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Metabolism of Benzphetamine and Clobenzorex in human urine

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ABSTRACT

The metabolism of the stimulants Benzphetamine and Clobenzorex in the human urine after a single oral dose are investigated. Several new hydroxymetabolites of Benzphetamine and Clobenzorex as well as the well-known metabolites - amphetamine, methamphetamine and desmethylbenzphetamine are found and identified in the collected urines upto 48th hour after administration. Conjugated urinary metabolites are determined after hydrolysis. Their structures are identified by gas chromatography/mass spectrometry using the different derivatisation procedures - methylation, silyllation, selective derivatization, cyclization with methaneboronic acid. The fragmentation of some type of different derivatives is discussed and the probable metabolism scheme of Benzphetamine and Clobenzorex is proposed.

Key words: Stimulants, Benzphetamine, Clobenzorex, Doping analysis, Urine, Metabolism, Gaschromatography/Electron impact Mass spectrometry.

INTRODUCTION

Many drugs, enclosed in the list of banned stimulants for athletes produce in human urine amphetamine as its primary metabolite and in minor extent of p-hydroxy-amphetamine. At the time, the literature data show that the applied substance could be change depending on the temperature and the duration of the sample storage [1]. Therefore the detection of amphetamine in the screening procedure of free stimulants could be submit to the analyst the

question: **What is the pattern substance?** For this reason the study gives attention to the detection of other metabolites that could be prove the origin of the administrated drug.

Benzphetamine, (S)-N, α - Dimethyl-N-(phenylmethyl) benzeneethanamine and Clobenzorex, (+)-N-[(2-Chlorophenyl)methyl]-α-methylbenzeneethanamine have similar chemical structures and an anorexic therapeutic effect.

The aim of this work is to describe the obtained results on the metabolism of Benzphetamine and Clobenzorex in collected urine samples up to 48 hours after oral administration of one therapeutic dose. The investigation was performed with extracts of urine samples obtained without hydrolysis and after enzyme hydrolysis by Helix Pomatia. The chemical conditions of screening procedures for free and conjugated stimulants and narcotics were used. The analytical determinations are performed by gas chromatography/electron impact-mass spectrometry (GC/EI-MS). In order of receiving a better idea about the structures of the detected metabolites were used the different derivatization procedures as methylation, silyllation, selective derivatization and cyclization with methaneboronic acid.

EXPERIMENTAL

Chemical and reagents

All reagents were of analytical grade. Double distilled diethylether stored in glass bottle in a refrigerator was used. Anhydrous sodium sulphate was dried at 300°C for 12 hours.

Stock standard solutions of Diphenylamine (DPA), Bamethane (ISTD), Amphetamine and methamphetamine were prepared in methanol. These solutions were stored at -20°C.

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), N-Methyl-bis-trifluoroacetamide (MBTFA) were obtained from Machery-Nagel and methyliodide and methaneboronic acid (MBA) - from Fluka.

β-Glucuronidase/arylsulphatase from Helix Pomatia was purchased from Sigma.

Excretion study

Two benzphetamine (25 mg) tablets Inapetyl®and a clobenzorex (30 mg) tablet Dinintel® were taken orally by healthy volunteers. The urine samples were collected up to 48 h and stored at 4°C until analysing.

Sample preparation

For free stimulants: 5 ml of urine were added to 50 µl of internal standard working solution to obtain a concentration of 0.5 µg/ml Diphenylamine (DPA) and alkalised by 0.5 ml of 20% NaOH (pH 14). Two ml of distilled diethylether and 3 g of anhydrous sodium sulphate were added to the solution. The sample was roller mixed for 30 min. Then, it was centrifuged at 2500 rpm for 5 min. The organic layer was transferred to another tube and concentrated to 1 ml by evaporation under the stream of nitrogen. 2 µl were injected into the GC.

For conjugated stimulants: Urine sample (5 ml) was added to 20 µl of internal standard working solution to obtain a concentration of 0.5 µg/ml bamethane (ISTD). Then, 1 ml of 1N acetate buffer (pH 5.2) and 50 µl of ß-glucuronidase/arylsulphatase from Helix Pomatia were added. The sample was vortex mixed, heated to 55°C for 3 h or for one night at 37°C in a block-heater. The sample was cooled to room temperature and extracted by 5 ml of distilled diethylether in a shaker for 20 min. Then, it was centrifuged at 2500 rpm for 5 min.
The organic layer was discarded. The aqueous phase pH was adjusted to 9.6 ± 0.1 with $NaHCO_3/K_2CO_3$ (1:2 w/w) and were added 0.5 ml tert-butanol, 5 ml of distilled diethylether and 3 g of anhydrous sodium sulphate. The mixture was extracted by shaking for 20 min, then centrifuged at 2500 rpm for 5 min. The organic layer was transferred in a derivatisation vial and evaporated to dryness under stream of nitrogen in a 50°C water bath. The sample was kept in a desiccator over P_2O_5/KOH for 30 min.

Derivatisation

Preparation of O-TMS, N-TMS derivatives: To the dried residue was added 50 µl MSTFA and heated for 20 min at 80°C. After cooling to room temperature 1 µl of the sample was submitted to GC/MSD.

Preparation of O-TMS, N-TFA derivatives: To the dried residue was added 50 µl MSTFA and heated for 5 min at 80°C. After cooling to room temperature 15 µl of MBTFA was added and heated again for 10 min at 80°C. The sample was cooled and 1 µl was submitted to GC/MSD.

Preparation of n-methylboronates: To the dried residue was added 100 µl solution of methaneboronic acid in dried acetone (c = 5 mg/ml) and the mixture was kept at room temperature for 15 min.
2 µl were injected to GC/MSD.

Preparation of methyl derivatives: To the dried residue was added 250 µl acetone, 50 µl methyliodide and 100 mg K_2CO_3. The sample was vortex mixed and heated for 3 h at 60°C.

2-3 μl were injected into GC/MSD.

Instrumental analysis

GC parameters:

Hewlett-Packard Gas Chromatograph, model 5890 serie II, NP Detector

column: HP Ultra -2, 17 m x 0.25 mm i.d., 0.32 μm film thickness

temperatures: injector -280°C, detector - 300°C and oven: initial 100°C - 2 min, final 290°C and rate of 20°C/min.

Carrier gas: Nitrogen - 1.12 ml/min., auxiliary -nitrogen at 28.5 ml/min, hydrogen: 4 ml/min, air-80 ml/min, split ratio: 1:10

GC/MS parameters:

HP 5890A GC coupled with HP 5970 MSD

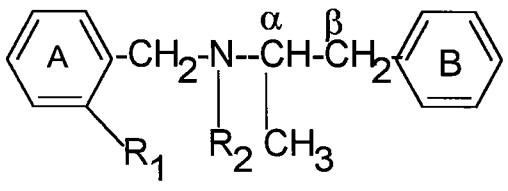
column: HP -5, 17 m x 0.25 mm i.d., 0.25 μm film thickness

temperatures: injector -280°C, transfer line - 280°C and oven: initial 110°C - 1 min, final 300°C and rate of 15°C/min.

Carrier gas: Helium - 1.3 ml/min, split ratio: 1:10

RESULTS AND DISCUSSION

The molecules of benzphetamine and clobenzorex have a close structure and built up of two aromatic rings and an aliphatic chain containing secondary amino group (I), in this connection was interested to be investigate and compare the metabolism of both substances.



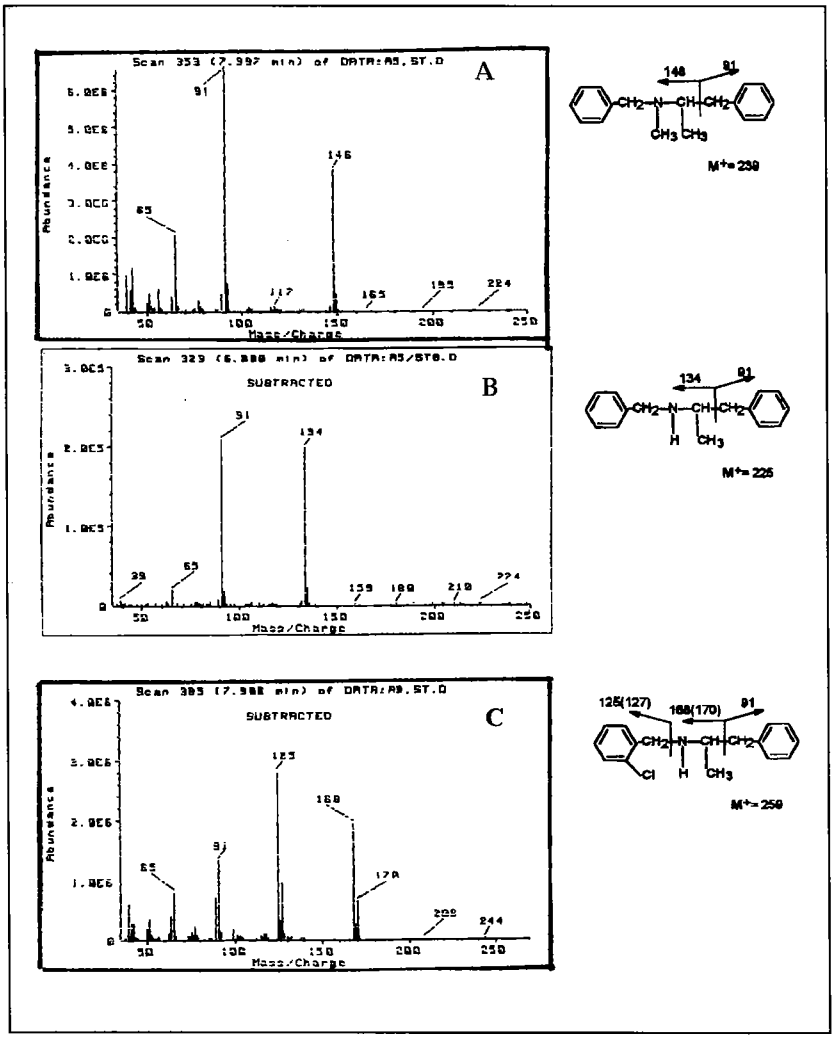
I

where: R_1=H, R_2=CH_3 for benzphetamine

R_1= Cl, R_2= H for clobenzorex

Their chemical structures permit the formation of great number of likely metabolites in the body receiving by demethylation, hydroxylation, O-methylation and/or splitting of the molecule. It is known that CYP3A4 enzyme catalyses N-demethylation of benzphetamine [2]. The hydroxylation following by O-methylation is possible also and can be performed on different places (para- or meta-) in the both aromatic rings - A or B and/or in β - position of the aliphatic chain.

On the Fig. 1 can be seen the electron impact (EI) fragmentations of benzphetamine and clobenzorex. Their similarity is based on their closed chemical structures. The mass spectra of both compounds have not representative molecular ions (M^+) but they possess intensive ion at m/e 91 resp. m/e 125 (127). The characteristic ions at m/e 148 for benzphetamine and 168 (170) for clobenzorex are dominating and formed from the cleavage of tropilium ion from M^+. The common specificity in the fragmentation and the presence of the chlorine atom in the chemical structure of clobenzorex have assisted for the following identifications of the found metabolites in studied urine samples.



The performed GC analyses of urine samples treated with usual procedure for free stimulants were confirmed the presence of known metabolites for benzphetamine [3] - amphetamine, methamphetamine and desmethylbenzphetamine and only amphetamine for clobenzorex. The gas chromatograms of the blank and studied urine samples are illustrated on Fig. 2.

Fig.1. *Mass spectra of underivatized Benzphetamine (A), Desmethylbenzphetamine (B) and Clobenzorex (C).*

The excretions of amphetamine and methamphetamine in urine samples were studied and the obtained results are shown on the Fig. 3. It is interesting to note that maximums on the excretion curves of amphetamine and methamphetamine are registered at about 12 hours after

administration of benzphetamine while the maximum of amphetamine excretion after the application of clobenzorex is at the 6th hours. The investigations were confirmed the presence of desmethylbenzphetamine in lower level. The EI fragmentation of desmethylbenzphetamine (Fig. 1c) is very closed to the benzphetamine.

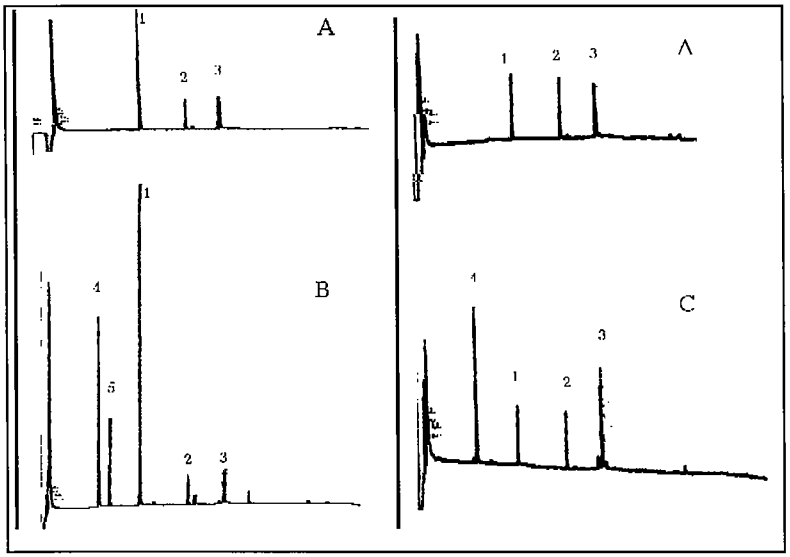


Fig.2. *GC/NPD chromatograms of blank urine (A) and urine sample after application of benzphetamine (B) resp. clobenzorex (C). The recorded peaks are: 1- nicotine; 2- ISTD -DPA; 3- caffeine; 4- amphetamine and 5- methamphetamine.*

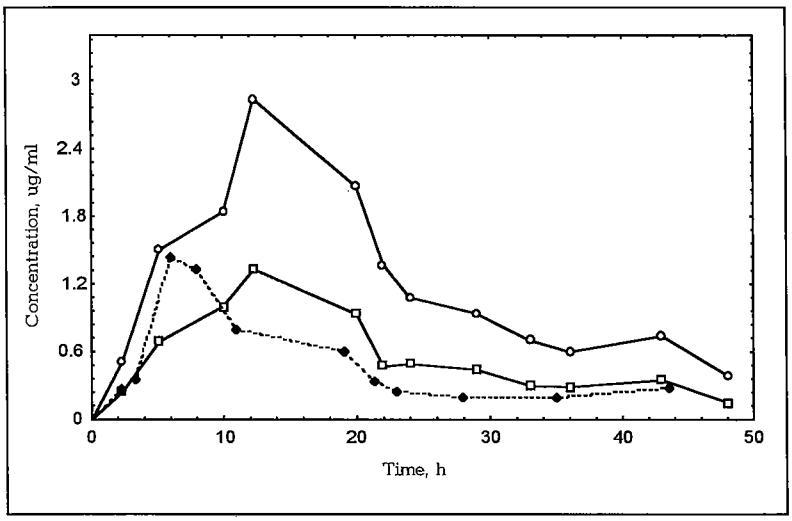


Fig.3. *Excretion of Amphetamine (-o-); Methamphetamine (--□--) after oral administration of Benzphetamine and Amphetamine (◆) after application of Clobenzorex.*

Analysing the urine samples under conditions for free stimulants were not found unchanged started substances or other metabolites. For this reason a set of experiments was carried out after hydrolyses of urine samples with Helix Pomatia. The obtained data from performed GC/MS analyses confirm the presence of conjugated metabolites of benzphetamine and clobenzorex as would be expected. The identification of the found metabolites and the confirmation of their structures were enabled by using different kind of derivatisation -

methylation, silyllation, selective derivatization (silyllation and acetylation) and cyclization with methaneboronic acid.

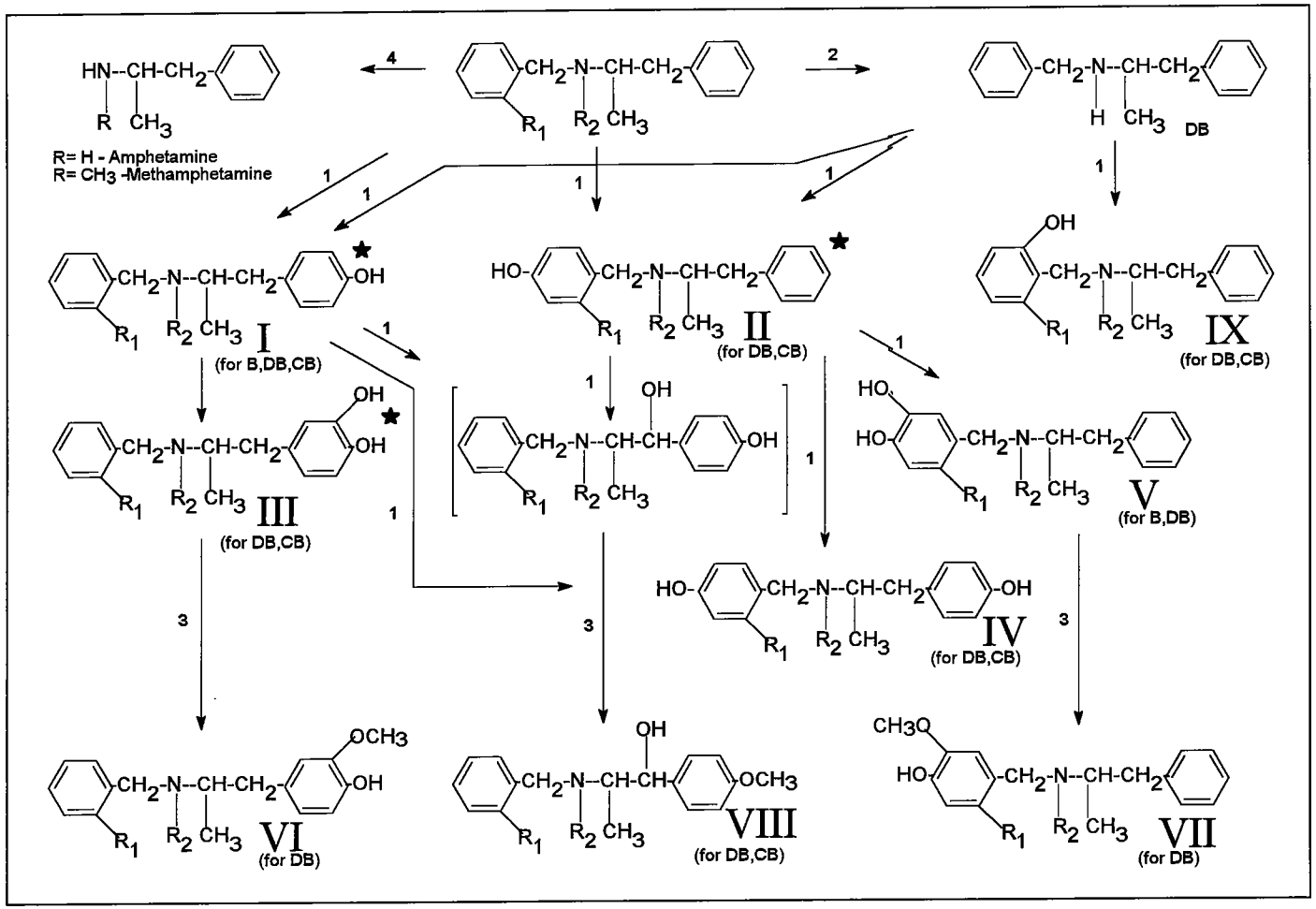
The probable metabolic pathway of benzphetamine and clobenzorex is based on the received results and presented on Fig. 4. The positions of hydroxy- and methoxy- groups in the metabolic structures are determined by interpretation of the obtained mass spectra from different derivatives and the corresponded retention times (Table 1). The most evident proofs for the structures were obtained by selective derivatization and cyclization by methaneboronic acid, that is why the next discussion concerns to these data.

Identification after selective derivatization

Monohydroxy metabolites

Figures 5 and 6 represent the mass spectra of both types of monohydroxymetabolites - I and II. It can be seen that they have common ions at m/e 91 resp. 125 (127) and similarly the specific ions formed according to the situation of the hydroxy group on A- or B-ring. On the other hand the compounds with the same type structure give a similar fragmentation. The presence of the ion at m/e 179 accompanied by specific ion at m/e 206 in the mass spectra of monohydroxy clobenzorex and desmethylbenzphetamine shows that the hydroxy group is situated on the outlying aromatic ring from the nitrogen atom (B-ring). The ions at m/e 230 (Fig. 5b) and m/e 264 (Fig. 5a) are formed by the cleavage of the ion 179 from M^+. The mass spectrum of hydroxybenzphetamine (Fig. 5c) confirms the position of hydroxy group in B-ring with the ions at m/e 236 (M^+- 91) and m/e 91, 148,179.

Figure 6 represents the mass spectra of another type of monohydroxymetabolites - Met. II which OH-group is disposed in A-ring. It can be seen that the basic ion is at m/e 213 (215) (Fig. 6a) resp. m/e 179 (Fig. 6b). The specific ion at m/e 325 (327) resp. m/e 291 produced by cleavage of the fragment $C_6H_5CH_2CH{=}CH_2$ from the M^+ (M^+ - 118) and the absent of ion at m/e 206 confirm the position of the OH-group on A-ring. The found quantity of Met. II for desmethylbenzphetamine is higher than for clobenzorex. It can be explained with the influence of chlorine atom in A-aromatic ring of clobenzorex under the hydroxylation.



R_1=Cl; R_2=H - Clobenzorex (CB)
R_1=H; R_2=CH_3 -Benzphetamine (B)
R_1=H; R_2=H -Desmethyl-benzphetamine (DB)
The major metabolites are pointed with ★.

Reaction
1- hydroxylation
2- N-demethylation
3- O-methylation
4- degradation

Fig.4 . Probable metabolic pathway of clobenzorex and benzphetamine

Table 1

Relative retention times and M^+ of detected Metabolites of Benzphetamine and Clobenzorex in hydrolysed urine samples after different derivatization: silyllation(MSTFA), selective derivatization (MSTFA/MBTFA) and cyclization with MBA

Meta-bolites	Clobenzorex						Benzphetamine						Desmethylbenzphetamine					
	MSTFA		MSTFA/ MBTFA		MBA		MSTFA		MSTFA/ MBTFA		MBA		MSTFA		MSTFA/ MBTFA		MBA	
	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+
I	1.20	419	1.36	443	n.d.	-	1.17	327	1.20	327	n.d.	-	1.28	385	1.26	409	n.d.	-
II	1.14	419	1.27	443	n.d.	-	-	-	-	-	-	-	1.27	385	1.25	409	n.d.	-
III	1.34	507	1.39	531	* 1.06/ 1.11	315	-	-	-	-	-	-	1.40	473	1.39	497	* 0.95/ 0.98	281
IV	1.40	507	1.47	531	n.d.	-	-	-	-	-	-	-	1.48	473	1.47	497	n.d.	-
V	-	-	-	-	-	-	1.25	415	1.27	415	0.86	295	1.39	473	1.37	497	0.92	281
VI	-	-	-	-	-	-	-	-	-	-	-	-	1.35	415	1.33	439	n.d.	-
VII	-	-	-	-	-	-	-	-	-	-	-	-	1.37	415	1.35	439	n.d.	-
VIII	1.27	449	1.37	473	* 1.04/ 1.08	329	-	-	-	-	-	-	1.38	415	1.36	439	* 0.94/ 0.97	295
IX					0.82	265	-	-	-	-	-	-					0.82	265

Remarks: *1. Bamethane was used as ISTD with next retention times - Bamethane (2 O-TMS, N-TMS) t_R = 7.92 min.; Bamethane (2 O-TMS, N-TFA) - t_R = 7.76 min.*

2. MBA derivatization was carried out with the presence of Pindolol as ISTD with t_R = 9.29 min. as Pindolol-boronate.

3. The abbreviation n.d. denote that compound does not react with MBA.

4. After cyclization with MBA the marked couple of RRT with (*) correspond to the isomers of metabolites.

Dihydroxy metabolites

Figure 7 represents the mass spectra of dihydroxymetabolites (Met. IV) of clobenzorex and desmethylbenzphetamine. The ion at m/e 91 resp. 125 (127) misses in both mass spectra. This fact confirms that both OH-groups are situated on A- and B-rings. In the mass spectrum of clobenzorex can be seen the ions at m/e 179, 206 and 213 (215). In the mass spectrum of desmethylbenzphetamine the ion at m/e 179 is more intensive than ion at m/e 206 (Fig. 7b). The quantity of dihydroxyclobenzorex is lower than those of desmethylbenzphetamine.

Figure 8 presents the mass spectra of another type of dihydroxymetabolites III and V that have two hydroxy groups situated on one of the rings. The intensive ions at m/e 267, 179 and the specific one for the structure of pattern substances (for clobenzorex -ion at m/e 125 (127) and for desmethylbenzphetamine - ion at m/e 91) confirm the above. The position of both hydroxy groups has established by specific ions as m/e 294 and 378. The ion at m/e 294 is formed by cleavage of the fragment $C_6H_5CH_2N$-TFA (for desmethylbenzphetamine) and $C_6H_4ClCH_2N$-TFA (for clobenzorex) from M^+ and is characteristic for OH groups on the B ring (Met. III). When OH groups are situated on ring A (Met. V) the cleavage of fragment $C_6H_5CH_2CH{=}CH_2$ (M^+ - 118) leads to formation of the ion at m/e 378 (Fig.8c). This way of fragmentation is similarly to that of Met. II (Fig. 6). Dihydroxybenzphetamine which hydroxy groups are on A-ring was detected in negligible quantity.

Hydroxy-, methoxy- metabolites

Figure 9 presents two mass spectra of O-TMS, N-TFA methoxydesmethyl-benzphetamine (Mets. VI and VII). The specific ion at m/e 209 gives a proof that O-TMS, OCH_3-groups are situated on the one of aromatic rings. The presence of intensive ion at m/e 91 and ion at m/e 179 (formed by a cleavage of OCH_3 group from the ion at m/e 209) are in according with above statement. The ions at m/e 236 and m/e 320 have determined the structural difference between two compounds. The specific ion at m/e 236 had formed by cleavage of fragment C_6H_5 CH_2N-TFA from M^+ when the O-TMS, OCH_3 - groups are situated on B-ring (Met. VII), while the ion at m/e 320 is specific for A-ring disposition of groups (Met. VI). This ion at m/e 320 is produced by cleavage of fragment $C_6H_5CH_2CH{=}CH_2$ from M^+ (Fig. 9b).

Identification after cyclization with methaneboronic acid

It is known that 1,2- or 1,3- aromatic- or aliphatic- diols and 1,2- or 1,3- aminoalcohols react with some of alkyl- and aryl boronic acids and the obtained boronate derivatives are stable compounds with good chromatographic properties. Their mass spectra are specific and give the information about the neighbourhood of two hydroxy- or hydroxy- and amino- groups [4-7]. The structures of the discussed above Mets. III and V were confirmed by mass spectra received after cyclization with methaneboronic acid. Figure 10 shows the mass spectra of both types of dihydroxymetabolites which have different fragmentation. The positions of both hydroxy groups on B-ring determinate specific the fragments at m/e 91, resp. 125 (127) and 160 (Fig. 10 a, b), while the position on A-ring - m/e 147, 91 and 190 (for dihydroxydes-methylbenzphetamine - Fig. 10c) resp. m/e 204 for dihyroxybenzphetamine.

The derivatization with methaneboronic acid has assisted for identification of another hydroxy- methoxy-metabolite (Mets. VIII) of clobenzorex and desmethylbenzphetamine. The hydroxy group is situated in β-position of the aliphatic chain and methoxy one is on B-ring. The specific ions are m/e 91 respectively 125 (127) and 173, 174 (Fig. 11a, b).

On Fig. 11c can be seen the mass spectrum of boronate derivative that is received from urine samples of benzphetamine and clobenzorex also. We assume that the metabolite IX is monohydroxy with hydroxy group in *ortho*-place on A-ring. The detection of the such metabolite at the same retention time from the urine sample of clobenzorex may be explain by a possible dehalogenation of the molecule.

Analysing the urine samples after reaction by methaneboronic acid were detected some couples of peaks with close retention times and equal spectra corresponding to those of Mets. III and VIII. According to the reported data [7,8] the methyl- or butyl- boronate derivatives of structural- and stereo- isomers can be separated under GC analysis on achiral stationary phases (OV-1, OV-17). The obtained data give us the reason to assume that these detected compounds are structural- or stereo-isomers.

CONCLUSION

Several new metabolites of benzphetamine and clobenzorex are established as a result of the conducted investigation. The structures of these compounds are discussed and the probable metabolism scheme is proposed. The mono- and dihydroxy- metabolites of the studied drugs are the major compounds excreted as conjugated in the urine.

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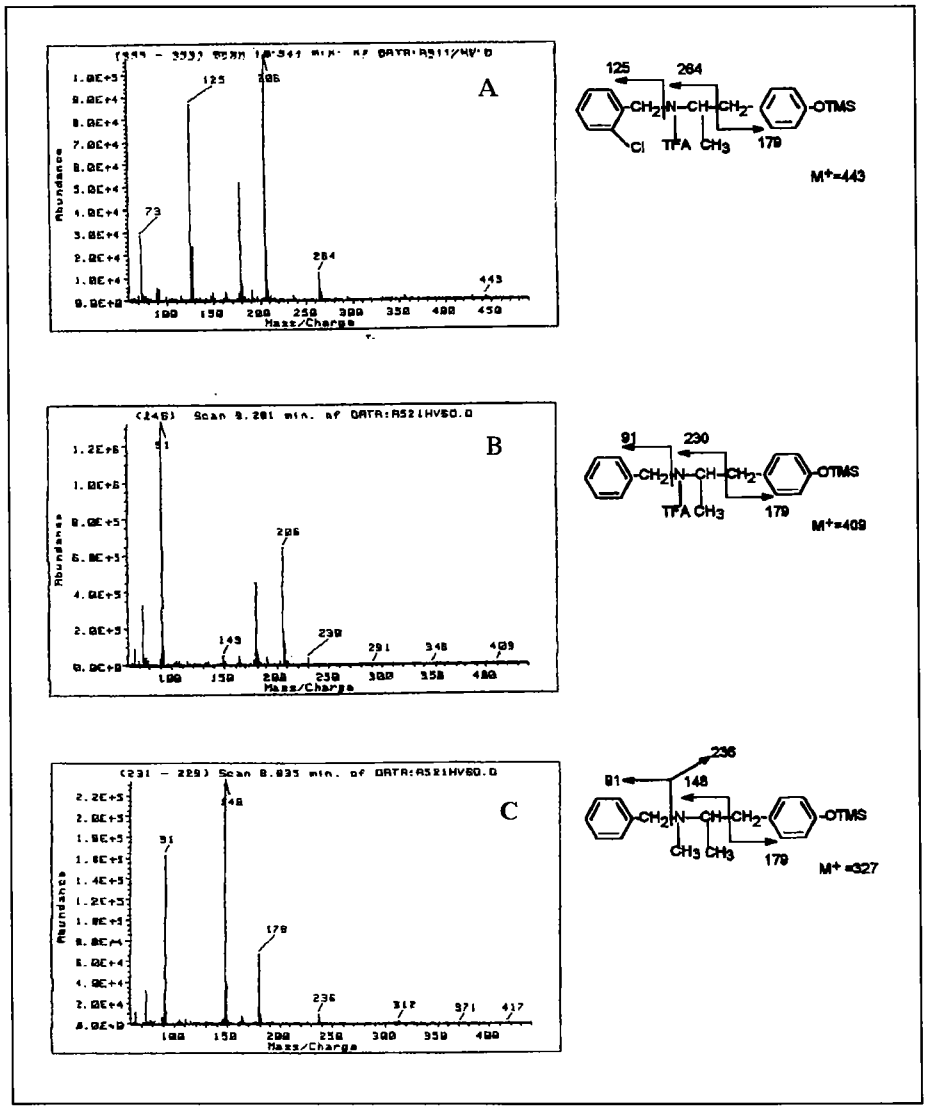

Fig.5. *Mass spectra of the monohydroxy metabolites* ***I*** *of clobenzorex (A), desmethylbenzphetamine (B) and benzphetamine (C) after derivatisation with MSTFA/MBTFA.*

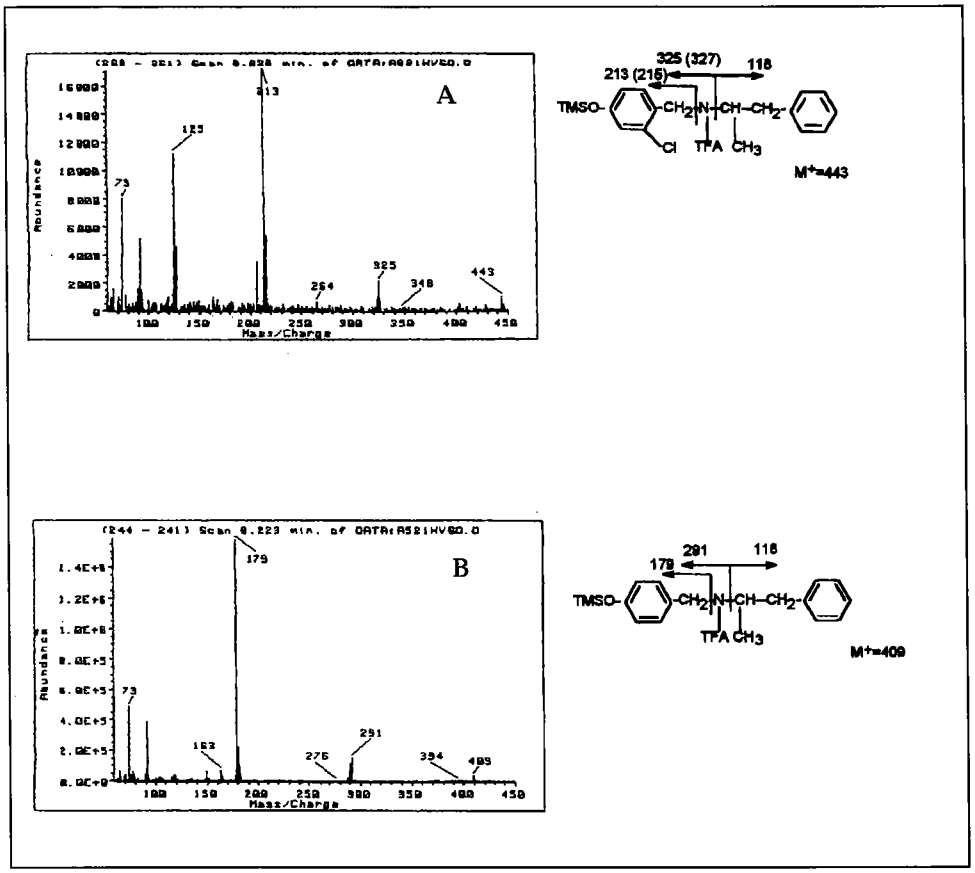

Fig. 6. *Mass spectra of the monohydroxy metabolites* ***II*** *of clobenzorex (A) and desmethylbenzphetamine (B) afer derivatisation with MSTFA/MBTFA.*

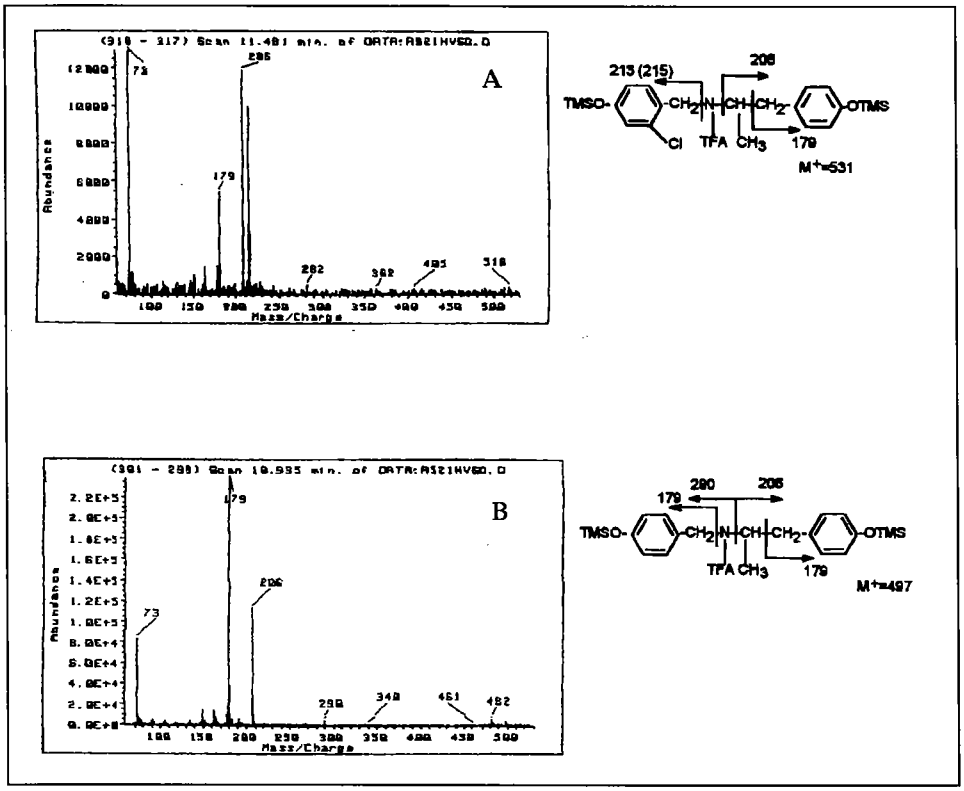

*Fig. 7. Mass spectra of the dihydroxy metabolites **IV** of clobenzorex (A) and desmethylbenzphetamine (B) after derivatisation with MSTFA/MBTFA.*

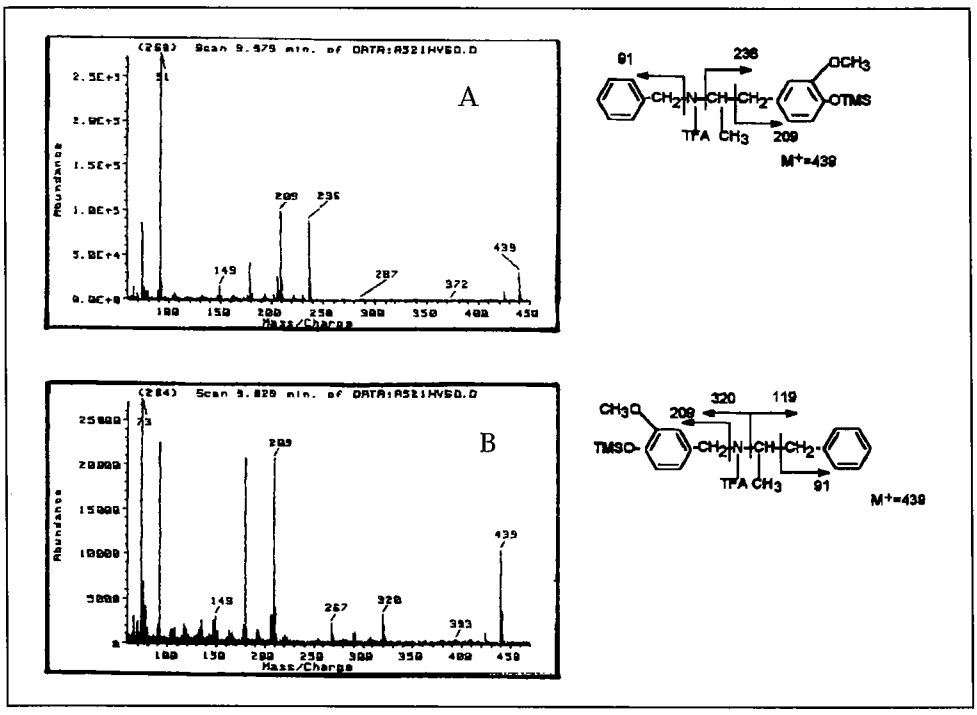

*Fig. 9. Mass spectra of the hydroxy- methoxy metabolites of desmethylbenzphetamine **VI** (A) and **VII** (B) after derivatisation with MSTFA/MBTFA.*

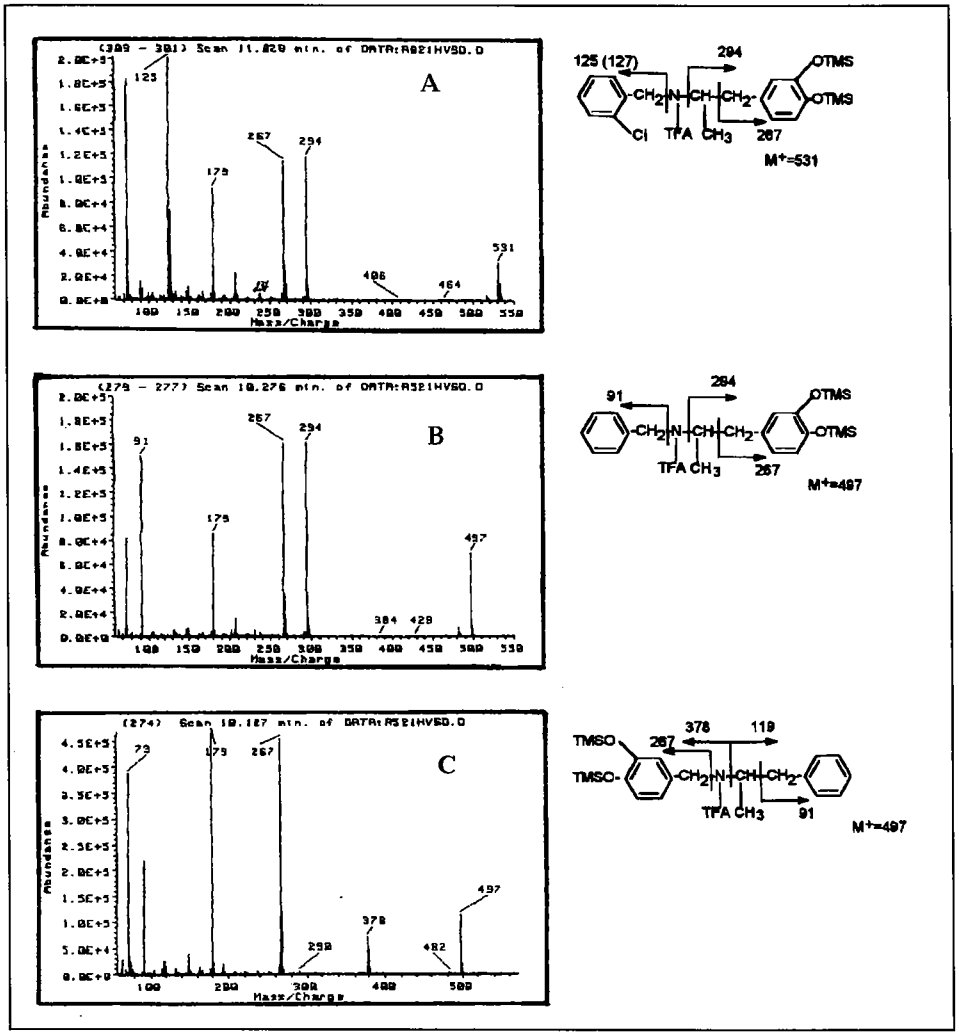

Fig. 8. *Mass spectra of the dihydroxy metabolites* ***III*** *of clobenzorex (A), desmethylbenzphetamine (B) and dihydroxymetabolite* ***V*** *of desmethylbenzphetamine (C) after derivatisation with MSTFA/MBTFA.*

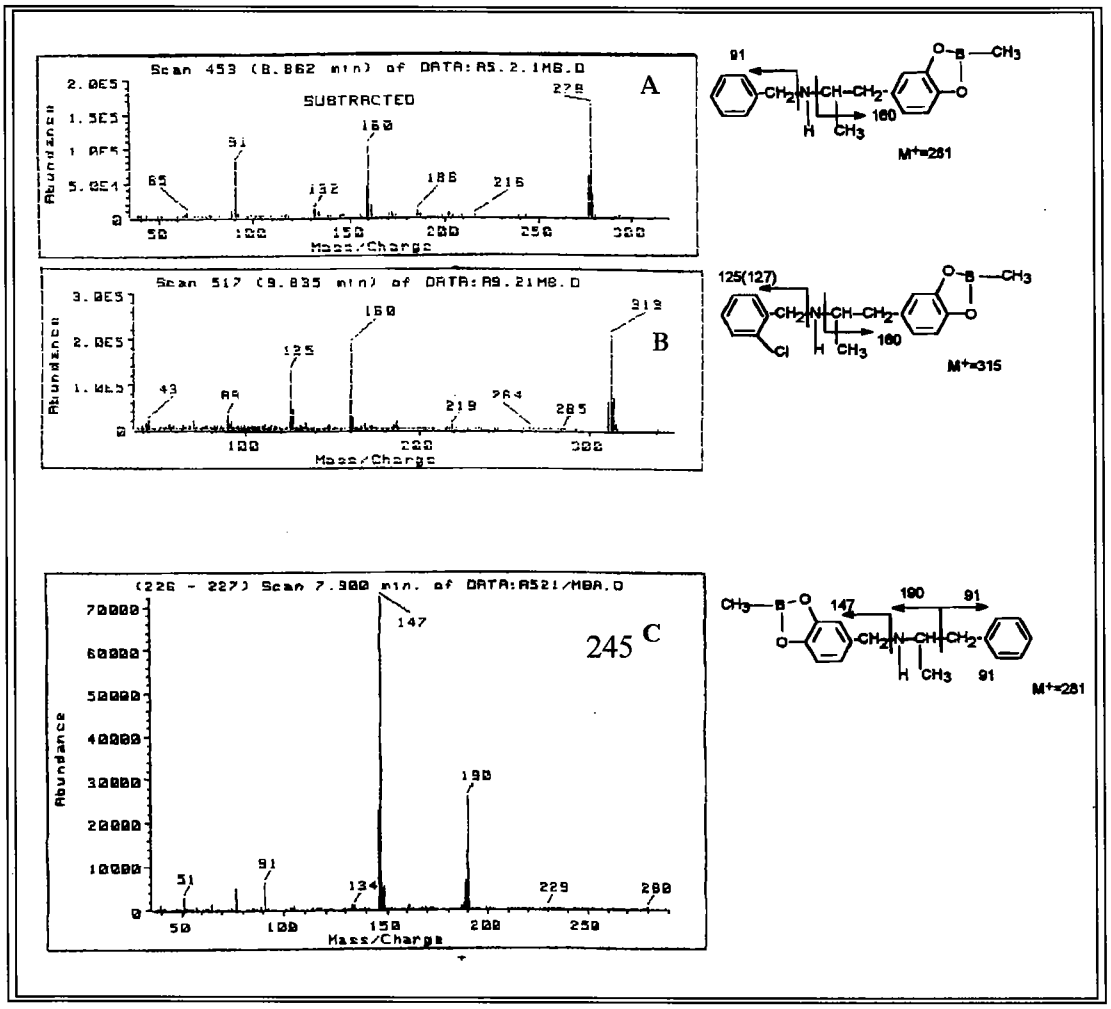

Fig.10. *Mass spectra of the dihydroxy metabolites* ***III*** *of desmethylbenzphetamine (A) clobenzorex (B) and dihydroxymetabolite* ***V*** *of desmethylbenzphetamine (C) after derivatisation with MBA.*

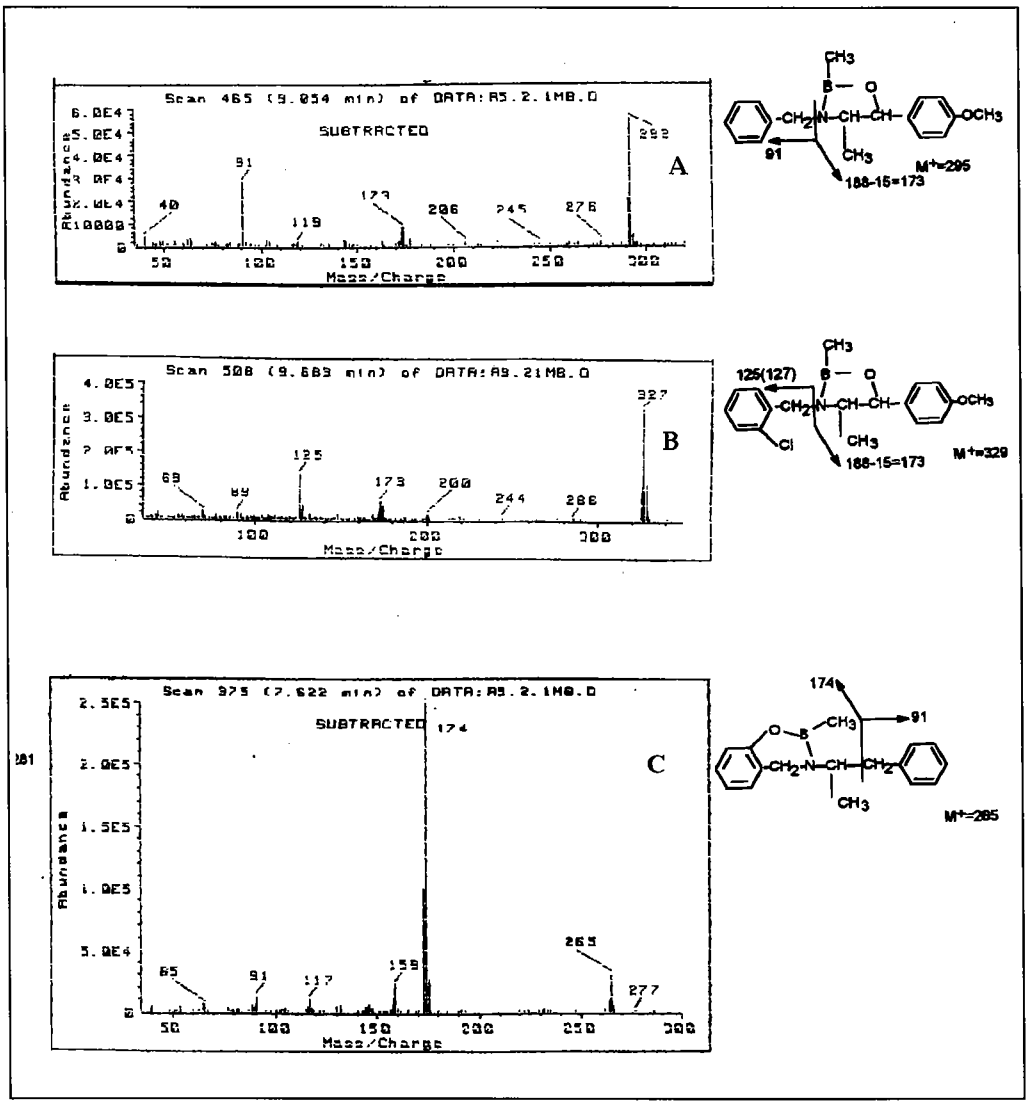

Fig.11. *Mass spectra of the hydroxy-methoxy metabolites* ***VIII*** *of desmethylbenzphetamine (A) clobenzorex (B) and monohydroxymetabolite* ***IX*** *of desmethylbenzphetamine (C) after derivatisation with MBA.*