METABOLISM OF PROXODOLOL STUDIED BY METHODS OF TLC, MASS SPECTROMETRY, AND NMR

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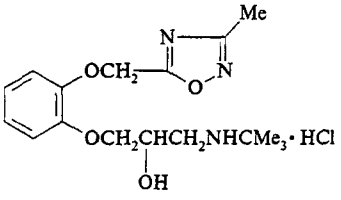
Proxodolol, or 5-[2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxymethyl]-3-methyl-1,2,4-oxadiazole hydrochloride, is a new β- andα-adrenoblocking drug created at the Center for Drug Chemistry – All-Russia Research Institute of Pharmaceutical Chemistry (Moscow) [1, 2].



The purpose of this work was to establish the structure of the main products of proxodolol bioconversion in rats using the methods of thin-layer chromatography (TLC), electron-impact mass spectrometry (EIMS), secondary-ion mass spectrometry (SIMS), and ^{1}H NMR spectroscopy.

EXPERIMENTAL PART

Experiments with animals. The study of proxodolol metabolism was performed on white mongrel intact rats weighing 200 – 300 g. Each test was carried out in a group of 8 – 10 animals. One day before testing, the animals were deprived of feeding and received only water. The test preparations were introduced perorally at a dose of 100 mg / kg in the form of an aqueous solution. Samples of urine were taken within 0 – 4, 4 – 7, and 7 – 24 h intervals after introduction of the drugs.

Extraction of metabolites from biological materials. Proxodolol and its metabolites were extracted either from primary urine samples at various pH values with solvents of different polarity (chloroform, benzene, ethyl acetate, methanol) or from urine samples dried in a rotor evaporator at 37°C. Completeness of the extraction was checked by TLC. Since the best results were obtained using dry urine samples, this technique was mainly used for the isolation of metabolites from biological materials. Both the drug and its major metabolites were rather completely extracted with chloroform. Polar metabolites were revealed upon subsequent extraction with ethanol.

Thin-layer chromatography. Proxodolol and its metabolites extracted with chloroform from urine samples were separated on TLC plates by elution in a benzene – ethanol – aqueous NH_4OH (25 %) system (45 : 15 : 1). The spots were visualized under UV irradiation and developed by Dragendorf's reagent. Separated phases were extracted from the thin layer by the method of descending elution [3] using methanol as the mobile phase.

Spectroscopic measurements. The electron-impact ionization mass spectra of proxodolol and its metabolites were obtained on a Varian MAT-112 chromato-mass-spectrometer (Germany) with direct sample injection in the ion source. The energy of ionizing electrons was 70 eV and the temperature of the ionization chamber, 180°C. The secondary-ion mass spectra were obtained on a Hitachi M-80A mass spectrometer (Japan). The samples were ionized in a glycerol matrix on a gold target bombarded with a primary beam of Xe^+ ions incident at an angle of 20° and having an energy of 8 eV. The pressure in the ionization chamber of the mass spectrometer was 0.2 Torr.

The ^{1}H NMR spectra were measured on a Varian XL-200 spectrometer (USA) with a working frequency of 200 MHz, using methanol-d_4 as the solvent and TMS as the internal standard.

SYNTHESIS OF MODEL COMPOUNDS

[2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]-acetamide hydrochloride (IV · HCl).

1) (2-Hydroxyphenoxy)acetamide. To 50 ml of concentrated aqueous ammonia solution, cooled with ice-cold water, was added by portions 15 g (0.1 mole) of 1,4-benzodioxin-

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2(3H)-one and the reaction mass was kept for 2 h. The precipitate was filtered and washed with water and cooled isopropyl alcohol to obtain 10.5 g (63%) of the target product purified by recrystallization from water; m.p., 129 – 130°C [4].

2) [2-(2, 3-Epoxypropoxy)phenoxy]acetamide. To a mixture of 5 g (0.03 mole) of (2-hydroxyphenoxy)acetamide in 15 ml of dioxane and 1.2 g of sodium hydroxide in 15 ml of water was added 15 ml of epichlorohydrin and the reaction mass was stirred for 3 h at 70 – 80°C. Then the solution was evaporated to dryness and the residue treated with anhydrous ether. The oily product crystallizes upon standing to yield 3.2 g of [2-(2,3-epoxypropoxy)phenoxy]acetamide (m.p., 104 – 107°C), which is used subsequently without additional purification [5].

3) [2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]-acetamide hydrochloride. A mixture of 1.6 g of as-obtained [2-(2,3-epoxypropoxy)phenoxy]acetamide, 5 ml of methanol, and 2.5 ml of *tert*-butylamine was boiled for 3 h and then evaporated. The residue was dissolved in acetone and treated with an ether solution of hydrogen chloride. The precipitate was filtered and recrystallized from absolute ethanol to obtain 1.1 g (44%) of IV · HCl; m.p., 153 – 154°C.

Found (%): C, 53.93; H, 7.42; N, 8.28. For $C_{15}H_{24}N_2O_4 \cdot HCl$ anal. calcd. (%): C, 54.11; H, 7.57; N, 8.42.

[2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]ac etic acid hydrochloride (III · HCl). To 30 ml of a 20% aqueous sodium hydroxide solution was added 2.25 g of amide IV and the mixture was heated with stirring for 5 h at 90 – 95°C. The mixture was cooled, acidified with hydrochloric acid to pH 6 – 7, and continuously extracted during 30 h with methylene chloride. Then the methylene chloride was distilled off and the residue triturated with acetone. The precipitate was filtered, dried in a vacuum desiccator, and recrystallized from from an isopropyl alcohol – hexane mixture. Finally, the product was dissolved in acetone and treated with an ether solution of hydrogen chloride to obtain 0.2 g of amino acid III hydrochloride; m.p., 118°C.

Found (%): C, 53.73; H, 7.20; N, 4.35. For $C_{15}H_{23}NO_5 \cdot HCl$ anal. calcd. (%): C, 53.97; H, 7.28; N, 4.20.

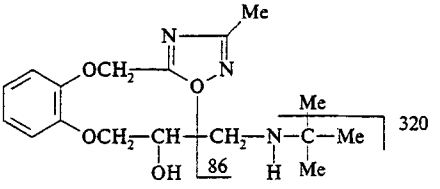
Study of the β- and α-adrenoblocking activity. The biological activity of proxodolol metabolites was studied on a group of male rats weighing 250 – 300 g narcotized with pentobarbital sodium (40 – 50 mg/kg, i.v.). The β-adrenoblocking action was determined by suppression of the positive chronotropic and depressor effects of isadrin (1 μg/kg, i.v.), and the α-adrenoblocking action was assessed by the influence on the processor effect of mesaton (0.1 mg/kg, i.v.).

RESULTS AND DISCUSSION

First we studied the laws of decomposition of the proxodolol molecule under conditions of electron-impact and secondary-ion ionization. It was established that the EIMS spectrum of proxodolol (Table 1) contains no peak of the molecular ion, but displays the characteristic peaks of ions caused by the chain decay of inderal.[2] The maximum peak intensity is observed for the amine fragment ($m/z = 86$). This fragment and the $[M - Me]^+$ ion ($m/z = 320$) are formed according to the following scheme:

TABLE 1. Characteristic Ion Peaks in the Mass Spectra of Proxodolol

Compound	EIMS, m/z (I_{rel})			SIMS, m/z (I_{rel})
	$[M–Me]^+$	$[M–Me–29]^+$	$CH_2=\overset{+}{N}HCMe_3$	MH^+
I	320 (12)	291 (9)	86 (100)	336 (100)
III	282 (23)	253 (15)	86 (100)	298 (100)
IV	281 (13)	252 (12)	86 (100)	297 (100)
V	296 (5)	267 (4)	86 (100)	312 (100)
VI	336 (7)	307 (6)	86 (100)	352 (100)



I

The fragmentation of proxodolol is also characterized by the elimination of 29 amu from the $[M–Me]^+$ ion (leading to a peak with $m/z = 291$), which can be related to the ejection of either CH_2NH or CH_2Me groups from the alkylamine substituent.

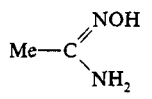
The SIMS spectrum of proxodolol shows an intense peak due to the quasimolecular MH^+ ion with $m/z = 336$.

Table 2 gives the parameters of the ^1H NMR spectrum of proxodolol. The presence of an asymmetric center in the inderal chain of the drug leads to significant nonequivalence of the protons of the CH_2 groups in this chain. The nonequivalent protons are manifested as two pairs of quartets (NCH_2 proton signal observed at a stronger field compared to that of OCH_2). The signal of protons of the $-OCH_2C\overset{//}{\backslash}$ group (remote from the asymmetric center) represents a broadened singlet with weak side components (AB type spectrum with $\Delta\delta\nu_0 / J \gg 1$).

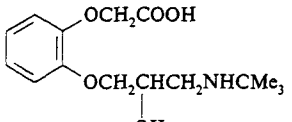
Since hydrolysis plays a considerable role in the process of bioconversion, we have studied the behavior of proxodolol in water and the HCl and NaOH solutions. It was found that the drug is stable with respect to incubation for 5 h at 80°C in water (pH 7) and in acid medium (pH 1), while heating in an alkaline medium (pH 10) results in hydrolysis of the oxadiazole ring with the formation of acetamide oxime (II) and

[2] The mass spectrum of proxodolol hydrochloride represents a superposition of the spectra of base and HCl [6].

[2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]acetic acid (III).



II



III

Compound II was identified by comparison with true acetamide oxime with respect to the chromatographic mobility, EIMS mass spectrum, and ^{1}H NMR spectrum (Table 2).[3]

The SIMS spectrum of compound III exhibits a peak of the quasimolecular MH^+ ion with $m/z = 298$. The EIMS spectrum retains the character of decay observed for proxodolol, showing no peak of the molecular ion. The maximum peak ($m/z = 86$) corresponding to the amine fragment $[CH_2{=}\overset{+}{N}H{-}CMe_3]$ and the peaks due to $[M{-}Me]^+$ ($m/z = 282$) and $[M{-}Me{-}29]^+$ ($m/z = 253$) indicate that compound III retains the inderal chain. In the ^{1}H NMR spectrum of this compound (studied in the form of hydrochloride), the signal due to protons of the $OCH_2C(=O)$ group shifts toward stronger field (by 0.71 ppm) as compared to the analogous signal in the spectrum of proxodolol, the signals of protons belonging to the inderal chain are retained, and the signal of the methyl group in position 3 of the oxadiazole ring is absent. These data suggest that the product of proxodolol hydrolysis has the structure of [2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]acetic acid (III). The final identification was performed by comparison with the model compounds obtained by direct synthesis.

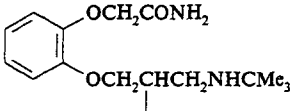
The SIMS spectra of the dry samples of urine obtained in the study of proxodolol metabolism in rats showed peaks of quasimolecular ions with $m/z = 352$, 336, 314, 312, 298, and 297 that were absent in the spectra of urine samples taken from control animals. The maximum intensity among these signals was observed for the peaks with $m/z = 336$ and 298. TLC patterns of chloroform extracts from urine samples taken 4, 7, and 24 h after proxodolol administration showed four intense spots at $R_f = 0.5$, 0.2, 0.12, 0.05 and weaker spots in the region of $R_f = 0.38 - 0.35$. The spots with $R_f = 0.05$, $0.38 - 0.35$, and 0.5 were absent in the chromatograms of urine extracts obtained from control animals.

The substance extracted from the spot with $R_f = 0.5$ was identified by the mass spectra as the base of nonmodified compound I. The SIMS spectrum showed an intense peak of the ions with $m/z = 336$, and the EIMS spectrum contained peaks with $m/z = 320$, 291, 86 having relative intensities identical with those in the mass spectrum of proxodolol. According to the TLC data, the nonmodified drug was present in all samples taken within 24 h after administration and its relative content decreased with time.

The compound extracted from the spot with $R_f = 0.05$ proved to be [2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]acetic acid (III). This assignment was based on the coincidence of data on the chromatographic mobility and the mass-spectrometric decay (Table 1) for the extracted substance and the model compound. As shown above, compound III is one of the major products of the alkaline hydrolysis of proxodolol.

According to the mass-spectrometric data, the TLC zone with $R_f = 0.12$ contains only substances of endogenic origin.

Substances extracted from the TLC zone with $R_f = 0.2$ contained, besides xanthine ($M^+ = 152$) of endogenic origin, a component whose SIMS spectrum (Table 1) showed a peak due to the MH^+ ion with $m/z = 297$. This signal indicates the presence of an even number of nitrogen atoms in the molecules of this metabolite. The EIMS spectrum of this compound (Table 1) contain peaks due to $CH_2\overset{+}{N}HCMe_3$ ($m/z = 86$). $[M{-}Me]^+$ (281), and $[M{-}Me{-}29]^+$ (252), which are characteristic of the chain decay of inderal. This combination of data allowed us to assign this metabolite the structure of 5-[2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]acetamide (IV). The proposed structure was confirmed by the coincidence of mass spectra and the values of chromatographic mobility observed upon comparison of the extracted substance with the model compound obtained by direct synthesis.



IV

Extracts from the TLC zone with $R_f = 0.38 - 0.35$ contained minor amounts of two compounds, the SIMS spectra of which showed the molecular ions with $m/z = 312$ and 352 (Table 1). These compounds apparently retained the inderal

[3] The mass spectrum of compound II contains a peak due to molecular ions with $m/z = 74$ (52), while the most intense peak with $m/z = 42$ belongs to $[CH_3{-}C{=}\overset{+}{N}H]$.

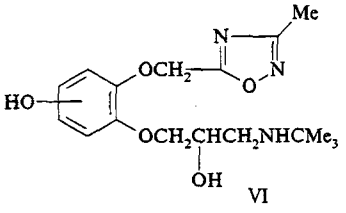
TABLE 2. Characteristics of ^{1}H NMR Spectra of Proxodolol, Products of Alkaline Hydrolysis, Metabolites

Compound	Chemical shift (δ, ppm) in methanol						
	$OCH_2C(=O)$	C–Me	Ar	OCH_2	—CH—	CH_2N	CMe_3
I	5.38 s	2.39 s	6.95 – 7.10	4.06 q 4.13 q	4.23 m	3.15 q 3.34 q	1.41 s
II		1.79 s					
III · HCl	4.67 s*		6.95 – 7.05	4.07 q 4.13 q	4.21 m	3.18 q 3.36 q	1.42 s
VIII		2.21 s				,	

* In the spectrum of methanol urine extract δ = 4.42 ppm, the other proton signals overlap with more intense signals of proxodolol I.

chain, because their EIMS spectra contained an intense peak of the amine fragment at $m/z = 86$ and the peaks of $[M–Me]^+$ and $[M–Me–29]^+$ ions (296, 267 and 336, 307, respectively).[4] We postulate that the compound with a molecular ion corresponding to $m/z = 311$ is a methylation product of metabolite III ($M^+ = 297$). Acid III has two possible methylation sites: the nitrogen atom of the inderal chain and the carboxy group. The former variant contradicts the mass-spectroscopic data, since the spectra contains no peaks due to the amine fragment $CH_2{=}\overset{+}{N}(Me)CMe_3$ with $m/z = 100$. Thus, the mass-spectrometric pattern of decay of the metabolite with $m/z = 312$ (MH^+) agrees with the structure of methyl ester of [2-(3-*tert*-butylamino-2-hydroxypropoxy)phenoxy]acetic acid (V). Methylation of acids in the course of bioconversion is a well-known [7], albeit rare, phenomenon, leading to the formation of lipophilic compounds.

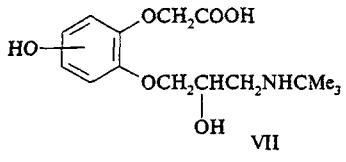
Another substance extracted from the zone with $R_f = 0.38 – 0.35$ has a molecular weight 16 amu higher as compared to that of the drug, which is indicative of one oxygen atom added to the drug molecule. Because the mass spectrum of this compound retains the ion peaks at $m/z = 86$, 336 ($M–Me]^+$), and 307 ($[M–Me–29]^+$) characteristic of thechain decay of inderal, it is obvious that the inderal chain is conserved. We postulate that the drug exhibits a metabolic conversion involving hydroxylation of the aromatic ring, which is typical of the aromatic compounds [7, p. 10]. Therefore, the metabolite with $m/z = 352$ (MH^+) can be assigned the structure of compound VI:



VI

According to the TLC data, the amount of metabolites V and VI is markedly lower compared to that of metabolite III.

The SIMS spectrum of the total methanol urine extract shows, besides the peaks of the metabolites considered above, a peak of the quasimolecular ion with $m/z = 314$ that can be attributed to compound VII:

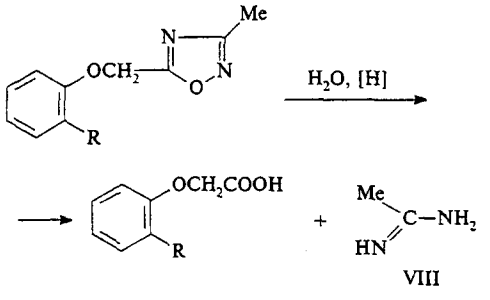


VII

According to the SIMS data, no conjugated forms of proxodolol metabolites are present in the system studied.

The ^1H NMR spectra of methanol urine extracts obtained from test animals showed eight intense signals (5 singlets and three multiplets) not observed in the spectra of control urine extracts (Table 2).[5] These signals (except for the singlet at 2.21 ppm) refer to the protons of nonmodified drug I and acid metabolite III. Note the upfield shift of the proton signal from the $OCH_2C\overset{//}{\backslash}$ group of compound III in the spectrum of the methanol urine extract ($\delta = 4.42$ ppm) compared to the analogous signal in the spectrum of chloride in methanol ($\delta = 4.67$ ppm). This may indicate both the existence of compound III in the form of a zwitterion and the formation of salts between compound III and amines (including proxodolol) present in the urine extract.

The singlet observed at 2.21 ppm was assigned to acetamidine VIII. This assignment was confirmed by comparison with the spectrum of a methanol urine extract with addition of acetamidine.[6] We believe that the formation of acetamidine is related to hydrolysis of the oxadiazole ring to acetamide oxime, followed by its reduction (acetamide oxime II formed upon the alkaline hydrolysis of proxodolol was not detected among the metabolites extracted from urine).



VIII

R = $OCH_2CH(OH)CH_2NHCMe_3$.

Thus, the bioconversion of proxodolol proceeds for the most part at the expense of scission of the oxadiazole ring with the formation of acid III, amide IV, and amidine VIII. There is also insignificant oxidation of the benzene ring in the drug molecule, with the formation of compounds VI and VII, and methylation of acid III. The inderal chain is subjected to no significant metabolic transformations.

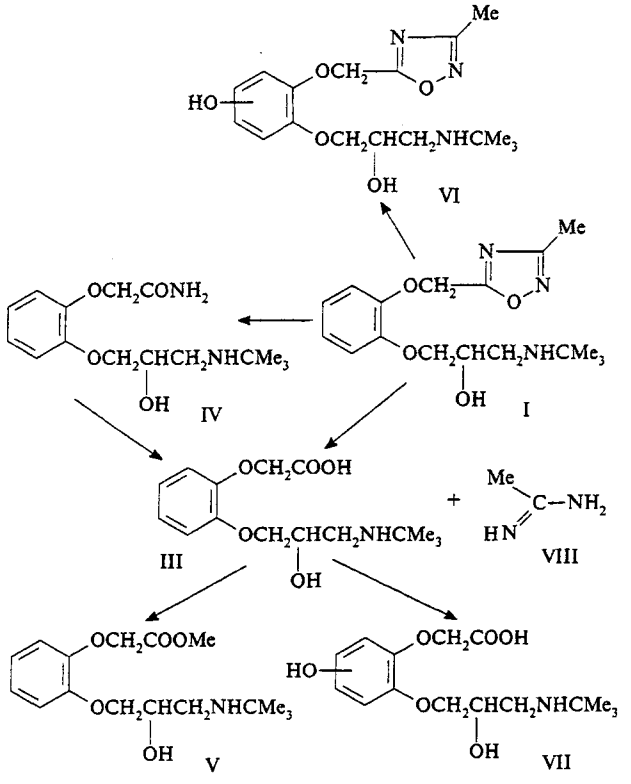
We have studied the biological activity of the main proxodolol metabolites, acid III and amide IV. It was found that compound III injected intravenously at a dose of 0.1 or 0.5 mg/kg does not modify the effect of isadrin, while a dose of 2 mg/kg suppresses the depressor and chronotropic effects of isadrin by 53 and 32%, respectively (averaged over three tests). Compound IV injected at a dose of 0.1, 0.5, or

[4] The two ions belong to different compounds, as indicated by a significant synchronous redistribution of relative peak intensities between the 296, 267, 336, and 307 pairs observed during the sample evaporation.

[5] In this experiment the extraction with methanol was preceded by extraction with ethyl acetate According to the mass-spectroscopic data, ethyl acetate takes away the major proportion of amide IV.

[6] Mass-spectrometric identification of acetamidine with $m/z = 58$ (M^+) is hindered because the region of small masses contains a number of peaks reflecting the fragmentation of endogenic substances.

2 mg/kg inhibited the depressor effect by 79, 71, and 60% (averaged over 16 tests) and the chronotropic effect by 46, 75, and 60%, respectively.



These data are indicative of the absence of β-adrenoblocking activity in compound III and the weak activity of compound IV (on the average, two orders of magnitude lower compared to the activity of proxodolol).

Investigation of the α-adrenoblocking activity of compounds III and IV at a dose of up to 10 mg/kg showed no influence on the pressor effect of mesaton, which is indicative of the absence of α-adrenoblocking properties in these proxodolol metabolites.

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