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## Benzbromarone - A Possible Masking Agent of Androgen Anabolic Steroids; Identification by GC/MS and its Action upon the Steroid Profile (1)

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### INTRODUCTION

Benzbromarone is an uricosuric with high potency and long lasting effect and is set on the European market under various names: desuric, exurate, uricovac M, azubromarone, nancaricin, norumurate, etc.[2].

Benzbromarone has the following structural formula (Fig. 1).

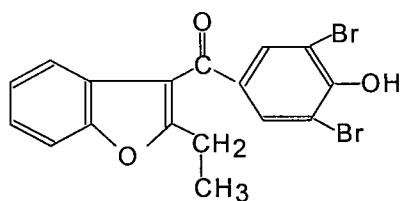


Figure 1. Benzbromarone structure

The therapeutical dose is generally between 40-80 mg per day. The uricosuric action is blunted by aspirin or sulfinpyrazone. The effect is intensified whenever large doses of benzbromarone are associated with orally administrated anticoagulant agents.

On the basis of a longitudinal study performed on the occasion of the doping controls, especially on the athletes of the disciplines requiring an increased muscular force and where the excessive use of anabolic agents is well known [8], the presence of benzbromarone was noticed in the urine samples [7].

This finding was the basis of the present study which has in view the action of benzbromarone upon some parameters of the steroid profile, especially upon the renal excretion of testosterone, and the influences of this drug on the detection of the androgen anabolic steroids abuse.

### MATERIAL AND METHODS

Three voluntary male subjects, who do not perform sport activities, took part in the experiment. A single dose of 100 mg of testosterone propionate was administrated to the first subject. Metandienone (20 mg/day) was administered to the second subject and stanozolol (5mg/day) to the third subject for 3 days each.. The urine samples for the identification of benzbromarone and the estimate of its action were collected from all the subjects before and after the administration of a dose of 100 mg of desuric and 24 hours after the anabolic treatment was stopped.

#### *Isolation of the steroids and benzbromarone*

The isolation of the steroid hormones from the urine samples was made using the procedure for the total fractions [1]. The quantity of urine used was 2 ml and *there were also used 20 μl deuterated internal standard obtained from the Doping Control Laboratory Cologne.*

In order to quantitate metandienone and stanozolol metabolites the urine samples collected from the subjects were passed through a Sep Pak C18 cartridge (previously washed with methanol and water). After washing with 5 ml of distilled water the steroids were eluted with 5 ml of methanol. To the methanolic eluates *20 μl of methyltestosterone* were added as internal standard. The solvent was

evaporated under a nitrogen stream; 1 ml of phosphate buffer (0.8 M, pH=7.0) and 20  $\mu$ l of  $\beta$ -glucuronidase (E. coli) were added and the mixtures heated at 37 $^{\circ}$  C for 12 hours. After the hydrolysis the samples were extracted and derivatized following the procedure used for total fractions [1,4].

### Derivatization for GC/MS

The dry residue is dissolved in 100  $\mu$ l of MSTFA/ $\text{NH}_4\text{I}$ /Dithioerythritol (1000:2:3) and heated for 15 min at 60 $^{\circ}$  C. 3  $\mu$ l are injected directly into GC/MS.

### GC/MS analysis

Instrument : GC/MSD HP 6890/HP 5972

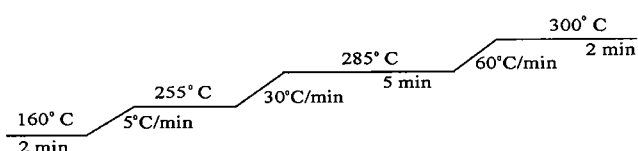
Column : CP - SIL 5CB (methyl silicone) 17 m length, I.D. 0.25 mm, film thickness 0.12  $\mu$ m;

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

Temperature program:

Injector temperature: 300 $^{\circ}$ C;

Interface temperature: 300 $^{\circ}$ C.



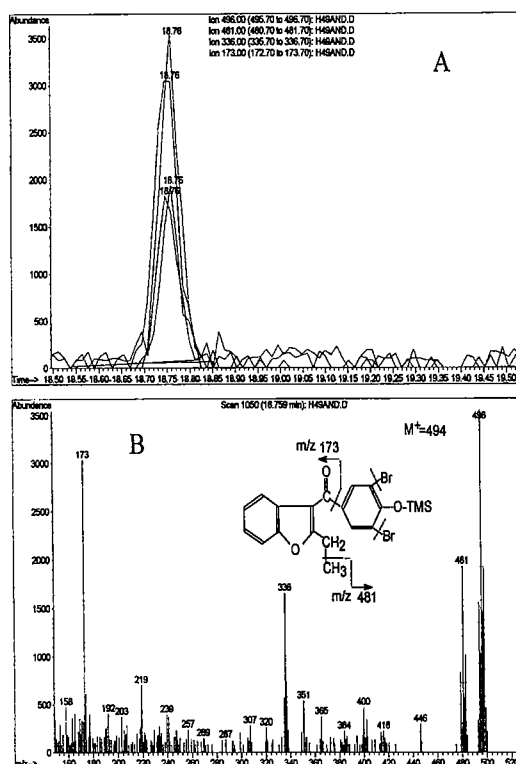
For the quantification of the endogenous hormones, epimetendiol (17 $\beta$ -methyl-5 $\beta$ -androst-1-ene-3 $\alpha$ ,17 $\alpha$ -diol) and 3'-hydroxy-stanozolol the selected ion monitoring (SIM) mode was used with specific ions and the method of calibration with deuterated internal standard.

## RESULTS AND DISCUSSION

### Identification of benzbromarone by GC/MS

Benzbromarone was isolated from urine following the procedure used for total fractions [1]. The dry residue was derivatized with 100  $\mu$ l from the mixture of MSTFA/ $\text{HN}_4\text{I}$ /Dithioerythritol (1000:2:3) and 1  $\mu$ l was injected in GC/MS, "SCAN" mode. The GC/MS conditions are those previously presented.

Figure 2 presents the EI mass spectrum of the mono-TMS derivative of benzbromarone. In the temperature program used the retention time is 18.76 min.



**Figure 2.** GC/MS analysis (full scan mode): A.Total ion chromatogram; B. Mass spectrum of urinary mono-TMS-benzbromarone

The mass fragments of the mono-TMS derivative of benzbromarone are:  $m/z$  494 ( $M^+$ ),  $m/z$  479,  $m/z$  336 and  $m/z$  173. The mass fragment  $m/z$  479 comes from  $M^+$ -15 and  $m/z$  336 is formed by a loss of bromine from  $M^+$ .

### ***Influence of benzbromarone upon some parameters of the steroid profile***

To one voluntary male subject a single dose of 100 mg of testosterone propionate was applied by intramuscular injection and 24 h after a dose of 100 mg of benzbromarone. The urine samples destined to the *establishment* of the steroid profile were collected before and after the administration of benzbromarone.

The *dosage* of the steroid hormones was made using the GC/MS Hewlett Packard (GC 6890/MS 5972) system [3].

The evolution of the following steroid hormones was supervised: androsterone, etiocholanolone, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, epitestosterone, testosterone, 11 $\beta$ -hydroxy-androsterone, 11 $\beta$ -hydroxy-etiocholanolone and pregnanediol.

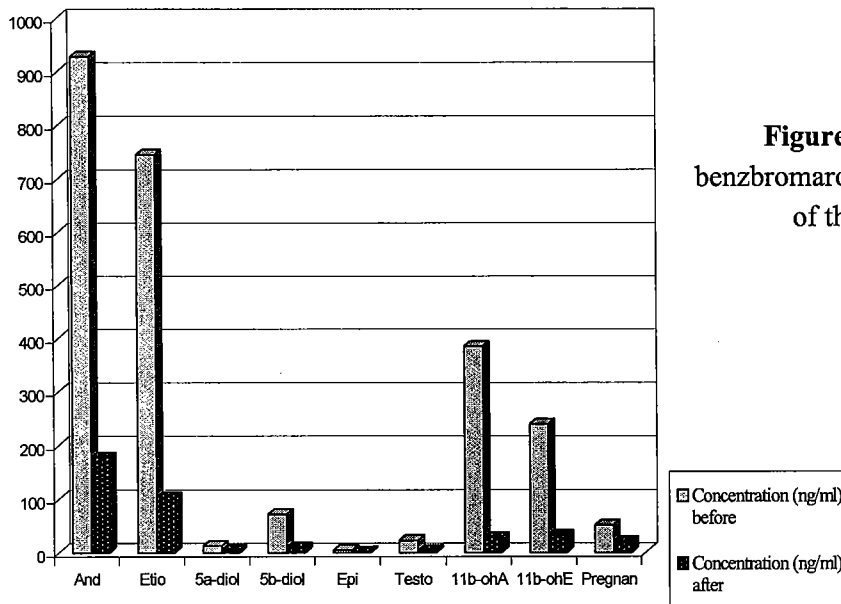
**Table 1** presents the retention times (RT), ions monitored, areas and concentrations of endogenous steroids before and after administration of benzbromarone

Abbreviations	RT	m/z	Concentration (ng/ml)		Area	
			Before	After	Before	After
AND	14.00	434	928.56	177.78	68450	13100
ETIO	14.14	434	745.58	103.79	56947	7928
5 $\alpha$ A-3 $\alpha$ ,17 $\beta$ D	14.24	241	32.15	10.17	3975	1257
5 $\beta$ A-3 $\alpha$ ,17 $\beta$ D	14.37	241	71.48	23.75	8972	2981
EpiT	15.13	432	20.5	6.17	1389	598
T	16.17	432	45.97	10	10200	2275
11 $\beta$ -OH-AND	16.18	522	385.45	28.14	12299	898
11 $\beta$ -OH-ETIO	16.32	522	239.37	32.67	3465	473
Pregnanediol	17.63	117	51.40	20.05	72034	28104

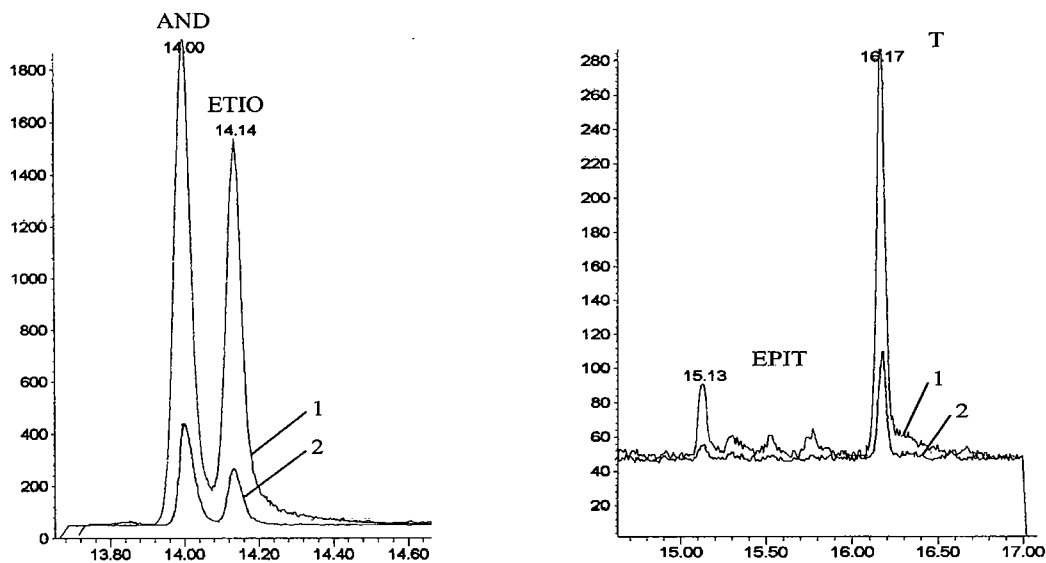
**Table 2.** The values of some parameters of the steroid profile before and after administration of benzbromarone determined in the total fractions

Parameter	Value before adm.	Value after adm.
AND / ETIO	1.21	1.65
5 $\alpha$ A-3 $\alpha$ ,17 $\beta$ D / 5 $\beta$ A-3 $\alpha$ ,17 $\beta$ D	0.44	0.42
T / EpiT	7.34	3.80
11 $\beta$ -OH-AND / 11 $\beta$ -OH-ETIO	3.54	1.89
AND / T	7.30	5.60

It can be easily noticed that the use of benzbromarone leads to a reduction of the renal excretion of all the steroid hormones monitored (Fig. 3).



**Figure 3.** The influence of benzbromarone upon the renal excretion of the steroid hormones



**Figure 4 and 5** Influence of benzbromarone upon the excretion of androsterone, etiocholanolone, testosterone and epitestosterone.

1. Chromatogram before administration;

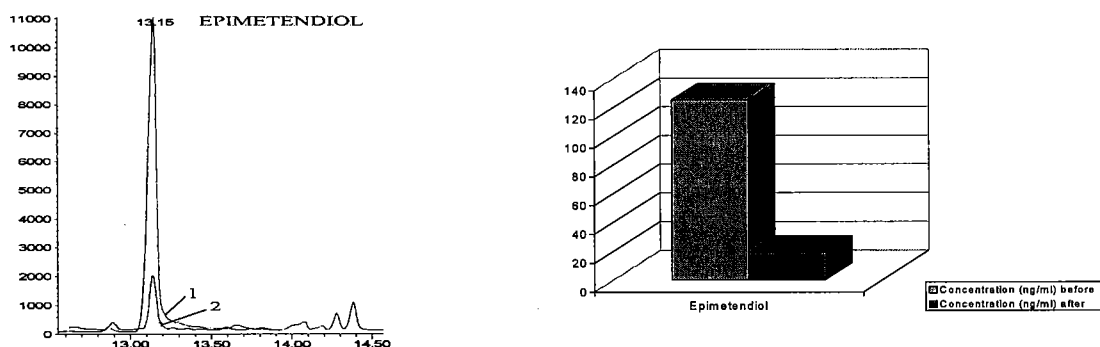
2. Chromatogram after administration.

Analysing the pair AND-ETIO (Fig. 4) we notice that, although the excretion of both hormones is suppressed after the use of benzbromarone, the ratio AND/ETIO increases from 1.21 to 1.65 (Table 2) as a result of the more accentuated decrease of etiocholanolone compared to that of androsterone. The ratios T/Epit are significantly decreased after the use of benzbromarone (Table 2 and Fig.5).

We believe that benzbromarone can mask the testosterone abuse by significantly decreasing the excretion of testosterone and of epitestosterone. Many times the concentration level of epitestosterone decreases under the detection limit. Another result was that the excretion of pregnanediol is approximately 2.5 times lower after the use of benzbromarone.

## Influence of benzbromarone upon the anabolic agents excretion (metandienone and stanozolol)

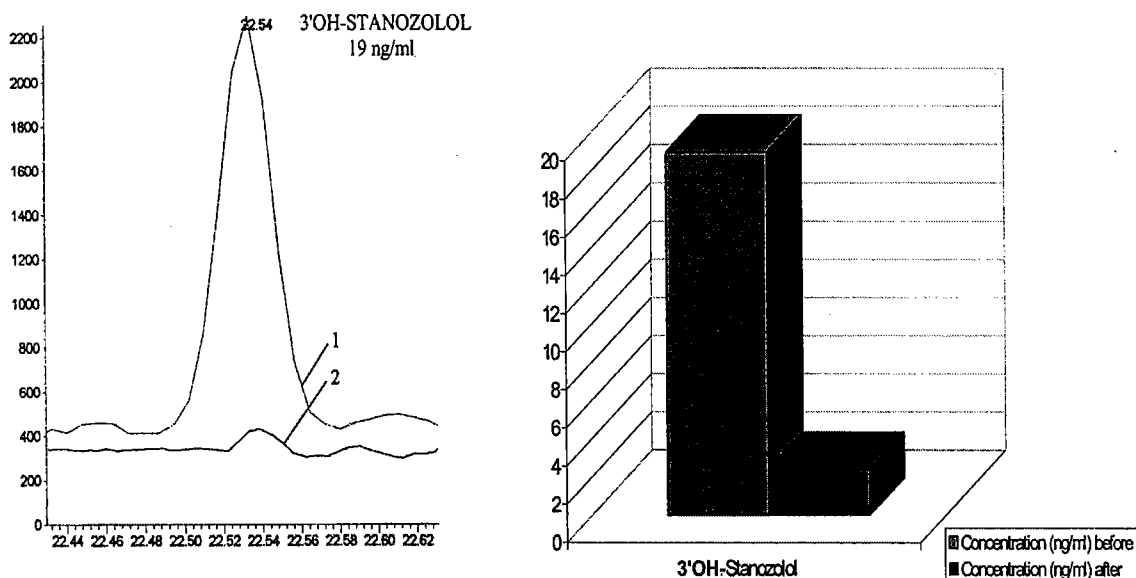
Taking into account the influence of benzbromarone upon the steroid profile, we *proposed*, as a natural step forward, to verify if it can influence the detection of the anabolic agents by “low resolution” mass spectrometry. So we studied metandienone and stanozolol, which are most frequently found in the samples collected for doping control. We point out that we are presenting here a preliminary case study and we are going to examine the influence of benzbromarone upon the level of excretion of the two steroids in a future paper.



**Figure 6 and 7** Influence of benzbromarone upon the renal excretion of epimetendiol.  
 1. Chromatogram before administration;      2. Chromatogram after administration;

**Table 3.** Retention times (RT), ions monitored, areas and concentrations of epimetendiol and 3'-hydroxy-stanozolol before and after administration of benzbromarone

Substance	RT	m/z	Concentration (ng/ml)		Area	
			Before	After	Before	After
Epimetendiol	13.15	143, 216, 358, 448	125	18.8	273034	41046
3'-OH-stanozolol	22.54	143, 254, 545, 560	19.0	2.4	47078	12094



**Figure 8 and 9.** Influence of benzbromarone upon the renal excretion of 3'-OH-stanozolol.  
 1. Chromatogram before administration;      2. Chromatogram after administration;

As it can be noticed from Fig.8 and 9, in both cases the concentration of the two metabolites is significantly decreased after the use of benzbromarone. After its administration the concentration of epimetendiol decreases from 125 ng/ml to 18.8 ng/ml (Fig. 7), while the concentration of 3'-OH-stanozolol decreases from 19 ng/ml to 2.4 ng/ml (Fig.9).

## CONCLUSIONS

- Benzbromarone reduces the urinary concentrations of epimetendiol and 3'-hydroxy-stanozolol which are excreted as glucuronides. One might expect that it will reduce the renal excretion of other androgen anabolic steroid metabolites.
- It could complicate the detection of the testosterone abuse by suppressing the concentration level of epitestosterone under the detection limit. Also the concentrations of androsterone and etiocholanolone are reduced that way they disturb the normal steroid profile.
- *For the elucidation of the influence of benzbromarone upon the renal excretion of anabolic steroids it is necessary the monitorization for a long period of time.*

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