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Journal of Chromatography A, 694 (1995) 415–423

JOURNAL OF CHROMATOGRAPHY A

High-temperature reversed-phase high-performance liquid chromatographic analysis of a synthetic copolymer on a non-porous support

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First received 7 September 1994; revised manuscript received 11 October 1994; accepted 11 October 1994

Abstract

High-temperature HPLC using a non-porous microparticulate reversed-phase column was evaluated for characterizing an exploratory magnetic resonance imaging polymeric contrast agent. High-efficiency separations were achieved allowing resolution of over 50 individual oligomers. Separations were optimized with respect to temperature and addition of neutral salt. Results were compared to those obtained with a traditional porous HPLC support as well as a macroporous perfusion-type column. Separation efficiencies were highest with the non-porous support followed in order by the macroporous and traditional porous packing. The utility of this technique for characterizing degradation of the polymer under stress conditions was demonstrated.

1. Introduction

The application of reversed-phase (RP) HPLC to characterize oligomeric and polymeric materials has been well documented [1–7]. For high-molecular-mass polymers, where the total number of species is very large and the differences in the chromatographic properties of different sized chains is negligible, resolution of individual polymer species is not feasible. In this case RP-HPLC has been restricted to purity determination for residual monomers or other low-molecular-mass impurities or assessment of chemical composition [5,8,9] for certain types of copolymers. However, the chromatographic properties of low-molecular-mass polymers are sometimes distinct enough to allow chromatographic resolution of the polymer into its constituent oligomers. This allows for a more detailed characterization of the chemical species composing the polymer in terms of molecular mass distribution and structural properties.

Examples of HPLC separations of oligomeric materials include polyethers [4–7,10], polystyrene [2], ethoxylated alkyl alcohols and carboxylic acids [11], polyethoxylated octylphenols [12,13] and various copolymers [14–16]. These separations have typically been conducted on traditional bonded phase porous silica packings in either the RP or, in the case of diol and amino bonded phases or bare silica columns, in the normal-phase mode. The advantages of elevated temperatures was noted in some RP-HPLC applications to improve resolution for poly-

0021-9673/95/$09.50
SSDI 0021-9673(94)00974-0

ethers [8] due to improvement in overall chromatographic efficiency in addition to a favorable conformational change in the polymer.

In the field of bioanalytical chemistry, there have been a number of improvements in chromatographic supports in recent years which have resulted in higher chromatographic efficiency and recovery for proteins and oligonucleotides. Of note are the development of small-particle non-porous or micropellicular supports [17–21] operated in either the RP or ion-exchange modes. These supports were designed to have improved mass transfer properties for these large biopolymers which translates in many instances into improved chromatographic efficiencies and recoveries. As with traditional HPLC packings, chromatographic efficiency is expected to increase with increasing temperature for these alternative HPLC supports. However, the instability of these biopolymers to heat has limited the use of these columns at elevated temperatures. Numerous types of wide-pore or macroporous HPLC supports have also been developed for biopolymer separations. Wide-pore columns were designed to allow greater access of the biopolymers to the bonded phase and eliminate the potential for any sizing effects. In the case of the macroporous supports, a second objective is to reduce band spreading as a result of slow diffusion through a porous matrix.

In this work, the application of non-porous and macroporous supports operated at elevated temperatures (up to 100°C) was investigated for characterizing a synthetic, copolymeric, exploratory magnetic resonance imaging (MRI) contrast agent. This agent was designed to have an extended in vivo half-life in the vascular compartment for certain types of diagnostic applications [22]. The samples examined in this work have average molecular masses ranging from 10 000 to over 30 000. The combination of the non-porous packing along with the elevated temperatures was investigated for examining the oligomeric distribution of different molecular mass samples as well as stressed samples of the polymer.

2. Experimental

2.1. Materials

Water was purified by a Barnstead water-purification system (Barnstead/Thermolyne). HPLC-grade acetonitrile was from J.T. Baker and HPLC-grade $NaClO_4$ was purchased from Fisher Scientific. All other chemicals were of reagent grade and from J.T. Baker.

Polymer samples were synthesized as depicted in Fig. 1. Details of the synthesis can be found elsewhere [22]. Molecular mass analysis of the samples was performed by aqueous size-exclusion chromatography. Samples of the polymer were degraded by heating at 80°C in either 0.1 *M* NaOH or 0.1 *M* HCl and neutralized prior to analysis.

2.2. HPLC instrumentation

All separations were conducted on a Hewlett-Packard (HP) 1090M liquid chromatograph controlled by a HP Vectra DOS-based personal computer work station. A 3 cm × 4.6 mm Hytach

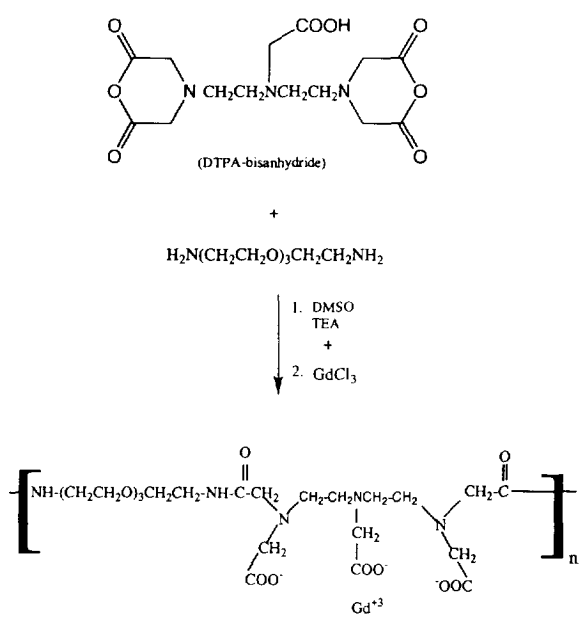

Fig. 1. Synthetic scheme for producing the MRI polymer.

C_{18} column based on a 2-μm non-porous silica support was purchased from Glycotech (Hamden, CT, USA). A 3 cm × 4.6 mm Poros R/H column based on a macroporous perfusion type support was obtained from Perseptive Biosystems (Cambridge, MA, USA). A Capcell PAK C_{18} SG, 120 Å, 15 cm × 4.6 mm column was obtained from Dychrom (Sunnyvale, CA, USA). Unless otherwise noted, solvent A was 400 m*M* $NaClO_4$ and solvent B was 400 m*M* $NaClO_4$ in a mixture of acetonitrile–water (20:80). The flow-rate was 1 ml/min and detection was accomplished at 200 nm. Polymer samples were prepared in the A solvent at 6 mg/ml. Injection volumes of 3 μl were used corresponding to 18 μg of polymer. The column temperatures and gradient profiles used are indicated in the text.

3. Results

3.1. Influence of temperature on efficiency and resolution

Fig. 2 displays a set of chromatograms obtained at different temperatures using the non-porous RP-HPLC column on a synthetic polymer sample of average molecular mass 30 000. A linear gradient from 0 to 100% B over 60 min was used in each case. A significant improvement in resolution and efficiency accompanied by decreasing retention is observed with increasing temperature. At the highest temperature, partial resolution of up to the first 50 or so oligomers is achieved. The characteristic pattern of peaks observed in these chromatograms is

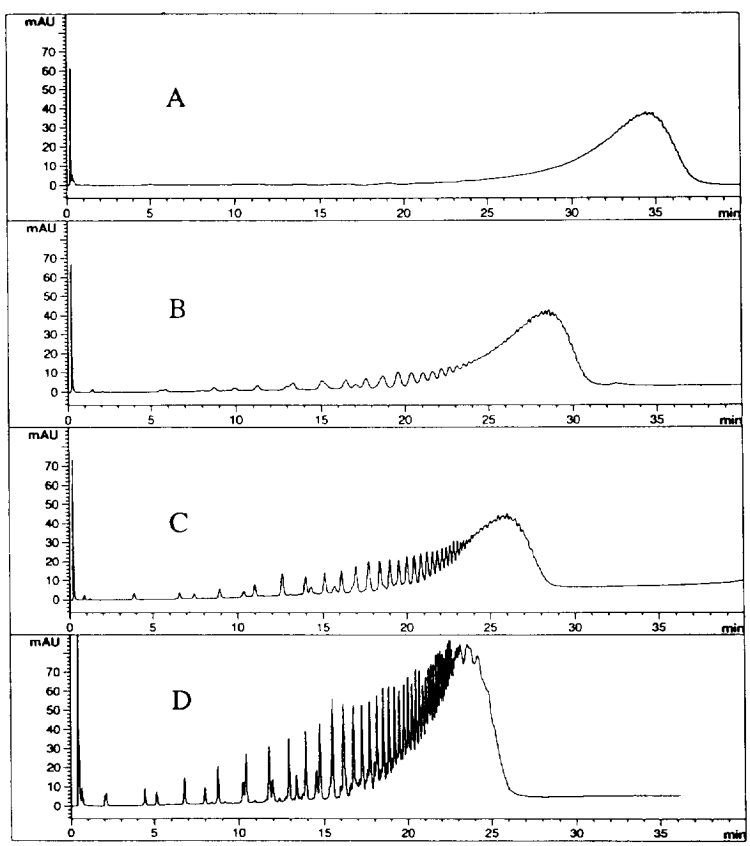

Fig. 2. Separations of a polymer of average molecular mass 30 000 at different operating temperatures: (A) 25°C, (B) 60°C, (C) 80°C and (D) 100°C. Gradient profile from 0 to 100% B in 60 min. Refer to Experimental section for other conditions.

interpreted to result from the distribution in molecular mass as well as the variability in the composition of end groups on each polymer chain which is anticipated for this condensation copolymer. This pattern was highly reproducible from analysis to analysis. In addition, the stability of the polymer at neutral pH values up to 121°C for at least 1 h has been established ruling out any type of on-column chemical degradation of the polymer.

3.2. Effect of neutral salt on retention

Fig. 3 contains chromatograms obtained under the identical conditions used in Fig. 2 except for the level of salt, $NaClO_4$, used in the mobile phase. This particular salt was chosen because of its high solubility in acetonitrile in addition to its low UV absorbance at the low wavelengths used in this work. In general, addition of salt increased retention of the polymer. This phenomenon can probably be explained by a "salting in" effect with the addition of increasing amounts of $NaClO_4$. An increase in the number of oligomers separated was observed in going from no salt to 400 m*M* salt. No significant improvement in resolution was attained at levels above 400 m*M* $NaClO_4$. In addition, this salt was also beneficial in minimizing any secondary ionic or silanophilic interactions between the silica support and the ionic termini or polar regions of the polymer. In the absence of any salt, the high-molecular-mass end of the polymer distribution tailed off slowly and did not return to baseline until about 45

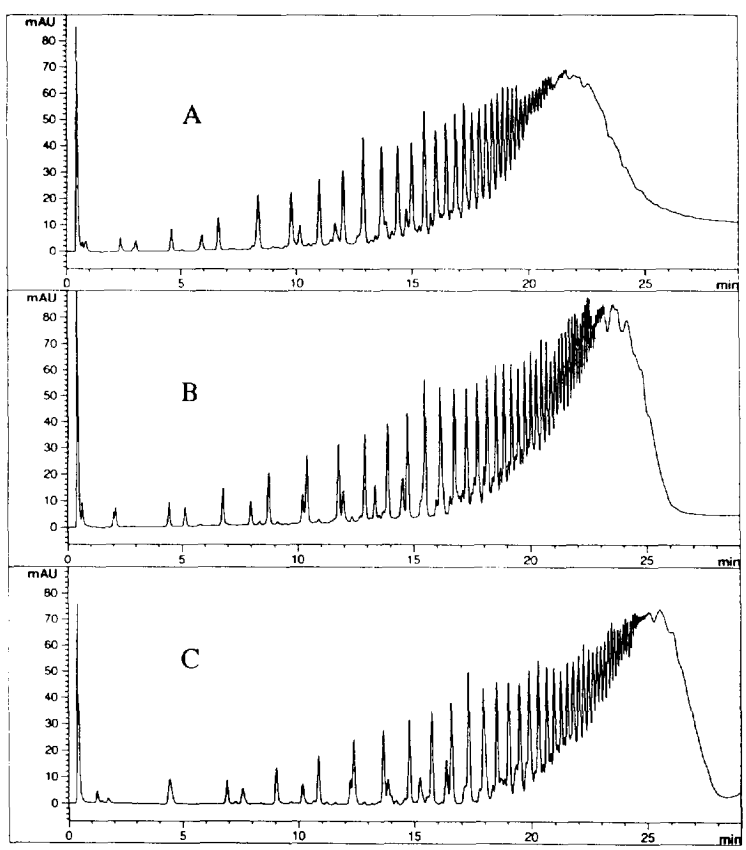

Fig. 3. Separations of a polymer of average molecular mass 30 000 at 100°C with different concentrations of $NaClO_4$: (A) no salt, (B) 400 m*M* and (C) 1000 m*M*. Other conditions as given in Fig. 2.

min. In the presence of salt, the high-molecular-mass portion of the polymer peak displayed no significant tailing.

3.3. Comparison of results on different types of packings

Fig. 4 compares the separation obtained at 100°C on the non-porous HPLC column to that obtained on a traditional porous C_{18} silica (120 Å) as well as a reversed-phase perfusion type (Poros) of packing. The Poros column contains a packing material composed of macropores of two size ranges: 800–1500 Å and 6000–8000 Å [23]. In terms of resolution and efficiency, a clear-cut advantage is observed with the non-porous packing material compared to the traditional porous column. The results with the Poros column were intermediate between the other two columns but, in general, this column showed significant improvement in resolution compared with the traditional C_{18} RP column. The lower retention of the polymer on the traditional pore size column compared to the macroporous column may indicate that the sample cannot fully penetrate the small pores of the former column. However, the retentive surface of these columns is different (C_{18} derivatized silica versus aromatic derivatized polystyrene resin) and, therefore, the inherent difference in hydrophobicities and capacities of these columns may account for the difference in polymer retention. Both the

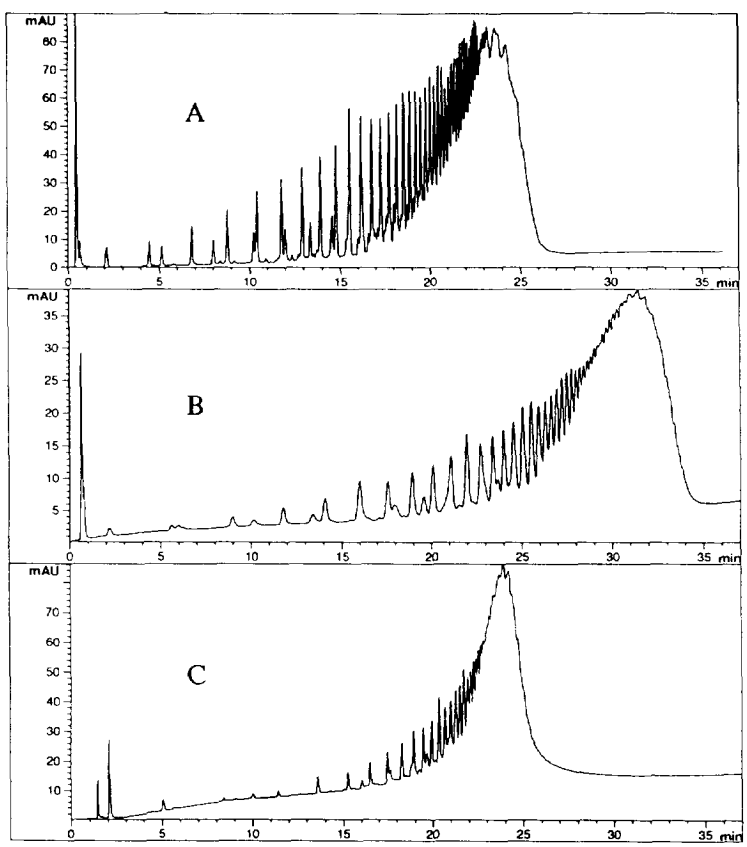

Fig. 4. Separations of a polymer of average molecular mass 30 000 on three different types of columns: (A) non-porous, (B) macroporous and (C) traditional porous RP-HPLC column. Operating temperature of 100°C and gradient profile of 0 to 100% B in 60 min. Other conditions as given in the Experimental section.

Poros and traditional C_{18} columns demonstrated an improvement in resolution with increasing temperature (data not shown) as was observed with the non-porous column.

Of concern in this work was the stability of the columns to high temperature. The manufacturer of the Poros column recommends an upper temperature limit of 80°C and so this column was used at elevated temperatures for only short periods. The other two manufacturers do not explicitly specify temperature limits. While the long-term stability of the non-porous column at 100°C has not been determined, daily use at these elevated temperatures for several weeks did not result in any noticeable deterioration in column performance.

3.4. Analysis of different molecular mass samples

The dependency of retention on molecular mass is substantiated based on analysis of polymer samples of different molecular mass. Fig. 5 depicts the chromatograms obtained on three different samples of average molecular masses 30 000, 15 000 and 8000 using the non-porous column at 100°C. These results demonstrate that for low-molecular-mass polymers (less than approximately 15 000) almost the entire distribution of polymeric species can be at least partially resolved. For higher-molecular-mass polymers, this technique is still useful for examining the lower-molecular-mass range of the distribution in

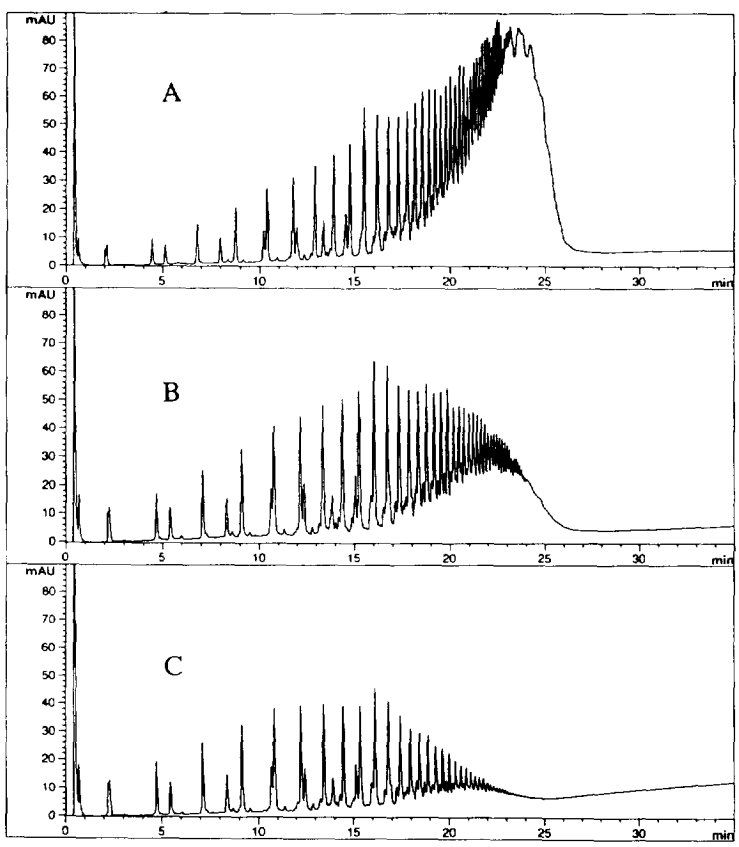

Fig. 5. Separations of polymers of average molecular mass (A) 30 000, (B) 15 000 and (C) 8000 on the non-porous RP-HPLC column operated at 100°C with a gradient of 0 to 100% B in 60 min.

addition to potential degradation products (see below).

3.5. Characterization of degradation products in stressed polymer samples

One of the potential uses of this methodology is to analyze for potential degradation products of the polymer as well as other low-molecular-mass by-products and residual starting materials. Polymer samples that were subjected to hydrolysis in acid or base at elevated temperatures were analyzed on the non-porous column at 100°C. Fig. 6 contains chromatograms of the base stressed polymer obtained at different time points. Substantially the same chromatograms in terms of oligomer profiles were obtained in the acid stressed samples although the rate of hydrolysis was slower in acid. With increasing hydrolysis time the oligomeric distribution in these stressed samples shifts to lower molecular masses. A distinct pattern of peaks is observed in these chromatograms and, in general, the pattern of low-molecular-mass oligomers formed on stressing differs somewhat from that originally present in the polymer. It is probable that degradation occurs primarily through hydrolysis of the amide linkages in the polymer, although other modes of degradation cannot be ruled out. In this case, one would expect to obtain a different compositional distribution in terms of end groups on individual polymer chains which

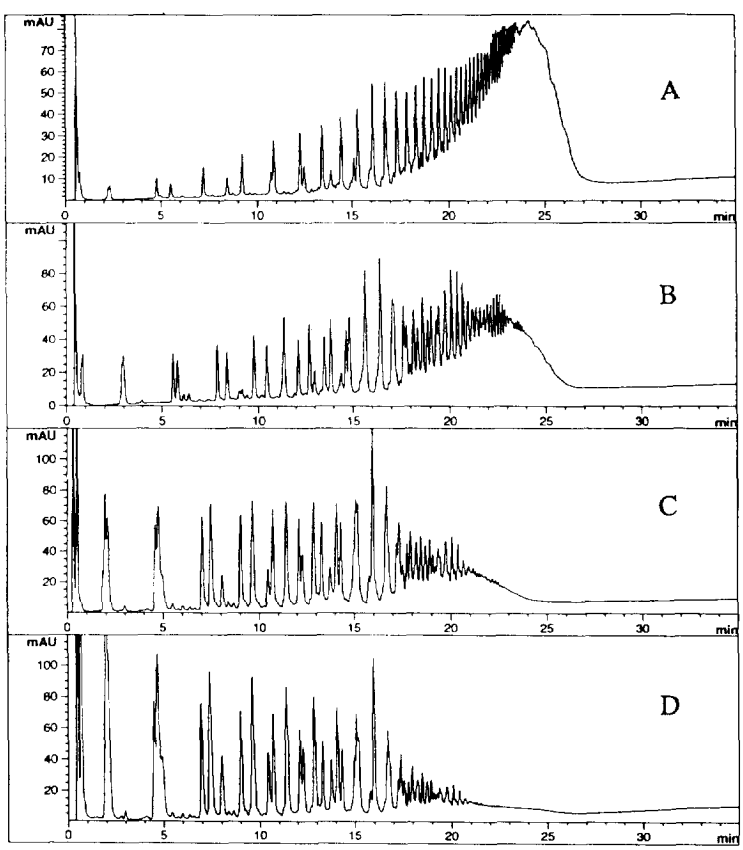

Fig. 6. Separations of a polymer of average molecular mass 30 000 stressed in 0.1 *M* NaOH at 80°C for (A) 0 min, (B) 5 min, (C) 15 min and (D) 30 min. Conditions as in Fig. 4 for the non-porous RP-HPLC column.

would change the pattern of peaks compared with the unstressed polymer.

4. Discussion

The high efficiency and resolution achieved here for individual oligomers in this polymer results from the combination of the small particle non-porous packing material in conjunction with high operating temperatures. The small particle size of the non-porous packing (2 μm) is expected to contribute some degree to the improved efficiency. The non-porous morphology of the particles should lend itself to an additional level of improvement in efficiency as a result of lowering the resistance to mass transfer. Finally, molecular diffusivity and sorption kinetics should increase with increasing temperature adding to the overall improvement in efficiency. These points are supported by the experiments documented here. In fact, the benefits of elevated temperatures when used in conjunction with non-porous supports has been predicted by Antia and Horváth [24] for the reasons enumerated here. In particular, their theoretical treatment of this subject matter highlighted these advantages for high-mass materials. Studies involving biopolymer separations on non-porous supports with temperatures as high as 80°C have been reported but in general the instability of these compounds to heat has limited the upper temperature used. The improvement in resolution obtained with the macroporous perfusion-type column compared to the traditional porous column is probably due to the superior mass transport through the macroporous support.

The use of high temperatures up to 80°C in conjunction with traditional porous supports has been reported for polyether-type oligomer separations [8]. In this case higher resolution with increasing temperature was concluded to result from the improvement in overall chromatographic efficiency with increasing temperature in addition to a favorable structural conformational change in the oligomers which resulted in better interaction of the oligomers with the bonded phase. Studies are ongoing to assess possible temperature induced conformational changes in the MRI polymer which might be contributing to the improved chromatographic efficiency and resolution observed in this work. However, preliminary differential scanning calorimetry studies could not substantiate any structural changes in the polymer with increasing temperature.

5. Conclusions

Oligomer separations for a synthetic copolymer with average molecular masses in the range 8000–30 000 have been achieved using a non-porous RP-HPLC column operated at elevated temperatures. The combination of the unique morphology of this type of column packing material in conjunction with high operating temperatures was shown to be necessary to achieve high-resolution separations of individual oligomers.

Acknowledgements

D. Ladd, R. Hollister, G. Wu and X. Peng of the Department of Medicinal Chemistry at Sterling-Winthrop are thanked for the preparation of the polymer samples.

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