Synthesis of New Methionine Derivatives for the Treatment of Paracetamol - Induced Hepatic Injury

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Abstract

The direct pharmacological properties of amino acids and the possibility of using them as carriers for other active pharmacological substances are well known. Methionine, being able to yield the methyl group, is very important in the treatment of hepatic diseases. Paracetamol acute poisoning causes liver injury in both humans and animals. The study is designed to synthesize some new methionine derivatives and to establish a possible correlation between the new structure and the pharmacological properties. To this end, acute experimental poisoning with Panadol® (paracetamol) was performed while, for the treatment of liver injury caused by this compound, two original synthesis derivatives of methionine, namely N-(m-nitrobenzoyl)-L-methionine and N-(m-aminobenzoyl)-L-methionine, were used. Male Wistar rats were administered Panadol® (paracetamol) per oral (7500 mg/kg). N-(m-nitrobenzoyl)-L-methionine (m-NBM) 50 mg/kg and N-(m-aminobenzoyl)-L-methionine (m-ABM) 50 mg/kg were given intraperitoneally, 30 minutes after Panadol® administration. Biochemical parameters such as SGOT, SGPT, serum bilirubin and glycemia were estimated to assess the liver function. Panadol® (paracetamol) poisoning produced an increase in serum transaminases, bilirubin and glicemia. These effects were reduced by treatment with m-NBM and especially m-ABM. These biochemical observations were supplemented by histopathological examination of liver sections. The results obtained with m-ABM were comparable with those reported on methionine, which is a recognised antidote in paracetamol poisoning.

Introduction

The unexpected development of the researches devoted to the physiological and biochemical action of aminoacids and of their derivatives – which permitted a large-scale application of such compounds in medicine – may be explained by the role they play in the organism as well as by the fact that their therapeutical qualities do not affect its vital mechanisms. Several investigations – cited in the literature of the field – have evidenced both aminoacids' direct pharmacological properties and their possible utilization as carriers for substances belonging to other phar-

macologically – active classes [1 - 10].

Among the compounds indispensable to the living organisms, one should mention methionine, an aminoacid present in the composition of several proteins and which, either alone or in mixtures with other aminoacids, plays an essential part in the vital processes. [11] Due to its labile methyl group, methionine acts as a transmethylation agent in the living organisms, being able of yielding its methyl group to some acceptors and thus being transformed into homocysteine.

Methionine's capacity of yielding its methyl group recommends it for the treatment of hepatic diseases. Consequently, methionine evidences a hepatoprotect-

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ive activity for numerous substances. [12-15] Along with other substances, methionine controls liver's metabolism [15] and increases the activity of some liver's enzymes, such as tirosinaminotransferase. [16]

Also, as a result of its stimulating role on the activity of certain enzymes in the organism, methionine has a positive influence on the antibacterian action of certain semisynthesis penicillins and cephalosporines. [17]

Methionine may give derivatives both to the aminic and carboxylic groups, and to the thioetheric one, most of them evidencing physiological activity. The amides and peptides containing methionine in their molecule are of interest both for their physiological action and for the fact that they may release in the organism the methionine necessary in either nutritional or therapeutical processes. [18,19]

The present paper discusses the synthesis of some new derivatives of methionine and establishes some correlation between the newly-formed structure and its pharmacological properties.

Experimental

Synthesis of N-(m-nitrobenzoyl)-L-methionine (m-NBM)

In a balloon equipped with a mechanical stirrer, 15 g (0.2 moles) L-methionine are introduced, at room temperature, into 300 mL water and 51 g (0.6 moles) sodium bicarbonate. The mixture is stirred until the components enter the solution, after which 19 g (0.1 moles) of the m-nitrobenzoic acid's chloride, dissolved in 100 mL anhydrous benzene, are introduced, for 1 hour, under intense stirring. On addition of the whole amount of benzenic solution, a white precipitate is formed, while the solution becomes red-violet. Stirring is continued for another hour, which is followed by vacuum filtration and washing with 100 mL distilled water, for dissolution

of the precipitate formed, the benzenic layer being removed through a separation funnel. The aqueous solution is treated – still under stirring – with 30% chlorhydric acid, up to a pH = 2.5, when the occurrence of a bulky, white precipitate may be noticed. The product is washed three times with 50 mL ethylic ether; after drying, the product is resumed in distilled water, then heated up to boiling and filtered at warm. On cooling, in the aqueous solution, a white precipitate is formed; after filtration and drying, this is melted at 170 – 172°C. Yield = 89%. The product is soluble – at cold – in methylic alcohol, ethylic alcohol, acetone, chloroform, dimethylformamide and – at warm – in water. It is insoluble in cold water, benzene, ethylic ether and oil ether. The product's elemental composition is listed in Table 1 and the IR and NMR spectra are indicated in Table 2.

Table 1
N-(m-nitrobenzoyl)-L-methionine elemental composition

$C_{12}H_{12}N_2O_5S$ (Molecular weight - M = 298)				
Elemental composition	C, %	H, %	N, %	S, %
Theoretical value:	48.31	4.73	9.39	10.78
Experimental value:	48.43	5.10	9.80	10.94

Synthesis of N-(m-aminobenzoyl)-L-metionine (m-ABM)

In a balloon equipped with an ascending refrigerator, 10 g (0.05 moles) N-(m-nitrobenzoyl)-L-methionine (m-NBM) and 40 mL of 35% ammonium solution are introduced. N-(m-nitrobenzoyl)-L-methionine shifts into solution as an ammonium salt. The mixture is bubbled for 3 hours in a flow of sulphuretted hydrogen and heated at 60-70°C. The solution becomes first red, then a precipitate – to be dissolved during the reaction – is formed.

When the reaction is over, the solution is cooled,

Table 2
Assignment of the absorption bands from m-NBM's IR and NMR spectrum

I.R. spectrum (KBr):				
3310 cm^{-1} ν_{N-H} amidic	1650 cm^{-1} $\nu_{C=O}$ amide	1540 cm^{-1}, 1350 cm^{-1} ν_{NO_2} asymmetrically and symmetrically	880 cm^{-1} disubstituted benzenic nucleus	725 cm^{-1} ν_S thioetheric
Spectrum ^{1}H-RMN (acetone, d^6, 300 MHz):				
δ (ppm) 2.12 (t, 3H, CH$_2$ – S –)	2.09 (S, CH$_3$)	2.44 (t, CH$_2$)	4.46 (t, CH)	8.21 (d, 1H, Ar)	8.37 (d, 1H, Ar)

then 30% chlorhydric acid is added up to a pH = 4.5, when the solution becomes opalescent (a result of sulphur's presence). The sulphur is removed through a three times stirring with carbon sulphide, into a separation funnel, then 20% chlorhydric acid is added into the aqueous solution up to a pH = 2.5 – 3, when a solid, violet product is separated. After filtration, drying and two-fold recrystallization from water, crystals – that are melted at 172 - 174°C – are obtained. Reaction's yield = 78%.

The product is soluble in acetone, dimethylformamide, water, ethylic and methylic alcohol, and insoluble in ethylic ether, petrolium ether, benzene. The product's elemental composition is given in Table 3, and the IR and NMR spectra in Table 4.

Table 3
N-(m-aminobenzoyl)-L-methionine elemental composition

$C_{12}H_{16}N_2O_3S$ (Molecular weight - M = 268)				
Analysis for:	C, %	H, %	N, %	S, %
Calculated:	53.73	5.97	10.44	11.94
Found:	53.95	6.16	10.79	12.22

Table 4
Assignment of the absorption bands from m-ABM's IR and NMR spectrum

I.R. spectrum (KBr):				
3210 cm^{-1} ν_{N-H} amidic	1710 cm^{-1} $\nu_{C=O}$ amide	1530 cm^{-1} band amide II	880 cm^{-1} disubstituted benzenic nucleus	755 cm^{-1} ν_S thioetheric
Spectrum ^{1}H-RMN (acetone, d^6, 300 MHz):						
δ (ppm) 2.5 (d, 2H, CH$_2$)	4.85 (t, 1H, CH)	5.6 (S, 2H, NH$_2$)	7.4 (t, 1H, Ar)	7.7 (d, 1H, Ar)	7.9 (d, 1H, Ar)	8.1 (S, 1H, Ar)

Determination of toxicity by the "up and down" method for establishing the lethal dose 50 (LD$_{50}$)

For determining LD$_{50}$ with satisfactory precision, a simple method, which uses few animals, known as the „up and down" method, described for the first time by Dixon and Mood was employed. [20]

From the beginning, a series of doses equally distributed on a geometric scale have been considered. An animal is injected with the lowest dose quite close to LD$_{50}$. If this animal dies, the second is injected with the lower, immediately following dose. If the first animal survives, the second is injected with the immediately following higher dose. The operation continues until the whole number of animals selected for study is injected.

For testing the toxicity of the newly sinthesized compuonds, m-NBM and m-ABM, male Wistar rats weighing 200-220 g and male mouses weighing 22-23 g were used. The animals had free access to standard diet and water.

The suspension was prepared using normal saline solution (on stirring at warm). Maximum concentration of the substance employed was of 14%. The substance was administered per oral (p.o.) and injected intraperitoneally (i.p.).

Study of the therapeutic effect of m-NBM and m-ABM in acute Panadol® (paracetamol) poisoning

The study complied with the ethical conditions established for animal experiments. [21] Male Wistar rats, weighing 180 ± 30 g, aged 12 weeks were used. The animals were exposed to a 12 hours light-dark cycle, under controlled environmental conditions (25 ± 1°C, humidity 55 ± 5%). Animals were fasted for 12 hours prior to the experiments, instead water was allowed *ad libitum*.

Forty rats were randomly divided into four groups (n = 10 for each group): group 1 received a 7500 mg/kgc dose of Panadol®, dissolved in 3 mL pathogen-free normal saline, p.o.; group 2, poisoned with Panadol® (7500 mg/kgc p.o.) was treated with m-NBM 50 mg/kgc dissolved in the same volume of normal saline, injected i.p. 30 minutes after toxic administration. Group 3 was poisoned with Panadol® (7500 mg/kgc p.o.) and treated with m-ABM 50 mg/kgc dissolved in the same volume of normal saline, injected i.p. 30 minutes after toxic administration. A

reference group (group 4) received an equivalent volume of saline.

All substances were administered between 8^{30} – 10 a.m. Heparinized blood samples were collected before the toxic administration, 48 hours and 7 days after toxic administration, under ether pro narcosis anesthesia, from the retro-orbitar plexus.

Glutamate pyruvate transaminase (SGPT) and glutamate oxaloacetate transaminase (SGOT) were determined using a standard semiautomatic analyzer (Cormay Multi, made in Poland), kits (Cypress Diagnostic Leuven, Belgium). Transaminases were assessed form heparinized plasma (37°C), and the results were expressed in international units (IU) per liter.

After 7 days, all rats were sacrificed by exsanguination *via* carotid section and liver specimens were collected for the histopathological study. Small pieces of liver tissues were fixed in formaldehyde, processed and embedded in paraffin wax, and sections of 3-4 microns in thickness were cut and stained with hematoxylin and eosin (HE). All liver specimens were examined by light microscopy, to evaluate cellular injury.

ANOVA analysis of variance was used to assess the presence of a significant difference among groups. Data are reported as mean ± standard deviation. Significance was taken as at $p < 0.05$.

Results and Discussion

For the obtention of physiologically-active products, the synthesis of N-(m-nitrobenzoyl)-L-methionine (m-NBM) was first performed, through condensation of m-nitrobenzoic acid's chloride with L-methionine, according to the following reaction:

NBM
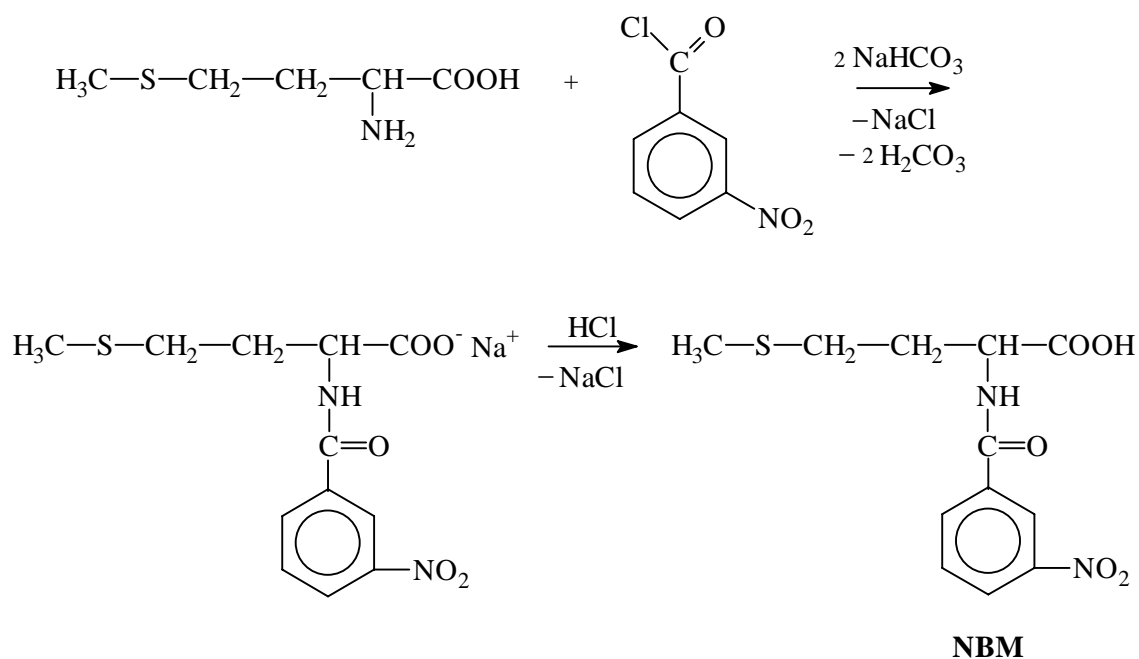
Addition of the acid chloride dissolved in anhydrous benzene in an aqueous solution of L-methionine and sodium bicarbonate led to the obtention of the sodium salt of N-(m-nitrobenzoyl)-L-methionine from which, through a treatment with chlorhydric acid, m-NBM was released.

Study of the reaction conditions permitted the conclusion that the optimum working temperature is between 20-25°C, the time for acid chloride's addition is of 30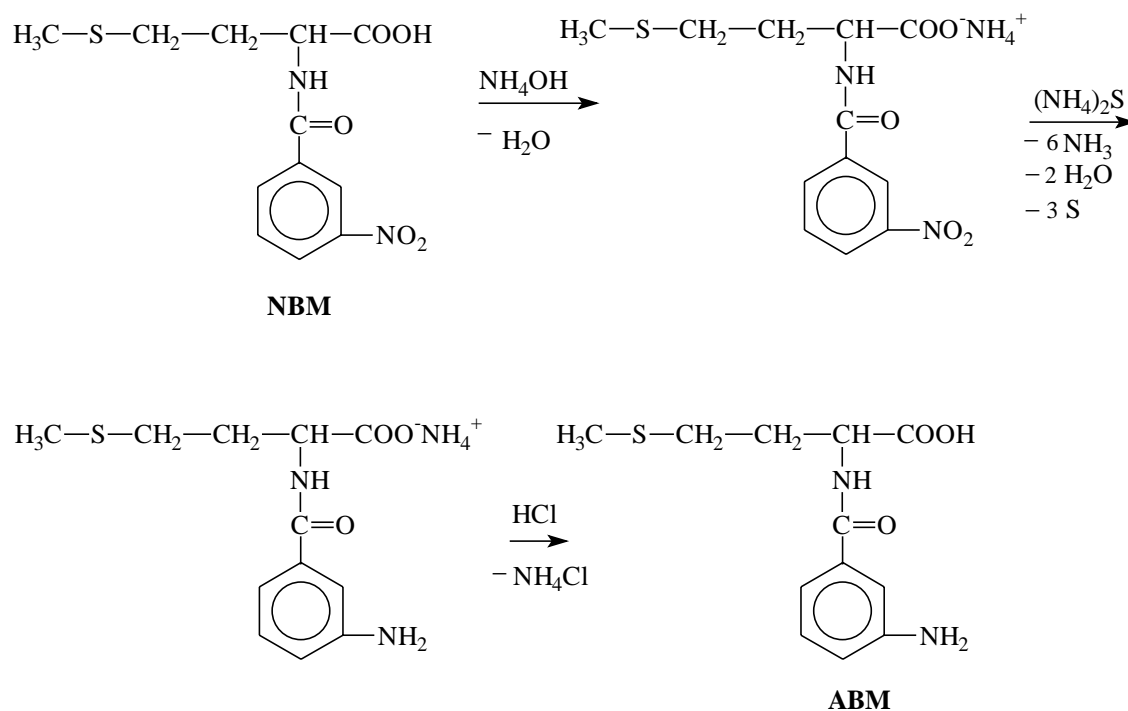 minutes, while the duration of contact should be of 2 hours, under strong stirring. All these gave a product which, after recrystallization from water on boiling, melts at 170-172°C, the yield being of 89%. The structure of this product has been confirmed by elemental chemical analysis, infrared spectra and nuclear magnetic resonance.

As the literature of the field presents the compounds that contain aminobenzoic acid in their molecule as especially active compounds from a physiological viewpoint, the authors of the study tried to

obtain.

N-(m-aminobenzoyl)-L-methionine (m-ABM) through the reduction of m-NBM with ammonium sulphide. Bubbling of sulphuretted hydrogen in a solution of m-NBM in 35% ammonium hydroxide leads to the obtention of the ammonium salt of N-(m-aminobenzoyl)-L-methionine. As, during the reaction, sulphur – which may impurify the final prod-

uct – results, its removal is performed through extraction in carbon sulphide, after solution's acidulation with chlorhydric acid, at pH = 4.5. After the removal of carbon sulphide, acidulation of the aqueous solution is continued up to a pH = 2.5, when N-(m-aminobenzoyl)-L-methionine (m-ABM) results. The reaction occurs according to the scheme presented in the following

$$H_3C-S-CH_2-CH_2-CH-COOH$$
NH
C=O
NO_2

NBM

$$\xrightarrow[-H_2O]{NH_4OH}$$

$$H_3C-S-CH_2-CH_2-CH-COO^-NH_4^+$$
NH
C=O
NO_2

$$\xrightarrow[\substack{-6\,NH_3 \\ -2\,H_2O \\ -3\,S}]{(NH_4)_2S}$$

$$H_3C-S-CH_2-CH_2-CH-COO^-NH_4^+$$
NH
C=O
NH_2

$$\xrightarrow[-NH_4Cl]{HCl}$$

$$H_3C-S-CH_2-CH_2-CH-COOH$$
NH
C=O
NH_2

ABM

The product obtained at an yield of 78%, after recrystallization from water at warm, melts at 172-173°C, its structure having been confirmed through elemental and spectral chemical analyses. [22,23]

Both compounds were tested as to their toxicity by the method described in the experimental part. The results were: *m-NBM - LD₅₀ = 414 mg/kgc; m-ABM - LD₅₀ = 490 mg/kgc (i.p.) and 900 mg/kgc p.o.*

The animals were observed for 14 days, then killed for the histopathological study (liver specimens). Microscopic examination of the liver, after administration of LD_{50} of m-NBM, showed minor steatosis in rats and steatosis and inflammation, with nuclear abnormalities of the hepatocytes, in mice (Fig. 1, 2). After administration of LD_{50} of m-ABM, no specific abnormalities were observed in liver specimens, either in rats, or in mice.

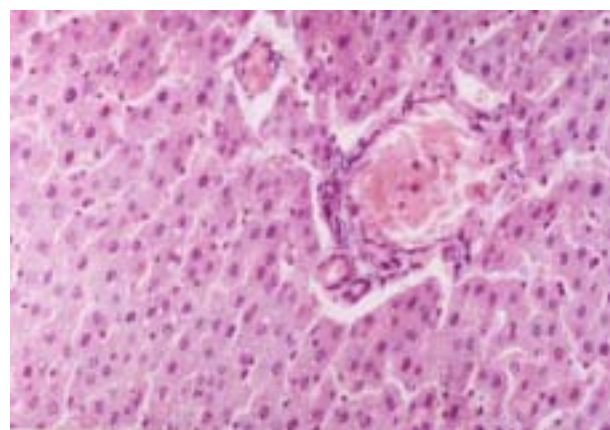Study of the effect of the newly synthesized methionine derivatives used for the treatment of liver

Fig. 1. Portobiliar area, hepatocytes with optic empty intracytoplasmatic vesicles (steatosis), rat, toxicity test for m-NBM. HE×40.

injury caused by acute Panadol® poisoning revealed the following aspects. Administration of m-NBM and m-ABM 30 minutes after toxic administration had a
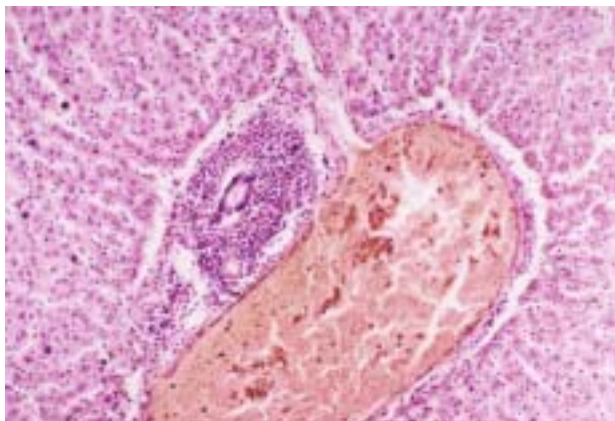
Fig. 2. Chronic inflammation in portobiliar area, pericentrivenular inflammation; hepatocytes with nuclear changes (nuclear distruction, chromatin condensation) and minor steatosis; mouse, toxicity test for m-NBM. HE×20.

significant hepatoprotective effect. SGOT and SGPT values were significantly decreased in groups 2 and 3, comparatively with group 1 (poisoned with Panadol®), both within 48 hours and 7 days after toxic administration, when the values of these enzymes got normalized ($p < 0.01$), as showed in Figs 3 and 4.
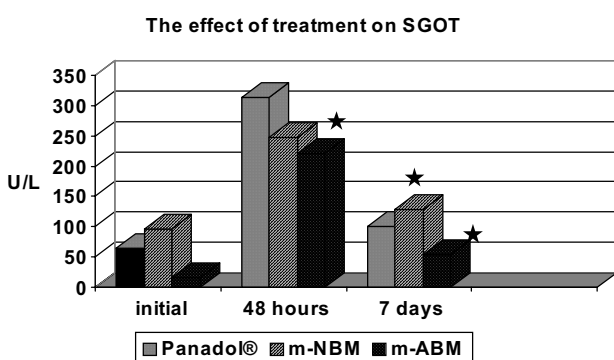
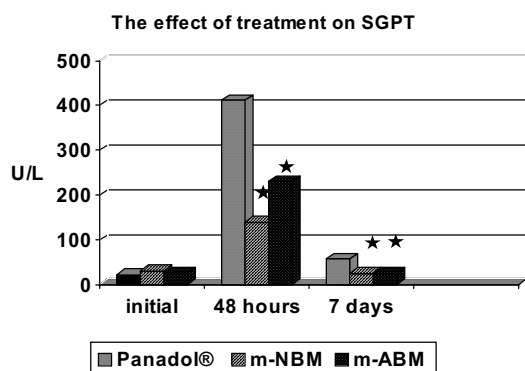Fig. 3. The effect of treatment on SGOT in Panadol® poisoning. ★ $p < 0.01$

Fig. 4. The effect of treatment on SGPT in Panadol® poisoning. ★ $p < 0,01$

A histopathological study showed, 7 days after paracetamol administration in group 1, typical changes of confluent centrilobular necrosis (Fig. 5). Group 2, poisoned with Panadol® (paracetamol) and treated with m-NBM, evidenced, after 7 days, minor hydropic changes and steatosis (Fig.6). Group 3, poisoned with Panadol® (paracetamol) and treated with m-ABM (Fig.7), evidenced no of liver injury (normal aspect of liver specimens), as it was the case with the control group 4.

In cases of paracetamol (acetaminophen) toxicity, the major pathways of paracteamol metabolism (i.e., conjugation with glucuronic and sulphuric acid) become saturated. Therefore, large amounts of paracetamol are oxidised to a highly reactive metabolite, N-acetyl-p-benzoquinone-imine (NAPQI) *via* the P450 system. This metabolite is detoxified by glutathione (GSH), to form 3-(GSH-S-yl) acetaminophen. [24] The glutathione level is reduced, and
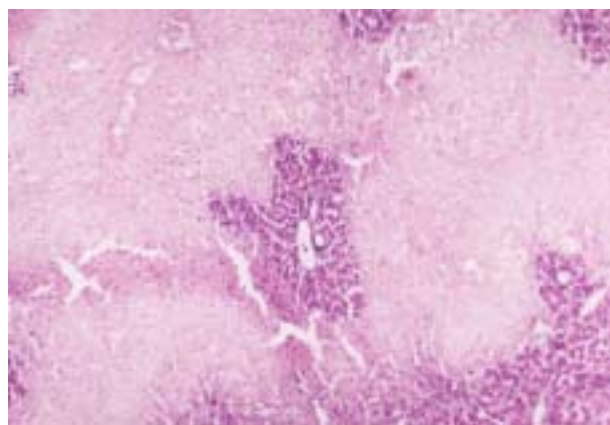

Fig. 5. Large areas of pericentrivenular necrosis, with preservation of periportal hepatocytes, poisoned rat, 7 days after toxic administration. HE×10.
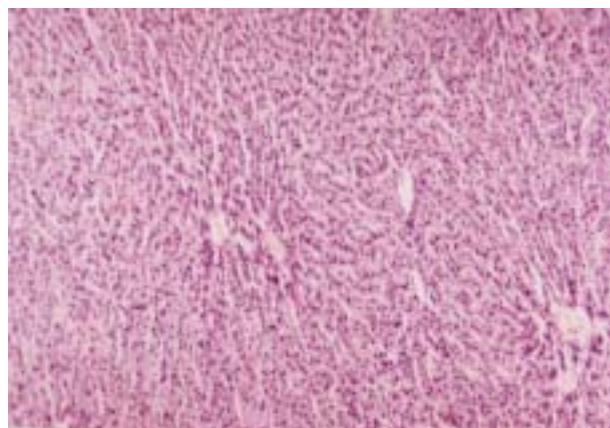

Fig. 6. Minor aspects of hydropic change, in poisoned and m-NBM – treated rat, after 7 days. HE×10.
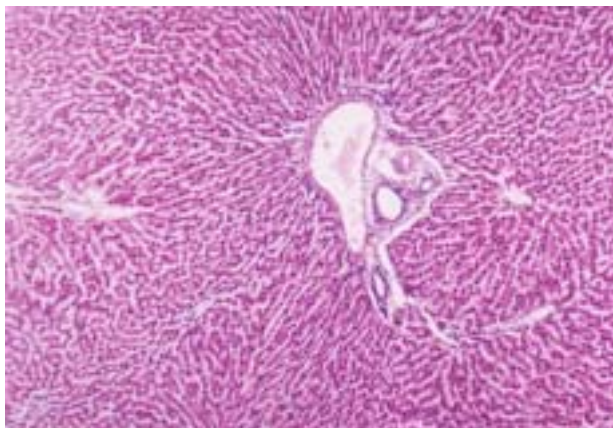
Fig. 7. Portobiliar area, normal hepatocytes and liver aspect, in poisoned and m-ABM – treated rat, 7 days after toxic administration. HE×10.

when it decreases below a critical value (30% of normal deposits), NAPQI binds covalently to macromolecules in hepatocytes, which results in cellular necrosis. [25,26,27,28] Necrosis begins in the centrilobular regions, because cells in this area have the highest levels of cytochrome P450. [29]

Our study on acute toxicity of Panadol® (paracetamol) had comparable results with those reported by Walker (1974), Dixon (1975) and Buttar (1976), cited by Prescott. [30]

The highest levels of serum transaminases, after a toxic dose of 7500 mg/kg of paracetamol, were obtained within 48 hours (20 times over the normal values), then the transaminase values decreased towards the reference ones, yet without getting normalized within 7 days from the beginning of the experiment, a moment in which all animals were sacrificed. No cases of mortality have been recorded during the 7 day experiment, because the clinical form of poisoning was medium, despite the large amounts of toxic substances used (7500 mg/kgc p.o.).

Groups 2 and 3, which were treated with methionine derivatives, had significantly lower values of serum transaminases, both within 48 hours and 7 days after toxic administration, comparative with group 1. Treatment with m-ABM had a hepatoprotective effect, as revealed by the lower transaminase levels during the experiment and by the normalisation of transaminases 7 days after toxic administration. There was also a significant difference between group 3, treated with m-ABM, and group 2, treated with m-NBM, in which the transaminases were lower, comparatively with group 1, which was statistically significant only for SGPT.

The biochemical results were confirmed by the histopathological study, which had, in group 1 (paracetamol only) similar results with those reported in literature. [30] Paracetamol administration caused significant necrosis of the centrilobular hepatocytes, and confluent hepatocyte necrosis, which agrees with the biochemical analyses. Extended hepatic necrosis induced by Panadol® poisonig was completly cured by the treatment with m-ABM, the histological liver aspect 7 days after the treatment being normal, similarly with the control group.

The same results were obtained when using methionine. The advantage would be that the dosis of m-ABM, represents 0.66% of the toxic dose of paracetamol used and also that no mortality occurred, while methionine is effective in doses up to 45.7% from the toxic dose of paracetamol, doses which reduce mortality at 3% in this type of acute poisoning. [31,32]

Treatment with m-NBM had a less important effect, because no necrosis was observed, yet the histological aspect 7 days after the treatment revealed minor hydropic change and steatosis. It is possible that these changes should be the consequence of the treatment with this compound, mostly because the histological examination in toxicity tests showed similar aspects. The results of the treatment with m-NBM are less encouraging than those with m-ABM and inferior to those with methionine.

Conclusions

The objective of the investigation was, on one side, the physico-chemical characterization and, on the other, testing of some new compounds with a methionine rest in their composition, for the preparation of substances possessing special biological properties.

A systematic study was developed on the condensation reaction between L-methionine and the m-nitrobenzoic acid's chloride, in conditions of the Schotten-Baumann reaction. Interpretation of the experimental data supported the elucidation of some significant aspects of methionine's acylation reaction.

Reduction of N-(m-nitrobenzoyl)-L-methionine was performed with ammonium sulphide, thus N-(m-aminobenzoyl)-L-methionine – a derivative whose molecule contains the rest of the aminobenzoic acid, a precursor of folic acid – being obtained.

Treatment with the methionine derivative m-ABM had a protective effect on liver injury caused by

Panadol® poisoning in rats, in our experimental conditions.

Treatment with methionine derivative m-NBM had a mild protective effect on liver injury caused by Panadol® poisoning in rats, reducing the extension of centrilobular necrosis.

These findings indicate that the methionine derivative m-ABM deserves further consideration as a potential option in the treatment of paracetamol acute poisoning.

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