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Amiloride – Detection and Excretion Study under Conditions of Steroid Screening Procedure

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INTRODUCTION

Amiloride -3,5 Diamino- N-(aminoiminomethyl) -6-chloropyrazine-carboxamide (Fig.1) is one of the potassium saving diuretics, which are clinically used in cases of potassium deficiency [1]. It is included in the list of banned substances of IOC [2].

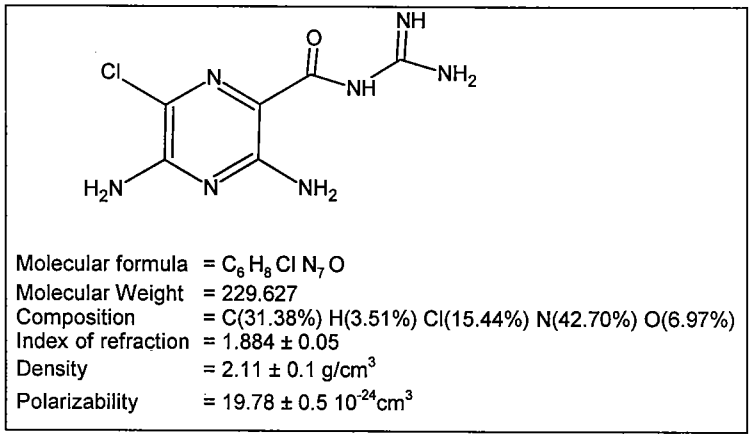


Fig.1. Structure formula and some theoretical constants of Amiloride.

One of the reasons for more rarely use of Amiloride as diuretic in sport is its effect of a low urinary flow [3]. The problems of detection of Amiloride by the diuretics screening procedure are known. The analytical difficulties result from its structure (very polar molecule containing guanidine and amino groups) which causes a limited solubility in usual organic solvents and problems with derivatization [4-7]. Therefore the aim of our work was to study the behaviour of Amiloride by methods of some screening procedures and to collect data of its excretion.

EXPERIMENTAL

1. Excretion study. An excretion study after an oral application of a therapeutical dose of Amiloride was performed. One volunteer ingested a single dose of 4mg of pure Amiloride. The urine samples were collected up to 70 hours after administration.

2. Sample preparation for excretion study.

	To 2ml urine add ISTD Methyltestosterone 40µl (25ppm) apply on XAD -2 column, wash with 2 ml of water elute with 3 ml of methanol, evaporated to dryness
ADD	1ml phosphate buffer 0.2M to pH 7.0 25µl β-glucuronidase from E.coli
HYDROLYSIS	1 hour at 55°C
ADD	100 mg K_2CO_3 to adjust pH 9.6 5 ml of diethyl ether
EXTRACTION	shake for 20 min.,centrifuge 5 min. evaporate to dryness
DERIVATIZATION	100 µl MSTFA/NH_4J/ethanethiol 1000:2:6 (v:w:v), 20 min. at 80°C, inject into GC/MS – 1 µl

3. GC/MS parameters

3) GC/MS:HP 5890/5970, carrier gas He - 1.8ml/min. flow, split ratio 1:10, Column Chrompack CP-Sil 5 CB, 0.25 mm i.D., 0.25 µm film thickness, Temperature program: 150°C/ 1 min./ 13°C/213°C; 2°C/220 °C; 20°C/300°C, Carrier: - 1 ml/min He; Split ratio 1:10; Transfer line – 280 °C; injector – 280 °C. SIM mode for enol-MSTFA derivatives (dwell time: 47 ms) – 293,300,302,308, 310,316, 318, 331, 333, 365, 380, 388,390,403,405,413,415,428,430,476, 478.

4. Extraction study.

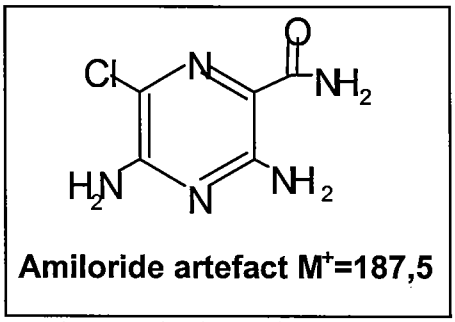
a) Liquid/Liquid extraction - Experiments were performed by 3ml spiked urine with 1 µg/ml Amiloride. Adjust the pH of urine to 9.6 with K_2CO_3/$NaHCO_3$ (2:1), add 5 ml of corresponding solvent and shake for 20 min. Centrifuge, freeze and transfer organic layer to a fresh tube. Dry the sample under a stream of nitrogen in water bath at 40°C. For HPLC analysis samples were dissolved with 200 µl of the mobile phase. The following organic solvents were tested: diethyl ether(DE), ethylacetate (EA) and tert-butylmethyl ether (t-BME).

b) Solid phase extraction (SPE) on XAD-2 - Spiked urine with Amiloride (1 μg/ml) was applied on a XAD-2 column. The preconditioning of the column and the elution of the sample were carried out as described in the excretion study - sample preparation. The methanol was removed under a stream of nitrogen and the residue was reconstituted in 200 μl of the mobile phase for HPLC.

c) HPLC conditions – Waters liquid chromatograph, column "NovaPack C18" 150x 39 mm, 4 μm, mobile phase phosphate buffer (pH=2.2), methanol and acetonitrile (85:10.5:4.5) (v/v/v), flow 0.7 ml/min, λ=280 nm.

RESULTS AND DISCUSSION

The GC property of Amiloride is very poor. One product with a lower molecular mass than that of the underivatized Amiloride was detected by GC/MS analysis. The mass spectrum of this product is shown in Fig.2 a). The retention time (2.26 min), intensive ions at m/e 187,189 and other fragments ions in the MS spectrum support the suggestion that this is an Amiloride artefact with following proposed structure:



Amiloride artefact M^+=187,5

Amiloride's methylation under standard conditions for diuretics, in the presence of acetone and K_2CO_3 do not create an appropriate medium for proceeding of this reaction.After derivatization for 5h at 60°C the GC/MS analysis gave rise to signals of the following compounds: Amiloride artefact, monomethylated, dimethylated and trimethylated Amiloride, hexamethylated and heptamethylated Amiloride. The experiment for extractive methylation of Amiloride was not successful. The other attempts for derivatization of Amiloride with MSTFA/MBTFA; MTBSTFA, CS_2 also failed. The spectra of the underivatized Amiloride artefact, the bis- and tetrakis-TMS-derivatives of Amiloride achieved by TMIS, shown in fig. 2.

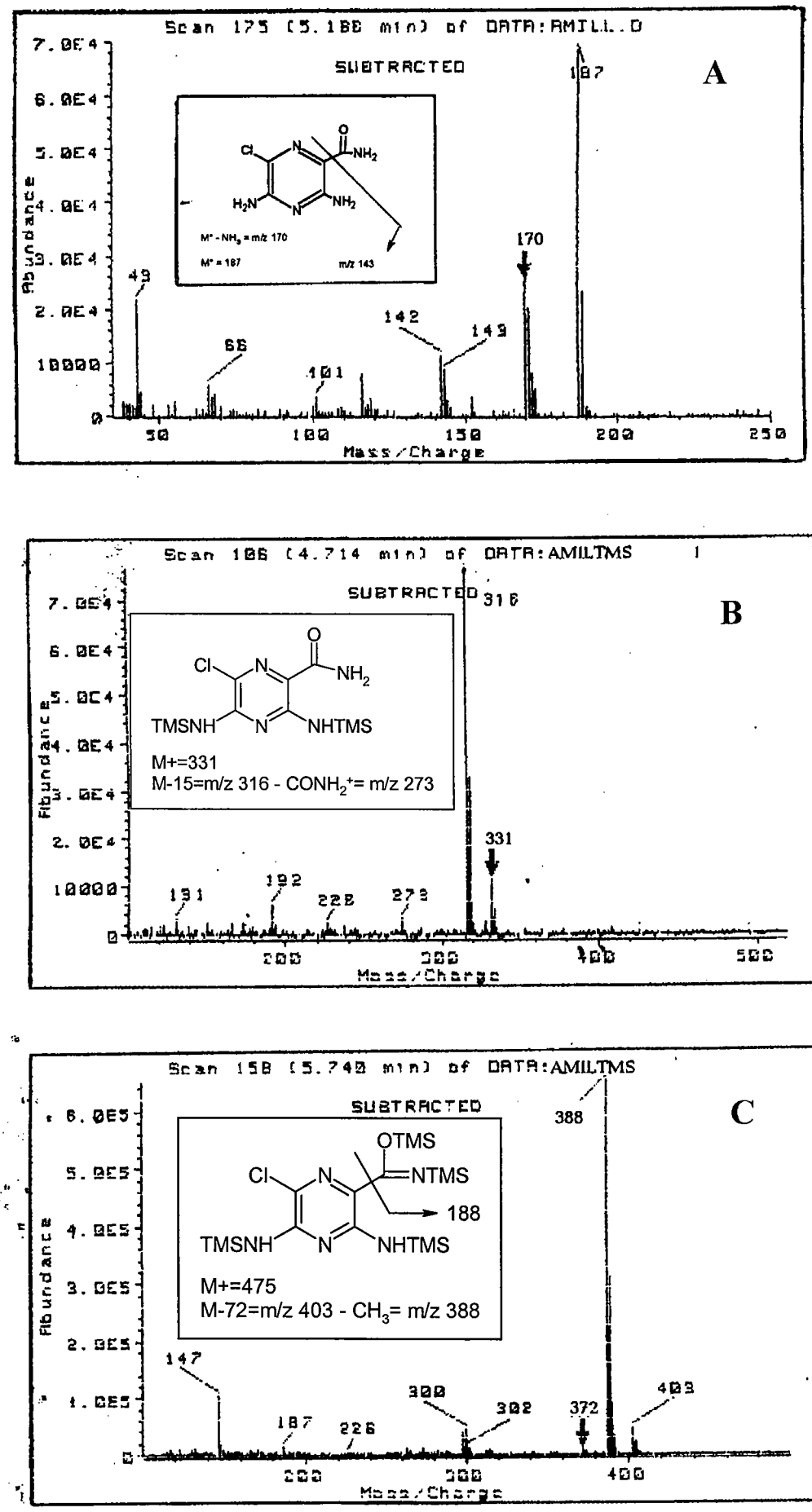


Fig.2. Mass spectra of Amiloride: A) underivatised; B) 2 TMS Artefact;C) 4-TMS Artefact.

2. Extraction of Amiloride.- Two chromatographic methods HPLC and GC/MS are used for comparison and determination of the extraction recovery of Amiloride. HPLC analysis of Amiloride was performed without derivatization, and unchanged substance was detected. Enol-TMS derivatization was used in combination with the GC/MS analysis and Amiloride artefact derivatives were detected. In spite of these differences the obtained results are comparable and tendencies are similar (Fig.3). The higher percent of extraction (23%) was obtained when ethylacetate used as extragent for L/L extraction. These results are comparable to those published in literature [8-9]. After applying SPE on XAD-2 column extraction recovery yielded to 10% (by GC/MS) and 12% (by HPLC).

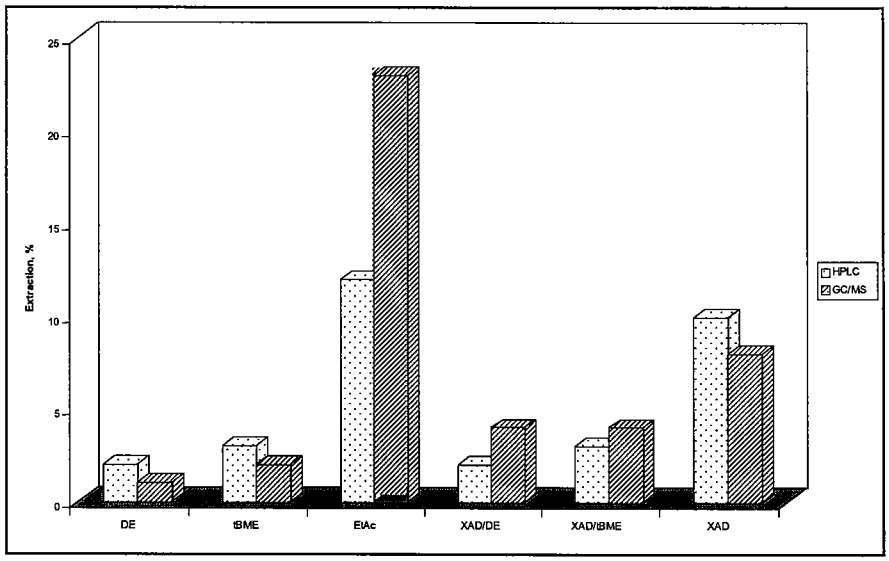


Fig.3. Percents of extraction from spiked urine with 1 μg/ml Amiloride.

3. Excretion study – The drugs containing Amiloride are usually combined with other diuretics as Triamterene or Hydrochlorothiazide [1]. This excretion study was performed after oral administration of a single dose (4 mg) of pure substance. Two excretion maxima were obtained – at 3 and 9 hours after administration (Fig.4). In urine samples up to 70 hours after application the Amiloride artefact was detected. Table 1 lists the values of urine parameters and urinary flow up to the first 24 hours after application.

Table 1

Hours after administration	Density, g/cm^3	pH	Urinary flow, ml
0	1.014	6.8	70
1	1.008	6.7	310
2	1.008	8.2	140

Hours after administration	Density, g/cm^3	pH	Urinary flow, ml
3	1.018	9.6	100
6	1.019	7.3	100
9	1.024	7.3	80
20	1.018	6.0	300
24	1.018	6.2	150

The limit of detection of the Amiloride artefact by the screening procedure of steroids is 8 ng/ml. The obtained results show the possibility to include Amiloride in the routine screening procedure of anabolic steroids.

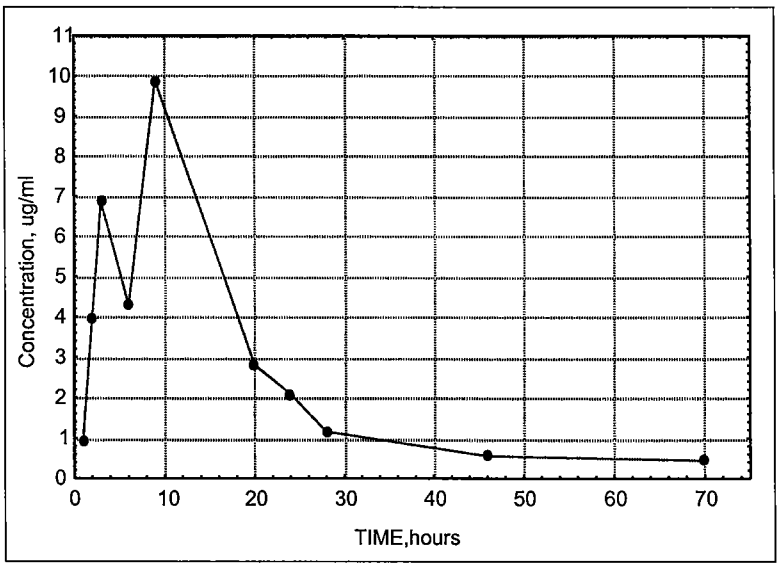


Fig.4. Excretion after oral administration of 4 mg Amiloride.

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