to the initial value: for sulfamonomethoxine 238 ± 21 μg, for sulfalene 240 ± 33 μg, and for sulfaethidole 259 ± 26 μg. On the basis of the data obtained, it can be considered that the distribution of the sulfanilamides studied in the blood occurs only in the plasma. A determination of the sulfanilamide concentration in the blood of rats and in the blood serum showed half as great a concentration of the preparations in the blood. For example, in the case of intramuscular injection of sulfamonomethoxine-meglumin into animals in a dose of 100 and 200 μg/kg, 82 ± 0.2 and 109 ± 1.6 μg/ml of the preparation was determined in the blood after 6 h, respectively, and 175 ± 3.3 and 216 ± 2.6 μg/ml in the blood serum (average data for 30 rats). Similar ratios were also obtained with other sulfanilamides. These data confirm the results described above for experiments *in vitro*. Consequently, erythrocytes in native blood practically do not bind sulfamonomethoxine, sulfalene, and sulfaethidole. It can be assumed that the interaction of the preparations with isolated erythrocytes in artificial medium does not reflect the principles of the true distribution of sulfanilamides in the blood. The deciding factor for the evaluation of the active concentration of the preparations in blood is a consideration of the degree of their binding in the plasma.

LITERATURE CITED

1. B. Davis, J. Clin. Invest., 22, 753-762 (1943).
2. B. B. Newbould and R. Kilpatrick, Lancet, 1, 887-891 (1960).
3. A. H. Anton, J. Pharmacol. Exp. Ther., 129, 282-290 (1960).
4. B. Qydis, Acta Path. Microbiol. Scand., 68, 81-92 (1966).
5. G. Ya. Kivman, É. A. Rudzim, and V. P. Yakovlev, in: Methods of Experimental Chemotherapy [in Russian] (G. N. Pershin, ed.), Moscow (1971), pp. 491-497.
6. N. P. Neugodova, G. Ya. Kivman, and I. Ya. Geitman, Khim. Farm. Zh., No. 1, 23-26 (1977).
7. L. M. Polukhina, in: Methods of Experimental Chemotherapy [in Russian] (G. N. Pershin, ed.), Moscow (1971), pp. 454-457.
8. G. Ya. Kivman, A. A. Firsov, N. P. Neugodova, et al., Khim. Farm. Zh., No. 3, 15-19 (1978).

METABOLISM OF PHENAZEPAM IN THE RAT ORGANISM

N. Ya. Golovenko, V. G. Zin'kovskii, A. V. Bogatskii, P. A. Sharbatyan, and S. A. Andronati

UDC 615.22:547.891.034

In previous studies [1, 2] we showed that in the bodies of white rats phenazepam is hydroxylated with the formation of metabolites containing the hydroxyl group in the 3-position and in the aromatic rings. In the indicated studies, the direction of the aromatic hydroxylation of phenazepam was not established. The purpose of the present investigation was a detailed study of the structure of the metabolites of phenazepam formed in the bodies of white rats as a result of hydroxylation of its aromatic ring.

EXPERIMENTAL

The experiments were conducted on male white rats weighing 180-240 g. The experiments received an interperitoneal injection of [^{14}C]phenazepam (1 Ci/mole) in Tween emulsion in a dose of 50 mg/kg. Urine specimens were collected for two days after administration of the preparation. The isolation of phenazepam and its free and conjugated metabolites was performed according to the method described earlier [1, 3]. Chromatographic purification of the extracts, as well as separation of the metabolites, were performed on Silufol UV-254 chromatographic plates [1]. The portions of the chromatograms containing phenazepam or its metabolites were cut out, extracted twice with alcohol and hot acetone, the eluates evaporated to dryness, and dried in a vacuum drying oven. The dry residues were investigated by physicochemical methods and subjected to hydrolysis with 6 N hydrochloric acid on a boiling water bath for 30 min. The hydrolysis products were extracted and freed of coextractive substances according to the method of [3]. The separation of benzophenones was performed in

I. I. Mechnikov Odessa University.

Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 14, No. 4, pp. 15-21, April, 1980. Original article submitted August 3, 1979.

0091-150X/80/1404-0208$07.50

TABLE 1. Physicochemical Properties of Phenazepam (I), Its Metabolites (II-IV), and the Corresponding Benzophenones (BP)

Metabolite	R_f*	UV spectrum λ_{max}, nm	IR spectrum, cm^{-1}	$E_{1/2}$, mV	Product isolated after hydrolysis
I:					
synthetic	0,85	229,322	1700	1075	BP-I
from organism	0,85	230, 320	1700	1075	BP-I
II:					
synthetic	0,45	—	1705	1100	BP-I
from organism	0,45	231, 322	1695	1125	BP-I
III, " "	0,15	231, 322	1695	1100	BP-III
IV, " "	0,08	243, 313	—	1100	BP-IVa, BP-IVb, and BP-IVc
BP -I:					
synthetic	0,85	205, 322, 263, 374	3350—3505	1325	—
from organism	0,85	204, 233, 263, 376	3340—3500	1300	—
BP -III, from organism	0,35	206, 230, 261, 386	3380—3495	1350	—
BP-IVa, " "	0,65	204, 232, 270, 380	3380—3495	1330	—
BP -IVb, " "	0,50	205, 230, 272, 378	—	1350	—
BP -IVc, " "	0,42	206, 241, 273, 396	3370—3515	1320	—

*For I-IV the solvent system was acetone—chloroform—30% ammonia (1:1:0.05); for BP-I to BP-IVc it was carbon tetrachloride—chloroform (2:2).

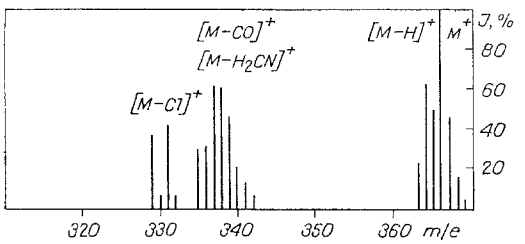


Fig. 1. Mass spectrum of the metabolite III.

the solvent system CCl_4—chloroform (1:1 and 1:2), as well as in diethyl ether and petroleum ether. The spots on the chromatograms were determined visually in UV light (253.7 and 328 nm). Qualitative determination of aromatic amines and aminophenols in the hydrolysis products of phenazepam metabolites was performed by the reactions of diazotization [3] and azo-coupling with α-naphthol, β-naphthol, and resorcinol (freshly prepared solutions from a reagent—acetone—water mixture 9:100:9900).

The metabolites and their hydrolysis products were investigated by polarography (PPT-1 polarograph) on a dropping mercury electrode in a mixture from acetate buffer pH 4.0 and DMFA (1:1), UV spectroscopy (Specord UV Vis) in alcohol, IR spectroscopy (Perkin-Elmer-577) in anhydrous CCl_4, and in KBr tablets, and by mass spectrometry on a MKh-1303 instrument.

RESULTS AND DISCUSSION

Analysis of Phenazepam Metabolites. An analysis of the radiochromatograms of extracts of the urine of rats that received [^{14}C]phenazepam showed that they contained four metabolites (I-IV [1]). The R_f values and physicochemical properties of substances I and II are evidence that they are identical with the initial phenazepam and its 3-hydroxy derivative, respectively (Table 1). The hydrolysis product of these compounds was 5-bromo-2-amino-2-chlorobenzophenone (see analysis of BP).

It was most difficult to identify compounds III and IV in connection with the absence of synthetic analogs of them. According to the data of polarographic reduction and the IR spectra (intense absorption in the region of 1680-1700 cm^{-1} CO in the heterocyclic ring), the indicated compounds contain an azomethine bond. On the basis of these data and the UV spectra (see Table 1), it was preliminarily concluded that III and IV have the structure of 1,4-benzdiazepines. However, we were able to obtain the most unambiguous information on the structure of these substances in mass spectrometric analysis of III, as well as an investigation of the hydrolysis products of III and IV.

Mass spectrometric decomposition of 1,4-benzidiazepin-2-ones has been studied in detail [4, 5]. The mass spectrum of III in the region of high values of m/e is presented in Fig. 1. In this case the peaks with m/e 364, 366, and 368 are due to molecular ions and are evidence of the presence of Cl and Br atoms. In comparison with phenazepam, the molecular weight of

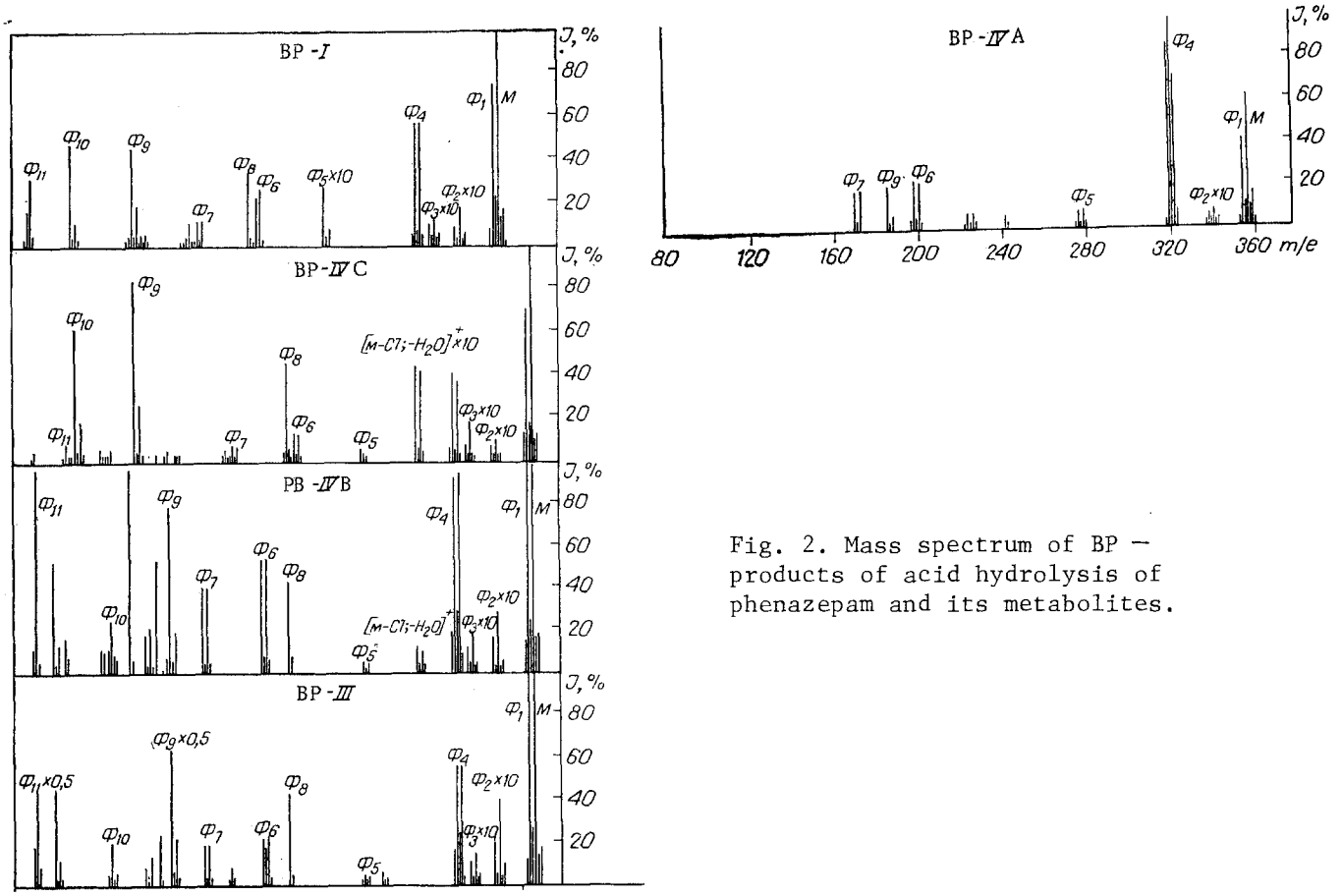
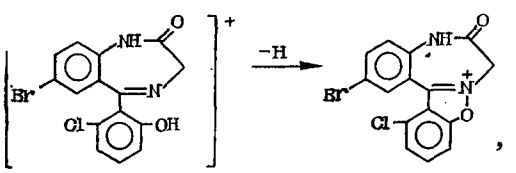


Fig. 2. Mass spectrum of BP — products of acid hydrolysis of phenazepam and its metabolites.

the metabolite is increased by 16 amu (mass of the oxygen atom). Evidently in the process of metabolism, one of the hydrogen atoms in the phenazepam molecule was replaced by a hydroxy group. The presence of peaks of the ions $[M-H_2CN]^+$ m/e 336, 338, and 340 is an unambiguous indication that substitution did not occur at the 3-position (as in the case of the 3'-hydroxy derivative). The peaks of the ions $[M-H]^+$ (m/e 363, 365, and 366), as well as $[M-Cl]^+$ (m/e 329 and 331), are due to processes of splitting out of H and Cl atoms from the corresponding molecular ions, while those of the ions with m/e 335, 337, and 339 are due to successive elimination of an H atom and a CO molecule.

No direct indication of the position of the hydroxy group was detected in the mass spectrum (for example, the peak of $[M-OH]^+$ was absent); if it had been present, we might have been able to speak of substitution in the ortho-position of the 5-phenyl ring. Nonetheless, the increase in the relative intensity of the peaks of the ions $[M-H]^+$ (62% in comparison with 55% for phenazepam) and the decrease in the relative intensity of the peaks of the ions $[M-Cl]^+$ (40% in comparison with 51%) are an indirect indication that the following process may compete with the elimination of Cl from the ortho-position of the phenyl ring of the molecule:



which was noted in [5] for 5-phenylbenzdiazepin-2-ones containing a methyl group in the ortho-position. Such a conclusion on the position of the hydroxy group in III was subsequently confirmed in an analysis of its hydrolysis product.

Establishment of the Structure of Phenazepam Metabolites According to Their Hydrolysis Products. Acid hydrolysis of phenazepam and its metabolites leads to the formation of the corresponding 2-aminobenzophenones. As a result of cleavage of the heterocyclic ring, some information on the structure of the initial metabolite is lost. In particular, it cannot be established whether the metabolites are derivatives of phenazepam or a product of it, oxidized

TABLE 2. Basic Directions of Decomposition of 5-Bromo-2-chloro-2-aminobenzophenone in a Mass Spectrometer

Ion	Index	Intensity, % of $[M]^+$	m/e	Notes
$[M]^+$	M^+	100	309	Molecular ion
$[M-H]^+$	Φ_1	24,4	308	Elimination of a hydrogen ion from M^+, most likely from the ortho-position of the chlorophenyl ring, with the formation of a cyclic structure
$[M-OH]^+$	Φ_2	1,6	292	Elimination of an OH radical, evidently from the enol form of M^+
$[M-CHO]^+$	Φ_3	1,5	280	Elimination of a CHO· radical, rearrangement process
$[M-Cl]^+$	Φ_4	59,6	274	Basic process, accompanied by metastable transition, mechanism of formation and structure similar to ions Φ_1
$[M-Br]^+$	Φ_5	1,9	230	Elimination of a bromine atom
$[M-C_6H_4Cl]^+$	Φ_6	25,4	198	Cleavage of the CO-Ar bond characteristic of BP
$[M-COC_6H_4Cl]^+$	Φ_7	11,4	170	The same
$[M-Cl, -Br]^+$	Φ_8	17,4	195	Elimination from Φ_4 ions
$[COC_6H_4Cl]^+$	Φ_9	38,4	139	Cleavage at CO-Ar bond
$[C_6H_4Cl]^+$	Φ_{10}	29,8	111	Cleavage at CO-Ar bond and elimination by Φ_9 ions of a CO molecule, which is confirmed by the metastable peak
$[M-COC_6H_4Cl, -Br]^+$	Φ_{11}	17,0	91	Elimination of a bromine atom under the influence of the Φ_7 ion

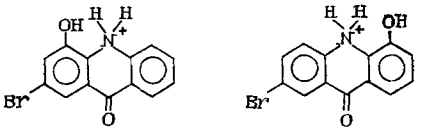
at the 3-position. Moreover, we cannot answer the question of whether there is a constriction of the benzdiazepine ring to a quinazoline ring. And, finally, the hydrolysis products of phenazepam and its metabolites (2-^{14}C) lose radioactive carbon, and their quantitative determination is hindered. However, a study of the structure of the hydrolysis products of 1,4-benzidiazepine metabolites permits a judgment of the directions of the processes of aromatic hydroxylation of preparations of this class.

In the hydrolysis of synthetic phenazepam and its 3-hydroxy derivative, as well as in those isolated from rat urine, a 2-aminobenzophenone (BP-I) was detected (see Table 1), whose diazotization product reacted with α- and β-naphthol and resorcinol to form colored (violet and red, respectively) azo-dyes. The hydrolysis product of III was a benzophenone (BP-III), which differed from the preceding. In a study of the hydrolysis products of IV it was found that the latter is not an individual compound, but a mixture of at least three substances. The physicochemical properties of the hydrolysis of IV — 2-aminobenzophenones (BP-IVa-c) are presented in Table 1.

Mass spectrometric decomposition of monosubstituted benzophenones has been studied sufficiently [5, 6], and consists of cleavage of bonds between the carbonyl and the aromatic ring with conservation of charge on one or another fragment being formed. Substituents practically do not affect the direction of decomposition, but only approximately regularly (corresponding to their own electronic properties) redistribute the electron density on the molecular ion formed and thereby change the relative intensities of the peaks of the molecular and fragmented ions. The presence of several substituents in benzophenone and especially Cl in the ortho-position substantially complicates the decomposition, and, consequently, the mass spectra as well (Fig. 2). The main directions of decomposition are presented in Table 2. Now let us compare the mass spectra of benzophenones obtained from the four isolated metabolites, and let us determine the basic changes in the structures relative to a standard (see Fig. 2).

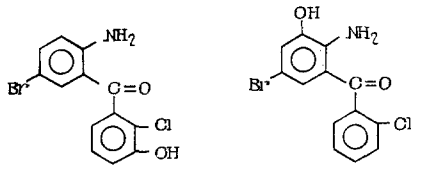
In the mass spectrum of BP-IV, the peaks of the molecular ions were shifted by 16 amu in the direction of larger values of m/e, which is evidence of the presence of hydroxyl in the molecule. The peaks Φ_{1-8} and Φ_{11} were correspondingly shifted by 16 amu, whereas m/e for the Φ_9 and Φ_{10} ions retained their values, which is evidence of the presence of a hydroxyl group in the arylamine ring in BP-IV. We should also note that ions Φ_4 $[M-Cl]^+$ also eliminate a water molecule (m/e 272-274), which was not observed in the mass spectrum of BP-I. Consequently, the hydroxy groups participate in this process.

In the mass spectrum of compound BP-IVb, the peaks of the molecular ions were also shifted by 16 amu in the direction of larger values of m/e. A corresponding increase in mass was observed for the ions Φ_{1-5} and Φ_{8-10}. The ions Φ_6, Φ_7, and Φ_{11} retained their position on the m/e scale. These changes in the spectrum in comparison with the mass spectra of the standard (BP-I) are evidence that the hydroxy group in the given benzophenone is in a chlorine-containing benzene ring. Just as in the case of BP-IVc, the Φ_4 ions lose a water molecule. On the basis of the fact that hydroxyl participates in this process, we should assume an equilibrium position of the hydroxy groups in the fragment ions Φ_4, and on the basis of this, we should ascribe the following structures to the ions Φ_4:



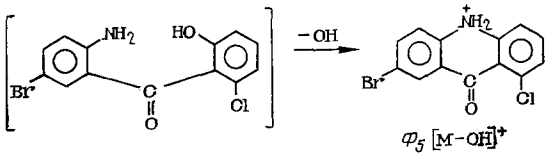
φ_4 (BP-4c) φ_4 (BP-4b)

In this case the elimination of a water molecule by the Φ_4 ions proceeds according to a monotypic mechanism with the participation of the hydrogen of the amino group. Thus, the $[\Phi_4-H_2O]^+$ ions proved extremely informative, despite the fact that their peaks in the mass spectra of both compounds had low intensity (4.4 and 11.3%, respectively), and they permitted the establishment of the precise position of the hydroxy group in these compounds (in a comparative study of their mass spectra).



BP-IVb BP-IVc

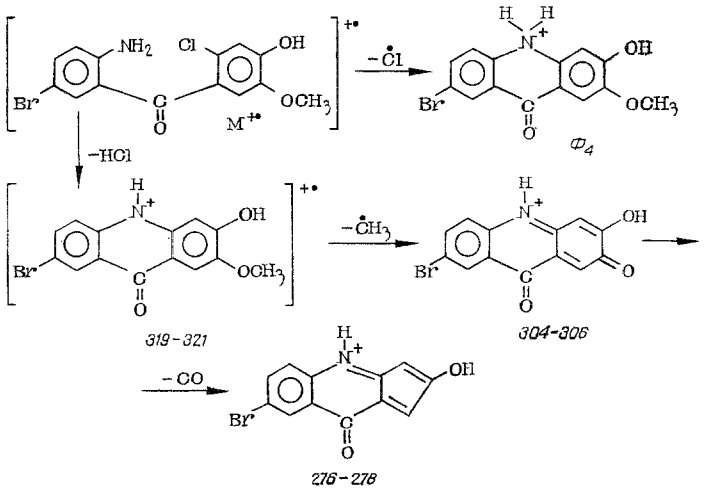
All the displacements of the values of m/e of the peaks of M^+ and fragment ions in the mass spectrum of BP-III (in comparison with BP-I taken as the standard) entirely coincide with the spectrum of BP-IVb (see Fig. 2). Consequently, in this benzophenone as well, the hydroxy group is in the chlorobenzene ring. However, this spectrum does not contain the peak of the $[\Phi_4-H_2O]^+$ ions, which indicates a different position of the hydroxyl in BP-III and BP-IV. Taking into consideration the fact that the relative intensity of Φ_2 $[M-OH]^+$ in the spectrum of BP-III was greatly increased (4.7% in comparison with 1.6% for BP-I), it can be assumed that in this case the hydroxy group is in the ortho-position of the chloro-substituted benzene ring. Moreover, in addition to the general pathway of formation of Φ_5 ions, a parallel process giving the same values of m/e can also occur.



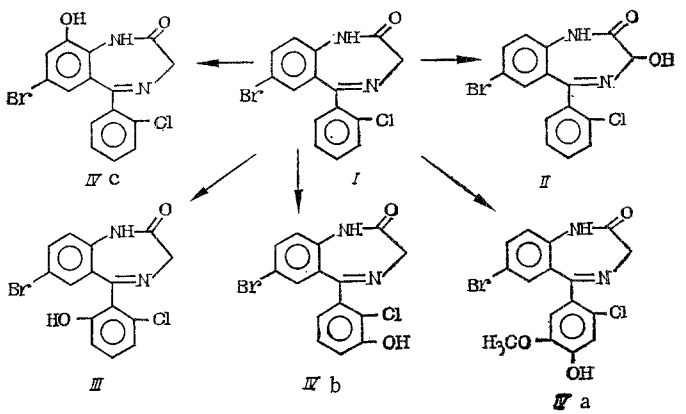
φ_5 $[M-OH]^+$

The molecular weight of BP-IVa was increased by 46 amu (see Fig. 2) in comparison with BP-I. This increase is due to a substituent (or substituents) in the chlorobenzene ring, since the peaks of the ions Φ_6 and Φ_7 (m/e 198-200 and 170-172), formed as a result of elimination of this fragment from the molecular ion, retained its position in the mass spectrum. At the same time, the peak of the Φ_6 ion was shifted by 46 amu in the direction of larger values of m/e. It is most probable that of all the possible combinations of elements with mass 46 (N_{O2}; H_2O; CH_2O_2; CH_6N_2; C_2H_6O; C_2H_8N), the elements of CH_2O_2 were added to the BP-I molecule in the process of phenazepam metabolism. On the basis of the fact that the molecular ions do not lose an OH group (the relative intensity of the peaks of the Φ_2 ions was not increased in comparison with the mass spectrum of BP-I), while the Φ_4 ions $[M-Cl]^+$ do not eliminate water or CH_3OH molecules, it can be assumed that the 3- and 6-positions are not substituted. Further decomposition of the Φ_4 ions consists of elimination of a hydrogen atom, characteristic of ionic structures in which the methoxy group is in conjugation with the charged center, followed by elimination of a CH_3 radical with m/e 304 and a CO molecule with m/e 276. Such a direction

of decomposition indirectly indicates that the methoxy group is in the 5-position, while the hydroxyl is a substituent in the 4-position. On the basis of this, the decomposition of BP-IVa can be represented by the following scheme:



Thus, on the basis of the literature data [1] and the results presented, the basic pathways of phenazepam metabolism in the bodies of white rats can be represented as follows:



All the metabolites of phenazepam form the corresponding glucuronides. The possibility remains that some of them (for example, IVa-c) contain a hydroxyl group in the 3-position of the heterocyclic ring, as is the case in the metabolism of diazepam [7], oxazepam [8], and lorazepam [9].

LITERATURE CITED

1. N. Ya. Golovenko, V. G. Zin'kovskii, A. V. Bogatskii, et al., Khim. Farm. Zh., No. 1, 21-26 (1979).
2. L. N. Yakubovskaya, A. V. Bogatskii, S. A. Andronati, et al., Ibid., No. 2, 85-89.
3. N. Ya. Golovenko and V. G. Zin'kovskii, Ibid., No. 1, 3-14 (1978).
4. W. Sadee, J. Med. Chem., 13, 475 (1970).
5. P. A. Sharbatyan, "Mass spectrometric investigation of benzodiazepines and their derivatives," Candidate's Dissertation [in Russian], Moscow (1965).
6. J. H. Beynon, Mass Spectrometry and Its Application to Organic Chemistry, American Elsevier (1960).
7. O. S. Anisimova, L. F. Linberg, and Yu. N. Sheinker, Mass Spectrometry in the Investigation of Drug Metabolism [in Russian], Moscow (1978).
8. M. A. Schwartz, P. Bommer, and F. M. Vane, Arch. Biochem., 121, 508-516 (1967).
9. S. Walkenstein, R. Wiser, C. Gudmundsen, et al., J. Pharm. Sci., 53, 1181-1186 (1964).