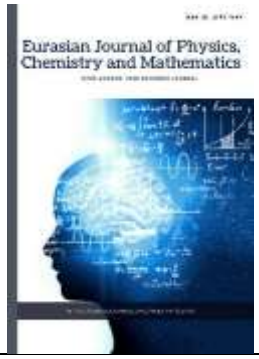


Anti-tuberculosis Conjugates Based on Polysaccharides

Shomurotov Shavkat Abduganievich
Institute of Bioorganic chemistry UzAS, PhD,
Department of Chemistry of Polysaccharides,
E-mail: shsha@mail.ru,

Mamatmusaeva Nilufar Erkinovna,
Tashkent Pharmaceutical Institute, PhD,
E-mail: erkinova81@mail.ru

Sagdullaev Bakhodir Takhirovich
Institute of Bioorganic chemistry UzAS, DSc,
Department of Chemistry of Polysaccharides,
E-mail: bsagdullaev29@gmail.com

Turaev Abbaskhan Sabirkhanovich
Institute of Bioorganic chemistry UzAS, DSc,akademik,
E-mail: abbaskhan@mail.ru

ABSTRACT

The research on the synthesis of polymeric complexes of polygalacturonic acid with anti-TB preparations having antituberculosis activity was conducted. The structures were ascertained and studied the physicochemical properties of polymeric systems. Pharmaco-toxicological studies have shown that polymeric complexes are less toxic than their low molecular analogues and Mycobacterium of tuberculosis show high sensitivity to the prepared preparations.

Keywords: polymeric complexes, tuberculosis, prolongation, polyglacturonic acid, isoniaside, ethambutol.

In connection with the specific treatment of tuberculosis - complex therapy (at the same time with several anti-TB preparations), duration of treatment, the need for the prescribing of multiple high doses of drugs and the emergence of this toxic and allergic complications – it is very important to find the method to reduce the dosage of anti-TB preparations. One way to improve the conditions of drug therapy is the creation and application of polymeric anti-TB preparations of prolonged action. Such long-acting anti-TB preparations are available including in polymer - carriers of known anti-TB preparations, which allows purposefully change their physico-chemical and medico-biological properties [1,2].

In this regard, were carried out the studies on obtaining the macromolecular drug delivery system by the addition of anti-TB preparations of hydrazide of isonicotinic acid (HINA) and ethambutol dihydrochloride (EBHC) to the macromolecule of polymer carrier. As the polymer carrier was used modified polygalacturonic acid (PGA) obtained by demethoxylation of citrus pectin [3].

Materials and methods:

UV spectra were recorded on a UV spectrophotometer «UV 1280" manufactured by «Shimadzu», Japan, in quartz cuvettes with 1

cm thick, in the range of 200 - 500 nm in aqueous solutions.

IR spectra were recorded on devices «Bruker Vektor-33", in the wavelength range of 500-4000 cm^{-1} in tablets with KBr.

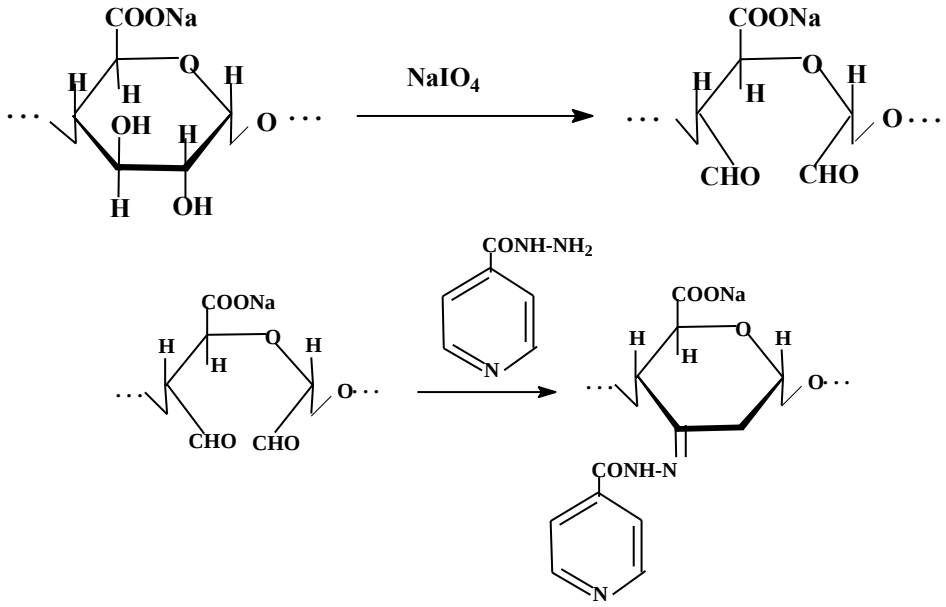
Synthesis of dialdehyde polygalacturonic acid (DAPGA). Periodate oxidation of PGA was performed at pH of 4.7 in 3% aqueous solution of PGA, at oxidant concentration of 0.5M and temperature of 25 ° C within 2.5 hours. Degree of oxidation of reaction in product was determined by iodometric method [1] and UV - spectrophotometric method [2] on flow rate JO_4^- of ions on intensity change of absorption band at λ = 222 nm.

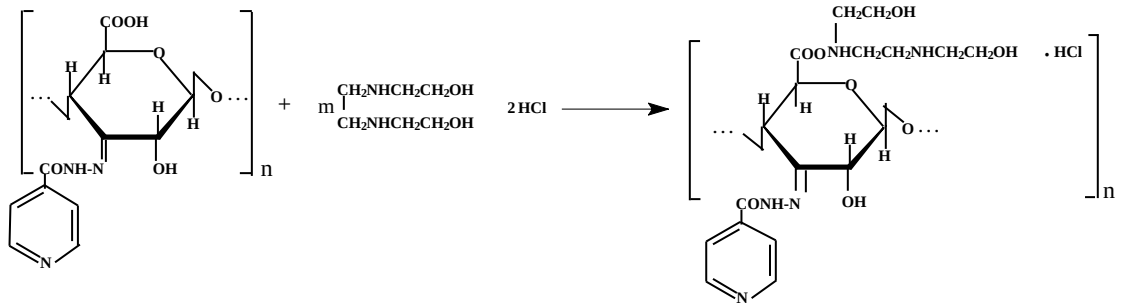
Synthesis of PGA with HINA. The reaction was performed in a three-necked thermostated flask equipped with thermometer and stirrer. Into the flask was placed 0.02 mole of DAPGA and 40 ml of an aqueous solution containing 0.06 mole of HINA and stirred vigorously within 60 min. at 25C. Then the reaction product was filtered, washed repeatedly with mixture of alcohol : water (1:1) and dried over P_2O_5 in a desiccator. The content of bound HINA was determined by the content of nitrogen.

Synthesis of PGA complex with HINA and EB. The reaction was carried out at pH 5.5 in 3% aqueous solution of the complex PGA with HINA at EBHC concentration of 0.5 M and temperature of 20 ° C for 60 min. The reaction mass was then dialyzed for 24 hours. The product was isolated by rotary drying. The content of HINA and EB was determined on nitrogen content and spectrophotometric method.

Results and discussion: We have studied the synthesis of polymeric complexes HINA and EB with modified macromolecule PGA, which was obtained by demethoxylation of citrus pectin (the content of OCH_3 groups, 7.6%)

Synthesis was carried out as follows.





DAPGA was obtained by periodate oxidation of PGA. The reaction was carried out homogeneously in aqueous solution of $NaJO_4$. The reaction products were precipitated with acetone. The control of the oxidation process was carried out on the spending of periodate-ion (spectrophotometric method) and on changing the content of aldehyde groups (iodometric method).

The dependence of the oxidation degree of the modified PGA on the reaction time, the molecular weight (MW) of PGA and ratio of reagents was studied.

Table1. - The dependence of the oxidation degree of PGA on the reaction time and MW at periodate oxidation.

(Reaction conditions: PGA : JO_4^-= 1: 1,5; t=20^{o}C; pH=4,0)

Time, hour	Molecular mass of PGA									
	48500		44000		40000		30000		15000	
	I*	S*	I*	S*	I*	S*	I*	S*	I*	S*
0,25	8,6	8,7	8,8	9,0	8,4	8,7	8,4	8,5	9,3	9,5
0,5	15,0	15,4	15,3	15,6	14,4	15,2	15,7	16,3	15,6	15,9
1,0	23,2	23,8	24,1	24,2	24,3	24,6	24,7	25,2	25,2	25,5
1,5	32,7	33,0	32,9	33,2	36,5	37,2	36,6	37,3	35,2	37,5
2,0	43,8	46,4	43,3	47,3	45,8	49,5	44,4	46,6	46,7	47,6
2,5	52,4	55,6	51,6	52,8	51,3	56,1	54,4	56,8	52,9	54,5
3,0	51,5	57,2	49,6	55,8	48,3	48,9	46,9	59,5	50,5	56,7
5,0	49,3	60,8	47,4	58,6	47,2	59,4	46,3	61,7	49,2	58,2

where: oxidation degree was determined: I*-iodometric method;

S*-spectrophotometric method*;*

As can be seen from Table 1, with increasing of the reaction time increases the degree of oxidation, in all samples it is nearly identical, suggesting that the MM almost doesn't influence on the degree of oxidation of PGA. Increasing the reaction time over 2.5 hours leads to some reduction in oxidation state (the content of aldehyde groups). Such a course of reaction, due to the fact that in addition to the main reaction proceeds adverse reaction - the oxidation of the resulting aldehyde groups to carboxyl.

Nucleophilic substitution reaction of DAPGA with HINA was conducted. The dependence of the interaction between HINA and DAPGA on the reaction conditions was studied.

Table 2. – Dependence of nucleophilic substitution of DAPGA with HINA on MW and the ratio of DAPGA : HINA.

(Reaction time 30 min., t= 20 ^{0}C, pH=5, content of oxidized parts 21 to 100 elementary parts)

Ratio of DAPGA: HINA, moles	**Molecular weight of DAPGA, dalton**				
	75670	**58530**	**34440**	**26570**	**11520**
	Content of bound HINA, moles %				
1:0,25	7,8	7,2	6,9	7,0	7,2
1:0,5	13,2	15,6	14,4	16,6	15,8
1:1,0	16,6	17,4	16,2	16,4	17,6
1:1,5	18,4	18,8	18,8	19,5	19,3
1:2,5	20,2	19,8	20,7	20,7	20,9
1:5,0	20,8	20,2	21,4	21,6	21,0

Effect of reagents ratio DAPGA:HINA on content of bound HINA (Table 2) shows that the increase of HINA in the reaction medium up to 2.5 mole leads to the increase in the content of bound HINA in polymer. However, further increase in its amount does not affect its content in the product, indicating the exhaustion of the aldehyde groups of the polymer matrix at certain ratios of components.

Nucleophilic substitution reaction of DAPGA with HINA passes homogeneously in aqueous solutions. Therefore MW of polymer hardly affects the nucleophilic substitution process. With increasing concentration of HINA increases the content of bound HINA, the limit value of bound HINA almost corresponds to the content of disclosed, pyranose parts, i.e., 50% of the total number of aldehyde groups.

This indicates that in condensation reaction is involved only one aldehyde group in DAPGA. The second aldehyde group reacts the reduction reaction of pyranose cycle. Similar data was previously obtained by reaction with dialdehydecellulose with phenylhydrazine [3]. To clarify this, the authors carried out a condensation reaction of dialdehyde triphenylmethylcellulose, in which is eliminated the formation of hemiacetal connection with tolilhydrazine (since all hydroxyl groups in C_6 are substituted). The authors demonstrated that during the condensation reaction of triphenylmethylcellulose with tolilhydrazine is proceeding the reduction of C_2-C_3 bond of glucopriranose cycle forming a compound containing arylnitrogroup in the third carbon atom.

The reaction of interaction of complex PGA-HINA with EBHC was studied. The reaction was carried out in aqueous solution at pH 5.5. At this pH the largest part of the carboxyl groups is in salt form. Thus, there is a reaction of ion binding of EB with macromolecule of PGA, as evidenced by IR spectroscopy method and elemental analysis. The resulting product is readily soluble in water.

In order to study the effect of the ratio of reagents on the content of bound EB in the macromolecule PGA-HINA reactions were carried out at different ratios of PGA-HINA:EB. Results are shown in Table. 3.

Table 3. – Dependence of content of bound EB on ratio of PGA- HINA:EBHC.

Mole ratio of PGA-HINA*: EBHC	**Nitrogen content, %**	**EB, moles %**
1:0,1	4,25	5,64
1:0,5	5,88	22,7
1:1	5,99	24,5
1:2	6,58	30,4
1:5	7,80	42,6

1:8	7,77	42,5

PGA- HINA ***-** *MW 12000 and containing 20,2 mole % of HINA with hydrozonic bond*.

The table shows that with increase in the ratio of PGA-HINA:EBHC increases the content of bound EB in the reaction product. It is interesting to note that even with a molar ratio of PGA-HINA:EBHC = 1:1 and large ratios the reaction does not proceed to full substitution of ions Na^+ to EB. The latter is due to steric difficulties of ion exchange along the chain of the macromolecule, and, of course, lower basicity of EB.

It is found that in the interaction reaction of PGA-HINA with EBHC molecular parameters of polymer-carrier hardly change. This is evidenced by results of a study of the characteristic viscosity and molecular weight determination of the original PGA-HINA and PGA-HINA associated with the EB. The characteristic viscosity, measured in 0.1 M NaCl solution, almost doesn't change and is in the range of 0.34 ± 0.5.

Pharmaco-toxicological properties of the obtained polymeric complexes were studied, in particular, the acute toxicity and anti-TB activity. The following polymeric complexes were studied:

- PGA-HINA-EB, containing 20.2 mole % of HINA and 25.6 mole % of EB;
- PGA-HINA, containing 20.2 mole % of HINA
- PGA-EB, containing 24.5 mole % of EB.

As a control was taken HINA with activity of 99% and EBHC with a content of active principle of 100% of the German company «Sigma chemical». Determination of acute toxicity of preparations was carried out in 100 white mongrel mice weighing 18-20 g of both sexes by intraperitoneal injection.

Experimental results were treated by variational statistics. LD_{50} calculations were performed according to the method of Litchfield and Wilcoxon.

On the basis of experiments were determined LD_{50}, which are summarized in Table 4

Table 4. – Composition of studied preparations and their LD_{50}

Composition	**HINA content**		**Ethambutol content**		**LD_{50} mg/ml**
	%	**Mole %**	**%**	**Mole %**	
HINA	100	100	-	-	224 (160÷300)
EB	-	-	100	100	1290 (1100÷1500)
CMC+HINA+EB	13,07	20,2	18,67	25,6	More than 5000
CMC+HINA	13,07	20,2	-	-	3600 (3200÷4300)
CMC+EB	-	-	17,85	24,5	More than 5000

Based on the obtained results, we can conclude that the tested preparations by single intraperitoneal administration can be assigned to different classes of toxicity: "HINA" - to the class of low toxic substances; "Ethambutol" - to practically non-toxic class of substances; "PT", "PTE" and "PEB" – to the class of non-toxic substances [4].

We conducted the study of sensitivity of Mycobacterium tuberculosis to the synthesized polymeric complexes [5]. The study was conducted under conditions in vitro in virulent strains of Mycobacterium tuberculosis H37Rv, Bovinus-8. Tests were conducted by the method of absolute concentrations with content of preparations in the medium of Lewenstein - Jensen 1mkg/ml and 10mkg/ml.

Table 5. - The sensitivity of Mycobacterium tuberculosis to the studied polymeric complexes

Preparations	**№ Cultures**							
	646	**824**	**831**	**956**	**806**	**743**	**552**	**765**
CMC+HINA+EB	+	+	+	+	+	+	+	+
CMC+HINA	+	-	-	+	+	+	+	-
CMC+EB	+	-	-	+	+	+	+	+

«+»- Sensitive to preparation; «-»- stable to preparation.

The results showed that to the complex CMC+HINA+EB all tested strains remained sensitive. To the CMC+EB complex of eight strains remained sensitive six cultures, to the CMC+HINA complex five strains of Mycobacterium cultures were sensitive and in all cases, the efficacy depends on active substance concentration in the preparation. The higher the concentration, the more effective its action on mycobacteria. Combinations of two preparations increase the inhibitory effect on mycobacteria.

Conclusions

Thus, in the course of the research were obtained macromolecular medicinal systems having anti-TB action. The reaction conditions were determined and ascertained the structures of the obtained macromolecular systems.

Pharmaco-toxicological studies have shown that strains H37Rv, Bovinus-8 show high sensitivity to the synthesized polymeric complexes, and also was observed the decrease in toxicity compared to the initial medicinal preparations.

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