigated, as it possesses different polarity properties.[10] By using this approach, the loss of ClTA could be reduced to 30%. However, the use of such a solvent still presents a significant disadvantage. Elimination of the non-aqueous phase is impractical. A more practical approach consists in the incorporation of an acidification step (1 M H_3PO_4), followed by centrifugation under cooling. In this manner, GC-MS results, as reflected in the applicability of the criteria,[7] indicated that the clean-up is more selective, resulting in a significant improvement in the recovery of gestagenic-type compounds.

Comparison of SPE procedures

Three types of extraction column were compared: silica, aminopropyl and octadecyl. The experimental conditions used by the different laboratories are listed in Table 2. These procedures present advantages and disadvantages. The single-column procedures, and particularly the aminopropyl 'filtration' procedure, seem attractive, considering their speed. However, on the latter column the recovery of Z was rather low, even though clean-up was very efficient. On a silica extraction column, results were unsatisfactory for EE2 and on a C_{18} column also for MPA and ClTA. Satisfactory results were obtained with the silica–aminopropyl combination procedure, as reflected by lower LODs for the different anabolic steroids (Table 1). The full scan spectra of ClTA (spiking level 2 ng g^{-1}; diagnostic ions 436, 438, 401 and 421) and NE (spiking level 0.5 ng g^{-1}; diagnostic ions 287, 446, 356 and 300) are presented in Fig. 2 and 3, respectively.

Recovery experiments

The radioactivity was measured under standard conditions by adding portions from extracts to a scintillation liquid (8 ml) in scintillation vials and counting in a beta counter. The results for the different labelled compounds are presented in Table 3. As can be observed, the recoveries are about 20%. The main loss, about 50%, occurs in the first step, namely, the primary

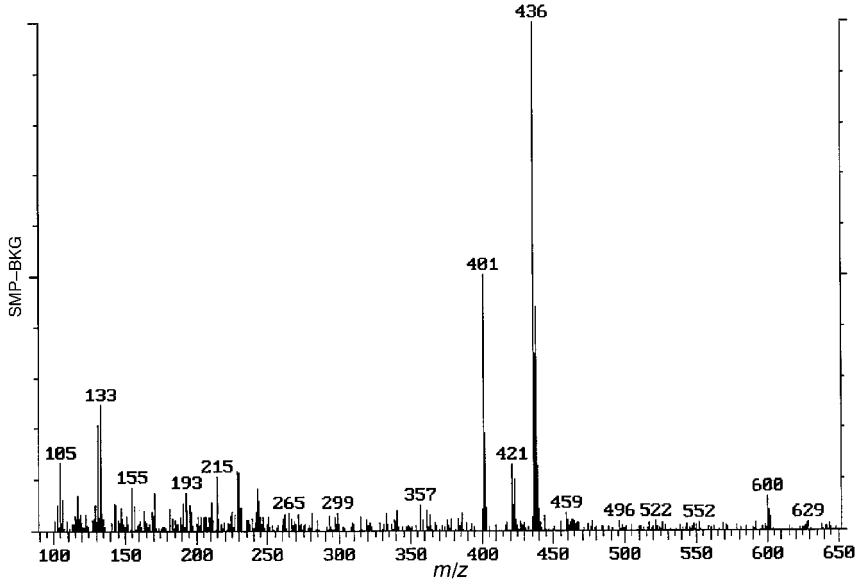

Fig. 2 Full scan spectrum of ClTA (spiking level 2 ng g^{-1}).

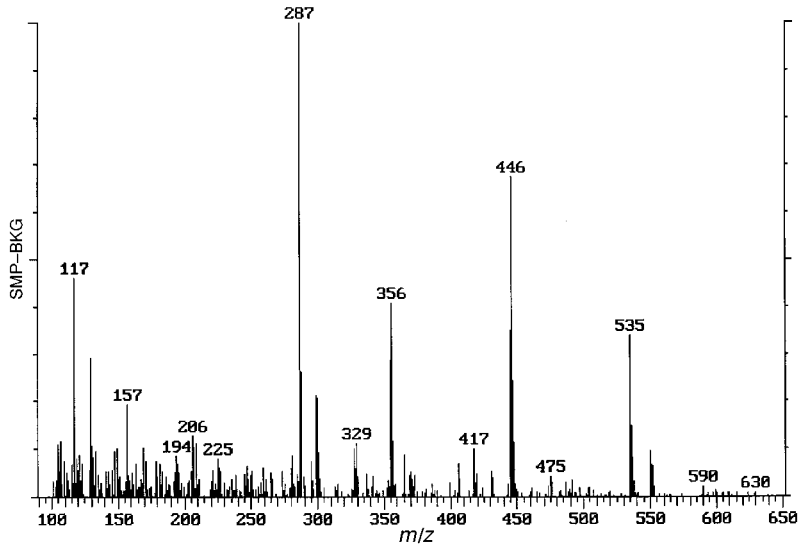

Fig. 3 Full scan spectrum of NE (spiking level 0.5 ng g^{-1}).

Table 3 Recoveries for the different labelled anabolic steroids in faeces

	Recovery (%)		
Step	[^{3}H]-EE2	[^{3}H]-bNT	[^{3}H]-MT
Methanol–water and acidification	48	51	51
Diethyl ether	41	46	47
Carbonate and water	41	38	39
SPE (Si/NH_2)	34	33	34
HPLC fractionation	19	19	17

extraction, probably due to coprecipitation. As a means to obtain better recoveries, matrix solid-phase dispersion (MSPD) seems attractive. This technique has previously been applied to kidney fat.[11] For MPA, recoveries of about 60% were reported using MSPD in combination with SPE. This will be investigated in the near future.

The authors acknowledge the financial support of the Institute of Veterinary Inspection and the technical assistance of Mrs. R. Schoenaers.

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Paper 8/05115A