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PHARMACOKINETIC PROFILE AND METABOLISM OF 2-ETHYL-6-METHYL-3-HYDROXYPYRIDINE IN RATS

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Summary. – *The wide spectrum of psychopharmacological effects of 3-hydroxypyridine derivatives was the base for investigating pharmacokinetics and metabolism of a representative agent from the antioxydant group, i.e. 2-ethyl-6-methyl-3-hydroxypyridine (compound I). Quantitative analysis of the unchanged substance was carried out by using HPLC method. Compound I was found to be quickly absorbed from the abdominal cavity of animals with semi-absorption period of 0,94 hours; its max concentration in plasma, liver and brain is measured in 2-3 hours during 24 hours. Using mass-spectrometry and synthesized standards witnesses, there were found and identified 5 metabolites of compound I, which were dealkylated and conjugated products. From metabolites found, greatest interest was paid to conjugate of the parent compound with phosphate (compound II). This metabolite can be found in substantial quantity only in animal liver. Its concentration in due intervals was higher than that of compound I. In blood, compound II was quickly tranformed in the initial compound, due to separation of phosphate. This explains the long-lasting effects of the parent compound in animals. The binding properties of compound I with cell membranes of liver and brain may explain its membranetropism, that probably defines compound I pharmacological activity.*

Riassunto (Profilo farmacocinetico e metabolismo della 2-etil-6-metil-3-idrossipiridina nei ratti). – *L'ampio spettro di effetti psicofarmacologici dei derivati della 3-idrossipiridina è stato la base dello studio della farmacocinetica e del metabolismo della 2-etil-6-metil-3-idrossipiridina (composto I) quale tipico rappresentante del gruppo degli antiossidanti. L'analisi quantitativa della sostanza immodificata è stata effettuata mediante HPLC. Il composto I, somministrato per via i.p. ai ratti, è stato rapidamente assorbito presentando un assorbimento del 50% dopo circa un'ora; la massima concentrazione nel plasma, fegato e cervello appare entro 2-3 ore. Mediante spettrometria di massa con uso di standards di sintesi sono stati identificati 5 metaboliti del composto I, derivati per dealchilazione e coniugazione. Tra i metaboliti, grande interesse è stato dato ai derivati per coniugazione con fosfato del prodotto base (composto II). Questo metabolita è stato trovato in considerevole quantità solo nel fegato. La sua concentrazione in alcuni intervalli risultava più alta rispetto a quella del composto I. Nel sangue, il composto II era rapidamente ritrasformato nel composto d'origine a causa della perdita del gruppo fosfato. Ciò spiega la lunga durata degli effetti del composto base negli animali. La capacità del composto I di legarsi con le membrane delle cellule epatiche e cerebrali può spiegare inoltre la sua attività farmacologica.*

Introduction

Search for physiologically active substances starting from hydroxyderivative six-membered nitrous heterocycles, vitamin B_6 structural analogs, resulted in the synthesis of some compounds, able to inhibit the excessive formation of free radicals. Such an action was found to be the major requisite for the application of these compounds as protective drugs for various illnesses.

After experiments in laboratory animals it has been ascertained that 3-hydroxypyridine has a wide spectrum of pharmacological activity: psychotropic (antistress, antihypoxic, anxiolytic, anticonvulsant) as well as antitumoural, geroprotective and radioprotective effects [1-4].

However, more research is needed to understand the mechanism of action of these compounds at the molecular level and further experiments are necessary in order to characterize the pharmacokinetic profile of 3-hydroxypyridine derivatives. It is well known that the final effects of drugs may depend not only from the native substance activity but also from its active metabolites [5-7].

Thus, we have studied pharmacokinetics and biotransformation of a representative drug of pyridine derivatives, i.e., 2-ethyl-6-methyl-3-hydroxypyridine (compound I).

Materials and methods

The experiments were carried out on non imbred albino male rats weighing 160-180 g. Compound I was injected intraperitoneally at the dose of 150 mg/kg. Its concentration in plasma, liver and brain was determined by means of high performance liquid chromatography, 15, 30, 45 minutes and 1, 2, 3, 4, 6, 8, 10 and 24 hours after drug administration. With the purpose of studying compound I binding capacity to cell membranes in animal's brain and liver, 2 volumetrical per cent of triton X-100 was added prior to extraction. The binding capacity of endoplasmic reticulum membranes in respect to compound I was studied in saccharose gradient by the method of centrifugation.

Metabolites were determined in liver at various time intervals and also in urine 1, 2 and 3 days after the administration of compound I. In order to determine metabolite glucuronoconjugates, a part of urine was incubated with β-glucuronidase enzyme (Spopha, CSR) 100,000 unit/ml at 37°C, pH 7.8 during 24 hours and then extracted.

The extraction of compound I and its potential metabolites was made by addition of 0.1 ml 0.05 M tris-HCl (pH 8.0) and of 0.2 ml of 20% SDS solution into 2.0 ml of plasma or urine and thorough stirring for 5 min. An equal volume of benzol was added to this mixture which was then shaken for 15 min with a further 15 min long centrifugation at 10,000 r.p.m. in order to separate water (lower) from benzol (upper) layers. The water layer was then carefully collected and subjected to the same extraction procedure as specified above. Water and benzol layers were brought together and steamed at 90°C and 70°C respectively. The procedure to obtain tissue extracts followed tissue homogenization in 4 volumes of 0.01 M tris-HCl, pH 7.70 at 0°C). Dawn's homogenizator followed by falling out of big size tissue fractions by way of centrifugation at 7,000 r.p.m. for 15 min the further extraction procedure is the same as that for plasma and urine as described above.

Dry residue obtained after evaporation was dissolved in 1 ml of 10% water-methanol, 25 ml of this solution was then put into chromatographic system "Altex-Tracor 970 A" injector and subjected to an analysis in "Alltech" column C-6000 (4.6 × 250 mm) in "Spherisorb-CN5S" steady phase, methanol-water system (1:9) being used as an eluent.

Chromatography conditions: eluent flow speed is 1.0 ml/min, Column temperature – 25°C.

Chromatograms were performed with an automatic recording per absorption at 296 nm long waves. Compound I and its metabolite fractions were identified by comparison with synthesized standards and on the basis of the data received by UV-spectrophotometric scanning and mass-spectrometry. Compound I and its metabolite substantial quantity in chromatographic fractions were determined by normalisation method using "Chromatopac C-R1A" integrator and interpreted within the limits of single-chamber model with suction.

Behavioral studies were performed in non-imbred albino male rats weighing 180-220 g (method of conflict situation) and in albino male mice weighing 18-20 g (animal farm "Stolbovaya").

The technical aspect has been specified in another paper [8]. Drugs to be studied were injected intraperitoneally 40 min prior to experiments. Statistical analysis was conducted according to Litchfield, Wilcoson and to Student's t test [9].

Results and discussions

Figure 1 shows the concentration of compound I in the native state in plasma, liver and brain, at different times after intraperitoneal (150 mg/kg) administration. Compound I absorption from the abdominal cavity occurred rather quickly with a half-absorption time equal to 0.94 h; maximum concentration in plasma was obtained in 3 hours. Maximum concentrations in brain, liver and plasma were achieved in 2-3 hours and were 146, 171 and 202 ng/ml.

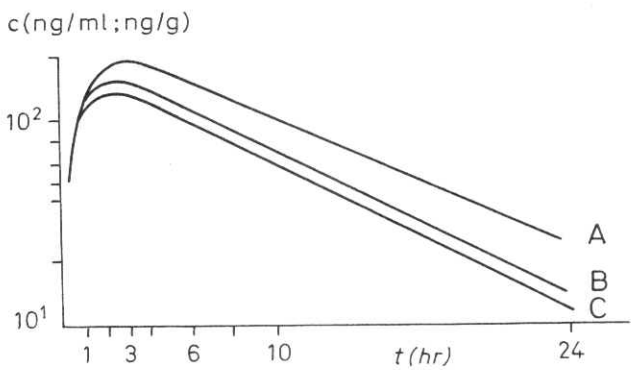

Fig. 1. – Kinetics of compound I in rat blood, liver and brain tissues after intraperitoneal injection (150 mg/kg).

A – concentration of the compound I in the blood;
B – concentration of the compound I in the liver;
C – concentration of the compound I in the brain.

Triton X-100 addition to brain and liver homogenates was followed by a considerable increase in compound I within appropriate time intervals (Table 1). Its maximum concentration in liver and brain increase more than twice.

Compound I accumulation in organs and its delayed release suggests that the compound binds to cell membranes of liver and brain. To prove this, we studied the binding capacity to liver and brain endoplasmic reticulum membranes (Table 2).

Thus, considerable concentrations of compound I are found in endoplasmic reticulum membranes of

Table 1. – *Pharmacokinetic parameters of compound I in rats*

Biomaterial	Parameters								
	Ka h^{-1}	$T_{1/2a}$ h	Ke h^{-1}	$T_{1/2e}$ h	V l	T_{max} h	C_{max} ng/l	Cl_p l/h	AUC $\frac{ng \times h}{ml \times kg}$
Plasma	0.74	0.94	0.11	6.38	533	3.0	202	57.8	2594
Liver	1.04	0.66	0.13	5.33	653	2.3	171	84,9	1767
Liver (Triton X-100)	0.80	0.85	0.10	6.97	309	2.9	363	30.7	4887
Brain	0.69	1.01	0.24	2.86	582	2.3	146	141.0	1061
Brain (Triton X-100)	0.35	1.96	0.36	1.92	147	2.8	373	52.8	2840

animal organs during 72 h, whereas lower concentrations were observed in cytosol only during 24 hours.

By studying compound I biotransformation in rats, 6 hydroxypyridine derivatives were detected (Fig. 2).

The identification proved compound I retention time on chromatogram (8.33 min) to coincide with that of the native substance. Furthermore, compound I mass-spectrum (Fig. 3) was found to coincide fully with that of initial substance with molecular ion m/e 137. The major fragments of compound I mass-spectrum were as follows: m/e 122 corresponds to compound I molecule CH_3 split out $[M\text{-}CH_3]^+$, m/e 109 to $[M\text{-}CO]^+$ and m/e 94 to $[M\text{-}CH_3\text{-}CO]^+$. Compound I substantial quantities were measured in urine in a small amount during the first and especially the second day, but no traces were detected during the third day.

Compound II substantial quantities were found only in animal liver with retention time on chromatogram of 9.45 min. Identification of the given metabolite mass-spectrum (Fig. 3) showed that it was formed due to phosphate added to the initial substance. Fragment m/e 81 corresponds to phosphate split out $[M\text{-}H_2PO_3]^+$ and m/e 47 fragment — to $[M\text{-}PO]^+$. To make identification more accurate,

Table 2. – *Compound I concentration following intraperitoneal administration (150 mg/kg) in various liver and brain subcellular fractions in rats*

Time after administration (hour)	Compound I content in endoplasmic reticulum membranes (ng/mg of protein)		Compound I content in cytosol fraction (ng/mg of protein)	
	liver	brain	liver	brain
2	380	307	110	95
24	140	102	32	40
48	80	64	—	—
72	30	50	—	—

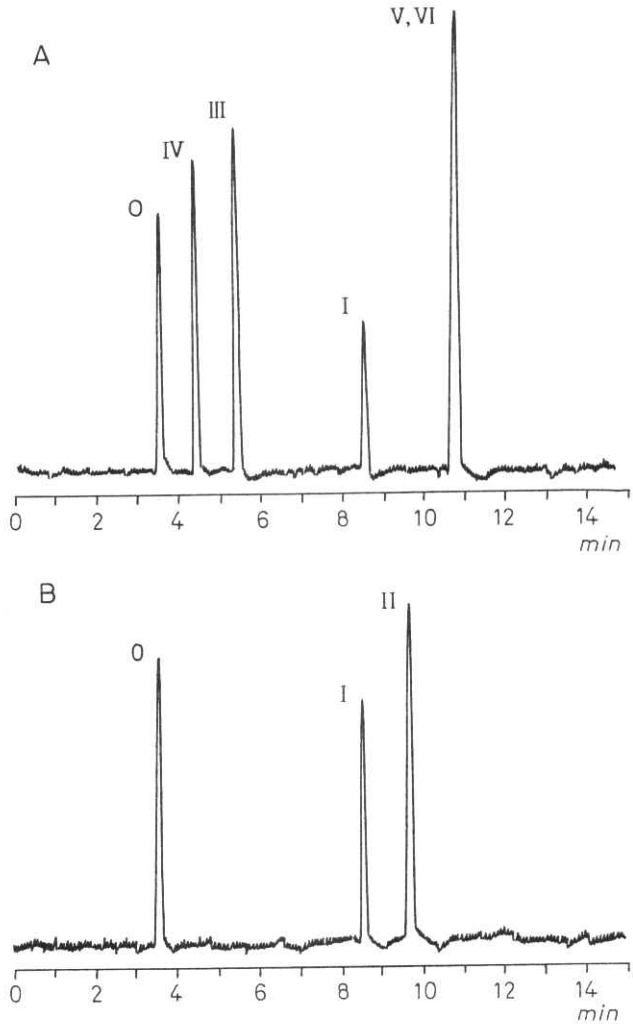

Fig. 2. – Chromatogram of compound I and its metabolites in urine (A) and liver (B).

0 – biological peak;
I – 2-ethyl-6-methyl-3-hydroxypyridine;
II – 2-ethyl-6-methyl-3-hydroxypyridine phosphate;
III – 2, 6-dimethyl-3-hydroxypyridine;
IV – 6-methyl-3-hydroxypyridine;
V – glucuronoconjugate 2-ethyl-6-methyl-3-hydroxypyridine;
VI – glucuronoconjugate 2-ethyl-6-methyl-3-hydroxypyridine phosphate .

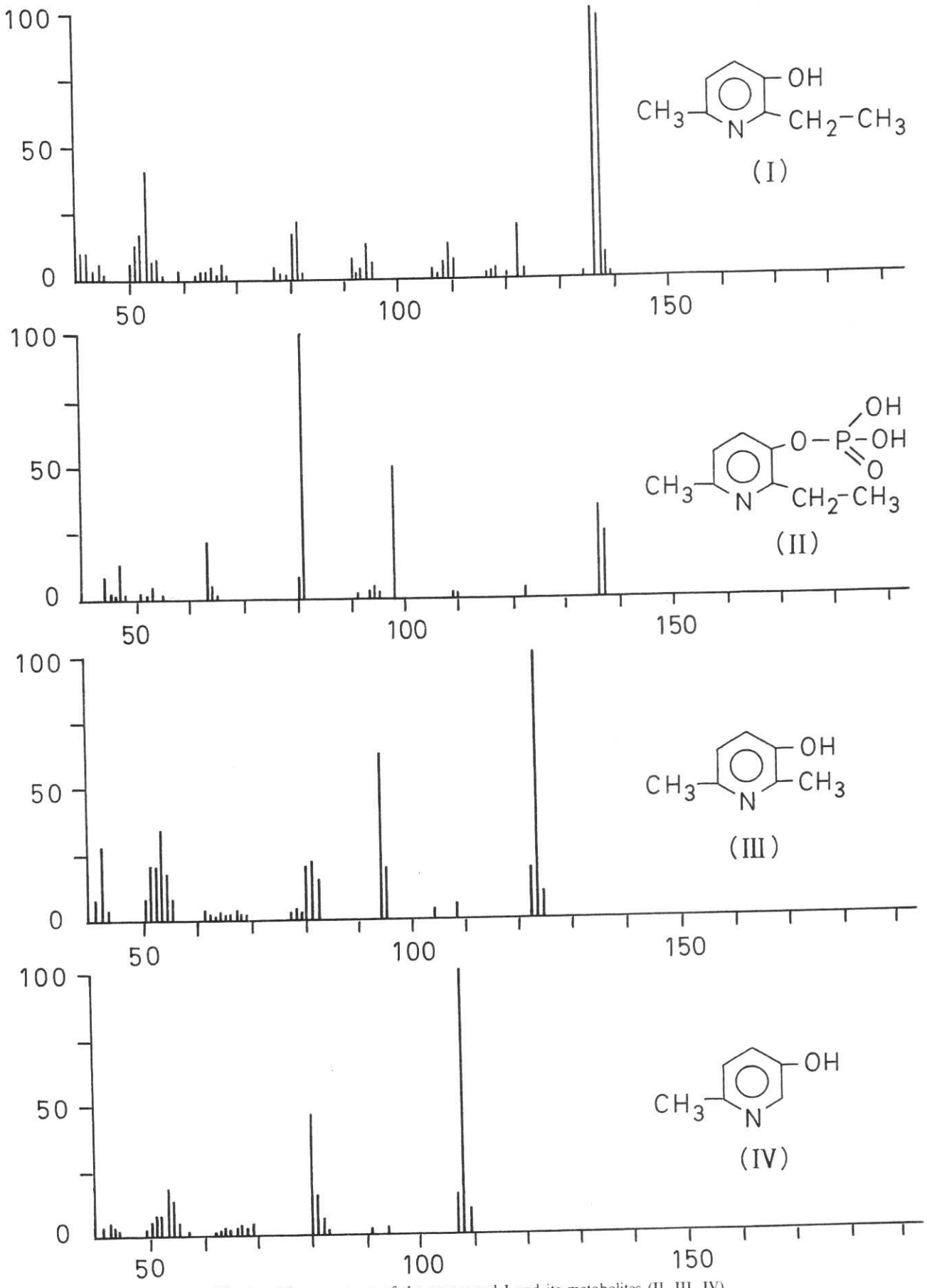

Fig. 3. – Mass-spectrum of the compound I and its metabolites (II, III, IV).
Compound's legend: see Fig. 2.

additional research has been carried out by administering radioactive orthophosphate ^{32}P side by side with the compound I into the rats. The results proved that at various time intervals after compound I and radioactive phosphate administration, metabolite II released from rat's liver contained considerable radioactivity that reaffirmed the metabolite structure. No other metabolites of compound I in liver were detected.

Metabolite III was found out (in considerable amounts) in urine during the first and second day; only traces of it were measured during the third day. Identification proved compound III mass-spectrum to fully coincide with the mass-spectrum (Fig. 3) of standard 2, 6-dimethyl-3-hydroxypyridine with molecular ion m/e 123. Chromatogram showed the metabolite III retention time and that of the above mentioned standard concurred and was 5.30 min.

Pharmacological investigation on metabolite III proved it to have a psychotropic effect similar to that of compound I.

Comparative studies of psychotropic properties of compound I and its metabolite III showed the metabolite to be not less active than the initial substance as anxiolytic revealed under conflict situation conditions. Both drugs at the dose of 100 mg/kg significantly increased the behavioral value in a conflict situation — a number of punished responses (water drinking in spite of schock received at the same time) (Table 3). An increase of the number of approaches to the water source without drinking and a locomotor activity enhancement were observed at the same time.

Table 3. – *Compound I anxiolytic activity compared to that of its metabolite (III) after their i.p. administration (100 mg/kg) in a conflict situation test*

Compound	Punished water drinkings	Comings up to water source	Locomotor activity
Control	1.7 ± 0.3	12.4 ± 3.8	15.1 ± 2.3
Compound I	9.3 ± 1.2 (*)	26.2 ± 6.8	20.2 ± 8.3
Compound III	8.1 ± 1.3 (*)	18.4 ± 7.1	16.2 ± 5.4

(*) $p < 0.05$ (Student's t test).

Just as compound I, its metabolite III also possesses certain other psychotropic effects: being antagonist of penthylentetrazol; having antihypoxic action, producing an inhibitory effect on locomotor activity and causing its impairment (Table 4). The metabolite showed a more potent antihypoxic activity and sedative effect than that of compound I, but it was found less active than compound I, in the antagonism of convulsions. Both compounds are toxic in a similar way (Table 4).

Table 4. – *Compound I psychotropic activity and toxicity in mice compared to that of its metabolite (III) after their i.p. administration (100 mg/kg)*

N.	Tests	Value	Compound I	Compound III
1.	Antagonism to pentylentetrazol	ED_{50} mg/kg	122 (102 – 146)	378 (302 – 436)
2.	Locomotor activity impairment	ED_{50} mg/kg	280 (233 – 336)	376 (302 – 436)
3.	Locomotor activity inhibition in actometer within 2 min	Effect in per cent (*)	38.2 ± 9.3	46.2 ± 12.4
4.	Antihypoxic action	Effect in per cent (**)	9.1 ± 1.4	18.1 ± 5.8
5.	Toxicity	ED_{50} mg/kg	430 (307 – 602)	420 (350 – 504)

(*) Locomotor activity inhibition expressed as percent with respect to controls (100%).

(**) Life duration increase in percent under conditions of hypoxia with respect to controls (100%).

Thus, metabolite III of compound I revealed a significant psychotropic activity which makes it possible to suggest that this metabolite is responsible of the pharmacological effect [6].

Metabolite IV was identified as 6-methyl-3-hydroxypyridine. This product was excreted in urines in equal quantities as metabolite III (according to peak area). While comparing compound IV mass-spectrum (Fig. 3) with that of standard, a full coincidence with the highest peak of molecular ion m/e 109 was found. Metabolite IV retention time and that of standard was 4.27 min. There were no traces of metabolite III and IV in liver and blood.

Having treated urine with β-glucuronidase the following were identified: metabolite V (retention time 8.33 min), i.e., a compound I glucuronoconjugate, and metabolite VI (retention time 9.45 min), i.e., metabolite II glucuronoconjugate: both were excreted with urines in equal quantities during the first and second day. Thus, metabolite II is excreted only in the form of glucuronide (compound VI), whereas compound I is excreted without any modification as well as in the form of glucuronoconjugate (compound V).

An outline of the possible compound I changes in rats was made up based on the excreted and identified metabolites of this compound (Fig. 4).

It is of interest that metabolite II traces are not found in blood. In this connection, we suggested that metabolite II goes from liver into blood with the splitted out phosphate in the form of unchanged substance (compound I). Additional work was carried out to prove this suggestion. Metabolite II was extracted from liver and added into the rat's blood incubated during 15 min at 37°C and extracted.

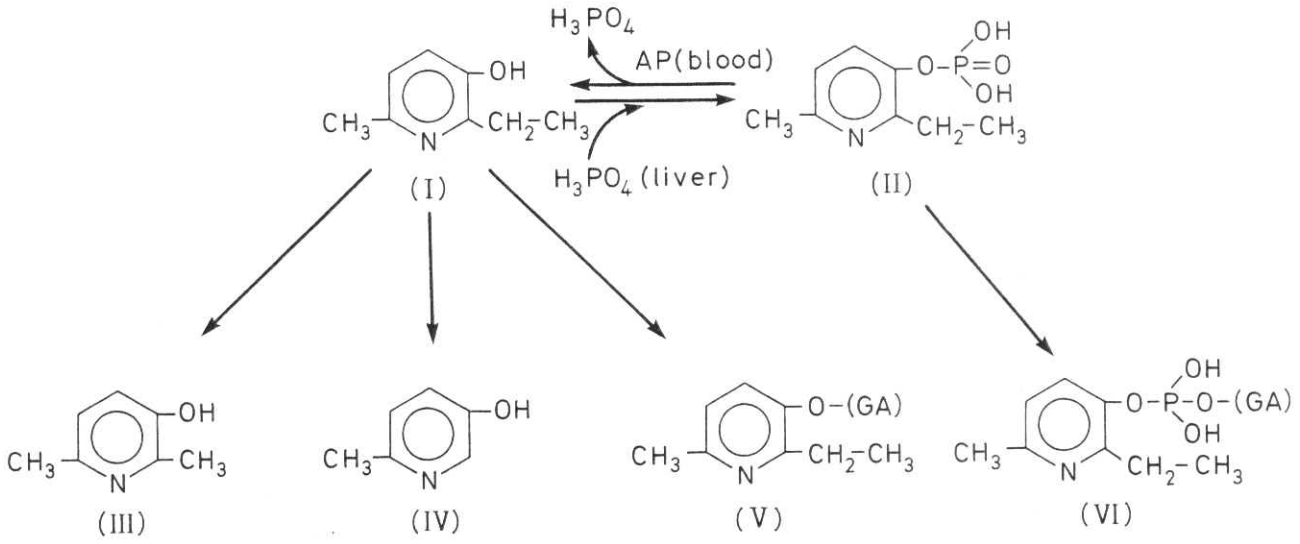

Fig. 4. – Scheme of possible transformation of compound I in rat.

AP – alkaline phosphatase;
GA – glucuronic acid.
Compound's legend: see Fig. 2.

Extract was analysed chromatographically; the chromatogram showed only one peak corresponding to invariable substance retention time (8.33 min). The same picture was observed after metabolite II incubation with alkaline phosphatase enzyme.

Thus, we suppose that metabolite II plays an important role in manifestation of compound I membranotropic and pharmacological effects, since on the one hand, it is a kind of phosphate accumulator, participating in cell energy metabolism, and on the other hand, it prolongs the parent compound life in the organism. For this reason, we studied metabolite II kinetics in rats.

Figure 5 shows that metabolite II, like compound I, was found in liver during 24 hours; metabolite II concentration being considerably higher that of the parent compound. In two hours metabolite II concentration in liver reached its maximum, up to 201 ng/ml. Triton X-100 added to the liver homogenates caused a considerable increase of metabolite II concentration in due time intervals (Table 5).

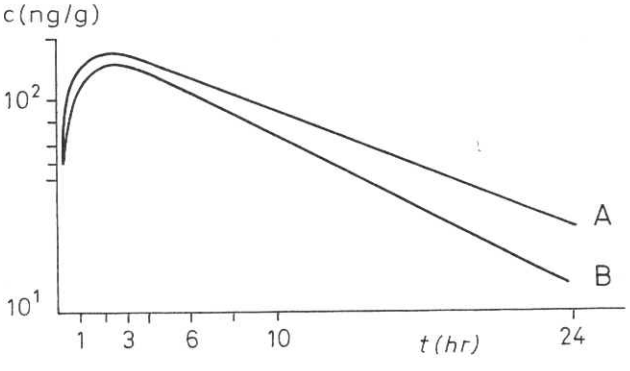

Fig. 5. – Kinetics of compound I an its metabolite II in rat's liver after i.p. injection in dose 150 mg/kg.

A – concentration of the compound II;
B – concentration of the compound I.

In this way, on the basis of the data obtained, we have ascertained that compound I was measured for a long period of time in cell membranes where very likely it changed physical and chemical properties, structure and functional activity of biomembranes, thus causing modulatory effects of the membrane level and pharmacological effects.

Among the five compound I identified metabolites, presented by dealkylated and conjugated products of transformation, we studied mostly metabolite III, i.e. 2, 6-dimethyl-3-hydroxypyridine, since it appeared to be pharmacologically active. From the view point of compound I pharmacological effect, metabolite II is of interest for the following reasons:

a) this metabolite is present in liver in great quantities during the first day after compound I injection;

b) getting into the blood from liver, this metabolite transforms into the initial substance able to add phosphate in liver, thus forming an hepatic store;

c) finally, adding triton X-100 to the liver homogenate, metabolite II concentration increases twice, suggesting metabolite binding with hepatic cell membranes.

Table 5. – *Pharmacokinetic parameters of compound I and its metabolite (II) in rats*

Compound	Parameters								
	Ka h^{-1}	$T_{1/2a}$ h	Ke h^{-1}	$T_{1/2e}$ h	Vd l	T_{max} h	C_{max} ng/l	Cl_p l/h	AUC $\frac{ng \times h}{ml \times kg}$
Compound I	1.04	0.66	0.13	5.33	653	2.3	171	84.9	1767
Compound I (Triton X-100)	0.81	0.85	0.10	6.97	309	2.9	363	30.7	4887
Compound II	1.23	0.56	0.085	8.19	613	2.3	201	51.9	2892
Compound II (Triton X-100)	1,30	0,53	0.089	7.81	291	2.2	424	25.8	5815

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