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RECENT ADVANCES IN DOPING ANALYSIS (9)

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Benzbromarone, a possible masking agent of anabolic androgenic steroids (2)

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INTRODUCTION

We reported, in the previous work [1], the influence of benzbromarone on the renal excretion of the testosterone and its degradation products. It could complicate the detection of the testosterone abuse by suppresing the concentration level of epitestosteron under the detection limit. Also, the concentrations of androsterone and etiocholanolone are reduced that way they disturb the normal steroid profile. It was also noticed that the intake change in the concentration level of other anabolic androgenic steroids, after the oral administration of benzbromarone.

In this paper, we are presenting evidence on the influence of benzbromarone, after its oral administration, on the renal excretion of some metabolites of metandienone and stanozolol, namely 6β-OH-metandienone, epimetendiol and 3'-OH-stanozolol, respectively, using the technique of GC/MS.

MATERIAL AND METHODS

Excretion studies with volunteers

Four volunteer male subjects, who performed no sport activities, participated in this experiment. The first two subjects, (subjects 1 and 2), were administered 30 mg of metandienone (*Naposim,* from Terapia Cluj, Romania) in a single dose, while the others, (subjects 3 and 4), were administered 20 mg of stanozolol (*Menabol,* from CFL Pharmaceuticals, India) in a single dose, too.

The next day, a dose of 100 mg of benzbromarone was given to the subjects 2 and 4.

The urine samples were collected from all subjects, before the administration and after this. The urine samples were collected everyday in the morning along a period of 7 days.

Isolation of the steroids

For isolation of the anabolic steroids from the urine samples, we used both the procedure of extraction by separating fractions (free and conjugated)[2] and the procedure of combined fractions [3]. The quantity of urine used was 2 ml and there were also used 20 µl deutereted internal standard obtained from the Doping Control Laboratory from Cologne.

Derivatization for GC/MS

For free fraction the dry residue is dissolved in 100 µl MSTFA/TMSC1/TMSIm (100:5:2), heated for 15 min at 60°C. 3 µl are injected into GC/MS. For combined fractions the dry residue is dissolved in 100 µl MSTFA/NH_4I/Dithioerythrytol (1000:2:3), heated for 15 min at 60°C. 3 µl are injected into GC/MS.

GC/MS analysis

Instrument: HP GC/MS (GC 6890/MS 5972).

EI ionisation energy: 70eV.

Capillary column: ULTRA 1, 17m length, ID=0.20mm, film thickness 0.11 µm.

Carrier gas: Helium 0.8ml/min; split injection mode (1:10).

Temperatures of injector and interface: 300°C.

Temperature program for free fraction: 2min 160°C, +10°C/min, 10 min 280°C.

Temperature program for conjugated and combined fractions: 2 min 160°C, + 5°C/min, 0 min 255°C, +30°C/min, 5 min 285°C, +60°C/min, 2 min 300°C.

The ions monitored for each metabolite were the following:

- 6β-OH-metandienone: m/z 143, 209, 281, 460;
- epimetendiol: m/z 216, 358, 448;
- 3'-OH-stanozolol: m/z 143, 254, 545, 560.

The quantification of the metabolites

The quantification of 6β-OH-metandienone, epimetendiol and 3'-OH-stanozolol was performed by GC/MS HP 6890/5980 in SIM mode, using the calibration method with internal standard [4,5,6]. We used methanolic solutions for each metabolite standards above mentioned, in concentration of 10 µg/ml. The concentrations in the samples are shown as in the Table 1.

Tabel 1. Concentration of the working solution and resulting concentrations per ml of urine

Nr.	working solution (10 µg/ml) (µl)	sample concentration (ng/ml)
1.	5	25
2.	10	50
3.	20	100
4.	30	150
5.	40	200
6.	50	250
7.	60	300
8.	70	350
9.	80	400
10.	90	450
11.	100	500

Results and discussions

Influence of benzbromarone on the excretion of 6β-OH-metandienone and epimetendiol

Identification of benzbromarone through GC/MS and characteristic mass spectra were widely presented in a previous study[1].

The benzbromarone was detected in urine for 2-3 days.

A single dose of 30 mg of metandienone was applied to two volunteer subjects. In the next day, a dose of 100 mg of benzbromarone was applied to one of the subjects.

Urine sample for dosage of two metabolites of metandienone were collected at the time intervals shown in the tables 2 and 3. Taking into account that the use of benzbromarone induces samples dilution, we used the correction depending on the density in order to calculate the concentrations [7]. Data from these tables were used to construct the plots presented in the figure 1.

Table 2. The dynamics of renal excretion of 6β-OH-metandienone and epimetendiol after application of 30 mg of metandienone (single dose)-**subject 1**

Probe number	Hours after administration	Density (g/cm^3)	pH	Concentration of 6β–OH-metandienone (ng/ml) free fraction	Concentration of epimetendiol (ng/ml) conjugated fraction
1	24	1.025	6.0	250.4	705.8
2	48	1.023	6.0	367.3	907.2
3	72	1.020	5.5	120.5	364.1
4	96	1.021	5.5	28.3	101.1
5	120	1.015	5.5	5	50.5
6	144	1.022	6.0	-	12.5
7	168	1.019	5.5	-	4.5

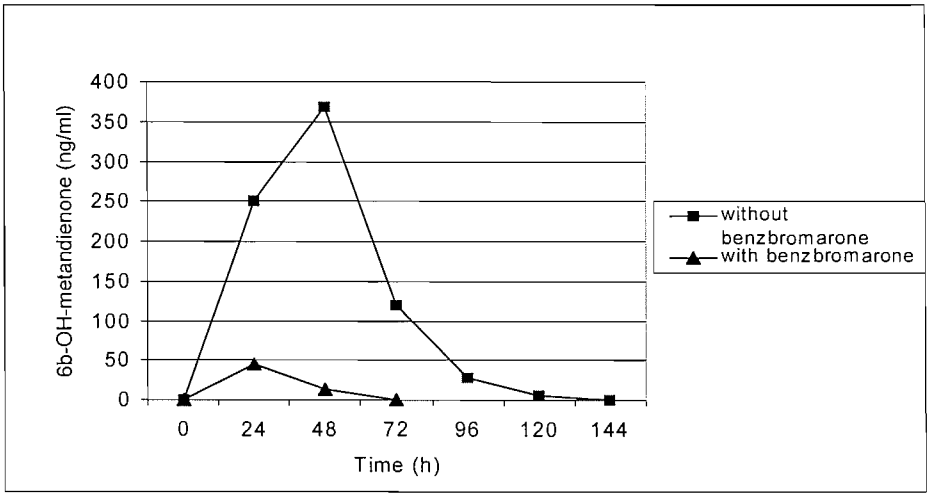

Fig.1 Excretion of 6β-OH-metandienone after oral administration of 30 mg. of metandienone **subjects 1 and 2**

Analysing the acquired data and the excretion curves for the two subjects, one may easily notice that the administration of benzbromarone significantly decreases the renal excretion of 6ß-OH-metandienone. Subject 1 wasn't administered benzbromarone and the 6 ß-OH metabolite could be dosed in about 120 h from the moment when metandienone administration was cut using benzbromarone. As far as concerns the subject 2, the 6 ß-OH metabolite could no longer be identified in 48 hrs after the administration of benzbromarone.

The renal excretion of epimetendiol is presented in the figure 2.

Table 3. The dynamics of renal excretion of 6β-OH-metandienone and epimetendiol after application of 30 mg of metandienone(single dose) and 100 mg benzbromarone, single dose -**subject 2.**

Probe number	Hours after administration	Density (g/cm^3)	pH	Concentration of 6β–OH-metandienone (ng/ml) free fraction	Concentration of epimetendiole (ng/ml) conjugated fraction
1	24	1.010	7.0	45.6	75.8
2	48	1.008	7.0	12.4	154.6
3	72	1.008	6.0	-	24.3
4	96	1.008	6.0	-	5.2

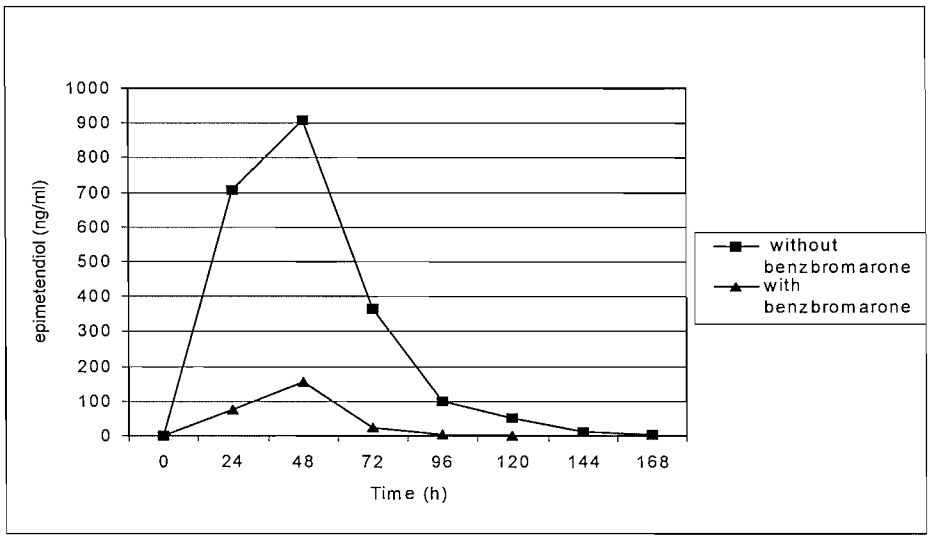

Fig. 2 Excretion of epimetendiol after oral administration of 30 mg. of metandienone

subjects 1 and 2

As far as concerning epimetendiol a cut of the renal excretion is also noticed under the action of benzbromarone, with the only exception that the identification and the dosing period increase up to 96 hrs as compared to 48 hrs for the 6 ß-OH-metandienone (Tables 2 and 3; Figure2).

Influence of benzbromarone on the 3'-OH-stanozolol excretion.

Stanozolol was administered in a single dose (20 mg) to two volunteer male subjects. In the next day, one of the subjects was administered 100 mg of benzbromarone. The acquired results for the two volunteer subjects are presented in tables 4 and 5 and the excretion rates of 3'-OH-stanozolol are illustrated in figure 3.

Table 4. The dynamics of renal excretion of 3'-OH-stanozolol after application of 20 mg of stanozolol(single dose) -subject 3.

Probe number	Hours after administration	Density (g/cm^3)	pH	Concentration of 3'-OH-stanozolol (ng/ml) combined fraction
1	24	1.018	5.0	308.9
2	48	1.020	6.0	257.8
3	72	1.022	5.5	56.3
4	96	1.023	5.5	18.5

Table 5. The dynamics of renal excretion of 3'-OH-stanozolol after application of 20 mg of stanozolol and 100 mg benzbromarone (both in a single dose) **-subject 4.**

Probe number	Hours after administration	Density (g/cm^3)	pH	Concentration of 3'-OH-stanozolol (ng/ml) combined fraction
1	24	1.010	5.5	103.7
2	48	1.007	6.0	36.8
3	72	1.008	6.0	19.6
4	96	1.005	6.5	-

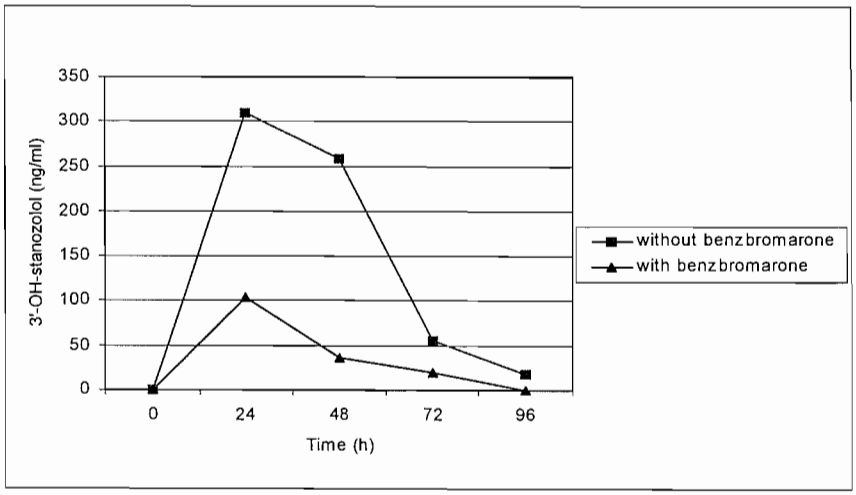

Fig. 3 Excretion of 3'-OH-stanozolol after the administration of 20 mg. of stanoizolol **subjects 3 and 4**

There was also noticed that the renal excretion of 3'-OH-stanozolol is diminished when benzbromarone is administered but the detection period through mass spectrometry is longer than in the case of the metandienone metabolites.

Conclusions

Our studies suggest that benzbromarone induces a decrease of renal excretion of the two metabolites of metandienone, shortening the period of their detection by the help of GC/MS.

In the case of stanozolol metabolite, a cut of the renal excretion was also found, when the benzbromarone was used, but the period of detection was longer than in the case of metandienone.

The obtained results suggest that the utilization of benzbromarone may make more difficult the anabolic steroids detection in the doping samples. We consider that in order to confirm these results, there are neccesary more profound studies on a larger number of volunteer subjects. At the same time, we should take into account the total excretion quantity.

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