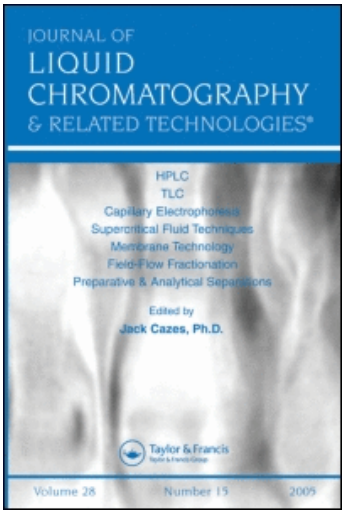

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Behaviour of Macromolecular Drug Carrier Poly(N-Vinyl Pyrrolidone-co-maleic Acid) and Its Bioconjugates at Different pH Values Investigated by Gel Permeation Chromatography

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To cite this Article Gyöffy, E. , Pató, J. , Horváth, A. , Érchegyi, J. , Teplán, I. , Kéri, Gy. and Idei, M.(1998) 'Behaviour of Macromolecular Drug Carrier Poly(N-Vinyl Pyrrolidone-co-maleic Acid) and Its Bioconjugates at Different pH Values Investigated by Gel Permeation Chromatography', Journal of Liquid Chromatography & Related Technologies, 21: 15, 2341 — 2353

To link to this Article: DOI: 10.1080/10826079808000542

URL: http://dx.doi.org/10.1080/10826079808000542

J. LIQ. CHROM. & REL. TECHNOL., 21(15), 2341–2353 (1998)

BEHAVIOUR OF MACROMOLECULAR DRUG CARRIER POLY(N-VINYL PYRROLIDONE-CO-MALEIC ACID) AND ITS BIOCONJUGATES AT DIFFERENT pH VALUES INVESTIGATED BY GEL PERMEATION CHROMATOGRAPHY

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ABSTRACT

Anionic poly(N-vinyl pyrrolidone-co-maleic acid) [referred later in the text as P], which is utilized in Drug Delivery Systems as macromolecular carrier of drugs was analysed by gel permeation chromatography. Anilide and quinoxaline derivatives were coupled to this drug-carrier to form conjugates, which were also analysed with gel permeation chromatography. Conjugate P-D1 was prepared by coupling the anilide derivative D1 [2-cyano 3-hydroxy 5-amino 2 pentenoyc (4-trifluoromethyl anilide)] to the carboxyl groups of the drug-carrier P, while

conjugate P-D2 was prepared by coupling the quinoxaline derivative D2 [(6`,7` dimethyl-1`-quinoxalinyl) 4-(2`amino) acetanilide] to the carboxyl groups of the drug-carrier P. Gel-chromatographic properties of the carrier P and its conjugates have been investigated on Biosil TSK 125 SW column in 0.25 N triethyl ammonium phosphate buffer of different pH values (2.25; 4.70 and 6.0). Applying appropriate pH (4.70) the method allowed us to differentiate between conjugate molecules carrying strong or weak residual carboxyl groups. Strong molecular dispersity resulting in wide, tailed chromatographic profiles could be detected in the case of the conjugates. Various distributions of the residual charges along the polymer chains, as well as presence of residual carboxyl groups of different acidity could be responsible for this molecular dispersity.

INTRODUCTION

One of the center of interest in pharmaceutical research is the preparation and characterization of different drug delivery systems. Biodegradable polymers used in design of Drug Delivery Systems might decompose in the human body releasing the drug coupled to them in the targeted area. These polymers are called carriers in the field of drug research. The advantages of conjugation of different compounds of medical importance (e.g. drugs, hormones, enzymes, toxins) to biopolymers or synthetic, biocompatible polymers initiated an intense research in the field of bioconjugate chemistry during the last two decades. Macromolecular carriers have been reported to increase stability of the attached small molecules to chemical or enzymatic degradation, and favorably alter their biodistribution resulting in prolonged duration of action and reduced toxicity.[1-3] Based on these observations, it is feasible to design drug-carrier conjugates for sustained release and controlled delivery. It has been demonstrated that enzyme-substrate interactions are characteristically influenced by the backbone.[4,5] Consequently, apart from the advantages mentioned above covalent coupling of inhibitors might result in increased effects as a consequence of increased stability of the enzyme-polymeric conjugate complex.

Tyrosine kinases play a key role in the signal mechanism of the cells and inhibitors are potent anticancer drugs.[6] In this study, aniline derivative 2-cyano 3-hydroxy 5-amino 2 pentenoyc (4-trifluoromethyl anilide) (designated as D1 in the followings) or quinoxaine derivative (6`,7` dimethyl-1`-quinoxalinyl) 4-(2`amino) acetanilide (designated as D2 in the followings) was conjugated to the anionic carrier molecule poly(N-vinyl pyrrolidone-co-maleic acid)

(designated as P in the following). Carrier P is a non-toxic, water soluble macromolecule which has been used for conjugation of bioactive molecules previously.[7,8] In this paper tyrosine kinase enzyme inhibitors mentioned before were conjugated to the carboxyl groups of the anionic carrier P. Well characterized and highly purified products are needed to perform comparative studies on the biological and pharmacological properties of the free drug molecules and their carrier-bound derivatives. As a consequence it is essential for laboratories working on this field to develop analytical methods of high accuracy and reliability applicable during the purification of such conjugates and for the characterization of the drug-carrier products.[9]

The molecular masses of the components in the crude product (carrier, free drug molecule [Tyrosine kinase inhibitor molecules in our case] and drug-carrier conjugates) cover a very wide molecular weight range (approximately from $5x10^2$ to $1\text{-}2x10^4$ Mw). Gel permeation chromatography seems to be an appropriate method for the characterization of the products with wide molecular mass distribution. In this study, anionic carrier poly(N-vinyl pyrrolidone-co-maleic acid) P and conjugates prepared with conjugation of 2-cyano 3-hydroxy 5-amino 2 pentenoyc (4-trifluoromethyl anilide) or (6`,7` dimethyl-1`-quinoxalinyl) 4-(2`amino) acetanilide to carrier P were characterized by gel permeation chromatography at different pH values.

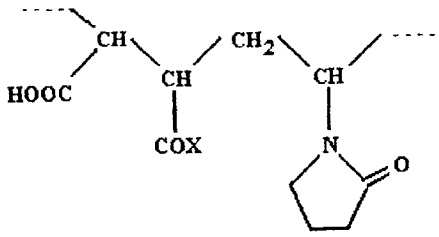
EXPERIMENTAL

Carrier P

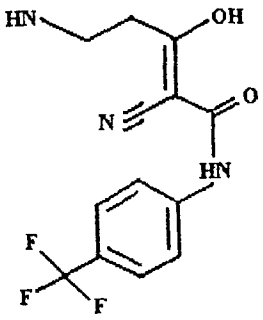
Poly(N-vinyl pyrrolidone-co-maleic acid) was synthesized and analysed as it was published earlier.[6,7] Its average molecular weight was 10 000 Mw. The macromolecular carrier was ultrafiltered after the synthesis through Amicon YM 02 membrane (Amicon, Witten, Germany; cut off limit 1000 Mw) to remove unpolymerized monomeric units. Chemical structure of carrier P is shown in Figure 1.

Tyrosine Kinase Inhibitor Molecules

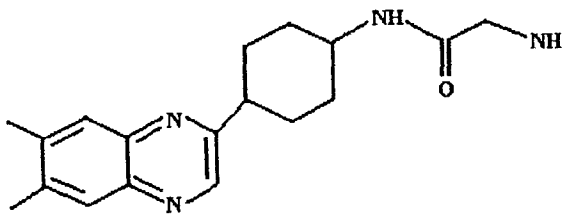
Synthesis of D1: 2-cyano 3-hydroxy 5-amino 2 pentenoyc (4-trifluoromethyl anilide) and D2: (6`,7` dimethyl-1`-quinoxalinyl) 4-(2`amino) acetanilide is submitted for publication (10). Chemical structures of the molecules are presented in Fig. 1.



P



D1



D2

Figure 1. Stucture of the carrier and tyrosine kinase molecules applied in this work. P: polyanionic carrier poly(N-vinyl pyrrolidone-co-maleic acid), group X: tyrosine kinase molecule conjugated to the carrier. X groups: D1: 2-cyano 3-hydroxy 5-amino 2 pentenoyc (4-trifluoromethyl anilide). D2: (6`,7` dimethyl-1`-quinoxalinyl) 4-(2`amino) acetanilide. Tyrosine kinase molecules D1 or D2 were coupled to the carrier P through their amino groups marked by asterisk.

Conjugates

The following conjugates have been synthesized by coupling of the tyrosine kinase molecules to the carrier P: Conjugate P-D1 was synthesized by coupling D1 to carrier P, degree of substitution: 12.3 % (mol ratio). Conjugate P-D2 was synthesized by coupling D2 to carrier P, degree of substitution: 13.8% (mol ratio). Synthesis of the conjugates is submitted for publication.[10] Degree of substitution was determined by UV spectroscopy. Both conjugates were ultrafiltered through Amicon YM 02 membrane (Amicon, Witten, Germany; cut off limit 1000 Mw) to remove unconjugated Tyrosine kinase molecules.

Chemicals

Ortho-phosphoric acid and triethylamine were purchased from Fluka (Buchs, Switzerland). Solutions were prepared of deionized, bacteria free water made by Elgastat UHP system (Elga Ltd., Bucks, England).

Gel Permeation Chromatography

Device: Varian 9012 Solvent Delivery System (Varian, Zug, Switzerland), Varian Polychrom 9065 Diode Array Detector, Rheodyne 7125 injector, column: Biosil (BioRad, Pharmacia, Uppsala, Sweden) TSK 125 column (300 x 7.5 mm + 50 x 7.5 mm guard column), V_o (void volume): 7.0 mL; samples: 1 mg/mL in eluent applied. Dissolved samples were filtered through Spartan (Schleicher and Schuell, Dassel, Germany) 13 disposable filters (0.45 μm); sample volume: 20 μL. Eluents for isocratic analysis (flow rate: 1.0 mL/min for each eluent): eluent 1: 0.25 N TEAP, pH=2.25; eluent 2: 0.25 N TEAP, pH=4.7; eluent 3: 0.25 N TEAP, pH=6.0.

RESULTS AND DISCUSSION

Behaviour of the Free Carrier Molecule at Different pH Values:

Figure 2 shows the chromatographic profiles of the free carrier P obtained at different pH values by gel permeation chromatography. GPC analysis performed at low pH (2.25) proved the polydisperse nature of the free carrier molecule P. Two main peaks eluted with elution times of 14.0 (peak I.) and 14.8 min (peak II.), respectively (Fig. 2.a). Elution time of peak II was not

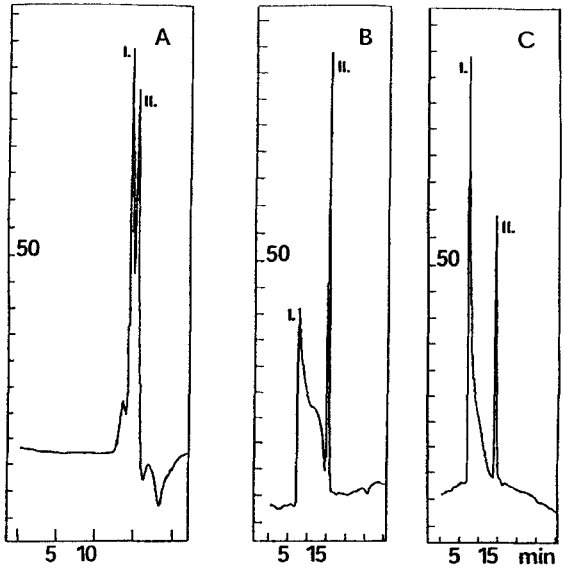


Figure 2. Gel permeation chromatograms of the free carrier P (poly(N-vinyl pyrrolidone-co-maleic acid) obtained at different pH values. A:pH 2.25; B: pH 4.7; C: pH:6.0. Abscissa: elution time in minutes, ordinate: absorbance (in milliAU units) detected at 205 nm. For other details see: Materials and Methods.

changed by changing the pH value of the eluent from 2.25 to 4.70. Contrary, the elution time of peak I. changed significantly by altering the pH value of the eluent. This peak at pH 4.70 moved towards shorter elution time: wide, tailed peak was obtained between 6 and 14 mins. Ratio of the peak II. (calculated on the basis of peak areas at a given pH value) was lower at pH 4.7 than at pH 2.25 (Fig.2.b).

The tendency observed between pH 2.25 and 4.70 was going on to pH 6.0. Ratio of peak II. diminished further compared to the peak ratio obtained either at pH 2.2.5 or pH 4.70. Components of peak II. eluted in a sharp peak even at this pH. The wide tailing of peak I. observed at pH 4.7 considerably diminished at pH 6.0 (Fig. 2.c). Polydispersity of the free carrier was detectable at all pH values applied.

As it is known from the works of Rios,[11] Dubin,[12] and Leyte[13] carboxyl groups of polyacids become more acidic than the respective monomeric carboxyl groups. The degree of this effect is influenced by the nature of the monomer units of the polymer chain. It was also found that the monomer units

play an important role in conformational behaviour of these polyelectrolyte polymers.[11,14] On the other hand, acidic strength (pK_a) of the carboxyl groups on the polymer chain is substantially influenced by the chain conformation. It was stated by Dippy[14] that there might be substantial differences between the acidic strength of meso- and racemic carboxyl groups: meso carboxyl groups are consistently weaker than the racemic ones. Based on these results, the presence of carboxyl groups of a wide range of acidic strength can be assumed along the polymer chain of polyanionic carrier P.

Free carboxyl groups of the polymer chain are supposed to be protonated mainly because of their suppressed dissociation at low pH value (2.25). In these circumstances the polymer is able to take the shape of a globular chain of relatively small Stokes radius because of the lack of repulsion. Consequently the elution volume is large; the polymer behaves as a species of a relatively small apparent molecular mass. At higher pH value (pH 4.7) free carboxyl groups of the macromolecular carrier chain are partially dissociated. Electrostatic interactions have substantial influence on the hydrodynamic behaviour of polyelectrolyte polymers. At medium or high pH these macromolecules assume extended conformation due to the repulsive electrostatic interactions between the charged groups. Molecules assume the random coil conformation (which is otherwise typical in the case of non-ionic polymers) if the ionization (dissociation) is suppressed.[15] Polyanionic electrolytes undergo a pH induced conformational transition from compact to an extended coil conformation because of the dissociation of the anionic groups. This transition results in increased Stokes radius and in decreased elution volume. Consequently the apparent molecular mass of the polymer seems to be greater at higher pH value than at lower one.

In full accordance with the effects mentioned above, changing the pH of the eluent induced substantial change in the chromatographic profile of the carrier P. Free carboxyl groups of the polymer chain were mainly protonated, because of their suppressed dissociation at low pH value (2.25). The polymer was able to take the shape of a globular chain of relatively small Stokes radius because of the lack of repulsion. Consequently, elution volume was large; the polymer behaved as a species of relatively small apparent molecular mass. Carboxyl groups were partially dissociated and the polymer chain assumed a partially extended conformation at pH 4.7. Consequently peaks of the polymer sample moved towards the shorter elution times at this pH.

At pH 4.7 (being very close to the pKa value of the acetic acid) all kind of the carboxyl groups of different acidity might be partially dissociated. The ratio of protonated carboxyl groups and dissociated ones (therefore the number of negative charges on the polymer chain) at a given pH value were determined

by the ratio of the strong and weak carboxyl groups. Since carboxyl groups of different acidity were on the polymer chain, it could be assumed that this fact contributed to the wide molecular dispersity obtained at pH 4.7.

A great majority of the free carboxyl groups of the macromolecular chain dissociated at pH 6.0. The strong repulsion between these negatively charged groups forced the polymer to take the shape of rigid elongated chain conformation. The Stokes radius of the polymer chain was further increased and the elution volume was further decreased at this pH. Consequently the apparent molecular mass of the polymer seemed to be greater at this pH than at lower ones. Because of the complete dissociation of the carboxyl groups at pH 6.0, dispersity of the polymer decreased and this change resulted in decreased tailing of the peaks (Fig. 2.c.). Because of the behaviour of the polymer mentioned above, the macromolecular carrier eluted with longer elution time (like a species with low apparent molecular mass) at low pH and eluted with shorter elution time (like a species with higher apparent molecular mass) at higher pH.

Elution time of peak II. was not influenced by the changes in the pH value of the eluent. Components of this peak eluted in sharp peak with the same elution time at pH 2.25 and 4.7, as well as at pH 6.0. This might reflect on the fact that the components eluted in this peak contained extremely strong acidic groups fully dissociated even at low pH (2.25). Increase of the pH value of the eluent did not affect the dissociation state of these groups and hence neither the charged state nor the conformation of the polymer chain was influenced by the change in the pH. That was in accordance with the fact that elution volume of these components was not affected by the changes of the pH of the eluent.

Because of the presence of chiral carbon atoms in the polymer chain the presence of diastereomers must be taken into consideration, too. The existence of diastereomers might also contribute to the wide molecular dispersity which resulted in wide tailed peaks obtained by GPC.

Behaviour of the Conjugates at Different pH Values

The chromatographic profile of the conjugate P-D1 at pH 2.25 was similar to that of the carrier but wider molecular dispersion was obtained. The elution time of the peak I. moved to 13.6 min and strong tailing of the peak II. was observed (Fig.3.a). These facts showed that molecular masses had become larger owing to the conjugation reaction.

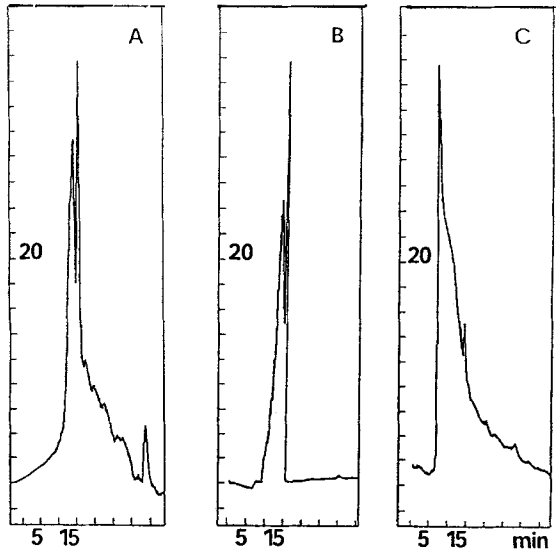


Figure 3. Gel permeation chromatograms of the conjugate P-D1 obtained at different pH values. A:pH 2.25; B: pH 4.7; C: pH:6.0. Abscissa: elution time in minutes, ordinate: absorbance (in milliAU units) detected at 205 nm. For other details see: Materials and Methods.

The GPC profile of the conjugate P-D1 was not significantly influenced by the increase of the pH of the eluent from 2.25 to 4.70 (Fig.3.b). At this pH value the elution times of the peaks decreased only slightly and the ratio of the peaks (based on the peak areas) were similar to those obtained at pH 2.25. To obtain considerable change in the chromatographic profile of the conjugate P-D1 the pH value of the eluent had to be raised significantly (from 2.25 to 6.0). At pH 6.0 the great majority of the components moved towards the shorter elution times (lower elution volumes) and they eluted between 7 and 15 min. giving only partially resolved and strongly tailed peaks (Fig.3.c).

The chromatographic profile of conjugate P-D2 obtained at pH 2.25 showed wide molecular dispersity. In consequence of the conjugation reaction, peaks significantly widened comparing to the peaks of the free carrier (parent compound) and the elution times of the peaks slightly decreased (Fig.4.a). This fact showed that the molecular mass became larger owing to the conjugation reaction. Separation obtained at pH 4.70 was considerably worse than that obtained at pH 2.25 (Fig.4.b).

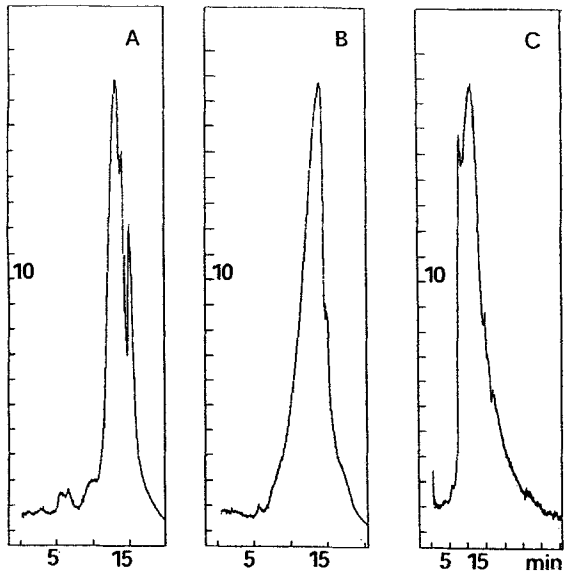


Figure 4. Gel permeation chromatograms of the conjugate P-D2 obtained at different pH values. A:pH 2.25; B: pH 4.7; C: pH:6.0. Abscissa: elution time in minutes, ordinate: absorbance (in milliAU units) detected at 205 nm. For other details see: Materials and Methods.

The chromatographic profile was essentially changed by altering the pH of the eluent from 4.70 to 6.0. The great majority of the components moved towards the shorter elution times (lower elution volumes) and eluted between 7 and 15 min. in strongly tailed, partially resolved peaks at pH 6.0 (Fig. 4.c).

The GPC profiles of both conjugates - similarly to the GPC profile of the free carrier - was sensitive to the changes in the pH of the eluent. Considering the fact, that the D1 or D2 molecules attached to the carrier did not possess dissociable groups after the conjugation reaction, this pH sensitivity referred to the presence of unreacted (free) carboxyl groups on the polymer chain. This is in full accordance with the fact that conjugates with substitution degree of approximately 13 % were synthesized.

On one hand, the elution volume of the conjugate must be lower than that of the free carrier because the conjugation reaction increased the molecular mass. On the other hand, the number of free (ionizable) carboxyl groups on the polymer chain and, therefore, the repulsion between the negative charges, diminished in the conjugate molecules, owing to the conjugation reaction.

Therefore, possibility of taking the globular shape is higher in the case of the conjugate than that of in the case of the free carrier molecule. Owing to this fact, the Stokes radius of the conjugate might be smaller than that of the free carrier. Consequently, the apparent molecular mass of the conjugate might seem to be smaller (or similar) than that of the parent molecule.

Peaks of the conjugate products were significantly wider than those of the parent compound; elution times decreased and new peaks with lower elution times appeared in the chromatograms referring to the wider molecular dispersity of the products. The molecular dispersity of the products was wider than that of the parent molecule because conjugate molecules of different substitution degrees might be produced during the course of the conjugation. Moreover, distribution of the conjugated D1 or D2 moieties along the polymer chain might differ in different conjugate molecules, even in the case of the same substitution degree. Consequently, the distribution of residual (unreacted, ionizable) carboxyl groups might also be different in the individual molecules. Moreover, there are carboxyl groups of different acidity (carboxyl groups of different pK_a values) on the backbone, as it was mentioned above. Because of the various distribution of the attached molecules, residual carboxyl groups of different acidity might be on the different chains of the conjugate, even in the case of the same substitution degree. This various distribution of charges along the polymer chain in individual molecules could lead to broad molecular dispersity resulting in wide peaks (especially at higher pH values). Therefore, the final polymeric product may contain conjugate molecules of different substitution degree and may contain conjugate molecules of the same substitution degree but of different distribution of attached D1 or D2 molecules and consequently different distribution of residual charges. These facts resulted in widening of the peaks of the conjugates, compared to the peaks of the parent free carrier molecule.

Conformation of macromolecules is strongly influenced by the charged state of the polymer chain. The higher is the number of charges on the polymer chain, the lower is the capability of taking a globular conformation instead of an elongated chain. Therefore, the conjugates investigated here behaved like molecules of a relatively small apparent molecular mass at a low pH, and behaved like molecules of a relatively high apparent molecular mass at higher pH value. It can be assumed that polymer chains of different number and different distribution of negative charges formed in the conjugation reaction resulting in wide molecular dispersity. This was especially obvious at pH 4.7, where the GPC method applied was able to distinguish between the molecules containing weak or strong residual carboxyl groups.

The GPC profiles of conjugates P-D1 and P-D2 were affected less by the changes in the eluent pH than that of the parent molecule was, because of the reduced number of the free carboxyl groups. The fact that pH of the eluent must have been raised to high pH (6.0) to get significant changes in the conformation (and elution profile) of the conjugates, proved that the residual free carboxyl groups on the polymer chain were weak ones.

CONCLUSIONS

The gel permeation chromatographic method reported in this paper can be used for the analysis of macromolecular carrier poly(N-vinyl pyrrolidone-co-maleic acid) as well as of its conjugates. Elution volumes of both the free carrier and the conjugates were considerably influenced by the pH value of the eluent because of the presence of ionizable carboxyl groups on the polymer chain. Both the macromolecular carrier and the conjugates eluted with longer elution times (low apparent molecular mass) at low pH value, and eluted with shorter elution times at higher pH value (high apparent molecular mass). Strong molecular dispersity resulting in wide, tailed chromatographic profiles could be detected in the case of the conjugates. Various distribution of the residual charges on the polymer chain and the presence of residual carboxyl groups of different acidity could be responsible for this molecular dispersity.

ABBREVIATIONS

GPC: gel permeation chromatography; TEAP: triethyl ammonium phosphate; P: anionic carrier poly(N-vinyl pyrrolidone-co-maleic acid); D1: 2-cyano 3-hydroxy 5-amino 2 pentenoyc (4-trifluoromethyl anilide); D2: (6`,7` dimethyl-1`-quinoxalinyl) 4-(2`amino) acetanilide; P-D1: conjugate molecule prepared by coupling D1 molecules to the drug-carrier P; P-D2: conjugate molecule prepared by coupling D2 molecules to the of drug-carrier P.

ACKNOWLEDGMENT

This work was supported by the OTKA Grant No.:TO 16730 and TO 7722 as well as ETT Grant No T-01 053/93.

REFERENCES

1. R. Duncan, Anti-Cancer Drugs, **3**, 175-210 (1992).

2. J. Kopecek, J. Contr. Release, **11**, 279-285 (1990).

3. L. Seymour, R. Duncan, J. Strohalm, J. Kopecek, J. Biomed. Mat. Res., **21**, 1341-1351 (1987).

4. M. Azori, J. Pató, E. Csákvári, F. Tüdos, Macromol. Chem., **187**, 2073-2086 (1986).

5. M. Móra, J. Pató, J. Contr. Release, **18**, 153-164 (1992).

6. Gy. Kéri, I. Mezo, Zs. Vadász, A. Horváth, A. Balogh, M. Idei, Gy. Bökönyi, T. Vántus, I. Teplán, J. Horváth, O. Csuka, Biochem. Biphys. Res. Comm., **191**, 681-689 (1993).

7. J. Pató, F. Tüdos, Makromol.Chem. Rapid Comm., **10**, 497-505 (1989).

8. M. Móra, J. Pató, F. Tüdos, Makromol. Chem., **190**, 1967-1975 (1989).

9. M. Idei, G. Dibó, K. Bogdán, G. Mezo, A. Horváth, J. Érchegyi, Gy. Mészáros, I. Teplán, Gy. Kéri, F. Hudecz, Electrophoresis, **17**, 1357-1360 (1996).

10. J. Pató, Gy. Kéri: submitted for publication in J. Chem. Soc.

11. H. Rios, L. Gargallo, D. Radic, Eur. Polym. J., **21(12)**, 1045-1049 (1985).

12. P. L. Dubin, J. Strauss, J. Phys. Chem., **74**, 2842-2858 (1970).

13. J. C. Leyte, M. Mandel, J. Polym. Sci., **A-2**, 1879-1886 (1964).

14. J. F. J. Dippy, S. R. C. Hughes, A. Rozansky, J. Chem. Soc., **48**, 2493-2512 (1959).

15. H. Rios, L. Gargallo, D. Radic, Journal of Polymer Sci., **24**, 2421-2433 (1986).

Received May 20, 1997
Accepted October 16, 1997
Manuscript 4496