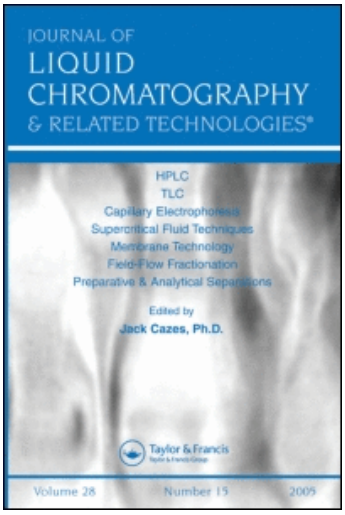

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MOLECULAR CHARACTERIZATION OF POLYMERIC ANTITUMOR DRUG CARRIERS BY SIZE EXCLUSION CHROMATOGRAPHY AND UNIVERSAL CALIBRATION

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ABSTRACT

The development of a Size Exclusion Chromatography (SEC) method for the molecular characterization of FCE 28068, a conjugate between a synthetic polymeric carrier and the antitumor drug doxorubicin is presented. The aim was an accurate and reproducible, yet relatively simple and rapid method for the routine quality control of production batches. A standard SEC method that utilizes just a refractive index detector and relies upon universal calibration with commercial narrow standards was found suitable. The development of this method implied the following steps: test of universal calibration in the chosen experimental conditions with commercial narrow

standards; fractionation of FCE 28068; test of compliance for the FCE 28068 fractions to universal calibration conditions; test of accuracy and reproducibility of the SEC measurement. The use of off-line light scattering and viscometry for the molecular characterization of standards and of FCE 28068 is discussed.

INTRODUCTION

Synthetic polymers are commonly used in many different biomedical applications, including prostheses, contact lenses, plasma expanders, wound dressing and pharmaceutical excipients.[1] The acquired knowledge about their biocompatibility and the experience gained in producing materials that comply with requests of regulatory authorities have encouraged studies about polymeric drug delivery systems, mainly in cancer chemotherapy.[2] Polymer-based drug delivery systems are usually designed to improve the pharmacokinetic profile of an antitumor agent. Compared to other macromolecular carriers (immunoconjugates, natural polymers), synthetic polymers are structures that can be tailor-made to optimize features such as molecular weight and inclusion of targeting moieties.

Poly-N-(2-hydroxypropyl)methacrylamide (PHPMA) is a biocompatible polymer, originally developed at the Prague Academy of Sciences as a plasma expander[3] and used later on for the preparation of a variety of water-soluble coniugates with antitumor agents (doxorubicin, daunorubicin, melphalan).[4-10] Generally, antitumor agents have been covalently bound to HPMA via a peptidyl spacer designed to be stable in bloodstream,[11] but cleavable by lysosomal enzymes inside the cells.[12] FCE 28068 is a PHPMA copolymer containing as a comonomer methacrylamide bound to doxorubicin via a tetrapeptidyl spacer (Gly-D,L-Phe-L-Leu-Gly).[4]

Doxorubicin, an antibiotic of the anthracycline group, is a well known antineoplastic agent used in the clinical treatment of leukemias, lymphomas, soft tissue and osteogenic carcinomas and solid tumors.[13,14] Nevertheless, its efficacy is restricted by a range of toxic side-effects, including cumulative cardiotoxicity.[15]

Improved antitumor activity of FCE 28068, compared to free drug, has been demonstrated preclinically, especially in solid tumor models.[16] Moreover, the stability of the linkage in the bloodstream reduces general toxicities such as cardiotoxicity in animal studies.[17] At present, the compound is under phase I clinical evaluation, where no cardiotoxicity has been observed, even at high drug-equivalent doses.[18]

The molecular weight of a drug-carrier conjugate and its molecular size under physiological conditions are important for effective function. They influence the accessibility of polymer to target cells other than phagocytes.[19] Besides, synthetic polymers are often non-degradable, therefore their molecular weight must be lower than the renal excretion threshold, otherwise they will be retained in the body.[20] The effect of molecular weight on plasma clearance makes the determination of the average molecular weight and of its distribution an important part of the analytical characterization of FCE 28068. Here, a conventional Size Exclusion Chromatography (SEC) method for such a characterization is presented: this method utilizes refractive index detection, and relies upon universal calibration with commercial narrow standards.

The aim was a relatively simple and fast procedure, complying with the batch quality control needs, for the accurate and reproducible determination of weight average-molecular weight (Mw) and of true molecular weight distribution (MWD). The study implied the following steps: choice of mobile phase and columns; check of universal calibration in the chosen experimental conditions, with narrow standards as polyethylenglycol/polyethylenoxide (PEG/PEO) and polymethylmethacrylate (PMMA); fractionation of PHPMA and of FCE 28068; check of universal calibration also for the narrow fractions of PHPMA and FCE 28068; check of the SEC measurement accuracy by comparison with results of independent light scattering determinations; check of the SEC measurement reproducibility. The study utilized some classic analytical techniques for the characterization of macromolecules in solution, such as multi-angle laser light scattering (MALLS) and viscometry both off- and on-line with the SEC system.

MATERIALS AND METHODS

Source of Polymers

PEO standards were purchased by Shove Denko. PEG and PMMA standards were provided by Polymer Laboratories. Both PHPMA and FCE 28068 fractions were obtained as described below.

PHPMA and FCE 28068 Fractionation.

Fractions of PHPMA prepared by chromatography on a 5x100 cm column containing a 1:1 mixture of Sepharose 4B and 6B (Pharmacia Biotech), were provided by Dr. K. Ulbrich, Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague. FCE 28068 was fractionated on a

5x100 cm column containing Sephacryl S-200 (Pharmacia Biotech); each fraction was further chromatographed on a 5x30 cm column filled with Sephacryl S-100 (Pharmacia Biotech). For both polymers the eluent was 0.1 M phosphate buffer, 0.5 M NaCl, pH 6.0; flow-rate: 1 mL/min for PHPMA and 5 mL/min for FCE 28068; temperature 22 °C; loaded sample concentration 1g/30 mL. For PHPMA a refractive index detector was used, whereas FCE 28068 elution peak was monitored off-line by optical density measurement at 480 nm on a 8451A UV-Visible detector (Hewlett Packard) after proper dilution.

The chromatographic peak of FCE 28068 was integrated and the integrated surface divided into 6 fractions of similar area. Tubes corresponding to each fraction were pooled and concentrated in an Amicon 8050 ultrafiltration cell on a Diaflo YM10 membrane (cut-off 10000) under nitrogen pressure. Eventually, fractions were desalted on a 2.6x14 cm column filled with Sephadex G-25 fine (Pharmacia Biotech), by elution at 1 mL/min and 22 °C, evaporated to dryness and dried in oven under vacuum at 50 °C for 3 hr.

Chromatographic Systems

Generally, for analytical purposes, a 150CV (Waters) system, composed by a liquid chromatograph equipped with an on-line single-capillary viscometer and a refractive index detector was used. Both detectors were exploited for the viscometric characterization, whereas just the latter was utilized for standard SEC analyses.

The "Expert Easy" software, version 2.0, was used for data acquisition and analysis. In some cases also a UV-visible photometer was used. Some experimental data were obtained with a 1090A chromatograph (Hewlett-Packard) equipped with an HP 1047A refractive index detector. For semi-preparative purposes, a Waters 625 chromatograph was utilized.

Chromatographic Experimental Conditions.

Two column sets were tested. The first one was composed by two identical PLgel Mixed C (Polymer Laboratories) columns in series. The second set was made by a Styragel HR4 and a Styragel HR3 column (Waters) in series.

Both sets were filled with a polystyrene/divinylbenzene matrix (5 μm particle size). Theoretical plate number was calculated with 1 % ethylene glycol as flow-marker. In spite of a lower efficiency (35,000 vs 40,000 plates/linear meter) the Waters Styragel columns were preferred for the higher reproducibility of chromatographic data.

A ternary mobile phase was used. N,N-dimethylformamide (DMF, Aldrich) was chosen on the basis of the polymer solubility and of the compatibility with commercially available columns; 0.01 M LiBr (Sigma) was added to avoid molecular aggregation, whereas 0.05 M acetic acid (Carlo Erba Analyticals) was necessary in order to prevent anthracycline chelation of metallic ions released by the chromatographic system. Flow-rate was 0.8 mL/min; column and detector temperature were 50 °C; the eluent was degassed with helium.

Light Scattering

Even though measurements were performed with a multiangle laser light scattering photometer (MALLS) Dawn DSP-F (Wyatt Technology Co.) in the static mode, a flow-cell (F2) was utilized to reduce the scattering volume. The MALLS instrument measures, through 15 detectors, the scattered light intensity over a broad range of angles (from 7° to 173° in methanol). The light source is a vertically polarized 5 mW He-Ne laser tuned at 632.8 nm. Data were analyzed by the software Dawn, version 2.04. More details on the instrument and data analysis are described elsewhere.[21,22]

Instrument calibration was carried out with toluene as a standard, assuming the Raileygh ratio value $R = 1.406\ 10^{-5}$. The angular normalization of photodiodes was obtained using an almost uniform low molecular weight PEG standard (Mp = 12,600 g/mol; dispersity, D = 1.04), assumed as isotropic scatterer. Light scattering of FCE 28068 in DMF is affected by luminescence phenomena, thus the used solvent for MALLS measurements was methanol (Baker) containing 0.05 M acetic acid. This solvent is incompatible with SEC columns, therefore light scattering measurements were performed only in batch mode. The refractive index increments, dn/dc, for PHPMA and FCE 28068 were determined with a Brice-Phoenix BP-2000-V differential refractometer at 25 °C in the above cited solvent.

Viscometry

Intrinsic viscosity ([η]) was generally determined on-line with the viscometer included in the Waters 150CV SEC system. Hardware and software data analysis of a SEC-viscometry system have been extensively reported in literature.[23,24] For some standards and polymer fractions, with [η] near the detector lower sensitivity threshold (≈ 0.1 dL/g), measurements were carried out in static mode, with an Ubbelhode capillary viscometer. The Mark-Houwink constants "k"and "a" of the narrow polymer fractions and of the SEC

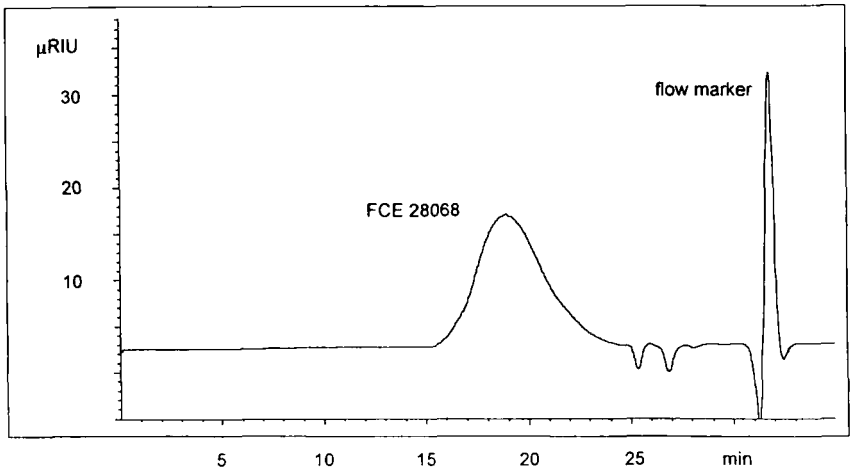

Figure 1. Size exclusion chromatogram of FCE 28068 (lot 0122; 2 mg/mL; injection volume 200 μl; filtered on Millipore 0.22 μm PTFE membrane) as obtained with a refractive index detector. Experimental conditions: see Materials and Methods. With a set of just two columns (Waters Styragel HR4 and HR3) impurity peaks (25-28 min), due to residual solvents, are completely resolved from the polymer peak (≈19 min). The peak at about 32 min is due to toluene, added as a flow marker.

standards were calculated by least squares fitting of [η] versus weight average molecular weight (Mw), as obtained from MALLS in batch mode. For SEC standards, only the molecular weight at peak apex (Mp) and dispersity were known. Still, for such narrow standards (D < 1.1), a log normal MWD can be assumed, and thus Mw could be calculated from Equation 1.

$$M_w = M_p \sqrt{D} \qquad (1)$$

RESULTS AND DISCUSSION

A typical chromatogram of a FCE 28068 sample, obtained as described in "Materials and Methods", is shown in Figure 1: the signal to noise ratio is quite high and the polymer peak (≈19 min) is well separated from peaks of low molecular weight contaminants (residual solvents: 25-28 min). The peak at about 32 min is due to toluene added as a flow marker.

Table 1

Manufacturer* Data and Viscometric Characterization of PEG/PEO and PMMA Narrow Standards

#	PEG/PEO Mp (g/mol)	PEG/PEO D	PEG/PEO [η] (dl/g)	PMMA Mp (g/mol)	PMMA D	PMMA [η] (dl/g)
1	860,000	1.17	4.2821	496,000	1.07	1.0250
2	570,000	1.10	3.0774	216,600	1.05	0.5465
3	270,000	1.09	2.0037	100,250	1.05	0.3390
4	160,000	1.07	1.3189	68,000	1.07	0.2438
5	85,000	1.06	0.8210	48,600	1.05	0.2040
6	45,000	1.07	0.5075	29,400	1.06	0.1431
7	21,000	1.12	0.3208	17,000	1.06	0.1002
8	12,600	1.04	0.2146	9,400	1.10	0.0679
9	4,100	1.05	0.1105	4,700	1.07	0.0503
10	1,470	1.05	0.0596	2,010	1.10	0.0386
11	960	1.03	0.0469	1,140	1.11	0.0320

* see Materials and Methods

Table 2

Mark-Houwink Equation Constants of PEG/PEO and PMMA Narrow Standards and of PHPMA and FCE28068 in SEC Mobile Phase at 50 °C

Polymer	$k \bullet 10^4$ (dl/g)	a
PEG/PEO	4.33	0.666
PMMA	1.12	0.692
PHPMA	1.46	0.650
FCE 28068	2.21	0.652

Viscometric Characterization of PEG/PEO and PMMA Standards

Seven PEO, four PEG ($960 < Mp < 8.6\ 10^5$ g/mol) and eleven PMMA narrow standards ($1140 < Mp < 4.96\ 10^5$ g/mol) were utilized for the universal calibration. Standard Mp and dispersity values (as reported by the manufacturer) are summarized in Table 1, together with intrinsic viscosity values, as measured with the SEC on-line viscometer. These values are in good agreement with the corresponding off-line measurements (not shown). The Mark-Houwink constants "k" and "a" for PEG/PEO and PMMA standards, in the chosen mobile phase at 50 °C were calculated from the data of Table 1, and are reported in Table 2.

Characterization of PHPMA and FCE 28068 Fractions

The refractive index increments, dn/dc, for PHPMA and FCE 28068 were 0.202 and 0.212 mL/g, respectively. The six PHPMA fractions (see Materials and Methods) showed a broad molecular mass range ($8.3\ 10^3 < Mw < 3.5\ 10^5$ g/mol) and a low dispersity ($1.13 < D < 1.3$). On the contrary, six FCE 28068 fractions covered a limited molecular mass range ($2.2\ 10^4 < Mw < 4.8\ 10^4$) and dispersity varied from 1.18 to 1.51. Every fraction was accurately characterized as far Mw and [η] are concerned. Corresponding values are reported in Table 3. In the same table, dispersity values are reported, as obtained with SEC. Values for the Mark-Houwink constants for PHPMA and FCE 28068 in the chosen SEC mobile phase at 50 °C were obtained from data in Table 3 and are reported in Table 2.

Check of Universal Calibration for PHPMA and FCE 28068

The universal calibration holds when polymers elute as a function of their hydrodynamic volume, that is proportional to the product between molecular mass and intrinsic viscosity.[25] Thus, to check the universal calibration validity means to verify that the calibration function $\log (M[\eta]) = f(V)$ (where V is the elution volume) is common to all the utilized polymers: PEG/PEO, PMMA, PHPMA, and FCE 28068.

If the universal calibration holds, a true (not nominal) MWD can be obtained, also when used standards are different from the analyzed polymer. In Figure 2, experimental data of the above cited polymers are reported, as obtained with the SEC described system, together with a 3rd degree polynomial data fitting (the calibration function). Deviation from the fitting function is evident for some of the data on low molecular weight samples: this is likely to be due to errors in the determination of

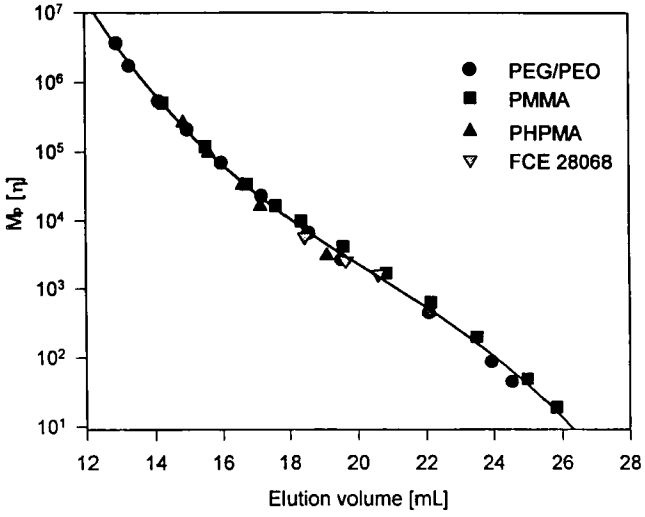

Figure 2. Test of universal calibration. Log (M_p [η]) values of four sets of polymers (PEG/PEO, PMMA, PHPMA, FCE 28068) are plotted versus their retention times: all these values are described with the same curve (a 3rd order polynomial), thus universal calibration holds in the chosen experimental conditions.

Table 3

Characterization of PHPMA and FCE 28068 Fractions

	PHPMA			FCE 28068		
#	Mp (g/mol)	D	[η] (dl/g)	Mp (g/mol)	D	[η] (dl/g)
1	349, 500	1.15	0.8138	48,200	1.51	0.1651
2	188,300	1.17	0.5585	45,500	1.36	0.1567
3	99,450	1.16	0.3539	37,300	1.38	0.1398
4	65,300	1.13	0.2637	32,400	1.24	0.1259
5	25,600	1.23	0.1349	28,200	1.20	0.1151
6	8,300	1.30	0.0637	22,000	1.18	0.0988

correspondingly low intrinsic viscosity values. Both the PHPMA and the FCE 28068 fractions follow the calibration function (as calculated with the narrow PEG/PEO and PMMA standards), thus it is true that these polymers elute accordingly to their hydrodynamic volume, and therefore they can be characterized by conventional SEC and universal calibration.

Characterization of Unfractioned FCE 28068 Polymer

Off-line characterization (by MALLS in static mode) of FCE 28068 (lot 0122) gave the following average values: weight average molecular weight Mw = $(3.84 \pm 0.04)\ 10^4$ g/mol; radius of gtyration $\langle s^2 \rangle_z$ 8.8 ± 1.4 nm. The radius of gyration value is near the low sensitivity limit of a MALLS instrument equipped with a He-Ne laser, and thus the datum precision is low. On the other hand, the Mw value is obtained with high precision. Data fitting was performed with the traditional Zimm graphic procedure, as shown in Figure 3.

SEC Measurement Accuracy

Comparison between "true" off-line determined Mw and [η] values, and the corresponding SEC on-line values for FCE 28068 (lot 0122) is reported in Table 4. The good agreement among these data confirms the validity of universal calibration for FCE 28068 polymer. Generally, improved accuracy of Mw values of FCE 28068 fractions, as determined with PEG/PEO standards (see Figure 4) led us to prefer this set of standards for the quality control method.

Heterogeneity in a copolymer composition can affect the MALLS determined Mw value, if the dn/dc value is not constant for all the sample molecules. In the FCE 28068 copolymer, the doxorubicin content was found to vary between about 6 % (in the lowest Mw fraction) and about 12 % (in the highest Mw fraction). Nevertheless, when two chromatograms are acquired for the same FCE 28068 sample, with two different detectors (RI for the whole copolymer and UV-visible tuned at 480 nm, the maximum wavelength for the doxorubicin chromophore), they are perfectly superposable.

Thus, by taking into account this identity, the low average molar fraction of doxorubicin in FCE 28068 and finally the relatively low difference between the dn/dc values of the polymeric carrier alone (PHPMA), and of the [drug-polymeric carrier] conjugate (FCE 28068) respectively, we can assume that the modest observed composition heterogeneity does not significantly affect the MALLS determined Mw value.

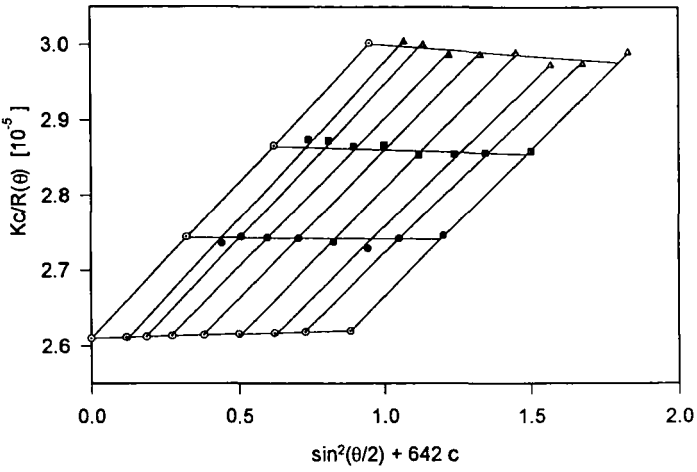

Figure 3. Zimm plot of a FCE 28068 sample (lot 0122), as obtained by MALLS in static mode. Solvent: methanol, 0.05 M acetic acid. Temperature: 25 °C.

Table 4

Accuracy of SEC Characterization of a FCE 28068 Sample (lot 0122)

$Mw^{(1)}$ (g/mol)	$Mw^{(2)}$ (g/mol)	$Mw^{(3)}$ (g/mol)	$[\eta]^{(4)}$ (dl/g)	$[\eta]^{(2)}$ (dl/g)	$[\eta]^{(3)}$ (dl/g)	$D^{(2)}$	$D^{(3)}$
38,400	37,200	40,850	0.1331	0.1286	0.1372	1.74	1.62

(1) as obtained by MALLS
(2) as obtained by SEC with PEG/PEO calibration
(3) as obtained by SEC with PMMA calibrations
(4) as obtained off-line with the Ubbelhode capillary viscometer

SEC Measurement Reproducibility

SEC measurement reproducibility was determined by analysing twice per week along one month a freshly prepared solution of FCE 28068 (lot 0120). Every time, a calibration curve with PEG/PEO standards was calculated. Average values of Mw and D, as obtained from 10 measurements, are 38,800

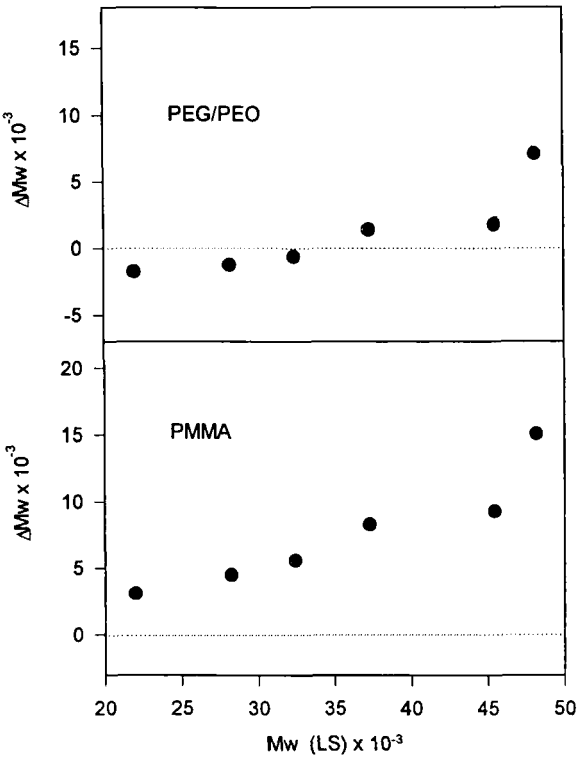

Figure 4. Test of method accuracy as a function of the used calibration standards. For six FCE 28068 fractions the difference between Mw obtained with SEC and the light scattering values (ΔMw) is plotted as a function of Mw. The two panels display results obtained with two sets of calibration standards.

and 1.84, respectively. The relative standard deviation (RSD% = 1.52 % for Mw and 1.38 % for dispersity) are well within the typical range for SEC determinations, and confirm the very good reproducibility of the method.

CONCLUSIONS

For routine Mw and MWD analysis on a given polymer, a two-step process works often well. In the first step, a commercial set of standards with known Mw value of each component is selected, the intrinsic viscosity of these standards in the chromatographic mobile phase is determined, and the universal calibration is checked.

In the second step, the universal calibration is used to analyze all the samples by a conventional SEC approach using only the refractive index detector. In the described work, the considerable amount of data obtained with several techniques (SEC, SEC-viscometry, MALLS, off-line viscometry) led to some meaningful conclusions. It is possible to characterize the FCE 28068 copolymer and the PHPMA homo-polymer through conventional SEC in organic mobile phase after calibration with commercial standards. Chromatographic separation of FCE 28068 and of PHPMA in the specified experimental conditions is fundamentally based on their hydrodynamic volume, thus universal calibration operates. The SEC method is rapid: forty minutes are needed for one chromatogram with a set of just two high-efficiency columns (35-40,000 theoretical plates). Both PEG/PEO and PMMA calibration give Mw and $[\eta]$ values close to those measured by light scattering in static mode and by off-line viscometry respectively. PEG/PEO calibrated SEC generally gives more accurate Mw values. Finally, reproducibility of the described SEC method (RSD % < 2 %) is very high, thus the method is suitable for quality control.

REFERENCES

1. C. G. Gebelein, **Advances in Biomedical Polymers,** New York, Plenum Press, 405, (1987).

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

3. L. Sprincl, J. Exner, O. Sterba, J. Kopecek, J. Biomed. Mater. Res., **10**, 953-63 (1976).

4. J. Kopecek, H. Bazilova, Eur. Polym. J., **9**, 7-14 (1978).

5. J. Strohalm, J. Kopecek, Angew. Makrom. Chem., **70**, 109-18 (1978).

6. P. Rejmanova, J. Labsky, J. Kopecek, Makromol. Chem., **178**, 2159-68 (1977).

7. K. Ulbrich, E.I. Zacharieva, B. Obereigner, Biomaterials, **1**, 199-204 (1980).

8. J. Kopecek, R. Duncan, J. Controlled Rel., **6**, 315-327 (1987).

9. B. Rihova, K. Ulbrich, J. Strohalm, V. Vetvicka, M. Bilej, R. Duncan, J. Kopecek, Biomaterials, **10**, 335-42 (1989).

10. J. Kopecek, J. Controlled Rel., **11**, 279-90 (1990).

11. P. Rejmanova, J. Kopecek, R. Duncan, J. B. Lloyd, Biomaterials, **6**, 45 (1985).

12. P. Rejmanova, J. Pohl, M. Baudys, V. Kostka, J. Kopecek, Makromol. Chem., **184**, 2009 (1983).

13. F. Arcamone, **Doxorubicin, Anticancer Antibiotics: Medicinal Chemistry,** a series of monographs, Vol. 17, Academic press, New York, 1981.

14. J. R. Brown, S. Heider Imam, in **Progress in Medicinal Chemistry**, G. P. Ellis, G.B. West Eds., Vol. 21, 196-236, Elsevier, Amsterdam, 1984.

15. C. Myers, **Anthracyclines in Cancer Chemotherapy**, H. Pinedo, B. Chabner Eds., Vol. 8, 52-64. Elsevier Science Publishers, Amsterdam, 1986.

16. L. W. Seymour, K. Ulbrich, P. S. Steyger, M. Brereton, V. Subr, J. Strohalm, R. Duncan, Br. J. Cancer, **70**, 636-641 (1994).

17. T. K. Yeung, J. W. Hopewell, R. Simmonds, L. W. Seymour, R. Duncan, O. Bellini, M. Grandi, F. Spreafico, J. Strohalm, K. Ulbrich, Cancer Chemother. Pharmacol., **29**, 105-111 (1994).

18. P. A. Vasev, R. Duncan, S. B. Kaye, J.Cassidy, Communication to the 8th European Conference on Clinical Oncology Cancer Research and Cancer Nursing, Paris, 29-10-1995.

19. R. Duncan, M. K. Pratten, H. C. Cable, H. Ringsdorf, J. B. Lloyd, Biochem. J, **196**, 49 (1981).

20. L. W. Seymour, R. Duncan, J. Strohalm, J. Kopecek, J. Biomed. Mater. Res, **21**, 1341-58 (1987).

21. P. J. Wyatt, I. Izisel, R. G. Parker, G. K. Wyatt, Proc. Int. GPC Symp., Itasca, IL, 168 (1987).

22. L. Nilsson, C. Jackson, P. J. Wyatt, Proc. Int. GPC Symp., Newton, MA, 166 (1989).

23. J. L. Ekmanis, Proc. Int. GPC Symp., Newton, MA, 1 (1989).

24. Y. Kuo, T.Provder, M. E. Koehler, Proc. Int. GPC Symp., Newton, MA, 54 (1989).

25. Z. Grubisic, P. Rempp, H. Benoit, J. Polymer Sci. B, **5**, 753 (1967).

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