



Vitamin B₁₂ Hydrophobic Derivative Exhibits Bioactivity: Biomedical and Photophysical Study

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Accepted: 28 October 2021

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Abstract

Vitamin B₁₂ (cyanocobalamin) and some of its hydrophilic derivatives are used as antidotes and also to treat megaloblastic anemia, nerve myelination disorders, and liver pathology. This work presents results of a comparative experimental study of cyanocobalamin and its derivatives aquacobalamin and heptamethyl ester of cyanoaquacobyrinic acid. In a model of thiosemicarbazide seizures in rats, aquacobalamin contributed to a lengthening of the seizure latency period while cyanocobalamin contributed to a decrease in the seizure latency period and to a decrease in seizures. Histological study of the brain samples showed that all investigated compounds exhibited an antispasmodic effect as well as neuroprotective and myelinating effects. For the first time it was shown that a derivative of vitamin B₁₂, which has hydrophobic substituents—heptamethyl ester of cyanoaquacobyrinic acid, also exhibits biological activity and, therefore, is of interest for further research. Analysis of changes in the electronic absorption spectra recorded during the interaction of aquacobalamin with thiosemicarbazide indicated possibility of direct interaction of thiosemicarbazide with aquacobalamin.

Keywords Vitamin B₁₂ · Aquacobalamin · Heptamethyl ester of cyanoaquacobyrinic acid · Thiosemicarbazide · Antidotes

1 Introduction

Vitamins represent important class of micronutrients vital for humans. Each vitamin affects the expression levels or function of a specific set of genes and proteins/enzymes [5] and thus has a certain range of specific biologic functions and certain compartmentalization in the organism. Vitamin

B₁₂, being compartmentalized predominantly in the liver, intestinal epithelium, bone marrow and nerve cells, is fundamental for folate metabolism (including neutralization of homocysteine), DNA methylation and nucleotide biosynthesis. Derangement of folate metabolism and DNA methylation that occurs against the background of vitamin B₁₂ deficiency contributes to carcinogenesis and megaloblastic anemia. In neurology, vitamin B₁₂ is used to treat pain and restore myelin sheath of neurons [15].

Currently, much attention is given to the study of the properties of vitamin B₁₂ derivatives which might expand the area of their practical applications. From the chemical point of view, vitamin B₁₂ (cyanocobalamin) is a unique natural cobalt complex which contains a substituted corrin ring and 5,6-dimethylbenzimidazole, a nucleotide base. Cobalt in vitamin B₁₂ can take different oxidation states: Co(III), Co(II), Co(I). Thanks to this, vitamin B₁₂ is able to act as a catalyst in redox processes. The biological functions of vitamin B₁₂ as well as the possibility of its chemical modification make it a promising tool in the design and development of new drugs. Aquacobalamin, by binding the cyanide base to form harmless cyanocobalamin, can also be used as an antidote for cyanide poisoning and overdose of sodium nitroprusside (in addition

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to the above-mentioned effects of vitamin B₁₂). [1]. Diaquacobinamide, in which the ribonucleotide base of aquacobalamin is replaced by a water molecule, can also act as an antidote for poisoning with cyanide [1], hydrogen sulfide [16], and methyl mercaptan [6]. Heptamethyl ester of cyanoaquacobyrinic acid (HECAA) forms strong complexes with cyanide and hydrogen sulfide in aqueous solutions [2].

The natural affinity of vitamin B₁₂ to the bone marrow and nerve cells can, in principle, be used for targeted delivery of drugs. Submicron capsules can reduce the loss of vitamin B₁₂ in the presence of oxidants [17] and during targeted delivery [3, 4]. Previously, vitamin B₁₂ was successfully encapsulated in micron-sized nano-engineered polymer capsules [13]. The process was accompanied by formation of two-dimensional and three-dimensional micro- and nanoaggregates [10, 20, 21, 29] due to the ability of such compounds to self-assemble [22–24, 26, 31]. These ensembles are formed in a confined space at nanolevel and their properties often differ from those of the initial compounds [11, 12, 14, 25, 27, 28, 30].

Chemoreactomic analysis is an effective method for a preliminary assessment of the potential pharmacological properties of the compounds [18, 19]. Chemoneurocytological analysis of vitamin B₁₂ derivatives shown, in particular, that cyanocobalamin and aquacobalamin can have significant neuroprotective effects [7]. One of the aim of the present paper is to corroborate these findings in an experimental study on rats. Derivatives of vitamin B₁₂ with hydrophobic substituents have not yet found wide application in medicine. So the other aim of the article is whether a) one of such derivatives exhibits biological activity and b) both derivatives under study in this work exhibit antidote properties towards thiosemicarbazide.

2 Materials and methods

The dicyanocobyrinic acid heptamethyl ester ((CN)₂Cby(OCH₃)₇) was prepared by refluxing a vitamin B₁₂ methanol/sulfuric acid (1.0 M) solution for four days (Salnikov D.S. synthesis). Cyanoaquacobyrinic acid heptamethyl ester (CN)(H₂O)Cby(OCH₃)₇ (Fig. 5a) was prepared by vacuum drying of (CN)₂Cby(OCH₃)₇ aqueous solution (pH 4.0 and 25 °C). The structure and purity of the ester were confirmed by ¹H NMR, elemental analysis, MALDI-TOF mass spectroscopy.

The values of the equilibrium constants, *K*, were obtained by fitting titration curve to Eq. 1:

$$A_t = (A_0 + A_\infty \times K \times [L]) / (1 + K \times [L]) \quad (1)$$

where *A*₀ and *A*_∞ correspond to absorbance values at 0% and 100% formation of complexes between thiosemicarbazide and vitamin B₁₂ derivative, respectively, and *A*_{*t*} is the absorbance at any thiosemicarbazide concentration [*L*].

The medical study was carried out using a model of thiosemicarbazide seizures in rats on 30 white male rats (weight 200–300 g). All the substances studied were injected intramuscularly at dose of 60 μg/kg for 18 days. The animals were divided into 5 groups: the first group (*n*=6, intact control); the second group (*n*=6, control with the model of primary generalized seizures); the third group (*n*=6, cyanocobalamin); the fourth group (*n*=6, aquacobalamin); the fifth group (*n*=6, HECAA). Cyanocobalamin was purchased (registration number P No. 015993/01, JSC “Borisovskiy Plant of Medical Preparations,” Republic of Belarus), the rest of the compounds were synthesized in our lab.

To study the anticonvulsant properties of the substances, an experimental model of primary generalized seizures caused by chemical exposure was reproduced [8, 9]. In the experimental groups (second, third, fourth, fifth), the seizure model was reproduced by intraperitoneal administration of thiosemicarbazide at a dose of 28 mg/kg of body weight. In all groups, the latent time to the first seizure, the number and the nature of the peculiarities of the seizures (flinching, arena running, clonic seizures, tonic–clonic seizures with lateral position and tonic extension ending in death) and mortality within 90 min were registered. Statistical data processing was carried out using the program “Statistica 10.0.”

Histological analysis of sectional material (rat brain) was carried out on a model of primary generalized seizures in rats caused by thiosemicarbazide. By means of craniotomy, the entire brain was removed and fixed in a 10% solution of neutral formalin, after 1 day, using frontal incisions, the area of the precentral gyrus of the forebrain, cerebellum, and brain stem were isolated. After the secondary fixation and washing of the material, the nerve tissue was wired (dehydrated) using 99% isopropyl alcohol. Subsequently, the pieces of the brain were embedded in paraffin, and histological Sects. 5–6 μm thick made on a Microm sled microtome were stained with hematoxylin and eosin. Duplicate sections were stained according to the Nissl method and impregnated with silver using a kit of reagents from BioVitrum (Russia).

Nissl staining was carried out as follows. Straightened sections of nerve tissue were placed in a 0.1% solution of toluidine blue or thionine, which was then heated twice until vapors appeared. After cooling, the tissue samples were rinsed in water and 70% alcohol then in 96% alcohol, xylene, balsam. The samples were stained with ready-made dyes in the kit “Toluidine Blue Nissl Modification” from the Biovitrum company (Russia).

Evaluation of pathological changes in the rat brain during modeling of primary generalized seizures took into account the degree of circulatory disorders, damage to the pathways, structural changes in pyramidal neurons of the cerebral cortex (cerebral hemispheres) and piriform neurons (Purkinje cells) of the cerebellum. The morphometric study of histological sections was carried out on a BioVision image

analyzer (Vision Microscopy, Austria) and consisted in counting damaged piriform neurons in the cerebellar cortex in 10 different fields of vision. Micrographs were obtained using an MC200 research microscope (Micros, Austria) and a DCM900 digital eyepiece camera (UK).

Photophysical studies of derivatives of vitamin B₁₂ were performed on a Cary 60 spectrophotometer in a sealed quartz cell. Preliminary comparative studies have shown that oxygen does not affect the interaction of the vitamin B₁₂ derivatives studied with thiosemicarbazide. Therefore, all further experiments were carried out in aerobic conditions.

3 Results

3.1 The medical study using a model of thiosemicarbazide seizures in rats

In animals of the control (second) group introduction of thiosemicarbazide at 28 mg/kg dose resulted in primary generalized convulsions in 100% of cases. The convulsions were manifested in the form of flinching, dressage running, clonic convulsions, tonic-clonic convulsions with lateral position, and tonic extension. Mortality in this group of animals was 100%.

The results of the study showed that the administration of cyanocobalamin (third group) at the indicated dose on the thiosemicarbazide seizure model significantly reduced the severity of convulsive seizures according to frequencies of the features “flinching” ($p=0.02$) and “tonic extension ending in death” ($p=0.027$) as compared to the control group (Table 1). The lethality of animals in the third group was 50%.

In animals injected with aquacobalamin (fourth group), the latency period to seizures significantly increased ($p=0.008$), the number of seizures ($p=0.01$) and the severity of seizures according to occurrence of tonic extension ending in death ($p=0.027$) were decreased in comparison with the control group (Table 1, Fig. 1a). The lethality of animals in the fourth group was 50% (Table 1, Fig. 1b). As a result of a comparative assessment of the duration, nature and severity of seizures in the third (who received cyanocobalamin) and the fourth (receiving aquacobalamin) groups of animals, no significant differences were observed.

In the fifth group of rats (injected with HECAA), the latency period to seizures was significantly lengthened ($p=0.006$) while the severity of flinching was decreased ($p=0.02$) compared to the control group of animals and to the third group (cyanocobalamin, see Table 1 and Fig. 1a). The lethality of animals in the fifth observation group was 67% (Table 1, Fig. 1b). As a result of a comparative assessment of the duration, nature and severity of seizures in the

Table 1 Assessment of the experimental groups

Group	Latency period prior to seizures (min)	Number of seizures	Survival time (min)	Severity of seizures				Duration of convulsions (sec)		
				1—flinching	2—“wild” arena running	3—clonic convulsions	4—tonic-clonic convulsions with lateral position	5—tonic extension	6—tonic extension resulting in death	
Control (2 nd group)	52.5 ± 3.21	3.67 ± 0.76	72.67 ± 5.98	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	0.67 ± 0.21	0.83 ± 0.17	161.0 ± 56.51
Cyano-cobalamin (3 rd group)	65.33 ± 5.67	2.83 ± 0.54	76.67 ± 8.11	0.33 ± 0.21* ²	0.83 ± 0.17	0.67 ± 0.21	0.83 ± 0.17	0.83 ± 0.17	0.17 ± 0.17* ²	118.67 ± 33.75
Aqua-cobalamin (4 th group)	73.67 ± 4.52* ¹	1.5 ± 0.22* ¹	74.67 ± 8.41	0.67 ± 0.21	1 ± 0.0	0.83 ± 0.17	1 ± 0.0	0.67 ± 0.21	0.17 ± 0.17* ¹	79.5 ± 16.81
HECAA# ¹ (5 th group)	71.83 ± 2.6* ³	2.0 ± 0.37	81.25 ± 0.55# ¹	1 ± 0.0	1 ± 0.0	1 ± 0.0	0.83 ± 0.17	1 ± 0.0	0.33 ± 0.21	103.83 ± 19.68

* 1—groups compared: control and aquacobalamin; * 2—groups: control and cyanocobalamin; * 3—groups: control and HECAA. # 1—groups: cyanocobalamin and HECAA

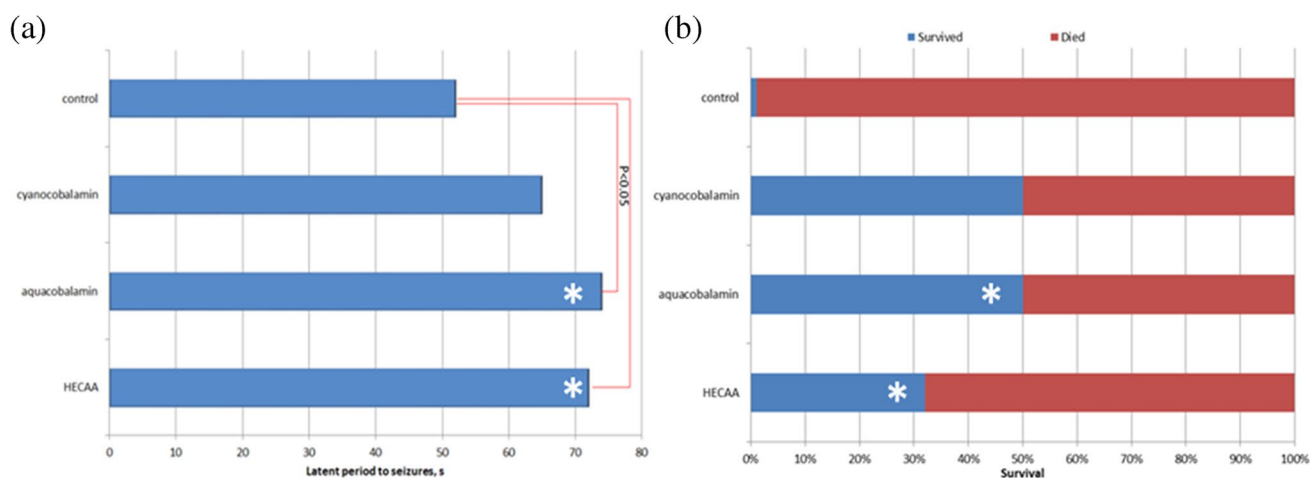


Fig. 1 Latent period to seizures (a) and survival (b) in comparison groups

fifth and the fourth groups, no significant differences were observed.

Thus, cobalamin derivatives studied on the model of primary generalized seizures in rats significantly tended to increase the survival rate and to decrease the severity and duration of seizures. At the same time, aquacobalamin contributed to a greater extent to the lengthening of the latent period of seizures and the number of seizures (which corresponds to a “faster” antidote-like action), and cyanocobalamin—to reduce the severity of seizures (which corresponds to a more “long-term” tissue-protective effect).

3.2 Histological analysis

A pathohistological study of the sectional material of rat brain took into account the degree of circulatory disorders, damage to the pathways, structural changes in pyramidal

neurons of the cerebral cortex (cerebral hemispheres), and piriform neurons (Purkinje cells) of the cerebellum.

In all observations of the control group (group No. 2), after the reproduction of primary generalized seizures, all parts of the brain had significant circulatory disorders characterized by stasis of erythrocytes in the capillaries, the formation of fibrin-erythrocyte thrombi in the lumens of small veins of the gray and white matter of the brain with the development of pronounced perivascular edema of the nervous tissue (Fig. 2a). In the pial and intracerebral arteries, there was a change in the contours of the elastic membrane of the intima against the background of narrowing of the vascular lumen, which characterizes persistent spasm of the arterial link (Fig. 2b).

Pathohistological examination of the gray matter of the cerebral hemispheres and cerebellum revealed the predominance of the ischemic type of neuronal damage. In many

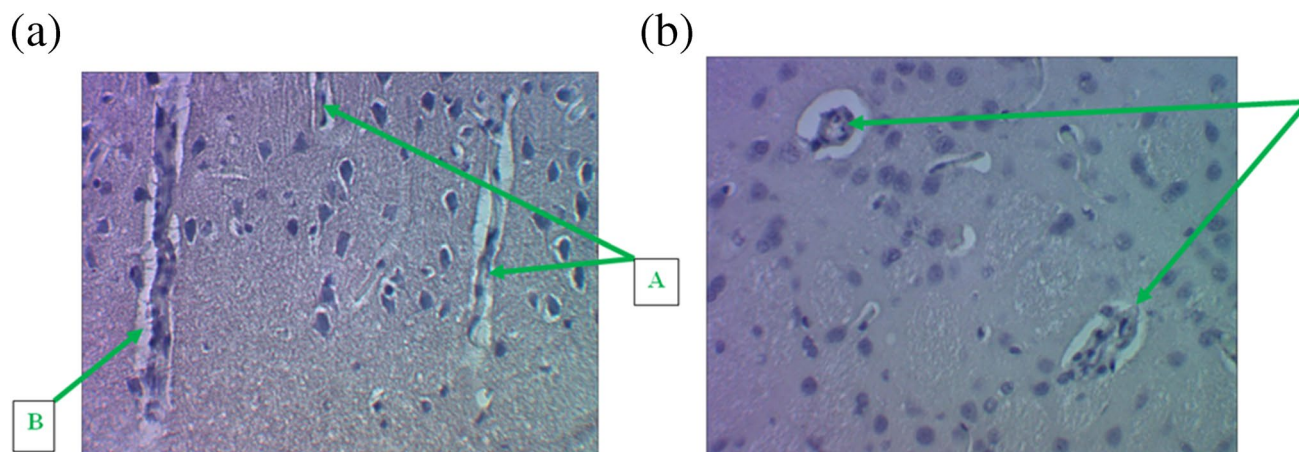


Fig. 2 Hemostasis (a) and spasm of intracerebral arteries (b) in microvasculature (A) and perivascular edema of the nervous tissue of the cerebral cortex (B). Staining with hematoxylin and eosin. Magnification $\times 480$

fields of vision there were whole groups of pyknomorphic and hyperchromic neurons with an increase in the activity of microglial elements in the damaged areas. When brain slices were impregnated with silver, myelin fibers were unevenly colored, indicating disintegration processes of the myelin sheath of the nerve fibers.

Pathohistological study of the effects of cyanocobalamin (group 3) showed a less pronounced diffused focal hemostasis of capillaries, a plethora of intracerebral and pial veins (Fig. 3a) and an increase in the lumen of arteries. Analysis of structural changes in neurocytes showed a decrease in the number of cells irreversibly damaged by ischemia and structural changes in nerve cells were more reversible as represented by focal fusion of the Nissl lumps (Fig. 3b). Myelinated nerve fibers in many fields of view retained clear contours, with only single foci of dechromination (Fig. 3c). A similar histological picture was observed in the other two

groups. With the introduction of aquacobalamin (group 4), a moderately expressed spasm of small.

Reversible changes in nerve cells in groups 3 and 4 prevailed over deep lesions that were characterized by swelling and deformation of the cell nuclei, diffuse small focal chromatolysis of the cytoplasm. The impregnation of the conducting pathways of the brain with silver in most cases showed the presence of only local damage to the myelin nerve fibers.

Injections of HECAA (group 5) corresponded to moderate circulatory disorders in the form of diffuse focal hemostasis in capillaries and venules, perivascular edema of the nervous tissue. Damage to the neurons of the cerebral hemispheres and cerebellum was of a diffuse focal nature with a predominance of ischemic changes in the form of hyperchromia and a decrease in the volume of cytoplasm while preserving the nuclei, a macroglial

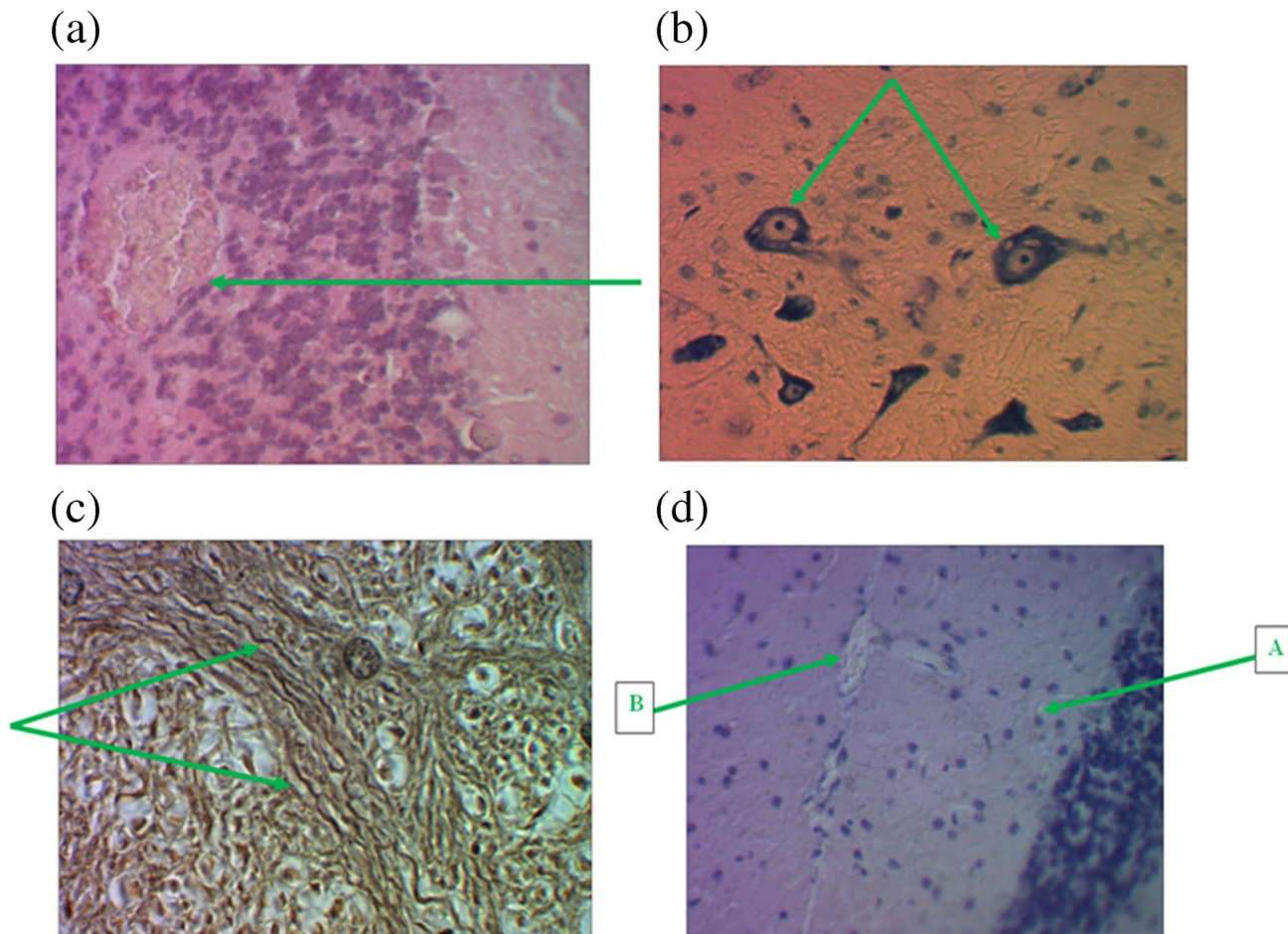


Fig. 3 Pathohistological study of the effects of cyanocobalamin (group 3). (a) Dilation, acute plethora of the venule of the molecular layer of the cerebellum. Staining with hematoxylin and eosin, Magnification $\times 480$. (b) Vacuolization of the cytoplasm, swelling of the axons of the neurons of the brain stem. Nissl staining with toluidine

blue, magnification $\times 1200$. (c) Myelin fibers of the white matter of the cerebral hemisphere without damage over a considerable extent. Silver impregnation, magnification $\times 1200$. (d) Hemostasis, pericapillary edema (A), plethora and venule dilation (B). Staining with hematoxylin and eosin, magnification $\times 480$

reaction with moderate astrocyte hypertrophy (Fig. 4a). Damage to myelin fibers was of a focal nature with loss of stained areas of insignificant extent (Fig. 4b).

The results of morphometric analysis showed significant differences only in comparison with the control group (group 2), while between groups 3, 4, and 5, corresponding to various derivatives of vitamin B₁₂, there were no significant differences (Table 2).

In general, the model of primary generalized seizures had morphological confirmation in all cases of observation and was characterized by circulatory disorders, edema of the nervous tissue, damage to neurocytes, and demyelination of the pathways. Administration of vitamin B₁₂ preparations to laboratory animals in groups 3, 4, and 5 prior to reproduction of the model did influence the levels of cerebral circulatory disorders and was characterized by a decrease in the severity of hemostasis in vessels and edema of the nervous tissue. All the compounds studied showed an antispasmodic effect, neuroprotective effect, and stimulated lower level of damage to the myelin fibers of the brain. In group 5 (HECAA), a relatively high neuroglia reactivity was revealed.

3.3 Photophysical studies of the interaction of thiosemicarbazide with vitamin B₁₂ derivatives

In accordance with the results of behavioral tests (Table 1), aquacobalamin in a greater extent contributed to the lengthening of the latent period of seizures and of the number of seizures (which corresponds to a “faster” antidote-like action of vitamin B₁₂). It is known that the derivatives of vitamin B₁₂ are used as antidotes against a number of compounds. In this regard, we hypothesized that the studied derivatives of vitamin B₁₂ can act as antidotes against thiosemicarbazide poisoning. To test this hypothesis, a study was carried out on the possibility of interaction of thiosemicarbazide with aquacobalamin (Fig. 5a) and heptamethyl ester of cyanocobalamin (Fig. 5b).

Spectral analysis indicated that the addition of thiosemicarbazide to aquacobalamin in aqueous solution at pH 6.86 resulted in disappearance of the peaks of the initial aquacobalamin spectrum (Fig. 5c, spectrum 1) and appearance of two new peaks at 304 nm and at 540 nm (Fig. 5, spectrum 2). The resulting electronic spectrum (spectrum 2) is characteristic of cobalamin complexes formed with the participation of the Co³⁺ ion. The experiments were performed in the

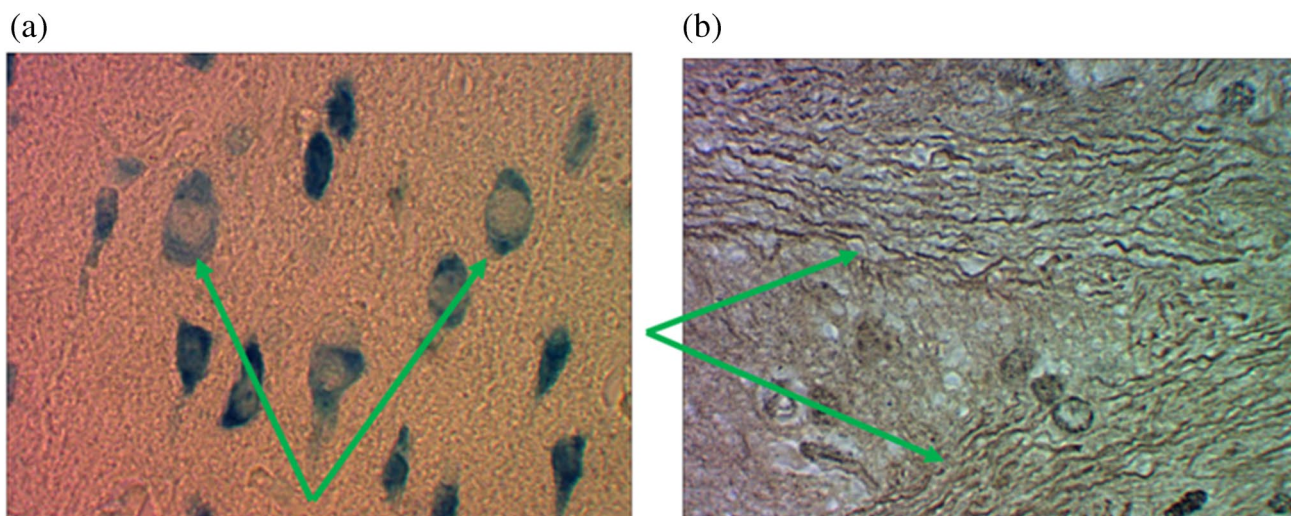


Fig. 4 Pathology of group 5 (HECAA). Silver impregnation. (a) Hypertrophied obese astrocytes. Magnification $\times 1200$. (b) Focal demyelination of nerve fibers. Magnification $\times 480$

Table 2 Results of morphometric analysis of structural changes in neurons in different groups

	Intact neurons	Reversible changes in neurons	Irreversible changes in neurons
2nd group (control)	27.8%	32.5%	39.7%
3rd group (cyanocobalamin)	32.4%	35.4%	32.2%
4th group (aquacobalamin)	31.7%	35.0%	33.3%
5th group (HECAA)	29.3%	35.9%	34.8%

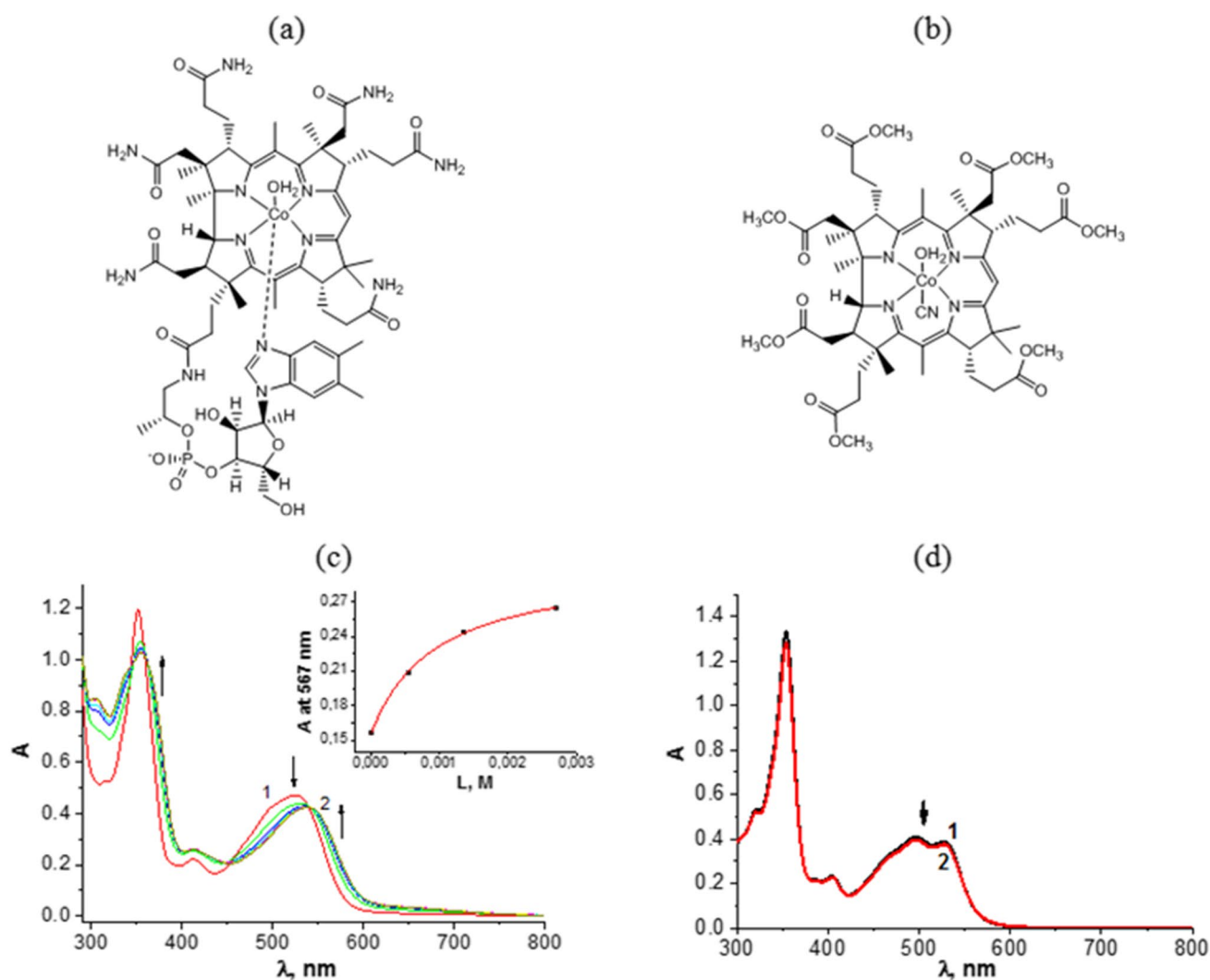


Fig. 5 Structures of vitamin B₁₂ (aquacobalamin, a) and heptamethyl ester of cyanoaquacobyrinic acid (b) Changes in the electronic absorption spectrum (pH=6.86, t=150C, reaction time—10 min) recorded during the interaction of 50 μ M of aquacobalamin (c) and

heptamethyl ester of cyanoaquacobyrinic acid (d) with 2.7 mM of thiosemicarbazide (the arrows show the direction of the change in optical density during the reaction). The insert in (c) shows dependence of absorption value at 567 nm on thiosemicarbazide concentration (L)

presence and absence of oxygen and we found that the presence of oxygen in the reaction medium does not affect the supposed reaction between thiosemicarbazide and aquacobalamin. Thus, the reaction probably involves complexation and not reduction of the Co³⁺ ion.

Formation of the complex between aquacobalamin and thiosemicarbazide is, most likely, reversible. The shape of the titration curve registered for this reaction strongly supports this supposition (Fig. 5c, insert). Fitting the titration curve to Eq. (1) gave the best fit with $K = 1 \pm 0.1 \text{ M}^{-1}$. Thus, the calculated binding constant was rather low. That is, thiosemicarbazide might bind to aquacobalamin in animal experiments only partially. In similar experiments, it was found that for a hydrophobic derivative of vitamin B₁₂ (HECAA) the binding with thiosemicarbazide was even weaker. Very small changes

were observed for this reaction (Fig. 5d). Because of it, the titration curve was not suitable for fitting by Eq. 1. In this case, we suppose that the equilibrium constant was 3 times weaker in comparison to that of aquacobalamin.

Thus, the effects of vitamin B₁₂ derivatives observed in the present series of experiments cannot be explained exclusively by direct interactions with the thiosemicarbazide molecule.

4 Conclusion

A comparative study of vitamin B₁₂, aquacobalamin and heptamethyl ester of cyanoaquacobyrinic acid on a model of thiosemicarbazide seizures in rats showed that

aquacobalamin, to a greater extent than the other compounds, contributed to the lengthening of the latent period of seizures, and cyanocobalamin—to reduction of the severity of the seizures.

The study of the rat brain showed that all compounds investigated exhibited an antispasmodic effect, neuroprotective and myelinating effects, which can partially be explained by antidote-like action of the compounds.

It is important that the derivative of vitamin B₁₂, which has hydrophobic substituents in its composition, heptamethyl ester of cyanoaquacobyrinic acid, also exhibits vitamin activity and, therefore, is of interest for further research, including for targeted delivery of the compound in capsules.

The results presented here are the second stage of a comprehensive project aimed at studying the possibility of targeted delivery of drugs based on vitamin B₁₂. At the first stage, before experimental studies on animals, a study was carried out by the method of chemoneurocytological analysis [7]. In addition, studies on the aggregate behavior and functional properties of compounds at the air–water interface, in thin films on solid supports and in capsules are started. At the third stage, a study of the biological effect of drugs in capsules on animals will be presented.

Acknowledgements This work was financially supported by the grant of the Russian Science Foundation (N20-12-00175), Ivanovo State University of Chemistry and Technology; synthesis of compounds was supported by a grant of the Russian Ministry of Education and Science (FZZW-2020-0008).

Funding This work was financially supported by the grant of the Russian Science Foundation (N20-12-00175), Ivanovo State University of Chemistry and Technology; synthesis of compounds was supported by a grant of the Russian Ministry of Education and Science (FZZW-2020-0008).

Declarations

Informed Consent All authors consent for publication of article.

Research Involving Humans and Animals Statement The protocol of study was approved by the Biomedical Ethic Expert Committee of Ivanovo State Medical Academy (protocol #2 of 12 February 2020) under the institutional and the international ethical guidelines. Injections and care were given in accordance with standard recommendations of veterinary practice by qualified personnel with additional checks for health and welfare of the animals.

Conflict of Interest None.

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