Enhancing the specificity of residue analysis for anabolics using HPLC clean up

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1. Introduction

For routine inspection kidney fat, urine and faeces samples have to be analysed for residues of estrogens, androgens, gestagens and related compounds. At this moment the number of target anabolics in Belgium is 28. This implicates the use of some multiresidue methods. The selection of such methods is not easy because each standard operating procedure has to be validated and all quality criteria have to be fulfilled for each analyte.

In document 93/256/EEC[1] general requirements for the methods to be used for detecting residues of substances having a hormonal or thyreostatic action are stated. One of the aspects of these quality criteria is the specificity of a method. Specificity is defined as the ability of the method to distinguish between the analyte being measured and other substances. This means that in this case the method must have the ability to distinguish between the analyte being measured at trace or ultra-trace concentrations and other substances possibly present at 10–10 000 times higher concentrations.

Two general approaches are possible for choosing the best method:

1. If available, a very specific detection technique (GC-MS, MS-MS, high-resolution GC-MS) could be chosen as detection technique. This could result in a reduction of sample pretreatment which is, at first sight, an advantage. However, it is not conceivable for a control lab to use these techniques in routine analysis: higher instrument cost, lower column lifetime and very likely quicker contamination of the system by using dirty extracts. As the number of substances of different parts to screen is high and not limited, one detection technique will never be even specific for all compounds.
2. A more extensive, but automated clean up procedure is the other option. HPLC fractionation, for instance results in several purified fractions each containing a limited number of anabolic compounds and matrix components. Each fraction may be analysed with a specific technique and if necessary with different techniques.

In this study two clean up techniques based on Liquid Chromatography: SPE (Solid Phase Extraction) and HPLC (High Performance Liquid Chromatography) are compared with each other. These are combined with two detection systems: HPTLC (High performance Thin Layer Chromatography) and GC-MS (Gas Chromatography Mass Spectrometry).

SPE ist used in many multi-residue screening procedures[2–5]. Groups fractionation with a combination of SPE columns is a more selective method in routine detection of various anabolics[6]. The use of the higher separation efficiency of HPLC[7] as clean up has the advantage that the specific fractions for one analyte or a selected group of analytes can be isolated.

2. Experimental

2.1 Apparatus

A homogenizer (e. g., Ultra Turrax, 20 000 rpm), a water-bath, a centrifuge equipped with centrifugation tubes of 450 ml (e. g., Beckman), a mechanical extractor (e. g., Stomacher), a rotary vacuum evaporator (e. g., Rotavapor), a N_2 evaporator (e. g., Vapotherm form LaborTechnik Barkley), extraction flasks of 100 ml and 250 ml, a solid-phase extractor (e. g., Baker), chromatographic tanks and a UV transilluminator (λ = 366 nm) were used. The sample applicator used was a semi-automatic Linomat IV (Camag). The LC system consisted of a Series 4 pump (Perkin-Elmer), an ISS-100 autoinjector (Perkin-Elmer), an automatic switching valve MUST (Chrompack), a Model 440 UV detector (Waters) and a Model 202 fraction collector (Gilson). The Gas chromatograph – Mass spectrometer was an ITS 40 ion trap (Finnigan MAT, USA). HPTLC plates were obtained from Merck (Darmstadt, Germany).

2.2 Reagents, reference compounds and solutions

All solvents were of analytical reagent grade or LC grade from Merck (Darmstadt, Germany), diethyl ether was obtained from Gifrer & Barbezat (Decines, France). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Machery-Nagel (Duren, Germany) and iodotrimethylsilane (ITMS) and dithioerythritol (DTE) from Pierce (Rockford, IL).

All hormonal standards were obtained from Steraloids (Wilton, NY, USA).

2.3 Solutions

Stock standard solutions were prepared in ethanol at a concentration of 1 mg ml^{-1}. For routine control purposes, working standard solutions were prepared by dilution of the stock standard solutions to 50 ng ml^{-1}. Buffer solutions: sodium acetate (0.04 M) pH: 5.2; sodium carbonate (10 %) pH ≤ 10.25. Glucuronidase-sulfatase enzyme suspension (Helix pomatia juice: 100 000 Fishman units/ml b-glucuronidase + 1 000 000 Roy units/ml sulfatase) was obtained from I. B. F. (Clichy, France). The following solvent systems were used to develop th HPTLC plates: 1 = n-hexane-diethyl-ether-dichloromethane (25 + 45 + 30); 2 = chloroform-acetone (90 + 10); 3 = cyclohexane-ethyl-acetate-ethanol (60 + 40 + 2.5); 4 = chloroform-n-hexane-acetone (50 + 40 + 10).

2.4 LC-Columns

Two semi-preparative C_{18}, Ultrasphere ODS columns (80 Å pore size, particle size 5 µm, 50 mm L x 10 mm I. D.; 250 mm Lx 10 mm I. D.) were obtained from Beckman Intruments. A C_{18} pellicular ODS guard column (particle size 37–53 µm, 30 mm L x 4.6 mm I. D.) from Whatman and a C_{18} MCH-10 cartridge precolumn (particle size 10 µm, 30 mm L x 4.6 mm I. D.) from Varian. SPE-columns „Bond Elut" were obtained from Varian Analytichem; Silica (Si)-3cc and Aminopropyl (NH2)-I cc. Before use, all these columns were conditioned with the appropriate solvents.

2.5 Solid phase extraction as clean up

For the regulatory control the SPE method in combination with 2D-HPTLC is used as screening procedure for the kidney fat samples. The overall scheme of the procedure is shown in Fig. 1A and described before[2,8]. Confirmation of the suspect samples follows from GC-MS analysis of the remainder of the extract or 2-D-HPTLC or GC-MS analysis after HPLC clean up (on one or two columns, see 2.6).

2.6 HPLC as clean up

The aim of this technique is to purify the primary extract by the isolation of different specific fractions. Each fraction contains a limited amount of analytes and also a limited amount of interferences. The fractionation windows are determined after injection of the appropriate standards.

HPLC on one column:

For confirmatory purpose one dimensional HPLC in recommended for the analysis of kidney fat samples. The overall scheme of this procedure is shown in Fig. 1B. The detailed methodology is described before [9,10].

HPLC on two columns in series:

For urine samples SPE clean up and also gradient elution on one single column is mostly not sufficient to achieve the specificity needed to fulfil all the identification criteria.

Therefore two HPLC columns were used in combination with a MUST switching valve-system in the heart-cutting mode. In this mode only a selected fraction (containing the anabolics of interest) of the eluate of the first column is diverted to the second column for further elution. This second chromatography results in much cleaner fractions which are collected for further detection on the presence of anabolic residues.

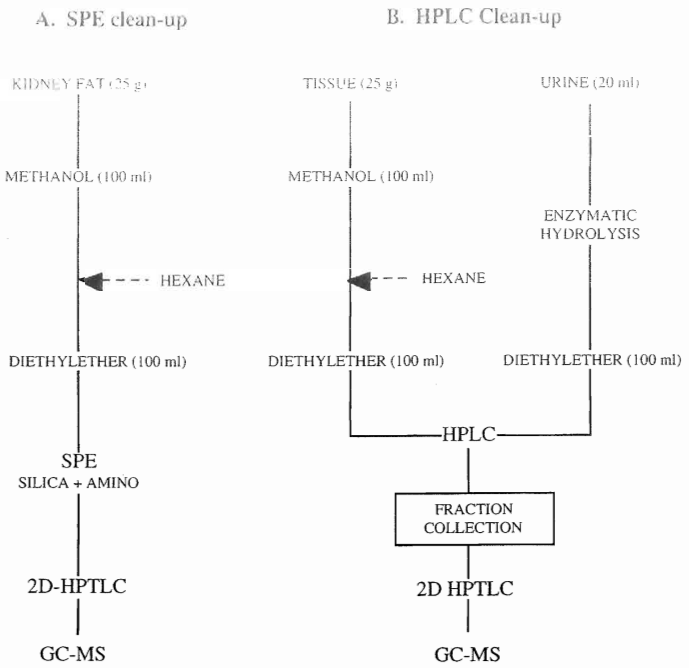

Fig. 1: Overall scheme of A: the SPE clean up and B: the HPLC clean up procedure

2.7 High-Performance Thin-Layer Chromatography

For screening purposes the extract is chromatographed on a precoated silica gel plate (10 cm x 10 cm) following the 4 x 4 elution technique[11]. The dry residue obtained after SPE or HPLC, is dissolved in 30 µl ethanol and 10 µl is spotted on the plate. Chromatographic development is performed with the solvent systems as described in "Solutions".

After fluorescence induction on the plate the spots are visualised by transillumination under UV radiation (λ = 366 nm) and identified by comparing the Rf values and the colours with those of the reference standards. Identification criteria for the qualitative determination of an analyte by TLC, in accordance with the EU-Directive 93/256/EEC:

1. The Rf value of the analyte should agree with the Rf value of the standard.
2. The visual appearance (color) of the analyte should be indistinguishable from that of the standard.
3. The centre of the spot nearest to that due to the analyte should be separated from it by at least half the sum of the spot diameters.
4. Two-dimensional development is obligatory.
5. Two-dimensional co-chromatography can give supplementary information and is obligatory in Belgium when there is an Rf variation greater than 3 %.

2.8 Gas Chromatography – Mass Spectrometry

The derivatization reagent mixture is MSTFA-TMSI-DTE (1000 + 2 + 2). Extracts should be evaporated to dryness prior to derivatization. To the

Table 1: **Results of the analysis of two kidney fat samples analysed by different methods (concentrations in parts per billion).**

Sample nr.	SPE clean up		HPLC clean up	
	HPTLC	GC-MS	HPTLC	GC-MS
IHE/93/164	MT** (>5) AP (>4) MPA (<2)	MT (>5)	MT (>2) AP (>4) MPA (<2) b-NT (<2) b-T (>2) a-T (<2)	MT AP MPA b-NT b-T a-T
IHE/93/165	MT** (>2) AP (>5) MPA (<2)	MT (>2) AP	MT (>2) AP (>5) MPA (trace*) CMA (trace*) b-NT (<2) b-T (<2) a-T (<2)	MT AP MPA b-NT b-T a-T

* = (trace means that the quality criteria are not completely fulfilled)
** = MT: Methyltestosterone; AP: Acetoxyprogesterone; MPA: Medroxyprogesteroneacetate; NT: Nortestosterone; T: Testosterone; CMA: Chlormadinonacetate

tube containing the extract, 25 µl of the reagent mixture are added and heated at 60 °C for 15 min. The tube is allowed to cool and then 1 µl is injected in the splitless mode into the GC-MS intrument.

The analyses were carried out on a Finnigan ITS 40 ion trap in the full-scan mode. The GC column used was a DB-5 fused silica (30 m x 0.25 mm I. D.) with a 0.25-mm film thickness and a carrier gas (helium) flow-rate of 1 ml min^{-1}. The temperature settings were as follows: injector, 260 °C; transfer line, 300 °C;, oven, programmed from 100 to 200 °C at 16.7 °C min^{-1} and from 250 to 300 °C at 4 °C min^{-1}, the final temperature of 300 °C being maintained for 3.5 min.

Identification criteria for the qualitative determination of an analyte by GC-MS.

1. The analyte has the same retention time as the reference.
2. The analyte shows a number of specific fragment ions:
   -minimum three ions with a S/N ≥ 3, intensity ratio's equal to chose of the reference and a minimum FIT-value of 800.

FIT = the degree to which the library spectrum is included in the sample mass spectrum. PURITY = similarity between the sample mass spectrum and the library mass spectrum. This parameter gives an idea of the coeluting peaks that are important to consider when analysing extracts of biological samples.

3. Results and discussion

Kidney fat samples

Several kidney fat samples from regulatory control are analysed with the two clean up procedures (SPE purification and HPLC fractionation) combined with two different detection techniques (HPTLC and GC-MS). The results obtained for two of them are summarised as an example in table 1.

Thin-layer chromatography:
After SPE purification only fraction has to be chromatographed. This is only an advantage when relatively high residue concentrations are present: methyltestosterone (MT) and acetoxyprogesterone (AP) (> 5 ppb) are detected both after SPE and HPLC purification (tab. 1). A concentration greater than 5 ppb is considered as relatively high for anabolics but depends upon the analyte (detection limit, Rf values etc.): medroxyprogesteroneacetate (MPA) for instance is detected after both purifications at concentrations smaller than 2 ppb. However, at lower concentrations the effect of interferences in some fractions becomes too important for other anabolics. In Fig. 2 it is shown that some anabolics as Nortestosterone (NT) and Testosterone (T) are detected in the two analysed samples only after HPLC clean up (on one column). In the SPE-eluates too many interferences were present on the final thin-layer chromatogram. These interferences disturb the chromatography and the signals from those analytes.

Gas Chromatography-Mass Spectrometry:
The HPTLC results are completely confirmed by GC-MS for the extract purified by HPLC. This is not the case after SPE clean up: in one sample only MT (in the other sample MT and AP) was confirmed by GC-MS (tab. 1). This ist most probably due to the observation that gas chromatography of the silyl derivatives of gestagens (as MPA and AP) is more easily disturbed by dirt than of other anabolics.
Moreover, according to the identification criteria used, the result of methyltestosterone as shown in Fig. 3 is a result "on the limit". Due to the co-elution of interferences, the library search gives a low fit value of 807 and a very low purity factor of 193. In the spectrum of the HPLC fraction, fit- and purity values of respectively 982 and 707 are recorded. This means that the analyst is in a much more comfortable position to evaluate this latter result.
Besides, in other HPLC fractions of that same sample, the presence of additional anabolic compounds (NT, T) was detected. These analytes were not found after SPE purification because of the effect of interferences.

Urine samples

Our experience in analysing real urine samples from the regulatory control is that the results obtained on fractions eluted from one HPLC column were not always satisfactory. Especially the first eluting fractions still contain too much impurities.

To improve these results we used a double chromatography combining two HPLC columns containing the same stationary phase (C18) but with different dimensions. The columns were coupled by a multiport switching valve system giving the possibility of heart-cutting as explained before.

Fig. 4 shows the GC-MS results of the same sample purified in the two modes. It can be seen that spectrum B is a mixture of different co-eluting peaks, including Nortestosterone. All specific ions (418, 403, 287, 182) are present but also a lot of noise because of the interferences. The interpretation of the results will become problematic when a lower concentration of this substance is present. Then the specific ions with the lower in-

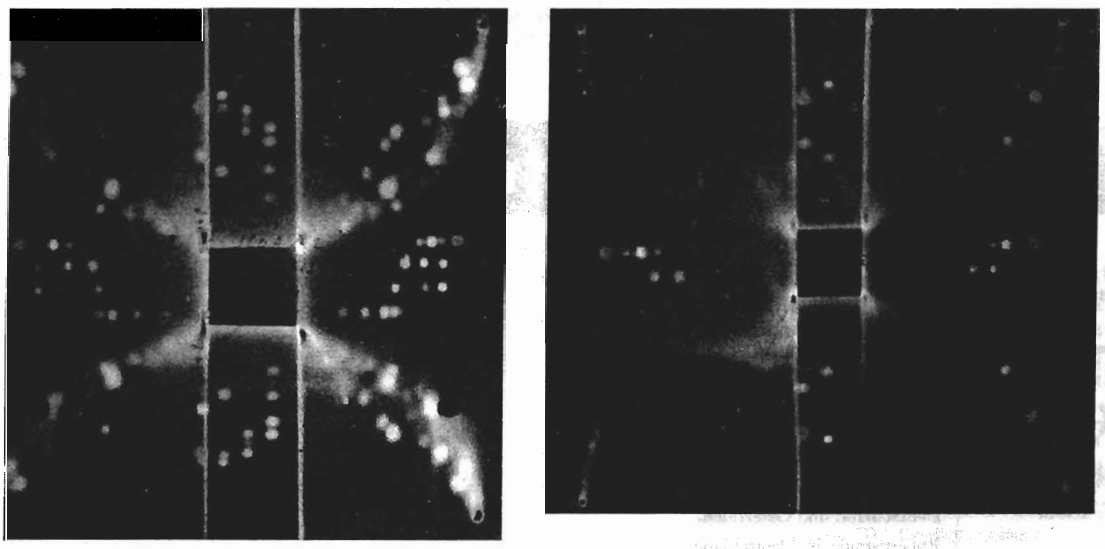

Fig. 2: Two-dimensional co-chromatography on silica plates of the extracts of the two fat samples. A: after SPE clean-up; B: after HPLC fractionation (only early eluting fraction is shown)

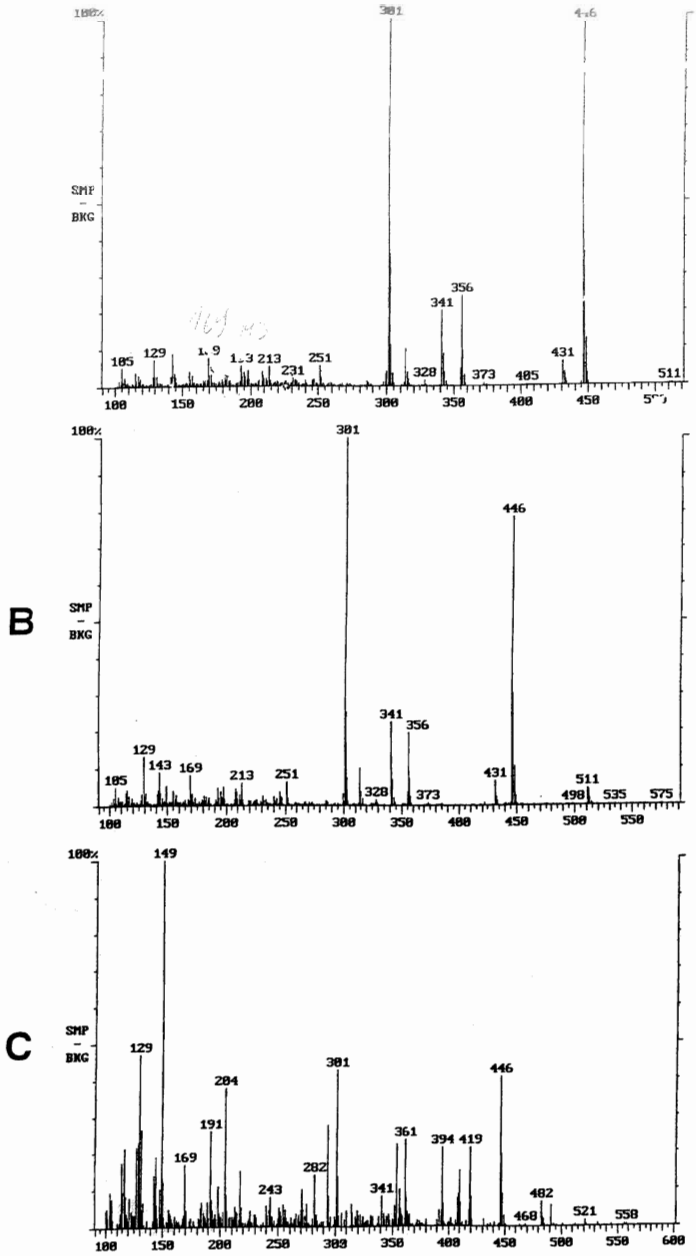

Fig. 3: GC-MS spectrum of methyltestosterone. A: standard; B: fat sample purified by HPLC; C: the same fat sample as B but after SPE clean-up

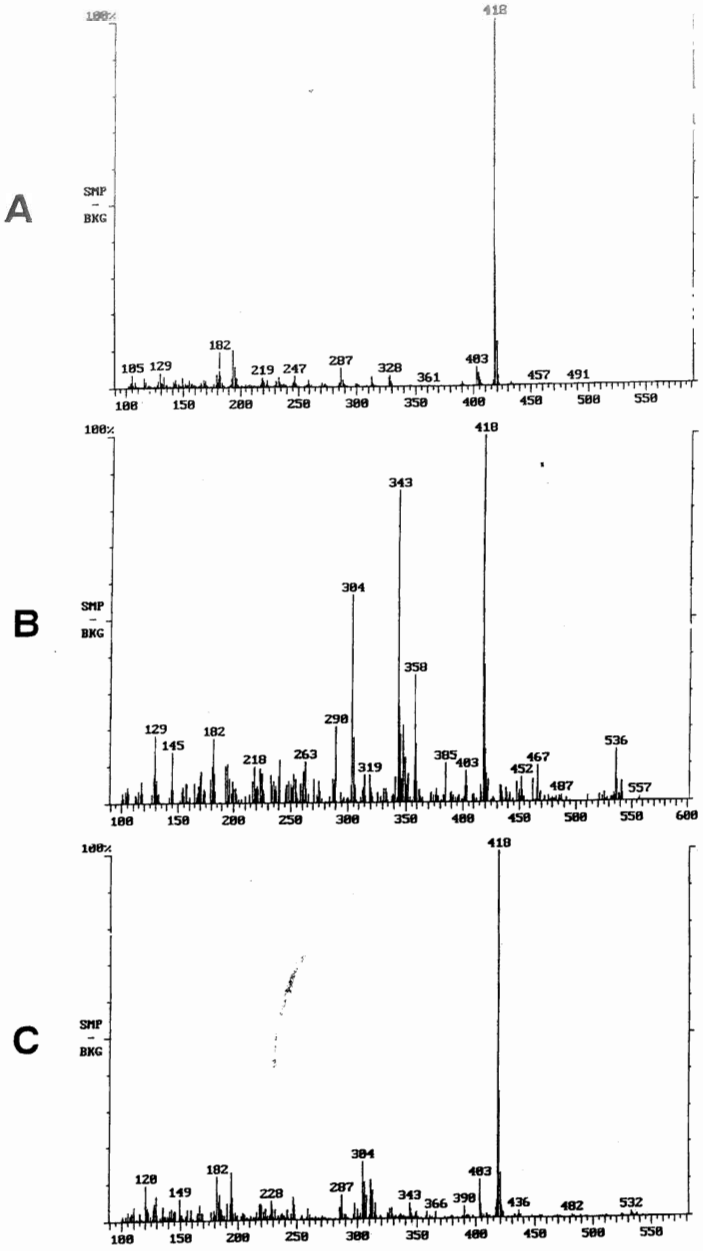

Fig. 4: GC-MS spectrum of α-nortestosterone. A: standard; B: Urine sample purified on one HPLC column; C: the same urine sample

tensities will be dominated by the noise and a false negative result will be produced.

In Fig. 4C we see that a pure spectrum is obtained after purification on a coupled column HPLC system. Lower limits of detection can be expected in proceeding this way.

4. Conclusions

The ability to distinguish the analyte, present in even trace or ultra-trace concentrations from interfering substances at much higher concentrations is an important parameter in the validation of an analytical procedure for residue analysis in biosamples.

In this paper it is demonstrated that the clean up of the primary extract needs special attention and adds a considerable value to the specificity of the method. Using HPLC with fraction collection as clean up step of the primary extract obtained from fat samples, it is proved that the interference effect obtained with these fractions is importantly smaller: SPE clean-up, even the combination of different specific columns didn't eliminate interfering matrix substances as well as HPLC clean up. For urine samples HPLC clean up on one column is not satisfactory: a two column system is necessary. This observation was valid for both TLC as MS detection. For laboratory economics the same two-column HPLC system could of course be used for both kidney fat samples and urine samples.

HPLC purification adds a considerable value to the specificity of the method and influences in a positive sense the reliability of the results. By minimising the interference effects the exact amount of sample introduced into the detection system may be enlarged and the limit of detection substantially decreased. In Belgium the use of HPLC clean up prior to detection of anabolics in excreta is mandatory.

Abstract

Some hyphenated techniques as GC-MS, LC-MS etc. are claimed to be so specific that they could be used after a minimum sample clean up. In our experience this is not (yet) true for the analyses of anabolics in complex matrices at the ppb (µg/kg) level. We obtained a more reliable result for fat and urine samples after HPLC clean up in comparison with a less extensive SPE purification.

Zusammenfassung

Es wird gesagt, einige gekuppelte Techniken, wie GC-MS, LC-MS und so weiter, seien so spezifisch, daß sie nach einem Minimum von Säuberung eines Musters angewendet werden können.

Aus unserer Erfahrung ist das (noch) nicht richtig für die Analysen von Anabolika in komplexen Nährböden auf dem ppb (µg(kg) Niveau. Nach der HPLC Säuberung haben wir, im Vergleich mit einer weniger ausgebreiteten SPE Säuberung, ein zuverlässigeres Resultat betreffs Fett- und Urinmuster erlangt.

Authors

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