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Methods for preliminary determination of pemetrexed in macromolecular drug-carrier systems

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Pemetrexed (PMX) is an antifolate drug utilized in the treatment of non-small cell lung cancer. For studies of potential macromolecular carriers for PMX, fast and precise methods were developed to determine the bound and free drug contained in investigated conjugate preparations. The analysis of the total amount of PMX in conjugates was based on absorption spectrophotometry. The linearity was found in the range of 4.697–46.97 μmol L^{-1} PMX. The limit of quantitation was 1.070 μmol L^{-1}. The method for the analysis of unbound PMX was based on size-exclusion chromatography and detection at 225 nm. This method shows linear range of 2.230–223.0 μmol L^{-1}. *LOQ* was 0.539 μmol L^{-1}. The proposed methods can be used both for the characterization of the polysaccharide based conjugates of PMX and for the determination of conjugate drug release profiles.

Keywords: pemetrexed, macromolecular drug-carrier, size-exclusion chromatography, ultraviolet spectroscopy

The search for new, innovative methods of disease treatment, especially for cancer, is an ongoing challenge in both industry and academia. Conjugates composed of high molecular mass natural or synthetic polymers and antineoplastic agents may exhibit a range of positive features, such as increased half-life in an organism, increased stability, reduced immunogenicity and intensified therapeutic efficacy (1, 2). The combination of drugs already used in therapy with high molecular carriers may lead to a new therapeutic quality (3).

Pemetrexed (PMX) is a multi-target antifolate. It is an antimetabolite of folic acid and the mechanism of its action is based on its ability to inhibit the activity of the enzymes involved in purine and pyrimidine synthesis: thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamideribonucleotideformyl transferase (GARFT) (4). After being transported into the cell *via* the reduced folate carrier or/and folate receptors, PMX is polyglutamated to the active pentaglutamide by a reaction catalyzed by polylpolyglutamate synthase (5). PMX has a wide usage in the treatment of pleural mesothelioma (6), non-small cell lung cancer (7) and prostate cancer (8). However, this drug demonstrates a

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range of disadvantages characteristic of low molecular compounds, such as fast metabolism and fast excretion from the organism, as well as adverse biodistribution and low selectivity of therapeutic use (9). Moreover, its clinical use is limited by dose-dependent toxicity. Solving these problems involves the coupling of PMX with macromolecular carriers, resulting in enhancement of the delivery and selectivity and improvement of the pharmacological properties of PMX.

There have been numerous scientific reports concerning another antifolate drug – methotrexate (MTX) – conjugated with a polysaccharide based, high molecular mass carrier such as mannan (10), dextranes (11), hyaluronic acid (12) or hydroxyethyl starch (HES) (13). *In vivo* studies have indicated that methotrexate conjugated with HES [a modified polymer, based on amylopectin, used clinically as a colloidal plasma volume expander (14)] exhibits high efficacy for the treatment of experimental tumors. Studies of HES as a carrier for MTX, conducted in our laboratory, are currently being extended by experiments with PMX covalently bound (ester bond) with HES. In order to investigate the potential of HES-PMX conjugates, a fast and precise method is needed to determine the total and free drug contained in investigated conjugate preparations.

The aim of this study was to develop novel methods for the determination of PMX in macromolecular conjugates. These methods were then used for characterization of the HES-PMX conjugate.

EXPERIMENTAL

Materials

The pemetrexed standard was purchased from Eli Lilly (USA). Sodium bicarbonate, analytical grade, was obtained from Avantor Performance Materials (Poland). HES-PMX conjugates were obtained according to the previously described methods (11, 13). The water used was produced by a Direct-Q3 UV system (Millipore, USA).

PMX standard preparations. – Different concentrations of PMX were prepared by diluting the stock solution of PMX (58.55 mmol L^{-1}) in 0.1 mol L^{-1} sodium bicarbonate.

Ultraviolet spectroscopy (UV)

All measurements were conducted on a Specord® 250 (Analytic Jena, Germany) spectrophotometer at ambient temperature. UV spectra were recorded in the range 210–350 nm, with a slit width of 1 nm and scan speed of 50 nm s^{-1}. Quartz cells with 1-cm light path length were used for measurements. Samples were prepared in sodium bicarbonate solution (0.1 mol L^{-1}).

Size-exclusion chromatography

Ultimate 3000 HPLC system (Dionex, USA) was used equipped with a DAD detector connected to a Superdex® 30 Peptide (GE Healthcare, UK) column (4.6 × 150 mm, 34 μm) at 22 °C. Sodium bicarbonate solution (0.1 mol L^{-1}) was used as the mobile phase at a flow rate of 0.4 mL min^{-1}. UV detection was done at 225 nm; injection volume was 10 μL. It was validated according to the following parameters.

Specificity. – Specificity was determined by analyzing the chromatograms of hydroxyethyl starch in comparison with the chromatogram of a mixture of hydroxyethyl starch and pemetrexed to confirm that none of the excipients interfered with quantitation of the drug.

Linearity and range. – Linearity of the methods was determined by measuring independent concentrations of the calibration standards of PMX.

The linearity of the method for the analysis of total PMX in preparations was determined by measuring fourteen independent concentrations (μmol L^{-1}) of the calibration standards (4.679; 7.019; 9.359; 11.70; 14.04; 16.38; 18.72; 21.06; 23.40; 28.08; 32.76; 37.43; 42.11; 46.79). Each concentration was measured at 225 nm and absorbance was plotted *vs.* concentration; regression parameters were evaluated. The linearity of the method for the analysis of unbound PMX in preparations was determined by the measurement of 11 independent concentrations (μmol L^{-1}) of the calibration standards (2.230; 4.461; 8.622; 13.38; 17.84; 22.30; 44.61; 89.22; 133.8; 178.4; 223.0). The calibration curve of the peak area *vs.* concentration was plotted and regression parameters were determined.

Accuracy. – In order to evaluate this parameter, three independent replicates of each sample (mixture of hydroxyethyl starch and pemetrexed) were measured and then compared to the theoretical concentration of PMX in the sample, with data calculated from the calibration curve. The result was presented as the percentage of recovery.

Precision. – Precision of the method was calculated by comparing the results for triplicates of one sample (mixture of hydroxyethyl starch and pemetrexed). The result was presented as RSD.

Limit of detection (LOD) *and limit of quantitation* (LOQ). – *LOD* was calculated based on a signal to noise ratio of 3:1 and *LOQ* based on a signal to noise ratio of 10:1.

Robustness. – Robustness was evaluated by analyzing the drug concentration (116.9 μmol L^{-1}) with variations in the temperature of the analytical column (15 and 35 °C), pH of the mobile phase (7.2 and 7.85) and flow rate (0.38 and 0.42 mL min^{-1}). Samples (mixture of hydroxyethyl starch and pemetrexed) were determined in triplicate for each variation of the method conditions. Results were compared with the previously reported results.

Determination of pemetrexed in HES-PMX conjugates

The conjugate was dissolved in $NaHCO_3$ (0.1 mol L^{-1}) and the total PMX was measured by the spectrophotometric method. Based on this result, the conjugate was diluted with $NaHCO_3$ 0.1 mol L^{-1} to a final concentration of 223.0 μmol L^{-1} (total PMX) and analyzed for unbound PMX by the gel chromatographic method. In addition, a quantitative analysis of the PMX released from the HES-PMX conjugate was performed. The conjugate was completely hydrolyzed in NaOH (0.1 mol L^{-1}) for 24 hours and then analyzed for unbound PMX by the chromatographic method. Total glucose contents in HES-PMX preparations were determined using the phenol-sulfuric acid method (15).

Analysis of the HES-PMX conjugate with PMX standard addition

The conjugate and PMX standard were diluted in $NaHCO_3$ (0.1 mol L^{-1}) to a final concentration of 339.9 μmol L^{-1} (total PMX 116.9 μmol L^{-1} was PMX standard) before chromato-

graphic analysis of unbound PMX in preparations. The drug content determined according to the method were reduced by the amount of free drug in the HES-PMX conjugate, and then the statistical significance was determined by Student's *t*-test.

RESULTS AND DISCUSSION

The analysis of total PMX in preparations was based on absorption spectrophotometry in 0.1 mol L^{-1} sodium bicarbonate at $\lambda = 225$ nm. Results of the validation of the method are presented in Table I. The method showed linearity in a concentration range of 4.679–46.79 μmol L^{-1} with a coefficient of determination of 0.9993. The accuracy of the method, expressed as a percentage of recovery, was found to be 96.2–101.7 %. Precision of the method, expressed as RSD, was found to be 0.9–5.5 %. *LOD* and *LOQ* of the method were found to be 0.3530 and 1.070 μmol L^{-1}, respectively.

Unbound PMX in preparations was determined by size-exclusion chromatography. Results of the validation of the method are presented in Table I. The results of specificity showed that there was no interference at the retention time of the drug from a mixture of hydroxyethyl starch and PMX (Figs. 1a and b). Moreover, the photodiode array detector indicated that the PMX peak was free from interference (purity index > 0.9999). Linearity concentration range was 2.230–223.0 μmol L^{-1} with a coefficient of determination of 0.9999. Accuracy of the method, expressed as recovery, was found to be 95.4–105.6 %. RSD was found to be 0.1–1.0 %. *LOD* and *LOQ* were found to be 0.1616 and 0.5386 μmol L^{-1}, respectively. Robustness was based on RSD values obtained by changing analytical parameters such as the analytical column temperature (15 and 35 °C), pH of the mobile phase (7.2 and 7.85) and flow rate (0.38 and 0.42 mL min^{-1}). The method was considered robust because the RSD values were lower than 0.9 %, as summarized in Table II. As expected, some variation in the retention time was observed, without compromising the drug content determination. The use of the standard addition method confirmed confidence interval of the added standard CI95 (116.3; 118.1; $p = 0.8597$).

Table I. Validation parameters for the analysis of PMX in macromolecular drug-carrier systems

Parameter	The method for the analysis of total PMX	The method for the analysis of unbound PMX
Linearity (μmol L^{-1})	4.679–46.79	2.230–223.0
Slope (mean ± SD)	0.02934 ± 0.00012	0.6874 ± 0.0009
Intercept (mean ± SD)	0.03834 ± 0.00314	–0.4432 ± 0.0901
Coefficient of determination (R^2)	0.9993	0.9999
Precision (RSD, %)	0.9–5.5	0.1–1.0
Accuracy (recovery, %)	96.2–101.7	95.4–105.6
LOD (μmol L^{-1})	0.3530	0.1616
LOQ (μmol L^{-1})	1.070	0.5386
PMX retention time (min)	–	8.887 ± 0.012 min
Molar absorptivity (ε, L mol^{-1})	29300 cm^{-1}	–

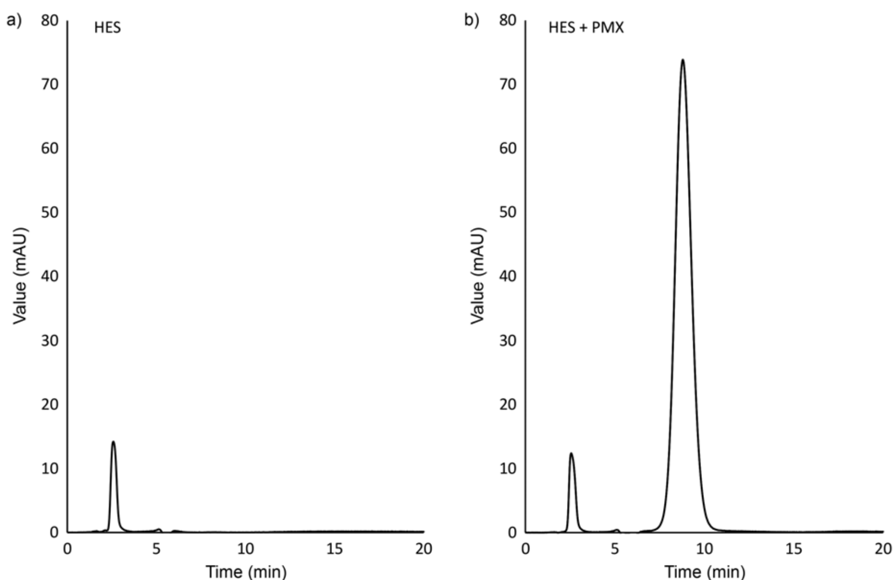

Fig. 1. HPLC chromatograms (λ = 225 nm) obtained for: a) hydroxyethyl starch and b) mixture of hydroxyethyl starch and PMX.

Table II. Robustness data for pemetrexed analysis[a]

Parameter	PMX content (%)	RSD (%)	Retention time (min)
Temperature (°C)			
15	100.0	0.71	9.560
22	100.2	0.11	8.810
30	99.8	0.86	8.093
pH of mobile phase			
7.20	102.1	0.19	9.730
7.85	102.1	0.66	9.800
8.50	100.2	0.11	8.810
Flow rate (mL min^{-1})			
0.38	103.1	0.18	9.213
0.40	100.2	0.11	8.810
0.42	96.0	0.13	8.337

[a] n = 3.

New methods have been used to determine both the overall and the covalently bound PMX in the HES-PMX conjugate. The conjugate contained 44.6 × 10^{-3} covalently bound PMX residued per anhydroglucose unit and there was 0.7 % of free (unbound) PMX in the final preparations (Table III, Figs. 2a and b). In addition, consistent results were obtained for the quantitative analysis of total PMX (after complete release from the HES-PMX con-

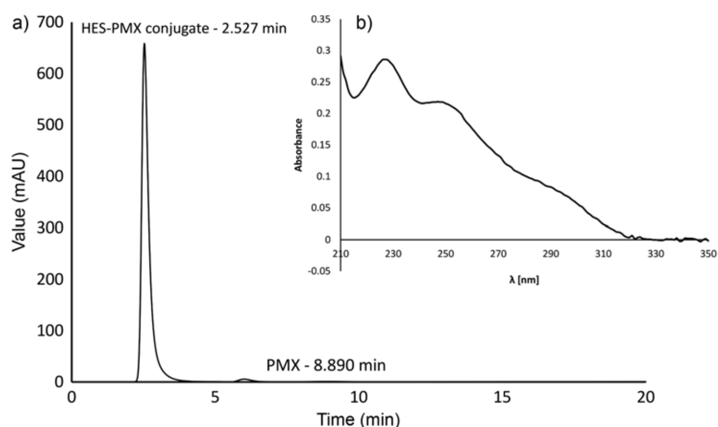

Fig. 2. a) Analytical size-exclusion chromatogram of HES-PMX conjugate, b) UV spectrum of HES-PMX conjugate.

Table III. HES-PMX conjugate characterization (final preparation)[a]

Parameter	PMX found
Total PMX (bound and unbound)[b]	8.914 μmol L^{-1}
Total PMX (after releasing from conjugate)[c]	8.997 μmol L^{-1}
Free PMX (unbound)[d]	0.06592 μmol L^{-1}
HES (glucose equivalent)[e]	198.3 μmol L^{-1}
SL[f]	0.0446

[a] For details see the experimental section.
[b] Total PMX content in the final preparation of HES-PMX conjugate, determined by UV spectrophotometry.
[c] Total PMX completely released from HES-PMX conjugate, determined by SEC.
[d] Free (unbound) PMX in the final preparation of HES-PMX conjugate, determined by SEC.
[e] Total glucose in HES-PMX preparation, determined by the phenol-sulfuric acid method.
[f] SL – molar substitution level by PMX on glucose units.

jugate) *via* size-exclusion chromatography and for the analysis of total PMX *via* absorption spectrophotometry (Table III). The difference between these measurements did not exceed 1 % PMX (below RSD of both methods).

CONCLUSIONS

The proposed methods can be used both for the characterization of the polysaccharide based conjugates of PMX and for the determination of conjugate drug release profiles.

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