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Immunoaffinity Chromatography in Isolation of Anabolic Steroids

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Introduction

The misuse of anabolic steroids in human sports has led to a ban of this group of substances by the International Olympic Committee (since 1976) and international as well as national sport federations. The misuse of an anabolic steroid is confirmed by gas chromatographic-mass spectrometric (GC/MS) anaylsis of urinary extracts [1,2,3,4]. The identification is based on comparison of the EI mass spectrum and GC retetention time of the isolated anabolic steroid and/or its metabolite with authentic reference substance. The detection limit of this method is limited by the sensitivity of the analytical approach (GC/MS) and the biological background of the isolated sample.

Therefore the isolation step is an important fact and the aim is to concentrate the anabolic steroids and their metabolites and to reduce the biological background. In routine screening for anabolic steroids urine is enzymatically hydrolyzed after isolation of steroids and their conjugates via XAD-2 adsorption [1] or C-18 cartridges [4] and then extracted with diethyl ether under basic conditions. With this unspecific isolation technique on one hand polar and less polar anabolic steroids are quantitatively isolated, but on the other hand a high number of natural occuring substances are extracted too, increasing the biological background. Especially when positive cases with low concentration of an anabolic steroid or metabolite have to be confirmed the EI mass spectra can be interferred by coeluted peaks.

A more specific isolation with immunoaffinity chromatography (IAC) using antibodies against anabolic steroids and their metabolites should enhence the detection limit of the GC/MS analysis by a reduction of the biological background. This technique was described by Van Ginkel et al. for detection of nortestosterone in bovine muscle [5] and for trenbolone and its metabolites in bovine urine [6].

For our investigations we started with an imunoaffinty gel against methyltestosterone which was prepared by P.Delahaut with a maximium binding capacity for methyltestosterone of 600 ng/ml of gel. First results are summerized in this paper: The specifity of this gel for other 17α-methyl anabolic steroids and the conformation of a stanozolol positve urine sample.

Experimental

Immunoaffintiy chromatography (IAC)

All experiments were performed using the same gel. Immunoaffinity gel (1 ml) with a binding capacity of 600 mg methyltestosterone was transferred to a Econo-column (Biorad.Code 7371010). The gel was stored in PBS buffer (120 mmol NaCl, 2.7 mmol KCl, 10 mmol Na_2PO_4 $2H_2O$, adjusted to pH 7.4 with 6N HCl) containing 0.1% of NaN_3 at 4°C.

Flow scheme for sample preparation via IAC column:

at room temperature

- IAC column was washed with 5 ml of bidestilled water
- IAC column substance or biological extract was dissolved in 5 ml of PBS buffer and applied
- IAC column 1. washing with 5 ml of 10% of ethanol
- IAC column 2. washing with 5 ml of 10% of ethanol
- IAC column 1. elution with 3 ml of 70% of ethanol
- IAC column 2. elution with 3 ml of 80% of ethanol
- IAC column 3. elution with 3 ml of 80% of ethanol
- IAC column washing with 10 ml of PBS puffer, 0.1%NaN_3
- IAC column stored at 4°C or used for next isolation

Experiment A: Methyltestosterone (750 ng) or steroid (750 ng) was dissolved in PBS buffer and applied to the IAC column. The column was washed and eluted as described above. To the washing phases and eluates were added 750 ng of [20,20,20-$^2H_3$]methyltestosterone as internal standard. The ethanolic eluates were evaporated to dryness under vacuo and then derivatized. The washing phases were extracted with 5 ml of diethyl ether, the etheral layer was concentrated to dryness and then derivatized.

Experiment B: Methyltestosterone (750 ng) and steroid (750 ng) were dissolved in 5 ml PBS buffer and applied together to the IAC column.The column was washed and eluted as described above. To the washing phases and eluates were added 750 ng of [20,20,20-$^2H_3$]methyltestosterone as internal standard. The ethanolic eluates were evaporated to dryness under vacuo and then derivatized. The washing phases were extracted with 5 ml of diethyl ether, the etheral layer was concentrated to dryness and then derivatized.

Experiment C: A stanozolol positive urine sample was isolated via IAC and compared with the isolated sample of the normal screening procedure.
The urinary excreted stanozolol and its metabolites (all excreted conjugated) were isolated as follows:
4 ml of urine was adsorbed on Amberlite XAD-2 polystyrene resin. The XAD-2 column (pasteur pipette, closed with a glass pearl, bed height 2 cm) was washed with 2 ml of bidestilled water and eluted with 2 ml of methanol. The methanolic eluate was evaporated to dryness and the residue was dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7. To the buffer solution 50 μl of ß-glucuronidase from E.coli (K12, Boehringer, 6800 Mannheim) was added and after 1 h at 50°C the buffer solution was alkalized with 250 μl of 5% potassium carbonate solution to pH 9-10 and the steroids were extracted with 5 ml of diethyl ether on a vibro mixer for 30 sec. After centrifugation the etheral layer was transferred and evaporated to dryness under vacuo.
For the normal conformation the extract was derivatized and for the additional IAC cleaning step the dry residue was dissolved in 5 ml of PBS buffer and applied to the IAC column. The first eluate was evaporated to dryness and derivatized.

Derivatization for GC-MS analysis

The dry residue is derivatized with 100 μl of MSTFA/NH_4I/dithioerythritol 1000:2:4 (v/w/w) and heated for 15 min at 60°C.
This reaction mixture is equivalent to a mixture of MSTFA/TMIS 1000:2 (v/v) [7].

GC-MS parameters

GC-MSD HP 5890 / HP 5970
carrier gas: helium 1 ml/min at 180°C, split 1:10,
Column: Hewlett-Packard; Ultra-1 fused silica, crosslinked methyl silicone (OV 1), 17 m, i.D. 0.2 mm, film thickness 0.11 μm.
temperature program: 180°C - 15°/min - 320°C,
SIM (selected ion monitoring) with m/z 446 for methyltestosterone,bis-TMS, m/z 449 for [20,20,20-$^2H_3$]methyltestosterone,bis-TMS, m/z 143 and 472 for stanozolol,bis-TMS and m/z 143, 254, 545, and 560 for 3'-hydroxy-stanozolol,tris-TMS and 4ß-hydroxy-stanozolol,tris-TMS.

Results and discussion

Stability and binding capacity of IAC gel

Only one IAC gel (1ml) was used for all experiments. This gel was tested each starting day for its maximum binding capacity for methyltestosterone. The results are listed in Table 1 and show a decrease from about 600 ng within the experimental time to 500ng.

Table 1: Maximum binding capacity of IAC gel (1 ml) for methyltestosterone (during investigation time)

maximum binding		maximum binding	
date	[ng]	date	[ng]
27.6.91	585	10.7.91	468
28.6.91	597	12.7.91	393
30.6.91	650	13.8.91	498
2.7.91	600	14.8.91	485
3.7.91	545	15.8.91	N.E.
4.7.91	497	20.8.91	468
5.7.91	451	12.9.91	507

N.E. not estimated

Binding capacity of 17ß-hydroxy-17α-methyl steroids

The immunoaffinty gel for methyltestosterone shows cross reactivity to other steroids especially to 17ß-hydroxy-17α-methyl steroids. The binding capacity of other steroids was estimated and compared with the binding capacity of methyltestosterone.
The maximum binding capacity for methyltestosterone and all other steroids was estimated using [20,20,20-$^2H_3$]methyltestosterone as internal standard. For this calculation the buffer solution (after passing the IAC gel), the washing phases and the eluates were analyzed. A typical IAC protocol for methyltestosterone, mestanolone, 6ß-hydroxymetandienone, oxandrolone, and 17-epioxandrolone is shown in Table 2.

Table 2: Immunoaffinity chromatography
of 750 ng of 17-hydroxy-17-methyl steroids

	PBS buffer[a]	Washingphase		Eluate		
		1.	2.	1.	2.	3.
Steroid	%	%	%	%	%	%
Methyltestosterone	0	22.5	4.8	67.6	5.1	0
Mestanolon	10.0	26.0	5.9	55.9	2.2	0
6ß-HO-metandienone	4.7	65.1	6.5	22.5	2.2	0
Oxandrolon	0	34.1	16.0	49.9	0	0
Epioxandrolon	62.0	38.0	0	0	0	0

% values are calculated relative to the applied amount of 750ng
a Buffer solution after passing the IAC gel

Table 3 displays the binding capacity of the investigated steroids in % of the binding capacity for methyltestosterone.
The high binding of 17α-methyl-17ß-hydroxy steroids show the specifity of the antibody for the D-ring. No binding is observed when the D-ring has a 17α-hydroxy-17ß-methyl configuration (17-epimetandienone, 17-epioxandrolone), a 17-keto configuration (androsterone) or 17α-hydroxy configuration (17-epitestosterone). Testosterone with a 17ß-hydroxy group is still bound to a high extend. The binding of 17ß-hydroxy-17α-methyl steroids is reduced when a further hydroxy group is located at position 11α (11α-hydroxymethyltestosterone), 11ß (fluoxymesterone), or 6ß (6ß-hydroxymetandienone).

Table 3: Binding capacity of 1 ml methyltesosterone IAC gel for steroids

Steroid	ng	%[a]
Methyltestosterone (I)	550	100
17α-Methyl-5α-androstane-3α,17ß-diol	364	66
17α-Methyl-5α-androstane-3ß,17ß-diol	433	79
17α-Methyl-5ß-androstane-3α,17ß-diol	273	50
17α-Methyl-5ß-androstane-3ß,17ß-diol	245	45
17-Epimethyltestosterone	35	6
17α-Methyl-5α-androstan-17ß-ol-3-one	435	79
11α-Hydroxymethyltestosterone	210	38
Metandienone	504	92
17-Epimetandienone	0	0
6ß-Hydroxymetandienone	180	33
Bolasterone	475	86
4-Chlorodehydromethyltestosterone	467	85
Fluoxymesterone	90	16
Furazabol	481	87
Oxandrolone	464	84
17-Epioxandrolone	0	0
Oxymesterone	568	103
Stanozolol	590	107
3'-Hydroxystanozolol	390	71
4ß-Hydroxystanozolol	174	32
4α-Hydroxystanozolol	260	47
Androsterone	19	3
Testosterone	440	80
Epitestosterone	13	2

a Binding capaciy calculated in % to the binding capacity of methyltestosterone at the experimental day

Binding of endogenous excreted steroids

The binding of endogenous excreted steroids to the IAC gel was investigated with a mixture of steroids (results see Table 4). Average steroid amounts were applied corresponding to 2 ml and 4 ml of urine.
The results show that normal excreted steroids with the exception of testosterone are less bound to the IAC gel.

Table 4: Binding of endogenous steroids to IAC gel (1 ml)

Steroid	amount added [ng]	amount bound to IAC gel [ng]
Androsterone	4000 / 8000	71.2 / 99.6
Etiocholanolone	4000 / 8000	46.2 / 89.8
5α-Androstane-3α,17ß-diol	160 / 320	N.E. / 22.0
5ß-Androstane-3α,17ß-diol	240 / 480	N.E. / 5.4
Testosterone	80 / 160	80.4 / 147.6
Epitestosterone	80 / 160	0.0 / 0.0
11ß-Hydroxyandrosterone	800 / 1600	0.4 / 1.6
11ß-Hydroxyetiocholanolone	400 / 800	1.2 / 2.4
Dehydroepiandrosterone	800 / 1600	6.8 / 10.0
Pregnandiol	2000 / 4000	61.4 / 96.8
Tetrahydrocortisol	2000 / 4000	0.2 / 0.4
Cortisol	40 / 80	0.0 / 0.0

Binding capacity for methyltestosterone 550 ng
N.E. Not estimated

Binding of 17ß-hydroxy-17α-methyl steroids in concurrence with methyltestosterone

A further investigation shows the binding of 17ß-hydroxy-17α-methyl steroids in concurrence with methyltestosterone. The steroid (750 ng) was applied to the IAC gel together with methyltestosterone (750 ng) both dissolved in 5 ml of PBS buffer. The results are listed in Table 5.

IAC isolation of 17ß-hydroxy-17α-methyl steroids from urine

In the first experiments with urine we spiked urine samples with standards and applied the urine to the IAC gel. But the GC/MS chromatograms showed no reduced background. The reason was a high adsorption of sugars and glucuronides by the gel itself.
To circumvent this problem urine samples were prepared as described for the normal screening procedure and then in addition isolated via IAC gel.
This work is still under investigation and we would like to present as first results the conformation of a stanozolol positive urine sample isolated by our normal procedure and by an additional IAC cleaning step.

Table 5: Binding capacity of 1 ml methyltestosterone immunoaffinity chromatography gel for steroids (A) and in concurrence with methyltestosterone (B)

Steroid	A %	B %
Methyltestosterone (I)	100	
17α-Methyl-5α-androstane-3α,17ß-diol	66	22
17α-Methyl-5α-androstane-3ß,17ß-diol	79	33
17α-Methyl-5ß-androstane-3α,17ß-diol	50	13
17α-Methyl-5ß-androstane-3ß,17ß-diol	45	7
17-Epimethyltestosterone	6	2
17α-Methyl-5α-androstan-17ß-ol-3-one	79	69
Metandienone	92	46
Bolasterone	86	40
Testosterone	80	11
Epitestosterone	2	1

A Binding capacity of steroid calculated to the binding capacity of methyltestosterone (see also Table 3)

B Steroid and methyltestosterone are applied together to the IAC gel, the amount of steroid bound is calculated relative to the amount of methyltestosterone bound.

The SIM-chromatograms are shown in Fig.1A (normal procedure) and Fig.1B (IAC isolation) and demonstrate excellent signals and a completely reduced background for the IAC SIM-chromatograms. The corresponding EI mass spectra of 3'-hydroxy-stanozolol,tris-TMS in Fig 2A. (normal procedure), Fig. 2B (IAC) and of 4ß-hydroxy-stanozolol,tris-TMS in Fig.3A (normal procedure) and Fig.3B (IAC isolation) show clear EI-spectra only for the IAC samples wheras the EI-spectra of the normal extracts are disturbed by coeluted signals.

The use of an IAC gel for methyltestosterone to isolate stanozolol and its metabolites from human urine is possible, even the extraction yield of 4ß-hydroxystanozolol is half of the extraction yield of 3'-hydroxystanozolol, but the reduced biological background enhenced the GC/MS confirmation of the isolated metabolites.

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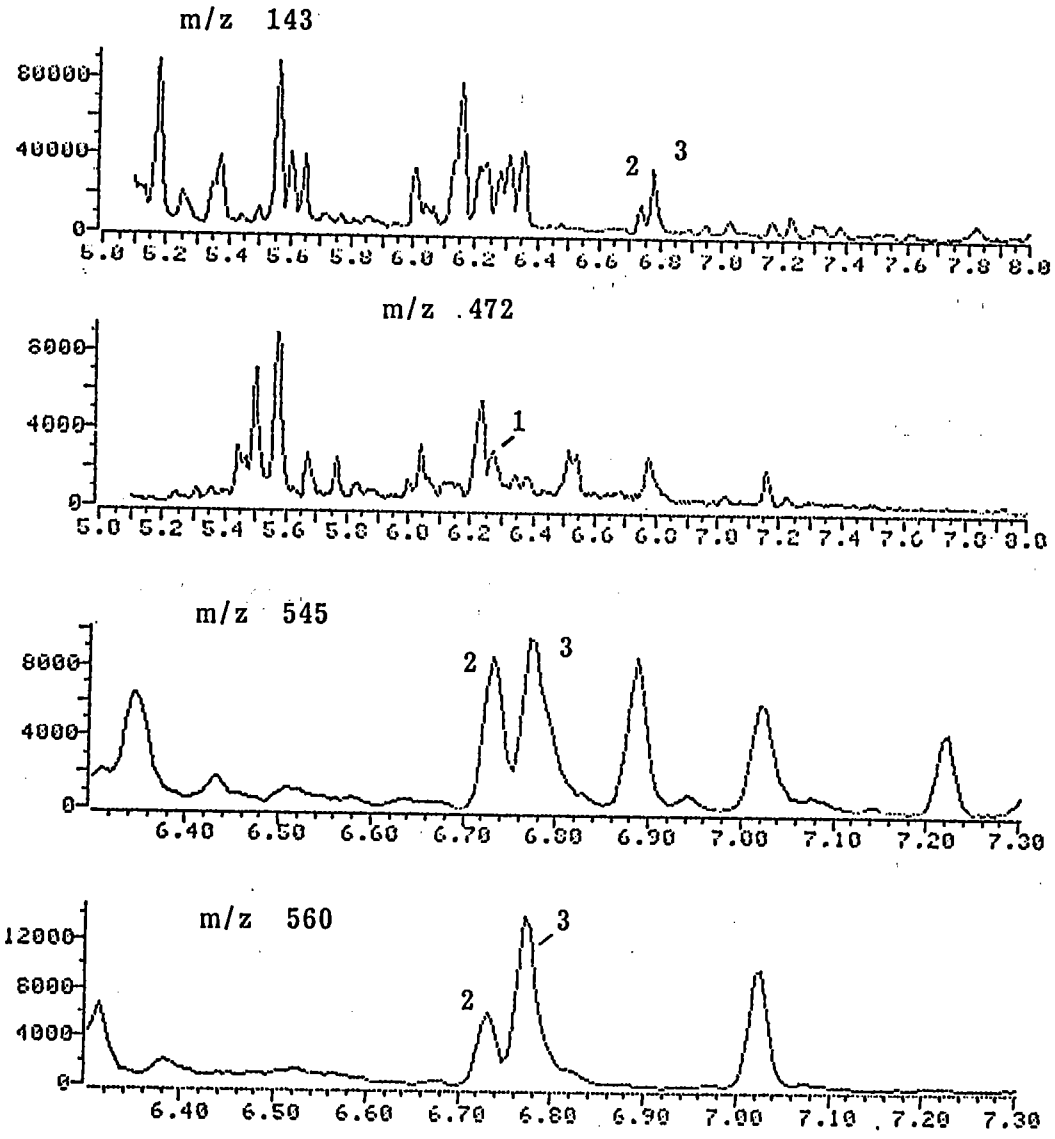
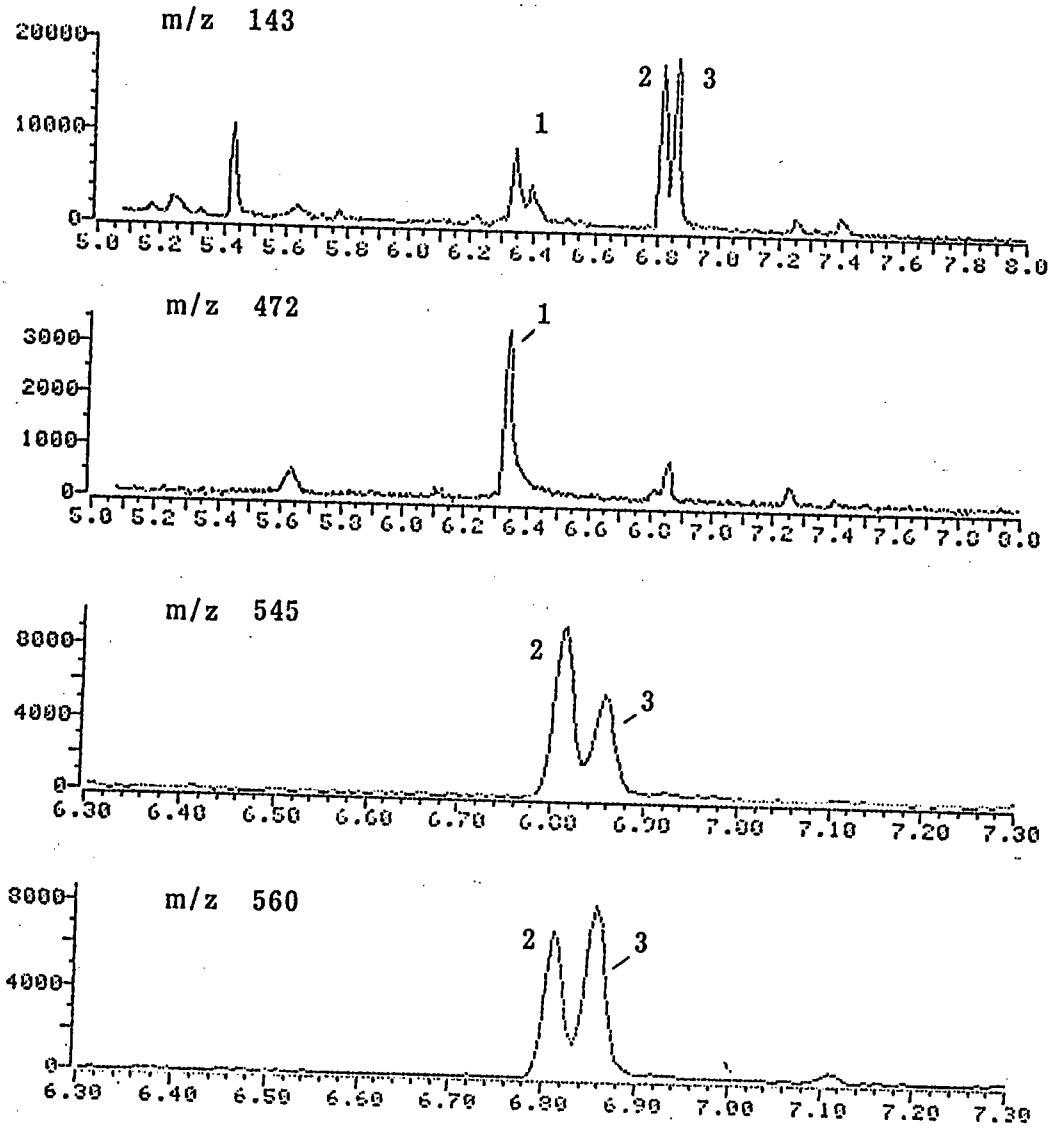


Fig,1A GC/MS SIM chromatogram of a stanozolol positive urine sample (normal extraction procedure), (1) stanozolol,bis-TMS (m/z 143, 472), (2) 3´-hydroxystanozolol,tris-TMS (m/z 143, 545 and 560), and (3) 4β-hydroxystanozolol,tris-TMS (m/z 143, 545, and 560)



Fig,1B GC/MS SIM chromatogram of a stanozolol positive urine sample (IAC isolation), (1) stanozolol,bis-TMS (m/z 143, 472), (2) 3'-hydroxystanozolol,tris-TMS (m/z 143, 545 and 560), and (3) 4β-hydroxystanozolol,tris-TMS (m/z 143, 545, and 560)

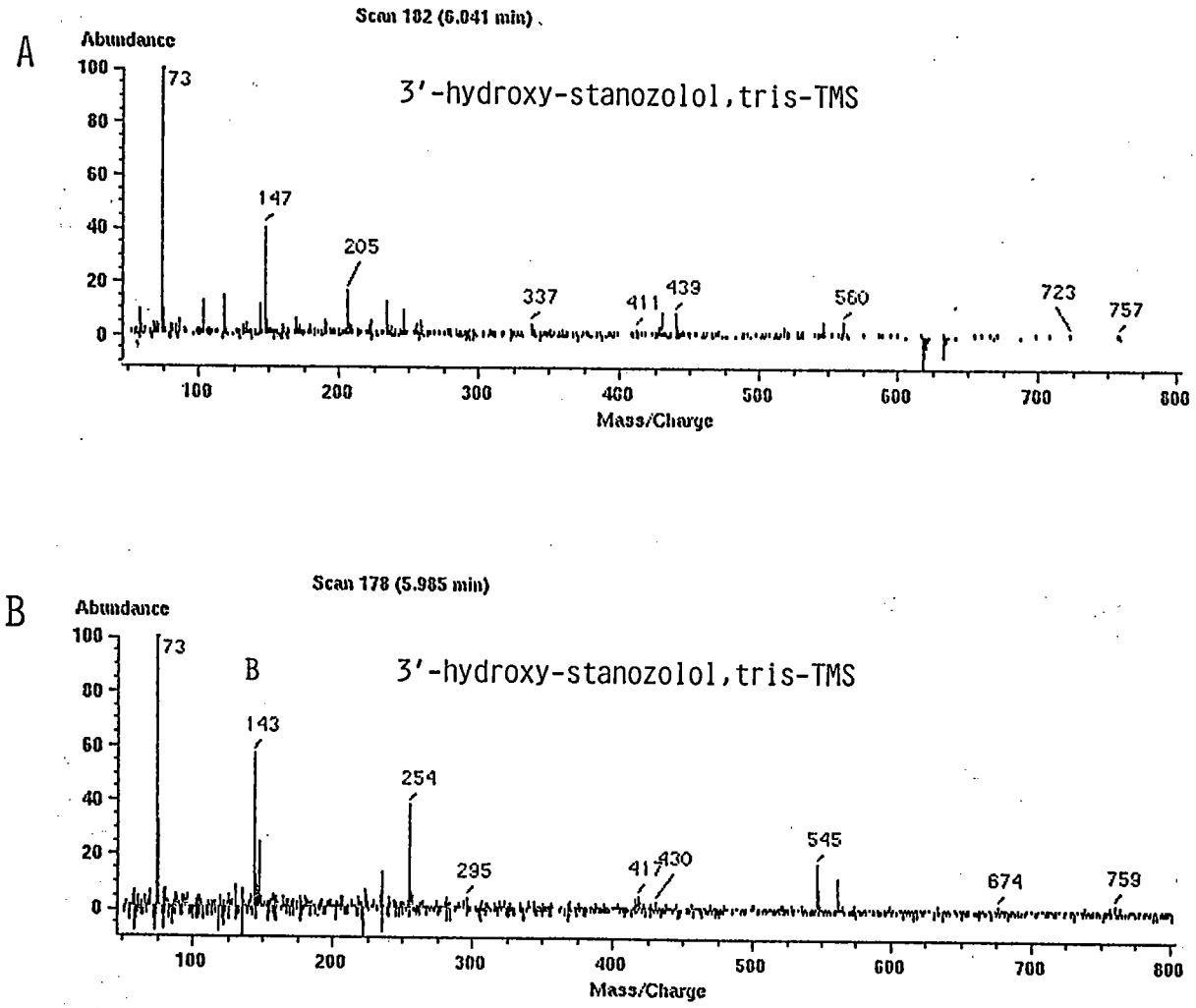


Fig.2 EI-mass spectra of 3'-hydroxystanozolol,tris-TMS obtained from a stanozolol positive urine sample
A) Normal sample preparation procedure (Fig.1A peak 2)
B) Sample preparation via IAC gel (Fig.1B peak 2)

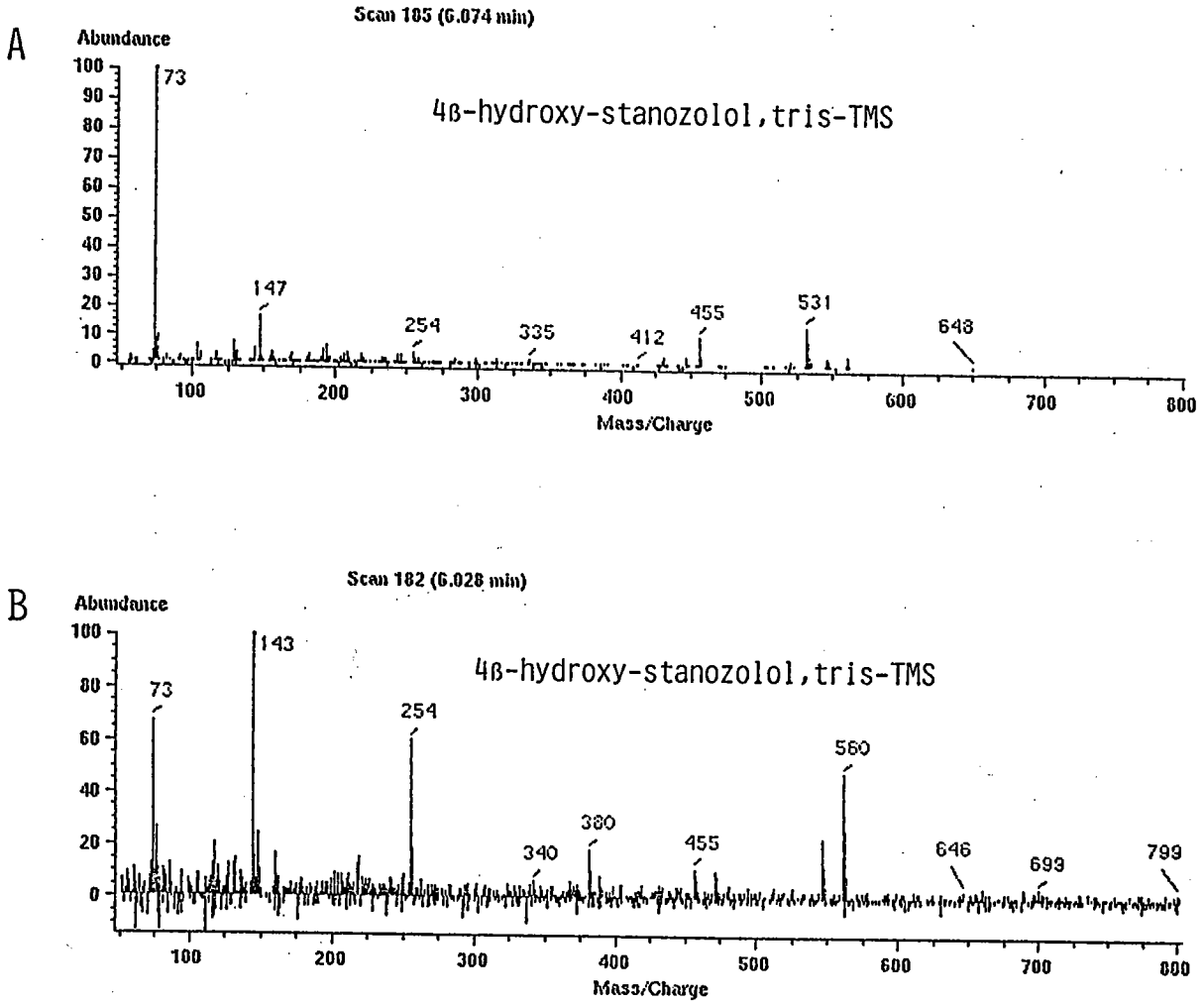


Fig.3 EI-mass spectra of 4ß-hydroxystanozolol,tris-TMS obtained from a stanozolol positive urine sample
A) Normal sample preparation procedure (Fig.1A peak 3)
B) Sample preparation via IAC gel (Fig.1B peak 3)