Gas Chromatography–Mass Spectrometric Confirmation of Anabolic Compounds in Injection Sites*

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Intramuscularly administered, many anabolic residues are present in injection sites in an esterified form. Routine high-performance thin-layer chromatography (HPTLC) results show different spots recognizable as the esters of a certain anabolic compound, *e.g.*, testosterone or estradiol, without giving the exact identity of the ester. Hydrolysis of the extract and respotting on HPTLC plates can confirm these spots to be esters owing to the absence of such spots on the plate after hydrolysis. The residues are then seen in their unesterified form. Identification is possible after respotting on reversed phase plates. As this method is complex and time consuming, an alternative method was tried out using gas chromatography–mass spectrometry (GC–MS). GC–MS analysis of injection site extracts is not only an independent method to confirm HPTLC results, but also provides the opportunity to identify the specific ester of the anabolic steroid. Identification is only possible when a standard of the ester is available for comparing the respective mass spectra. This report presents the mass spectra of some esters of testosterone, estradiol and nortestosterone found during the confirmation analysis of 35 injection sites by using GC–MS.

Keywords: Gas chromatography–mass spectrometry; anabolic steroid; long chain esters of steroids; injection site

Introduction

In the European Union (EU), the use of hormones as growth promoters has been made illegal.[1,2] Despite an intensive regulatory control program, the abuse of these compounds is still very popular in several EU-member states.

Mixtures of endogenic and exogenic anabolic steroids, the so-called cocktails, can be injected intramuscularly. This results in injection sites with a high concentration of residues of these products.[3] The consumption of these highly contaminated injection sites (mainly in minced meat) can pose a considerable threat to human health.

In routine analysis these injection sites are analysed using a high-performance thin-layer chromatography (HPTLC) method in the '4 × 4' mode.[4] Using this technique, the esters of the anabolic compounds are not always identified individually, but more as a group of the esters of a specific compound. To identify the esters with a greater certainty it is necessary to hydrolyse these compounds and to perform a second HPTLC analysis. The absence of the spots after hydrolysis confirms the earlier presence of esters of certain anabolic steroids. Because this is a rather complicated and time consuming way of analysing injection sites, it would be preferable to perform the analysis by gas chromatography–mass spectrometry (GC–MS). GC–MS data also provide results with a greater certainty about the identity of a compound (*i.e.*, retention time, mass spectrum).

We studied a large number of injection sites for the presence of anabolic steroids and their esters and attempted to identify them by comparing the mass spectrum obtained with the spectra of several standards of esterified anabolic compounds.

Experimental

Apparatus

The following apparatus was used: extraction flasks (10–20 ml), nitrogen evaporator, reacti-term heating module, and autosampler vials [*e.g.*, Chromacol 07-CPV (A) (Welwyn Garden City, Herts., UK)] and a Magnum Ion Trap System (Finnigan-MAT, San Jose, CA, USA) comprising a: Finnigan-MAT A200S GC Autosampler, a Varian 3400 GC with 1077 capillary split/splitless injector (Palo Alto, CA, USA), and a Finnigan-MAT Magnum Ion Trap Mass Spectrometer with electron impact.

Reagents and Reference Components

The long chain esters of the steroids were obtained from Steraloids (Wilton, NY, USA) whereas the other compounds (see Table 1) were purchased from Sigma (St. Louis, MO,

Table 1 Prevalance of anabolic residues in injection sites: βT, beta-testosterone; βT-E, beta-testosterone esters; βE_2, beta-estradiol; CITA, chloro-testosterone acetate; βE_2-E, beta-estradiol esters; PG, progesterone; E_2B, estradiol benzoate; STAN, stanozolole; MT, methyltestosterone; FMT, fluoxymesterone; Mebol, methylboldenone; DhPA, algestone acetophenide; βNT, beta-nortestosterone; EE_2, ethinylestradiol; MPA, medroxyprogesterone acetate; βTB, beta-trenbolone; CP, caproxy-progesterone; and αTB, alpha-trenbolone

Name	*N** (%)	Name	*N* (%)	Name	*N* (%)
βT	32 (35)	E_2B	17 (18)	βNT	3 (3)
βT-E	32 (35)	STAN	14 (15)	EE_2	2 (2)
βE_2	28 (30)	MT	9 (10)	MPA	2 (2)
CITA	27 (29)	FMT	6 (6)	βTB	2 (2)
βE_2-E	26 (28)	Mebol	5 (5)	CP	1 (1)
PG	22 (24)	DhPA	3 (3)	αTB	1 (1)

* *N* = absolute number.

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USA). MSTFA [*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide] was obtained from Macherey Nagel (Düren, Germany) and TMSI (Iodotrimethylsilane) from Janssen Chimica (Geel, Belgium). DL-Dithiothreitol was purchased from Sigma-Chemie (Brussels, Belgium).

$MSTFA^{2+}$ was prepared by dissolving 1‰ (per mil) TMSI and a tip of a spatular point amount of reductant dithiothreitol in MSTFA.[5]

All solvents used were of analytical-reagent grade and were obtained from Merck (Darmstadt, Germany).

Solutions

Stock solutions of anabolic steroids were prepared at a concentration of 200 ng μl^{-1} in ethanol. Ten-fold dilutions of these stock solutions resulted in working solutions with concentrations of 20 ng μl^{-1}.

Derivatization and GC–MS Conditions

Anabolic steroids were derivatized using $MSTFA^{2+}$: the sample or 10 µl of standard solution (200 ng) was transferred into an autosampler vial (700 µl) and dried under a nitrogen stream. $MSTFA^{2+}$ (50 µl) was added, the contents were mixed and heated for half an hour at 60 °C. A 1 µl (4 ng) volume is injected into the GC system.

The initial GC–MS conditions were: 100 °C, raised to 250 °C at a rate of 15 °C min^{-1}, to 320 °C at a rate of 3 °C min^{-1}, held at 320 °C, 1.5 min (total program *ca.* 35 min). The injector temperature was 260 °C, and the transfer-line, 300 °C. The column used was a Hewlett-Packard Ultra-2 of dimensions: 25 m × 0.20 mm i.d. and, film thickness, 0.11 µm (Avondale, PA, USA). The aquisition method that was used was 1 scan s^{-1} over 25 min (mass range: 80–650 amu, filament-multiplier delay 600 s).

Table 2 Molecular masses (*M*) and diagnostic ions of the 12 available standards in our laboratory: E_2; estradiol; NT; nortestosterone; and T, testosterone

Name	*M*	M_{deriv}*	Diagnostic ions (intensity)
E_2-Benzoate	376	448	105 (100), 358 (25), 448 (12)
E_2-Cypionate	396	468	468 (100), 327 (11), 454 (1)
E_2-Diacetate	356	428	314 (100), 254 (9), 297 (2)
E_2-Dipropionate	384	456	328 (100), 254 (11), 311 (2)
E_2-Valerate	356	428	428 (100), 327 (12), 413 (3)
NT-Benzoate	378	450	450 (100), 105 (40), 436 (4)
NT-Decanoate	428	500	500 (100), 329 (6), 194 (5)
T-Acetate	330	402	402 (100), 388 (12), 343 (8)
T-Benzoate	392	464	464 (100), 105 (47), 450 (10)
T-Cypionate	412	484	485 (100), 470 (17), 343 (14)
	(413)	(485)	
T-Decanoate	442	514	515 (100), 343 (5), 501 (4)
	(443)	(515)	
T-Priopionate	344	416	416 (100), 402 (10), 343 (4)

* M_{deriv} = *M* after derivatization.

Table 3 The deducted molecular masses (*M*) of some testosterone-esters (T-esters)

Name	*M*	M_{deriv}*
T-Butyrate	358	430
T-Valerate	372	444
T-Caproate	386	458
T-Enanthate	400	472
T-Caprylate	414	486
T-Fenylpropionate	420	492
T-Cyclohexylpropionate	426	498
T-Nonanoate	428	500
T-Tosylate	442	514
T-Undecylenate	456	528
T-Laurate	470	542
T-Hexyloxyfenylpropionate	520	592

* M_{deriv} = *M* after derivatization.

Results and Discussion

The indirect linking of HPTLC and GC–MS for the determination of the mass spectrum of the suspect substances could not be performed analogously to the determination of thyreostatica. Thyreostatic drugs are released in their original form after dipping of the TLC plate for visualization of the residues. Thus, it was very easy to interfere HPTLC to GC–MS by scratching the suspected spots from the plate and transferring them into an autosampler vial for derivatization.[5] Otherwise, visualization of steroids occurs after dipping in an H_2SO_4 solution. This reaction results in the conjugated steroids fluorescing. Therefore, it was not possible to extrapolate the procedure described for thyreostatic drugs towards the steroids. Confirmation of HPTLC results of steroids had to be performed starting from the extract of the sample.

The identification of anabolic steroids is based on their specific colour after dipping of the plate and the R_F values as seen after the two-dimensional development. As GC–MS is not yet introduced as an official analytical method for the detection of steroid residues in Belgium, only the positive HPTLC results are confirmed using a GC–MS method. Thus, we get a second confirmation based on the retention time of the GC-analysis and the full-scan mass spectra. This interfacing of two techniques gives the final results a very high reliability.

Besides use as a confirmation technique, GC–MS data can be used for the identification of new illicit residues or their metabolites. In comparison with the short chain esters (methyltestosterone for instance), the long chain esters of the anabolic steroids (testosterone, estradiol and nortestosterone) do not leave the column within the time limit of the temperature programmation of the GC, used for routine analysis. Acquisition was prolonged for 10 to 30 min after injection instead of the usual 20 min. A delay time of 10 min after injection was held unchanged because none of the anabolic residues eluted that early.

Twelve standards of long chain esters of anabolic steroids were analysed by GC–MS. It is striking to observe that for all of the esters the molecular ion after derivatization was present as a base peak in their spectra with the exception of estradiol-benzoate, estradiol-diacetate and estradiol-dipropionate. Table 2 shows the molecular ions and the diagnostic ions of these 12 esters.

Because the standards of the esters, mostly found in

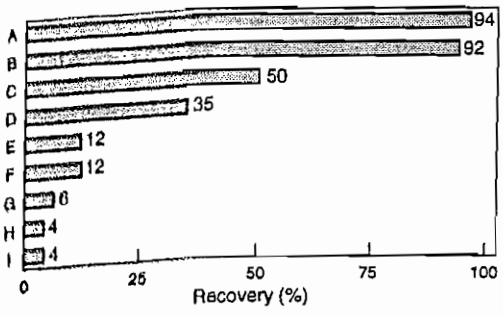

Fig. 1 GC–MS confirmation and identification of HPTLC results for T- and E_2-esters. A, T-cypionate; B, E_2-benzoate; C, T-decanoate; D, E_2-valerate; E, T-acetate; F, T-propionate; G, T-phenyl propionate; H, E_2-cypionate; and I, E_2-dipropionate.

injection sites,[3,6] were not all available in our laboratory, their identities were deducted from the molecular ion after derivatization (Table 3).

The molecular masses given in Table 3, were derived from the spectra of the available standards by treating them as compounds of a homologous series. For all of the testosterone-esters available the derivatized molecular mass was found in their spectra as the base peak, but this was not the case for some esters of estradiol. The molecular mass after derivatization is determined as the molecular mass of the original compound enlarged with the mass of 1 TMSI group (mass = 72). This procedure is extrapolated from the available standards towards the other esters of testosterone by taking into account the different substituted chains. According to the length of the chain, a longer retention time could also be expected. These data have to be interpreted bearing in mind that they are a guide line for further investigation with the standards of the concerned compounds. The identity of a residue can only be assured when a proper standard is available for comparison.

Of all the injection sites analysed in our laboratory in 1993, 25% tested negative for residues of anabolic compounds, leaving 75% as positive samples. The most commonly found anabolic residues are summarized in Table 1. Of the positive injection sites 85% contained one or more esters of testosterone and/or estradiol. Thirty-five extracts of injection sites, that tested positive for esters with HPTLC, were examined using GC–MS. The results are summarized in Fig. 1.

It is apparent that testosterone-cypionate was by far the most commonly found residue in injection sites testing positive for testosterone-esters (T-E) (94%), followed by testosterone-decanoate, present in 50% of all T-E positive samples by HPTLC. The other esters were less well represented. Estradiol-benzoate represented 92% of the analyses testing positive for esters of estradiol, followed by estradiol-valerate with 35% of all estradiol-ester positive samples. The occurrence of a couple of other estradiol-esters was even less (4%).

The absence of some other esters such as testosterone-butyrate, -enanthate, -tosylate and several others, could be owing to the lack of a proper standard.

The spectra of some esters identified in this paper (Fig. 1) are summarized in Fig. 2.

Conclusions

As could be expected, it was not as simple to interface HPTLC to GC–MS for steroid residue confirmation as for thyreostatic

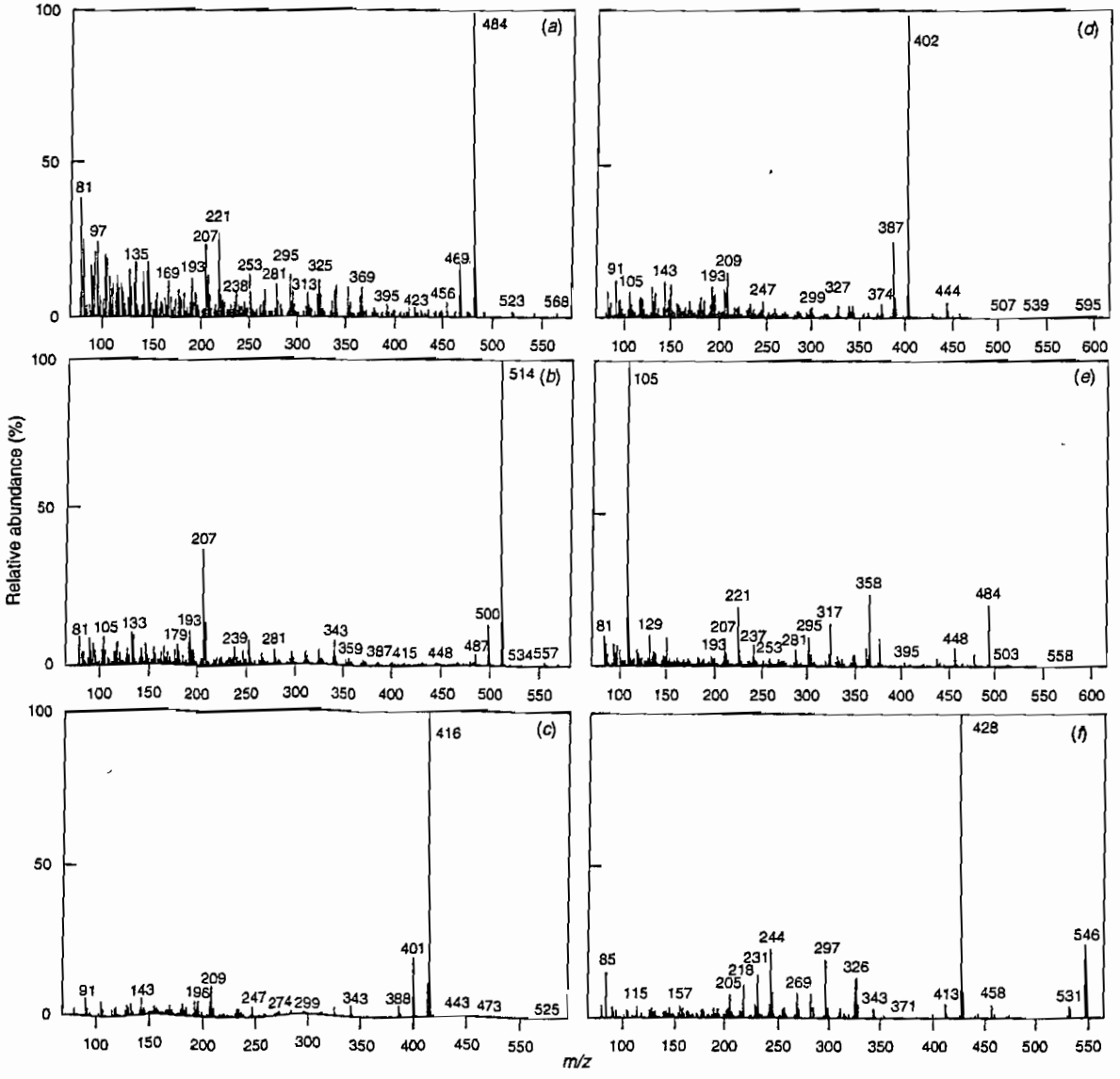

Fig. 2 Full scan mass spectra of some identified esters: (*a*) T-cypionate; (*b*) T-decanoate; (*c*) T-propionate; (*d*) T-acetate; (*e*) E_2-benzoate; and (*f*) E_2-valerate.

drug residues. GC–MS analysis had to be performed after the derivatization of the extract.

The interfacing of GC–MS to HPTLC was not only interesting for confirmational purposes but also gave the opportunity to identify unknown products as residues of recently introduced growth promoters or their metabolites. Besides the specific colour and R_F values on the plate, additional information was obtained in the form of a retention time value and the specific full-scan mass spectrum to obtain the best analytical results. This report has concentrated on the occurrence of long chain esters of the natural anabolic steroids in injection sites. Of the positive injection sites (analysed using HPTLC as well as GC–MS) 85% contained esters of estradiol and/or testosterone. Estradiol-benzoate (92%) and testosterone-cypionate (94%) were predominantly present in the positive samples. Other esters found in smaller percentages included estradiol-valerate, -cypionate and -dipropionate and testosterone-decanoate, -acetate and -propionate. Esters of nortestosterone were not found in the injection sites involved in this study. This could be an illustration of the decrease in the application of nortestostrerone and its esters in cattle fattening.[7]

Of all of the injection sites analysed during last year, 75% tested positive for one or more residues of anabolic steroids. This illustrates that the abuse of hormonal growth promoters is still prevalent. The use of GC–MS is certainly a progress in the battle against the illicit use of growth promoters in livestock production, not only for confirmational purposes but also for the identification of recently introduced compounds.

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