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Investigation on Products of Thiazide Diuretics in Human Urine

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Abstract

The thiazide diuretics as the other diuretics can be used illegally in sport with the aim to avoid a positive doping result or to reduce the athlete's body weight to reach better results in lower weight category. On this base, they have been included in the special lists of forbidden substances - diuretics, lists approved by the International Olympic Committee for the 1988 Olympic Games.

The thiazide diuretics are substances with the structure of 1, 2, 4- bensothiadiazine-7-sulphonamide 1,1-dioxide with chloro or trifluoromethyl substitutes at 6th position and different other substitutes at 3rd position. The most of them are hydrogenated at 3rd and 4th position. This general chemical structure determines to a certain extent their close or similar pharmacological actions and in all the possibility of similarity of their metabolites in urine samples.

The urine was investigated by gas chromatography/mass spectrometry after oral administration of five thiazide diuretics, hydrochlorothiazide, cyclopenthiazide, trichlormethiazide, polythiazide and bendroflumethiazide. The liquid-liquid extraction of the collected urine samples and derivatization with methyliodide were used.

The metabolism pathway of these diuretics leads to the universal products which were identified by mass spectral analysis.

Keywords: Thiazide diuretics, Metabolism, Urine analysis, Capilary GC-MS, Doping control

Introduction

The Medical Commission (MC) of the International Olympic Committee (IOC) has included the diuretics in the list of the forbidden substances for use in the sports since the 1988 Olympic Games. The healthy athlete can use the diuretics for the desire to reduce his body weight in order to qualify for a lower weight category and have better chances for success or to manipulate his urine to avoid a positive doping result. On the ethical and healthy reasons the use of diuretics has banned.

Irrespective of the motives of the usage, the diuretics can have negative consequences into the athlete body. About 6% of the body mass is water in the form of blood, lymph and intracellular fluid. Naturally this water contains many dissolved substances, first of all mineral (sodium, potassium and other) salts with precisely defined composition. The constancy of the composition of the body fluids is of vital importance. By forming urine the kidneys have a function to maintain the constant amount of water and salts in the body. The diuretics promote the excretion of water and electrolytes by the kidneys. The health risk of disturbing the normal water-ellectolyte balance and the break-down of the kidney and liver are quite real (1).

According to the pharmacological properties, the diuretics are classified as carbonic anhydrase inhibitors, "loop" diuretics, potassium saving diuretics, thiazides diuretics and others. All pharmacological effective diuretics primary increase the excretion of electrolytes which secondarily bind water and therefore increase the urine flow. The pharmacological effect of thiazides (2) is an inhibition of sodium and chloride reabsorption in the kidney tubules. They also promote potassium excretion.

The thiazides diuretics produce a more prolonged diuresis than the "loop" diuretics which plasma half-life of elimination is about one hour (3). The thiazide diuresis is initiated within about 2 hours after administration, reaches a maximum in about 6 hours and lasts for 24 to 48 hours. Thiazide with a plasma elimination half-life from 3 to 26 hours are excreted over a longer period of time and can be detected even longer.

The most of the diuretics are excreted unchanged to a high extent, except mefruside and etozolin which are completely metabolized (3). It is known by literature (4-7), the unchanged thiazides excrete for 20 to 30 % after oral administration. The prolonged diuresis and lower percent of detected unchanged thiazide diuretics give the reason to cause a speculation of metabolism of thiazides diuretics.

This paper reports a gas chromatographic/mass spectrometric (GC/MS) characterization of the biotransformation products of the thiazide diuretics. Their mass spectra are discussed. They may be used for the confirmation the usage of the thiazide diuretics in the doping analyses.

Experimental

Drug administration

Tablets with hydrochlorothiazide:

Triampur®, Veb Arzneimittel WERK, Dresden, 12.5 mg hydrochlorothiazide and 25 mg Triamterene;

Moduretik®, FROSST Pharma, 50 mg hydrochlorothiazide, 5.7 mg amiloride hydrochloride. $2H_2O$ and 5 mg amiloride hydrochloride;

Hypotiazide®, Budapest, 100 mg hydrochlorothiazide.

Tablet with a 4 mg trichlormethiazide, Esmarin ®, Merck.

Tablet with a 0.5 mg cyclopenthiazide, Cyclomethiazide®, Borisof' Pharmacie.

Tablet with 2 mg polythiazide, Drenusil®, PFIZER GmbH.

Tablet with 5 mg bendroflumethiazide, Sinesalin®, ICI Pharma.

All tablets were taken by a healthy volunteers. The urine samples were collected up to 48h and stored at 4°C until analysing.

Sample preparation

The extraction of the collected urine samples was performed as follows: 5 ml of the urine was added by 20 µl of internal standard (IS) stock solution of mefrusife with a concentration 24.88 µg/ml and 0.1 g solid phosphate buffer to adjust pH 7. Then 0.5 g of anhydrous sodium sulfate was added and after mixing the sample was extracted with 5 ml of diethyl ether for 20 min. Centrifuge at 2500 rpm. The extract was dried with a slow stream of nitrogen and analyzed after derivatization.

Derivatization

The dried residue was dissolved in 250 µl of acetone. 40 µl of methyliodide and 100 mg of potassium carbonate were added. The solution was heated in a heating block at 60°C for 3h. A 3-µl aliquot of methylated product was injected into the GC/MS.

GC/MS amalysis

The GC/MS analyses were performed on a Hewlett-Packard 5995C equipment, electron impact ionization with 70 eV. The column was fused silica capillary, crosslinked 5% phenyl methyl silicone (SE-54), 15 m length, 0.2 mm I.D., film thickness 0.33 µm; carrier gas - helium with 0.93 ml/min, splitless injection mode; temperature program: initial temperature 190°C, rate 30°C/min up to 320°C for 7 min.

The electron impact mass spectra were recorded in full Scan mode from 35 to 550 amu.

Results and discussion

The thiazide diuretics have a base chemical structure of 1, 2, 4- bensothiadiazine-7-sulphonamide 1,1-dioxide (Fig.1) with a presence of the chlorine or trifluormethyl substutute at 6th posittion. The most of them are hydrogenated at 3rd and 4th position and have different substitutes at 3rd position. Some of them have a methyl group at 2nd position. According to these differences, some thiazide diuretics are presented in Table 1.

Figure 1. Base chemical structure of thiazide diuretics

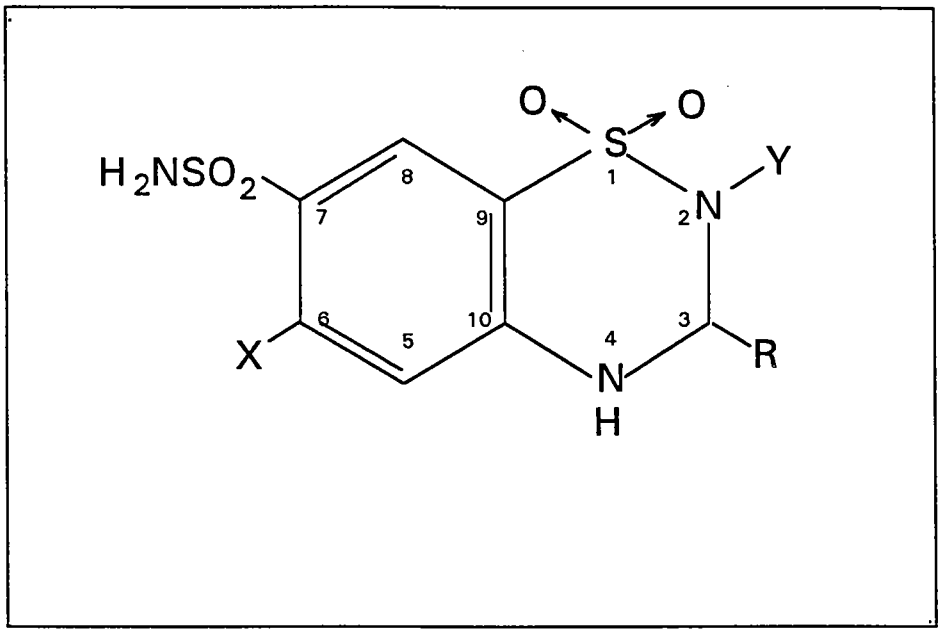

Table 1. The chemical structure of thiazide diuretics

Diuretic	X	Y	R	hydrogenation at 3rd-4th position
Chlorothiazide	Cl	H	H	no
Benzthiazide	Cl	H	CH_2SCH_2Ph	no
Hydrochlorothiazide	Cl	H	H	yes
Cyclothiazide	Cl	H		yes
Trichlormethiazide	Cl	H	$CHCl_2$	yes
Cyclopenthiazide	Cl	H	CH_2-⬡	yes
Epithiazide	Cl	H	$CH_2SCH_2CF_3$	yes
Althiazide	Cl	H	$CH_2SCH_2CH=CH_2$	yes
Butizide	Cl	H	$CH_2CH(CH_3)_2$	yes
Methyclothiazide	Cl	CH_3	CH_2Cl	yes
Polythiazide	Cl	CH_3	$CH_2SCH_2CF_3$	yes
Hydroflumethiazide	CF_3	H	H	yes
Bendroflumethiazide	CF_3	H	CH_2Ph	yes

The electron-impact fragmentation of the free form of seven thiazide diuretics, hydrochlorthiazide, hydroflumethiazide, bendroflumethiazide, cyclothiazide, cyclopenthiazide, polythiazide and methyclothiazide have been interpreted by Casy (8) (Fig. 2).

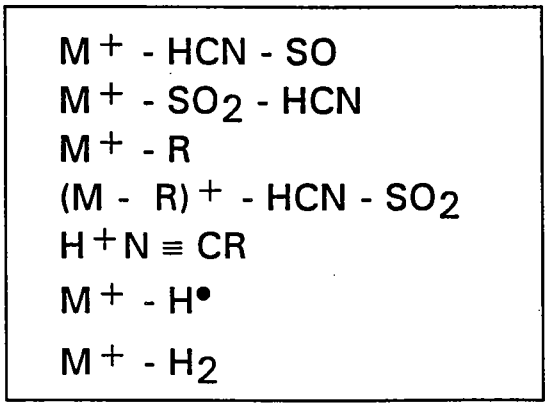

Figure 2. Base electron-impact fragmentation of free thiazide diuretics

The hydrochlorthiazide (M^+=297, 55%) and hydroflumethiazide (M^+=331, 90%) give prominent molecular ion peaks followed by loss of a hydrogen radical. The next fragmentation proceeds by the sequential loss of HCN (M-28) and SO, or SO_2 and HCN. The thiazide diuretics with a substitutes at 3rd position give mass spectra with M^+ intensities in the range 5-8%. Base peak in their mass spectra is formed by loss of this substitute. The most of them forme an $H^+N \equiv CR$ fragment. The pathway can be rationalized by loss of HCN, and HCN plus SO_2 from $(M-R)^+$ ions. The ions in the mass spectrum of the chlorothiazide, the di-3,4-dehydroanalogue of hydrochlorthiazide arise through loss of HCN followed by H· or H_2 from the molecular ion. In mass spectra of all thiazides with 6-chloro substitute, the ions containing ^{35}Cl were all accompanied by their heavier isotope counterparts in appropriate intensity ratio. Those, which have trifluoromethyl group at 6th position give many concomitant fragments formed by loss of fluorine or hydrofluoric (HF) ion.

The pathway of fragmentation of the methyl derivatives of thiazide diuretics (9, 10) is similar to the fragmentation of their free form. In our investigation we have used the methylation as a derivatization method.

The collected urines after oral administration of a single theurapeutic dose were treated and analysed as described above. We have detected the tetramethyl derivatives of hydrochlorothiazide and bendroflumethiazide in a large intensity while the polythiazide has found as a trimethyl derivative in a very small quantity.

In the urine samples of trichloromethiazide and cyclopenthiazide we have discovered no presence of parent compounds but we have found an unknown compound (X) (Fig. 3). The most of the fragments in the mass spectrum have indicated for the presence of chlorine. The peak on the total ion chromatogram has decreased when the time of methylation has been

prolonged or the sample has been injected with methelute (Fig. 3). At the same time, the amount of some other peak (**Y**) has increased. The both peaks have closed retention times and their appearance on the total ion chromatogram was earlier than those of the derivatived parent compounds (Fig. 3). The compound **X** has a longer retention time (t_r). The relative retention time (RRT) of the compounds are 0.88 and 0.92.

The compounds X and Y were found also in the urine samples of hydrochlorothiazide and polythiazide. The mass spectra of the two unknown compounds are shown in Fig.4.

Base peaks in the spectra are ions at m/z 220 and m/z 234. The peaks at m/z 44 (31%) in both spectra are very clear. Each fragment in the spectrum of compound **Y** has 14 amu more than the corresponding in the spectrum of compound **X**. The pathway of their fragmentation is probably identical. More of the fragments contain chlorine and the isotope ratio corresponds to the presence of one atom in the molecule.

Theses unkown substances also was obtained in the urines of polythiazide and hydrochlorothiazide. The last diuretic has no substitute at 3rd position. Therefore we have supposed that the origin of these substances are based on the structure of hydrochlorothiazide. The molecular weight of free hydrochlorothiazide is 297 and of tetramethylhydrochlorothiazide is 353. Its molecular ion is intensive (Fig. 4) contrary to the low percent of molecular ion in the spectra of the 3-subsituted thiazide diuretics.

We have accepted the peaks at m/z 341 and m/z 355 as molecular ions of the methylated derivatives. If the compounds X and Y are trimethyl- and tetramethyl derivatives, they posses 2 protons more than trimethyl- and tetramethyl hydrochlorothiazide. On this base we discuss the mass spectra of the compounds X and Y.

The pathway of the fragmentation passes through loss of a dimethylamino group from the both molecular ions forming a clear peak at m/z 44 and weak peaks at m/z 297 and m/z 311. The peaks at m/z 234 and 248 are formed by loss of fragment with 107 amu. The following loss of HCN creates the peaks at m/z 206 and m/z 220 which have different intensities. The substance Y has the peaks at m/z 139, m/z 140, 141 and m/z 142. The fragment at m/z 125 in the mass spectrum (X) was formed from the ion $ClPh^{+}NH_2$. This ion is missing in the mass spectrum of the tetramethyl derivative. It is obvious there is a small difference between both mass spectra

The amount of the trimethyl derivative in the all analysed urine samples was higher than the tetramethyl substituted and that might be explained with some difficulty of methylation. The presence of strong peak at m/z 44 may be explained, probably with a different distribution of the electrons in the molecule.

Accounting the differences between the fragments in the mass spectra of the two compounds and their gas chromatographic behaviours, we have accepted them as a substance with a different level of methylation.

The same results we have obtained after a solid-liquid extraction. On this grounds we have accepted this compound as products of the investigated chlorothiazide diuretics.

Analysing the collected urine samples of bendroflumethiazide, we have discovered a compound (Z) with a similar pathway of fragmentation so as written above and a lower RRT=0.74 comparing to parent compound. The retention times of the investigated thiazide diuretics are presented in Fig. 5. Tha found compound have lower retention time. Probably they have lower polarity than parent compounds. The mass spectrum of compound Z is shown in Fig. 6. It can seen no fragments showing for a chlorine presence.

We have applied the same considerations used in the previous discussion on the found metabolite of chlorothiazide diuretics. Now we have accepted that the substance is trimethyl derivative and the peak at m/z 375 is its molecular ion. So it has 2 protons more than those of trimethylhydroflumethiazide with a molecular ion at m/z 373. The pathway of the fragmentation passes through loss of a dimethylamino group from the molecular ion forming an intensive peak at m/z 44 and a weak peak at m/z 331. The base peak at m/z 268 was formed by cleavage of fragment with 107 amu. The following loss of HCN creates the peak at m/z 240. The fragment with m/z 159 was formed from the ion $F_3CPh^+NH_2$. The presence of the fluorine in the structure might proofed by peaks at m/z 356 (M^+-19), m/z 140 (m/z 159-19). The fluorine leads to many concomitant fragments.

All these results obtained during our investigation have given us the courage to propose the following structures (Fig. 7) of the found products from the chloro- and trifluoromethyl thiazide diuretics.. They have two protons more than the structure of hydrochlorothiazide or hydroflumethiazide. We have speculated on their formation.

In the following investigations we have studied the excretion of thiazide diuretics. In Fig. 8 there are presented the excretion curves of bendroflumethiazide and its "metabolite" in the urine samples. The unchanged substance is about 48% and its quantity correlates with the literature data (10). The unchanged bendroflumethiazide appears in the urine samples for 2 hours and has a maximum at 4th hour. Its amount decreases gradually to 28 hours.

The quantity of the metabolite is considerably (24%). The metabolite has detected in the urine samples for 2 hours after administration and has reached a maximum at about 13th hour. The amount of the metabolite decreases from 22 h to 28 h. but can be detected till 48th h. The excretion of bendroflumethiazide metabolite may give the explanation of low percent of found unchanged substance in the urine samples after administration of a single oral dose. The obtained results are in accordance with the established diuretic effect (1,2). The bendroflumethiazide produces a response in about 3 hours and the diuresis is maintained for about 20 hours.

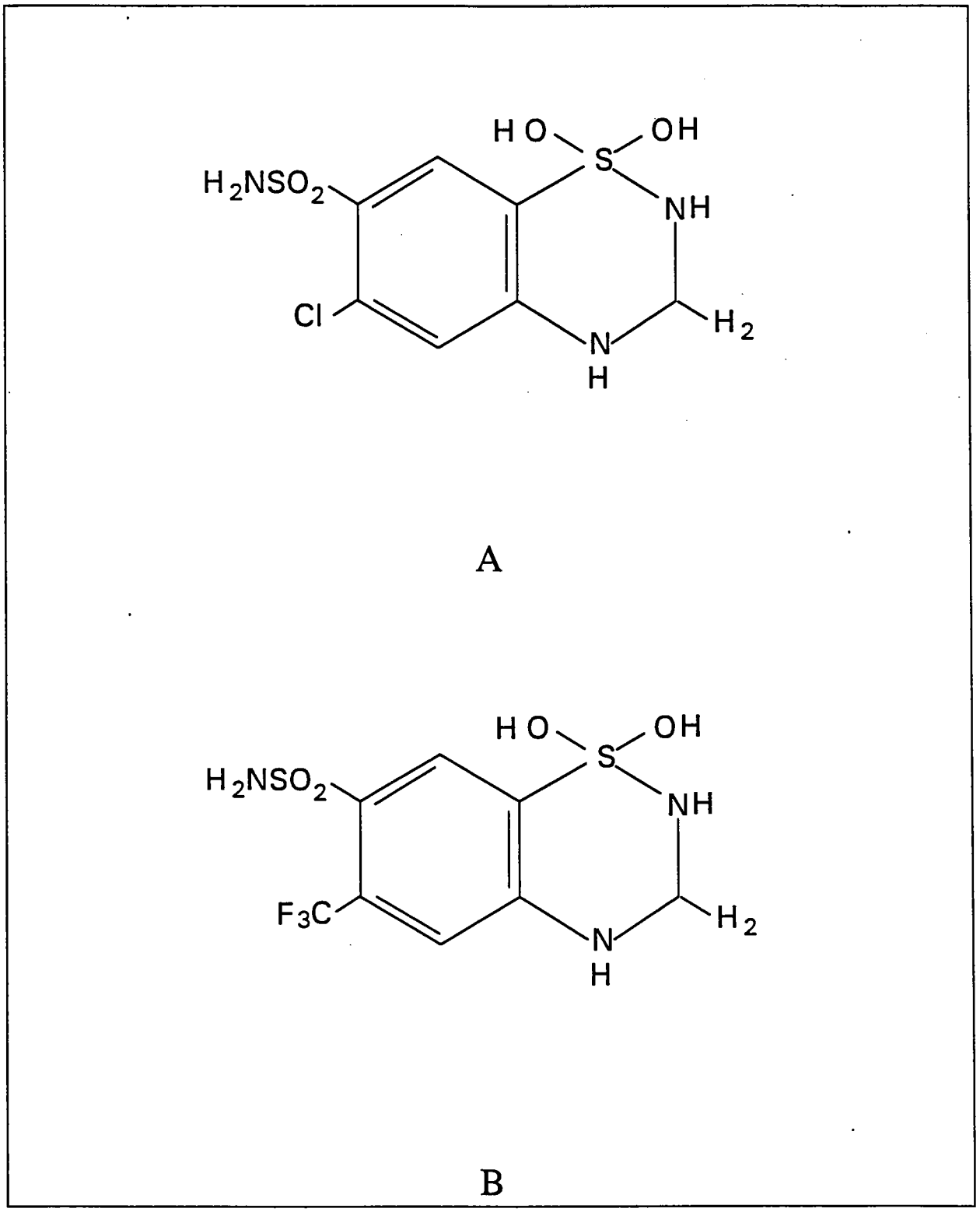

Fig. 7. Proposed structures for the found compounds in urine of chhloro-thiazide diuretics (A) and benroflumethiazide (B)

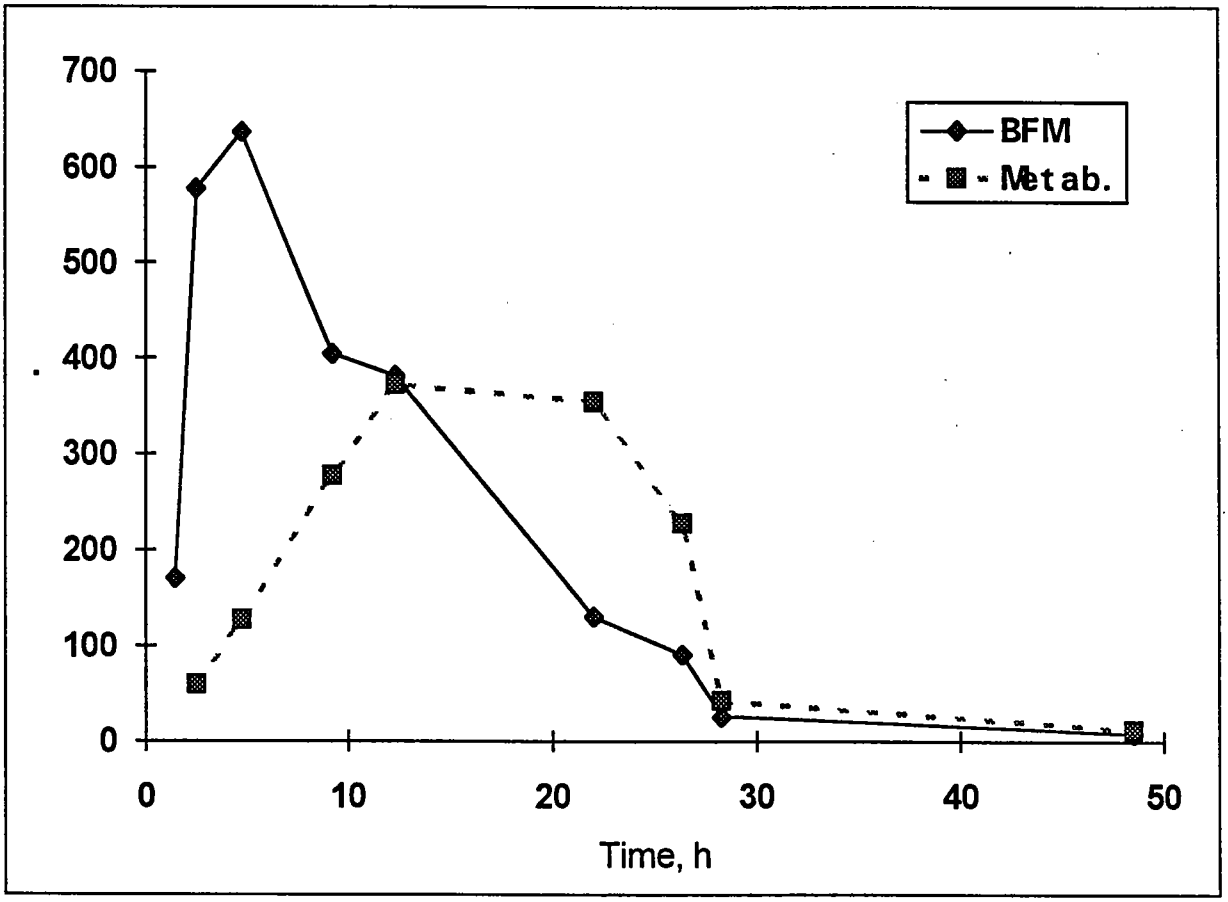

Figure 8. Excretion curves of amount of bendroflumethiazide and found metabolite in the urines after a single oral dose (5 mg)

The presence of the metabolites of chloro- and trifluoromethyl- thiazide diuretics are in accordance with their established pharmacological properties, laster initiation and prolonged diuresis.

Conclusion

The metabolism of the chloro- and fluorocontaining thiazide diuretics passes through loss of a substitute at 3rd position followed probably by reduction of the sulfure at 1st position. The obtained results can be used in the screening of diuretics in doping analyses.

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