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HPLC study on the stability of bendamustine hydrochloride immobilized onto polyphosphoesters

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ABSTRACT

Novel water soluble polymer complexes of bendamustine hydrochloride, a bifunctional alkylating agent with antimetabolic and cytotoxic activity, were developed using biodegradable polymer carriers—poly(oxyethylene H-phosphonate), poly(methyloxyethylene phosphate) and poly(hydroxyoxyethylene phosphate). Bendamustine hydrochloride was immobilized onto polyphosphoesters via covalent, ionic and hydrogen bonding. The structure of the complexes formed was elucidated by ^{1}H, ^{13}C, ^{31}P NMR and FT-IR spectroscopy. The chemical stability of bendamustine hydrochloride in the novel complexes was studied by HPLC analysis based on a validated method with appointed analytical parameters such as specificity, repeatability, limit of quantitation, limit of detection and linearity. The results from the HPLC indicate that in neutral (pH 7) and alkaline (pH 9) media bendamustine hydrochloride in the polymer complexes is more stable than the pure bendamustine hydrochloride. The enhanced stability of the immobilized drug is explained with the drug interaction with the polymer carriers or their degradation products.

1. Introduction

The macromolecular approach, i.e. application of appropriate polymers for drug immobilization, chemically conjugated or physically bound to a polymer chain, can improve some characteristics of low molecular drugs, already approved and used in practice, as well as can impart new valuable properties thus affording possibility novel polymeric drug forms to be developed [1]. Polymer chemistry has contributed in various ways to the present progress in biology, biochemistry, medicine and pharmacy, providing new, highly specified materials. Synthetic polymer formulations are becoming more and more attractive as delivery vehicles because of the great flexibility regarding: (i) the type and size of the bioactive molecules or agents delivered, (ii) the degree of carrier loading and (iii) the immobilization techniques applied. Biodegradable polymers afford an additional advantage—clearance of the implanted or injected drug carriers from the organism. The use of biocompatible polymers as drug carriers is a well-known and widely studied approach [2,3]. The essence of the method consists in the formation of macromolecular drug through physical entrapment or chemical attachment (ionic or covalent bond) of biologically active substance to a polymer template. The drug is then released by simple diffusion, desorption or bond scission. Among the numerous macromolecular systems potentially available as drug delivery vehicles the polymers with phosphoester repeating units in the backbone are particularly interesting because of their biocompatibility and structural resemblance to natural biomacromolecules like nucleic acids [4–8]. The biodegradability of these phosphoesters is induced by hydrolysis or enzymatic scission of the ester bonds [9]. Poly(alkylene H-phosphonate)s and their derivatives poly(alkylene phosphate)s have been actively investigated for pharmaceutical application such as polymer carriers of drugs [10,11] and genes [12–14]. They are especially attractive materials due to the relative easiness of their preparation from commercially available building blocks, the variety of molecular weights attainable and the relatively narrow molecular weight distributions of the polymers formed [15]. Poly(alkylene H-phosphonate)s have the following advantages [15] (i) they are water soluble; (ii) the drug-carrying capacity is not limited to two end functional groups; (iii) the reactive P–H group in repeating units allows chemical immobilization of drugs under mild reaction conditions; (iv) the presence of highly polar P=O group in repeating units affords possibility for physical immobilization of drugs; (v) possibility of hydrophilic/hydrophobic balance control; (vi) they can be regarded as degradable and

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biocompatible synthetic polymers; (vii) they can be designed to have nontoxic building blocks; (viii) they can be administrated over a wider molecular weight range because after hydrolysis, the low molecular PEG will be safely excreted—the most important potential advantage of polyphosphoesters; (ix) easy to prepare in industrial scale; (x) similarity to biomacromolecules such as nucleic acids. Poly(oxyethylene H-phosphonate)s are biodegradable and have low cytotoxicity [16,17], their hydrolysis leading to harmless low molecular products [18].

Bendamustine (4-{5-[bis-(2-chloroethyl)amino]-1-methyl-1*H*-benzimidazol-2-yl} butanoic acid) is a bifunctional alkylating agent with an atypical structure that includes a benzimidazole ring, an active nitrogen mustard fragment and a residue of butanoic acid. Besides biotransformation [19–22], bendamustine, similar to other nitrogen mustards, undergoes degradation by hydrolysis. Two hydrolysis products of bendamustine have been detected, namely monohydroxy and dihydroxy derivatives (4-{5-[(2-chloroethyl)-(2-hydroxyethyl)amino]-1-methyl-1*H*-benzimidazol-2-yl}butanoic acid and 4-{5-[bis-(2-hydroxyethyl)amino]-1-methyl-1*H*-benzimidazol-2-yl}butanoic acid) [23]. Because of the hydrolytic degradation in aqueous solutions, nitrogen mustards are often supplied for administration in a lyophilized form that requires reconstitution, usually in water. The reconstituted product should be administered to a patient as soon as possible. The lyophilized formulation of bendamustine contains degradation products that may occur during manufacturing of the drug substance and/or during the lyophilization process to make the finished drug product. The procedure has been optimized and a lyophilization formulation of bendamustine in 30% tertiary-butyl alcohol/water saturated solution has been proposed in order to shorten the time for reconstitution and decrease the degradation products [24].

We undertook the present study on the immobilization of bendamustine hydrochloride onto water soluble polymer carriers as an alternative approach to improve drug stability in an aqueous medium. Recently, new bendamustine polymer complexes using polyphosphoesters as drug carriers have been designed and prepared. Augmented drug efficacy has been observed after immobilization [25]. It has been assumed that the incorporation of bendamustine in a polymer complex stabilizes its molecule against degradation. The purpose of this study was to investigate *in situ* the chemical stability of the novel polymer complexes in dependence of the physiological pH values (2, 7 and 9) and time. This motivated the present HPLC study aimed to evaluate the chemical stability of the immobilized drug compared to the non-immobilized agent. The results of the HPLC analysis under different conditions—pH and type of the polyphosphoester are presented and discussed. The results of these studies can be useful in the pharmacokinetic investigation of the new drug formulations.

2. Experimental

2.1. Chemicals and reagents

Bendamustine hydrochloride (Ribomustin®, Ribosepharm) substance, Bendamustine hydrochloride (Ribomustin® CRS, Ribosepharm) reference substance was a gift from the producer. Carbon tetrachloride, dichloromethane, acetonitrile, and methanol were Aldrich products and dried following standard procedures and distilled before to use. Acetonitrile HPLC grade, distilled water R (see Reagents (R), European Pharmacopoeia 6th edition), acetic acid R, ethanol 99.98 vol.% R were used. Buffer solutions were prepared according European Pharmacopoeia, 6th Edition, as follows:

(i) a buffer solution pH 2.0—6.57 g of potassium chloride were dissolved in water R and 119 ml of 0.1 M hydrochloric acid were added, then the solution was diluted to 1000 ml with water R;
(ii) a buffer solution pH 7.0—1000 ml of a solution containing 1.8% (w/v) of disodium hydrogen orthophosphate and 2.3% (w/v) of sodium chloride, a sufficient amount (about 280 ml) of a solution containing 0.78% (w/v) of sodium dihydrogen orthophosphate and 2.3% (w/v) of sodium chloride was added to adjust the pH;
(iii) a buffer solution pH 9.0—6.20 g of boric acid were dissolved in 500 ml of water, adjusted to pH 9.0 with 1 M sodium hydroxide (about 41.5 ml) and diluted to 1000 ml with water).

All reagents were analytical grade.

All ^{1}H, ^{13}C and ^{31}P NMR spectra were measured on a Bruker 250 MHz spectrometer in D_2O, DMSO-d_6, $CDCl_3$ and CD_3OD solutions. The infrared (IR) spectra were recorded on Bruker-Vector 22 FT-IR spectrophotometer in KBr tablets.

2.2. Synthesis of poly(oxyethylene H-phosphonate) **1**

The synthesis of poly(oxyethylene H-phosphonate), **1**, has been described previously [26]. The product was obtained as a waxy solid.

^{1}H NMR ($CDCl_3$, δ (ppm)): 6.86 (d, $^{1}J_{(P,H)}$ = 716.2 Hz, 1H, P–*H* repeating unit), 6.79 (d, $^{1}J_{(P,H)}$ = 708.8 Hz, 1H, P(*H*)OCH_3 end group), 6.74 (d, $^{1}J_{(P,H)}$ = 690.3 Hz, 1H, P(*H*)OH end group), 4.19 –4.07 (m, 4H, $CH_2OP(O)OCH_2$), 3.62–3.55 (m, 50H, CH_2OCH_2); $^{13}C\{H\}$ NMR ($CDCl_3$, δ (ppm)): 70.43 (CH_2OCH_2), 70.03 (d, $^{3}J_{(P,C)}$ = 5.8 Hz, $POCH_2CH_2$), 64.57 (d, $^{2}J_{(P,C)}$ = 6.2 Hz, $POCH_2CH_2$); ^{31}P NMR ($CDCl_3$, δ (ppm)): 11.17 (d of sextet, $^{1}J_{(P,H)}$ = 708.8 Hz, $^{3}J_{(P,H)}$ = 10.5 Hz), 10.47 (d of quintet, $^{1}J_{(P,H)}$ = 716.2 Hz, $^{3}J_{(P,H)}$ = 9.9 Hz), 8.37 (d of t, $^{1}J_{(P,H)}$ = 690.3 Hz, $^{3}J_{(P,H)}$ = 10.97 Hz); IR (KBr) (cm^{-1}): 2437 P–H (stretching), 1252 P=O (stretching), 1107 P–O–C, C–O–C (stretching).

2.3. Synthesis of poly(methyloxyethylene phosphate) **2**

A typical procedure is described in Ref. [26]. Yield: 93%.

^{1}H NMR ($CDCl_3$), δ (ppm): 4.14–4.10 (m, 4H, CH_2OPOCH_2), 3.71 (d, $^{3}J_{(P,H)}$ = 11.14 Hz, 3H, $POCH_3$), 3.66–3.58 (m, 50H, CH_2OCH_2); $^{13}C\{H\}$ NMR (CD_3OD), δ (ppm): 70.43 (CH_2OCH_2), 70.03 (d, $^{3}J_{(P,C)}$ = 5.8 Hz, $POCH_2CH_2$), 64.57 (d, $^{2}J_{(P,C)}$ = 6.2 Hz, $POCH_2CH_2$), 55.36 (d, $^{2}J_{(P,C)}$ = 5.9 Hz, $POCH_3$); $^{31}P\{H\}$ NMR (CD_3OD), δ (ppm): 1.86, 0.77, 0.06 (phosphate structures); IR (KBr), (cm^{-1}): 2677 (P–OH), 1260 ν (P=O), 1170 ν (P–OCH_3), 1102 ν (CH_2–O–CH_2), 1036 ν (P–OCH_2).

2.4. Synthesis of poly(hydroxyoxyethylene phosphate) **3**

Poly(hydroxyoxyethylene phosphate) was synthesized as described in Ref. [26]. Yield: 91%.

^{1}H NMR (D_2O), δ (ppm) 3.95–3.89 (m, 4H, CH_2OPOCH_2), 3.65–3.59 (m, 50H, CH_2OCH_2); $^{13}C\{H\}$ NMR (D_2O), δ (ppm): 70.43 (CH_2OCH_2), 70.03 (d, $^{3}J_{(P,C)}$ = 5.8 Hz, $POCH_2CH_2$), 64.57 (d, $^{2}J_{(P,C)}$ = 6.2 Hz, $POCH_2CH_2$); $^{31}P\{H\}$NMR (D_2O), δ (ppm): 1.26; 0.00 (phosphate structure); IR (KBr) (cm^{-1}): 2677 P–OH (stretching), 1291 and 1259 (P=O), 1102 (CH_2–O–CH_2), 1036 (P–OCH_2–).

2.5. Immobilization of bendamustine hydrochloride onto poly(oxyethylene H-phosphonate)

Bendamustine hydrochloride (0.06 g; 1.5×10^{-4} mol), acetonitrile (10 ml), triethylamine (0.03 ml; 1.8×10^{-4} mol) and carbon tetrachloride (10 ml) were placed in a three-necked flask equipped

with a magnetic stirrer, inlet for inert gas and a reflux condenser. A solution of **1** (2.39 g, 3.7×10^{-3} mol of repeating units) in acetonitrile (15 ml) was added drop-wise within 3 h. The reaction was allowed to proceed for 40 h at room temperature (22 °C) by bubbling a stream of dry argon with vigorous stirring. The product was dried at 30–40 °C under reduced pressure.

^{1}H NMR (DMSO-d_6), δ (ppm): 7.5 (d, 1H, C(7)*H*), 6.93 (d, 1H, C(4)*H*), 6.91 (dd, 1H, C(6)*H*); 3.83–3.67 (m, 15H (NCH_3 and $(ClCH_2CH_2)_2N$ and CH_2OPOCH_2), 3.62–3.39 (50H, OCH_2CH_2O), 2.37 (t, 2H (CH_2COOH), 1.97 (q, 2H, (CH_2CH_2 CH_2COOH); ^{13}C {H} NMR (DMSO, δ (ppm)): 174.3 (C(a)), 152.4 (C(2)), 145.6 (C(5)), 133.5 (C(h)), 125.6 (C(i)), 113.4 (C(6)), 112.4 (C(7)), 96.0 (C(4)), 70.3 (OCH_2CH_2O), 70.05 (d, $^{3}J_{(P,C)}$ = 7.7 Hz, $POCH_2CH_2$), 63.94 (d, $^{2}J_{(P,C)}$ = 5.76 Hz, $POCH_2CH_2$), 53.0 (C(f)), 41.6 (C(g)), 32.9 (C(b)), 31.0 (C(e)), 24.5 (C(c)), 22.0 (C(d)); ^{31}P {H} NMR (DMSO-d_6, δ (ppm)): 5.38; 2.32; 1.23; IR (KBr), (cm^{-1}): 1719 C=O, 1250 P=O, 1216 C–O, 1171 P–O–C.

2.6. Immobilization of bendamustine hydrochloride onto poly(methyloxyethylene phosphate)

Poly(methyloxyethylene phosphate) **2** (0.17 g, 2.5×10^{-4} mol repeating units) was dissolved in ethanol (5 ml) at room temperature. A solution of bendamustine hydrochloride (0.1 g; 2.5×10^{-4} mol) in ethanol (5 ml) was added drop-wise to the polymer solution at constant stirring, room temperature and stream of inert gas. After 30 min the solvent was evaporated under reduced pressure.

^{31}P{H} NMR ($CD_3OD + CDCl_3$, δ (ppm)): 1.86; 0.77; 0.061 IR (KBr) (cm^{-1}): 1701 C=O, 1256 P=O, 1106 CH_2–O–CH_2, 1038 P–OCH_2.

2.7. Immobilization of bendamustine hydrochloride onto poly(hydroxyoxyethylene phosphate)

A solution of bendamustine hydrochloride (0.124 g; 3×10^{-4} mol) in ethanol (5 ml) was added drop-wise to a solution of **3** (0.2 g; 3×10^{-4} mol repeating units) in ethanol (5 ml) at constant stirring and room temperature. After 30 min the solvent was evaporated under reduced pressure.

^{31}P{H} NMR ($CDCl_3$), δ (ppm): −0.10; −0.60.

IR (KBr) (cm^{-1}): 1701 C=O, 1257 P=O, 1107 CH_2–O–CH_2, 1036 P–OCH_2.

2.8. Chromatographic system and conditions

Liquid chromatograph Shimadzu LC – 10 Advp equipped with 4.6 mm × 250 mm column Tracel Excel RP-18, ODS with particle size 5 μm and detector SPD 10 AVvp – UV-VIS with fixed analytical wavelength was used in the measurements.

Mobile phase was prepared by mixing of filtered and degassed acetonitrile, water and acetic acid in ratio 200:50:0.05 v/v/v respectively; 233 nm analytical wavelength; column temperature 25 °C; flow rate about 1 ml/min.

2.9. Solution preparation for the HPLC study

Reference solution (*a*). 10 mg bendamustine hydrochloride reference substance were dissolved in a 100 ml volumetric flask in a solvent mixture (mobile phase). The concentration of obtained solution was 100 μg/ml;

Reference solution (*b*). A dried residue of bendamustine polymer complex containing 10 mg bendamustine was dissolved in 4.0 ml ethanol 99.98 vol.%. This solution was diluted with distilled water to obtain the active compound concentration of 100 μg/ml;

Reference solutions (*c*). Solution of poly(oxyethylene H-phosphonate): 100 mg of the polymer were dissolved in 1.6 ml ethanol 99.98 vol.%; solution of poly(methyloxyethylene phosphate): 96 mg of the polymer were dissolved in 1.0 ml ethanol 99.98 vol.%; solution of poly(hydroxyoxyethylene phosphate): 66 mg of the polymer were dissolved in 1.0 ml ethanol 99.98 vol.%.

Sample solution (*a*). 10 mg bendamustine hydrochloride dried substance were dissolved in a 100 ml volumetric flask in a solvent mixture (mobile phase). The concentration of the obtained solution is 100 μg/ml.

Sample solutions (*b*). To dry and weighed samples, three from each of the bendamustine polymer complexes, containing 10 mg bendamustine hydrochloride were added 10.0 ml buffer solution with pH 2 or 7, or 9. The obtained test solution was heated at 37 °C and continuously stirred. 0.1 ml aliquots from the sample mixture were taken, diluted to 1.0 ml with mobile phase and immediately injected in the chromatograph. The study was prolonged to the obtaining of unchangeable remainder.

3. Results and discussion

3.1. Synthesis and characterization of poly(oxyethylene H-phosphonate)

Poly(oxyethylene H-phosphonate) **1** was synthesized via polytransesterification reaction of dimethyl H-phosphonate with poly(ethylene glycol) with number average molecular weight 600 g/mol (PEG 600). The synthesis has been described previously [26]. The structure of the reaction product **1** was confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. The number average molecular weight of the polymer product **1** of 7 200 g/mol was calculated using the ratio between the integral intensity of the signal for the P–H groups in the repeating units (δ = 6.86 ppm, $^{1}J_{(P-H)}$ = 716.2 Hz), that of the end diester –P(*H*)OCH_3 (δ = 6.79 ppm with $^{1}J_{(P-H)}$ = 708.8 Hz) and that of the monoester –P(*H*)OH (δ = 6.74, $^{1}J_{(P-H)}$ = 690.8 Hz) groups. The latter as identified in the NMR spectra were obtained probably due to partial hydrolysis of the end diester H-phosphonate groups.

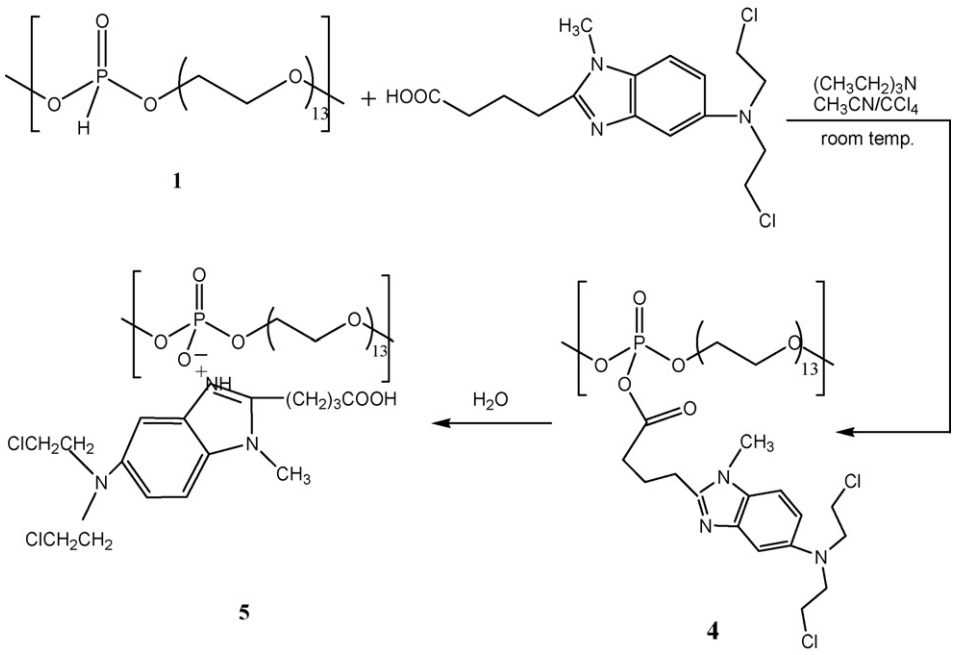
3.2. Synthesis of poly(methyloxyethylene phosphate) *2* and poly(hydroxyoxyethylene phosphate) *3*

Quantitative conversion of **1** into **2** or **3** was achieved via Atherton–Todd reaction using methanol or water, respectively, as reagents [26]. The structure of the polyphosphoesters **2** and **3** was proven by IR, ^{1}H, ^{13}C, ^{31}P NMR spectroscopy. In the ^{1}H NMR spectra of the two products, **2** and **3**, the signals of P–H protons from the starting **1** were lacking—an indication for the quantitative transformation of the P–H groups into methoxy and OH, respectively. In addition, in the ^{1}H and ^{13}C {H} NMR spectra of **2** new doublets appeared at 3.71 ppm ($^{3}J_{(P,H)}$ = 11.14 Hz) and at 55.36 ppm ($^{2}J_{(P,C)}$ = 5.9 Hz), respectively, resulting from the formation of methyl ester moieties (P–OCH_3) due to the oxidative coupling reaction. The ^{31}P{H} NMR spectra of **2** and **3** displayed signals in the spectral region (between 1 and −1 ppm) typical for phosphate structures [27].

3.3. Immobilization of bendamustine hydrochloride onto poly(oxyethylene H-phosphonate)

Bendamustine hydrochloride was immobilized onto **1** using Atherton–Todd reaction conditions (Scheme 1).

Carbon tetrachloride and acetonitrile were used as solvents. The results from IR, ^{1}H, ^{13}C, ^{31}P NMR spectroscopy confirm the struc-



Scheme 1. Reaction pathway for immobilization of bendamustine hydrochloride onto **1**.

ture of the reaction product **4**. Bendamustine is attached to the polyphosphoester via covalent bond.

3.4. Immobilization of bendamustine hydrochloride onto poly(methyloxyethylene phosphate)

It is well-known that the strongly polar phosphoryl (P=O) group participates in hydrogen bonding. Bendamustine hydrochloride was immobilized onto polyphosphoester **2** via hydrogen bonding between the P=O groups of **2** and hydrogen atom of COOH group in the drug molecule (Scheme 2).

The ^{31}P NMR spectroscopic data showed that a partial hydrolysis of P–OCH_3 groups occurred and the formed acidic P–OH groups of polyphosphoester **2** react with bendamustine hydrochloride to form the corresponding salt. The structure of **6** was confirmed by IR and ^{31}P NMR spectroscopy.

3.5. Immobilization of bendamustine hydrochloride onto poly(hydroxyoxyethylene phosphate)

The interaction of bendamustine hydrochloride with **3** leads to the formation of the salt **7** (Scheme 3). The structure of the polymer complex **7** was confirmed by ^{31}P NMR and FT-IR analyses.

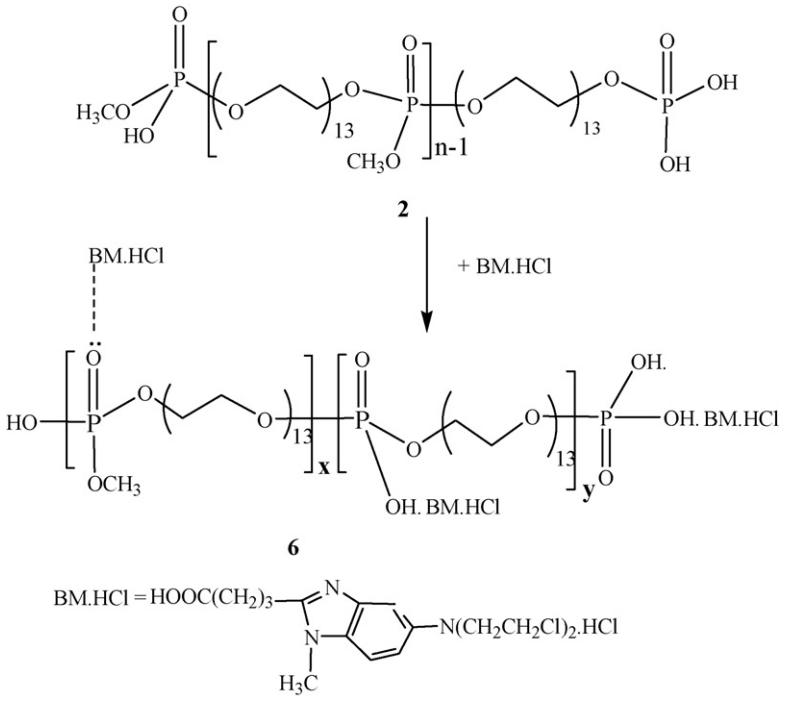
3.6. Validation of the HPLC method for bendamustine hydrochloride determination

For the validation of the HPLC method some analytical parameters were studied:

- *Selectivity*. Solution placebo containing all supplements without active substance was prepared. There are no peaks in the chromatogram obtained from this solution.
- The standard mixtures containing bendamustine hydrochloride and bendamustine hydrochloride polymer complexes were prepared. The chromatograms of these solutions show that the capacity factor is between 0.8 and 0.9. With validated HPLC method we substantiated the original molecule bendamustine hydrochloride against reference substance. The determination of the capacity factor is used for system suitability test along with limit of detection and limit of quantitation in accordance with Eur. Pharmacopoeia requirements about validation of HPLC methods.
- *Repeatability*. Six repeated measurements of a sample solution with a test concentration of 100 μg/ml were performed. The standard deviation (S.D.) and relative S.D. (R.S.D.) are presented in Table 1.
- *Limit of detection*. 9.6 μg/ml, established on the base of ratio noise–signal—1: 3.
- *Limit of quantitation*. 34 μg/ml, established on the base of ratio noise–signal—1: 10.
- *Linearity*. The parameter linearity was studied in concentration interval 50–150% from the theoretical quantity. The results are presented in Fig. 1. The correlation coefficient was found to be 0.9987.

3.7. Comparative study on the chemical stability of bendamustine hydrochloride immobilized on polyphosphoesters and in non-immobilized form at different pH of the aqueous solution

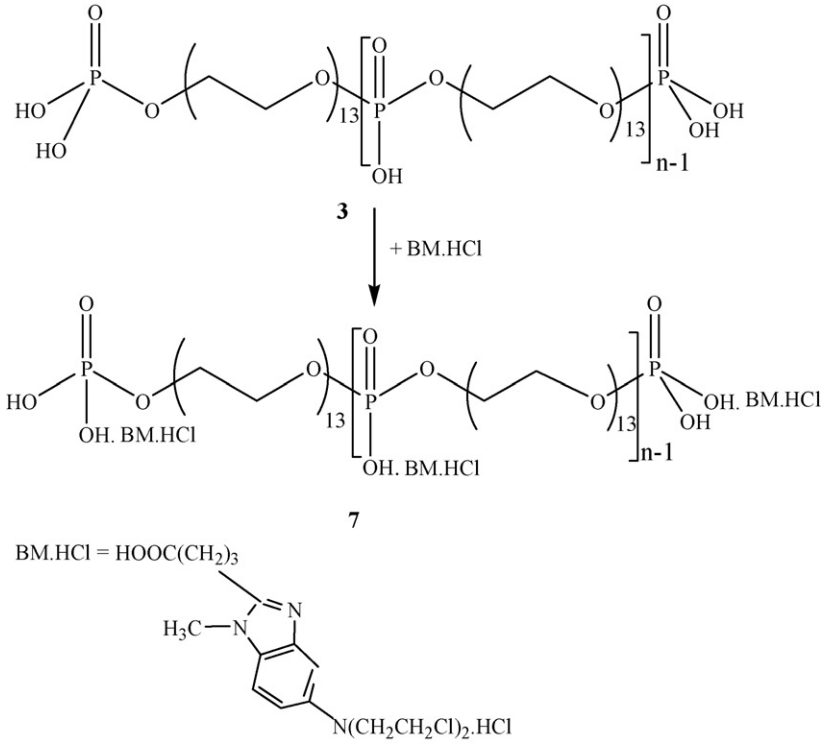
In the conjugate **4** bendamustine is bound to the polymer carrier via a phosphoacyl bond. The anhydride linkage is hydrolytically unstable and in an aqueous medium it degrades to hydroxyphosphate (P–OH) and carboxylic groups (Scheme 1, complex **5**). Depending on the pH of the solution the drug molecules could remain attached to the polyphosphoester chain via hydrogen or ionic bonds, or dipole–dipole interactions. In the case of complex **6** the phosphoester moieties in the carrier backbone present triester phosphate groups that could undergo hydrolytic degradation leading to diester (hydroxyphosphate (P–OH)) groups without or with chain breakage. The results of a kinetic study on poly(methyl ethylene phosphate) hydrolysis showed that under acidic conditions the hydrolysis of the side methyl group proceeded faster whereas in basic and neutral solutions both the main chain and side groups degrade with similar rates [18]. Therefore, the hydroxyphosphate moieties, which are structural units of the carrier chain in complex



Scheme 2. Reaction pathway for immobilization of bendamustine hydrochloride onto **2**.

7, can also present in the complexes **5** and **6** after dissolution. The hydroxyphosphate groups are rather strong acids. The pK_a value of diethyl phosphoric acid is 1.39 ± 0.05 [28].

Bendamustine molecule contains both acidic and basic groups and being an ampholyte it will participate in complex acid–base equilibria. The ratio of the different charged forms will depend on pH of the buffer solution. To our knowledge there are no data published concerning the pK_a values of the drug. Therefore the values were calculated using the ACD Labs software and compared to the experimental ones of compounds similar to fragments of the drug molecule. The estimated value for the dissociation constant of the carboxylic group (p$K_a(2) = 4.50 \pm 0.10$) in bendamustine is very close to the experimentally determined pK_a value of butanoic acid which is 4.84 (35 °C) [29]. The calculated



Scheme 3. Reaction pathway for immobilization of bendamustine hydrochloride onto **3**.

Table 1
Repeatability of the measurement (a test concentration of 100 μg/ml was used)

Number	Measured concentration of bendamustine HCl (μg/ml)	$\bar{X}$ (μg/ml)	S.D. (μg/ml)	R.S.D. (%)
1	98	103.(3)	8.64	8.36
2	104			
3	102			
4	96			
5	100			
6	120			

value for pK_a(3) of the conjugated acid in the imidazolyl residue is 6.36 ± 0.31 which is also in correspondence with that determined for 2-ethylbenzimidazole (6.27 (25 °C), 6.14 (35 °C) [30]. The protonated amino group in the bis(chloroethyl)aminoaryl fragment is the strongest acidic group in bendamustine molecule. The reference data that have been found concern *N*,*N*-di-β-chloroethylaniline which pK_a value was determined to be 3.26 [31]). Hence, the pK_a value under question can accept lower values than 3.26 due to the effect of the condensed benzimidazole structure (the calculated p$K_a(1) = -2.59 \pm 0.70$). That means it dissociates at lower pH than the carboxylic one. Thus, under acidic conditions (pH 2) the imidazole residue of the drug is protonated while the carboxylic function is in acid form whereas in neutral (pH 7) or alkaline (pH 9) solutions the equilibrium is shifted to the species with basic nitrogen atoms in the imidazole ring and dissociated carboxylic groups.

The HPLC data of the bendamustine hydrochloride solutions reveal that in acidic medium (pH 2) the retention time and the area under the bendamustine peaks have remained constant over a period of 300 min (not shown) whereas in neutral or basic solutions (pH 7 and 9) the drug degrades fast. Its concentration decreases considerably—the area of the peaks decreases with approximately 2 log units for 5 min and after 60 min it is reduced with about 3 log units (Figs. 2 and 3). The observations support the fact that the acidic HCl/KCl solution does not afford the necessary conditions for the spontaneous hydrolysis of the bis-chloroethyl moiety in aqueous media yielding monohydroxy and dihydroxy bendamustine [32–34] contrary to the nucleophilic substitution which proceeds at pH 7 and 9.

The results of the HPLC analysis of the solutions of the conjugate **4**, complexes **6** and **7** at pH 2 have shown that the drug is chemically stable in all of the three solutions over a period of 300 min. The retention time and the areas under the peaks corresponded to that observed in the chromatogram of the reference bendamustine hydrochloride solution. The drug concentration was determined to be approximately 100% of the theoretical one. Drug–polymer

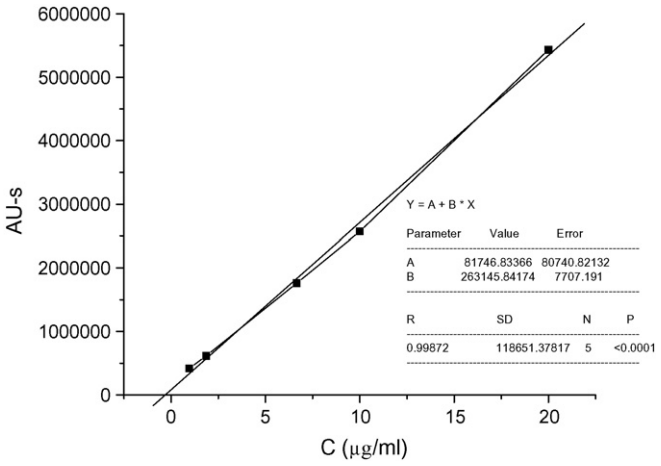


Fig. 1. Linearity of bendamustine hydrochloride.

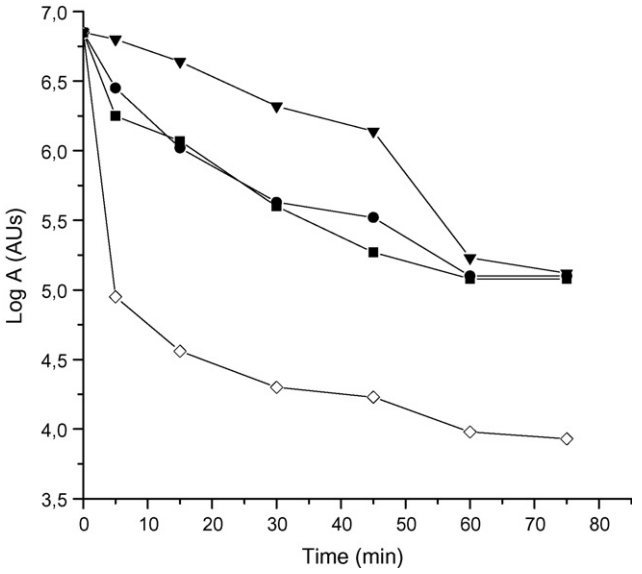


Fig. 2. Stability data of bendamustine and the drug–polymer complexes at pH 7 where (◊) bendamustine (pure substance); (■) conjugate **4**; (▼) complex **6**; (●) complex **7**.

interactions are possible through electrostatic attraction between the positively charged drug molecules and the phosphate anions (approximately 80% of hydroxyphosphate groups are dissociated at pH 2) and via hydrogen bonding of the highly polar phosphoryl groups in the polymer backbone and the carboxylic groups of bendamustine. In summary, the drug stability is preserved in the

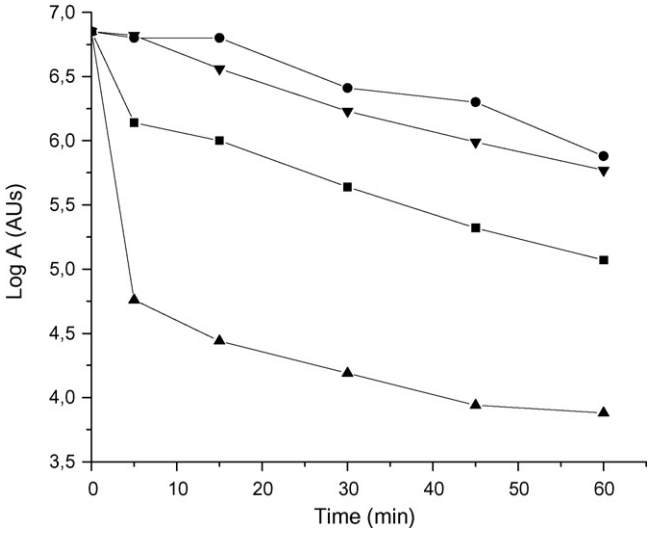


Fig. 3. Stability data of bendamustine and the drug–polymer complexes at pH 9 where (◊) bendamustine (pure substance); (■) conjugate **4**; (▼) complex **6**; (●) complex **7**.

presence of the polymer carrier at pH 2 assuming the acidic environment of the solution is the main factor.

The results of the HPLC analysis of the bendamustine–polymer complexes at pH 7 and 9 are summarized in Figs. 2 and 3. The concentration profiles undoubtedly display higher stability of bendamustine hydrochloride after immobilization compared to the non-immobilized drug. The degradation process is retarded and a sharp decrease in the drug concentration over the first 5–10 min after dissolving of its complexes has not been detected. In all cases the area under the peak decreased less than 2 log units after 60 min of drug incubation at 37 °C. An exponential decay of drug concentration was observed over that period of time. The correlation between the experimental curve and the theoretical one is excellent —0.97. The experimental data evidenced the favorable effect of the polymer carrier on drug stability, the reasons for which could be explained with polymer–drug interactions affording protection to the bioactive agent against hydrolytic degradation. A common observation was that after 60–75 min of sample incubation the estimated quantity of bendamustine was at a concentration level near to the limit of detection which was maintained for a very long time interval—about 300 min. Because of that the data obtained over the period from 75 min to 300 min were not representative and did not correspond to functional decay.

The single reason cannot be the chemical immobilization of the drug, i.e. the conjugate **4**, as it is known that the phosphoacyl linkage is very susceptible to hydrolytic degradation. Moreover, the HPLC data reveal increased stability of the drug even physically incorporated in the matrix of polyphosphoester **2** (i.e. complex **6**). Then the question about the nature of the interactions contributing to the enhanced drug stability arises taking into account that the formation of ionic complexes or hydrogen bonding between bendamustine molecules and the polyphosphoester are not favored at pH 7 or 9. It is assumed that the polymer globules create local microenvironment that is preferred by the drug molecules to the aqueous solution bulk. Though the hydroxyphosphate moieties in complexes **5** and **7** dissociate at pH 7 and 9 it could be presumed that the macromolecular chain conformation is closer to a globule than to extended coil due to the low charge density along the chain. Thermodynamically favored interactions between the amphiphilic polyether segments and the unprotonated benzimidazole skeleton bearing the bis-(2-chloroethyl) amino residue could play significant role in the formation of molecular associates. These intermolecular species carry negative charges that repel the attack of the OH^- on the nitrogen mustard fragment and the result is retardation of drug degradation.

The role of the non-specific polymer–drug interactions is supported by the fact that at pH 7 bendamustine incorporated into the polymer matrix of poly(methyloxyethylene phosphate) displays higher stability compared to the non-immobilized drug or included in the conjugate **4** and complex **7**. The poly(methyloxyethylene phosphate) macromolecules are less hydrophilic in comparison with the polyphosphoester backbones built up of hydroxy phosphate moieties. Therefore, the hydrophobic interactions between the polymer chain and the bis-(2-chloroethyl)amino residue of the drug are stronger and contribute to its stabilization, especially in the beginning of the experiment when carrier degradation is negligible. At pH 7 the curve corresponding to the behavior of the conjugate **4** follows similar trend-line to that of the complex **7**. It is expected result taking into account that the hydrolysis of the phosphoacyl linkage between the drug and the polymer generates system with similar composition and structure to that of complex **7**.

At pH 9 some differences in the drug concentration profiles (Fig. 3) are observed as compared to the corresponding ones at pH 7 (Fig. 2). This is result from different behavior of the drug complexes at higher pH of the solution, probably due to variation in the rate constants of hydrolysis of the carrier chains incorporating different phosphate groups. As it was mentioned above in basic solutions both the main chain and side groups of poly(ethylene methyl phosphate)s degrade with similar rates which are approximately 5 times higher than those in neutral medium [18]. That means that the polyphosphoester chains, especially in the case of complex **6**, are likely to undergo hydrolytic degradation that will lead to decrease in the carrier molecular weight and therefore variation in the polymer–drug interactions.

4. Conclusion

A comparative HPLC study on the chemical stability of bendamustine hydrochloride immobilized onto polyphosphoester carriers has been performed for the first time. The HPLC method used in the experiment was validated in respect of the main analytical parameters such as selectivity, repeatability, limit of detection, limit of quantitation and linearity. It has been found that in aqueous solution at pH 7 and 9 the newly synthesized polymer complexes of bendamustine hydrochloride display higher drug concentration levels than the non-immobilized drug. The enhanced bendamustine stability after immobilization is explained with drug interactions with the polymer carriers and formation of molecular associates that protect bendamustine against chemical transformations. The obtained experimental data and their analysis could contribute to the future investigation on the mechanism of bendamustine action and could have a practical impact in terms of a manageable hydrolytic profile of the drug.

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