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Preparation of monodisperse porous polymethacrylate microspheres and their application in the capillary electrochromatography of macrolide antibiotics

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Abstract

Monodisperse poly(glycidyl methacrylate–divinylbenzene) microspheres were prepared by a simple one-step dispersion polymerization process. Examination of the polymeric microspheres showed that they had a mean particle diameter of 3 μm and dual pore size distribution with mean pore diameters of 300 and 800 Å. The microspheres were functionalized by introducing quaternary ammonium/octadecyl groups to obtain positively charged beads in a wide pH range. The functionalized beads were packed into fused-silica capillary having 50 μm inner diameter and used to separate erythromycin derivatives by capillary electrochromatography (CEC). These samples require gradient elution when separated by high-performance liquid chromatography (HPLC) or micro-HPLC, but with the new columns isocratic elution suffices for their separation by CEC. The column efficiency ranged from 40 000 to 50 000 theoretical plates. © 2002 Published by Elsevier Science B.V.

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1. Introduction

At present most columns employed in capillary electrochromatography (CEC) are fused-silica capillaries with functionalized silica packings serving as the stationary phase [1–7]. Usually they are tailor-made for use in the reversed-phase or the electrostatic interaction mode of high-performance liquid chromatography (HPLC). CEC with silica based stationary phases is carried out with mobile phases having neutral or alkaline pH, where the silanol groups are dissociated and a high electrosmotic flow (EOF) is generated upon application of an electric field. Thus with charged sample components, especially with basic compounds, electrostatic and silanophilic interactions [8,9] between the deprotonated silanols and the charged solutes may complicate the separation and strongly tailing peaks are often seen on the electrochromatograms.

Organo–polymeric stationary phases have some favorable attributes, such as chemical stability over the entire pH range [10,11]. In particular the versatility of the surface chemistry of polymers makes them well suited as column packings for the separation of charged large biomacromolecules [12–14]. Vinyl–divinylbenzene polymers were introduced for gel permeation chromatography in 1964 [15] and have found numerous applications in various branch-

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es of HPLC [12–14,16–18]. Most of the supports are acrylic or styrenic beads. Most recently columns with a macroporous monolithic stationary phases have generated some interest in CEC [19–22]. Nonetheless, stationary phases based on porous organo–polymeric support have rarely been used so far as the stationary phase for CEC [6,23]. In this study we describe the preparation of monodisperse microspheres by dispersion polymerization [24–29] and their functionalization for use in the CEC of macrolide antibiotics.

2. Experimental

2.1. Materials

Fused-silica capillary tubing of 50 μm I.D.×375 μm O.D. with a polyimide outer coating was purchased from Quadrex Scientific (New Haven, CT, USA). Glycidyl methacrylate (GMA), *N*-methyloctadecylamine, dimethylformamide (DMF), iodomethane, [3-(trimethoxysilyl)propyl]octadecyldimethylammonium chloride, and dimethyl sulfoxide (DMSO) were from Aldrich (Milwaukee, WI, USA). Divinylbenzene (DVB) (85%) was from Dow (Midland, MI, USA). Analytical reagent-grade monobasic, dibasic and tribasic sodium phosphates were from J.T. Baker (Phillipsburg, NJ, USA) and azobisisobutyronitrile (AIBN) (98%) from Pfaltz and Bauer (Waterbury, CT, USA). Phosphoric acid (85%), sodium hydroxide and potassium hydroxide were of analytical reagent grade from Mallinckrodt (Paris, KY, USA). HPLC-grade methanol, ethanol, acetone and acetonitrile (ACN) were purchased from Fisher (Fair Lawn, NJ, USA). Except DVB, the materials were used without further purification. Water was purified and deionized with a NANOpure system (Barnstead, Boston, MA, USA).

Roxithromycin (RXM) was purchased from Sigma (St. Louis, MO, USA). Spiramycin (SM), oleandomycin (OM) and josamycin (JM) were obtained from Fluka (Milwaukee, WI, USA). Clarithromycin (CAM) and azithromycin (AZM) were gifts from Pfizer (Groton, CT, USA). Erythromycins A, B and C and *N*-demethylerythrymycin A were from European Pharmacopeia (Strasbourgh, France). Erythromycin enol ether was received from Abbott (North Chicago, IL, USA). Erythromycin was purchased from the USP Convention (Rockville, MD, USA).

2.2. Preparation of monodisperse porous polymethacrylate microspheres

DVB was first purified by washing with 10%(w/v) aqueous solution of sodium hydroxide, where the inhibitor was extracted into the aqueous phase. The DVB layer was separated and further washed with water. It was then dried by using anhydrous sodium sulfate for 1 h. The drying agent was filtered off and the purified DVB was ready to be used as the monomer in polymerization.

Then, 20 ml of DVB, 480 ml of acetonitrile and 500 mg of AIBN were added to a four-neck 1-l round-bottom flask equipped with a mechanical stirrer, condenser, nitrogen inlet and thermometer. The mixture was first purged with nitrogen for 10 min under stirring. With continuous stirring and nitrogen purge the mixture in the flask was heated in a 60°C oil bath for 4 h. Then, 10 ml of GMA and 200 mg of AIBN were added and the mixture was heated in 70°C oil bath for 16 h with continuous stirring and nitrogen purge. At different times during polymerization the suspension in the flask was sampled by microscopic observation using an Olympus BH-2 optical microscope (Olympus, Woodbury, NY, USA) with the transmitted light illumination method with typically 100-fold magnification. When the microscopic inspection showed that the size of the mature beads reached about 3 μm, the mixture was cooled to room temperature, filtered and washed thoroughly in a sintered-glass filter with acetonitrile and methanol. The beads obtained were dried under vacuum at room temperature for 4 h.

2.3. Characterization of polymethacrylate beads

2.3.1. Particle distribution by microscopy

The particle size distribution of the beads was measured by using a model ISI SS-40 scanning electron micrograph (International Scientific Instruments, Santa Clara, CA, USA) operated at 10 kV and at a filament current of 40 mA. The beads were mounted by placing them on an aluminum stud via a

double-sided carbon tape (Electro Microscopy Sciences, Ft. Washington, PA, USA). Then they were sputter-coated with a gold/palladium alloy using an SPI sputter (SPI Supplies Division of Structure Probe, West Chester, PA, USA) for 25 s at 30 mA to prevent charging.

2.3.2. Pore size distribution by the BET method

The adsorption–desorption isotherms were measured by using a model Autosorb-1C (Quantachrome, Boynton Beach, FL, USA) static volumetric instrument at −196°C. Prior to the measurements, the samples were degassed at 200°C to a residual pressure lower than 10^{-2} Pa. The Baratron (0.1–1000 Pa) pressure transducer was used for low pressure measurements.

2.4. Preparation of CEC columns

2.4.1. Functionalization of the polymethacrylate microspheres by introducing quaternary ammonium functions

The chemical reaction involved in this functionalization process is shown schematically in Fig. 1. Beads I were suspended in 10 ml solution of 20% *N*-methyloctadecylamine in acetonitrile. The mixture was sonicated for 30 min, purged with nitrogen for 10 min and then stirred at 70°C for 2 h with a condenser and a nitrogen inlet. The resulting beads II were washed with acetonitrile and ethanol and then refluxed with 5 ml of iodomethane and 5 ml of ethanol under nitrogen for 10 h and washed with methanol to obtain the cationic beads III.

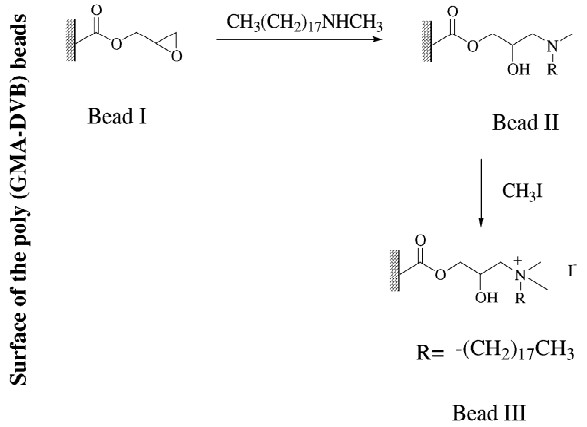


Fig. 1. Functionalization of the surface of the poly(GMA–DVB) beads to form the cationic bead III.

2.4.2. Pretreatment of the capillary inner wall

The capillary was washed and filled with 1 *M* NaOH, sealed at both ends using a butane flame burner (Veriflo Air-Gas Torch, Macalaster Bicknell, New Haven, CT, USA) and heat-treated in an oven at 120°C for 2 h. Thereafter, the capillary was washed with deionized water then methanol and dried at 120°C with nitrogen stream.

The NaOH treated capillary was filled with a solution containing 10% (v/v) of [3-(trimethoxysilyl)propyl]octadecyldimethyl ammonium chloride in DMF. After both ends were sealed, the capillary was heated in the oven at 110°C for 6 h. Then it was washed with deionized water, methanol and dried with a nitrogen stream.

2.4.3. Column packing procedure with cationic microspheres

The CEC column was packed with the functionalized cationic microspheres (Bead III) using a modified literature procedure [30]. Typically a capillary of 350 mm length was employed. The capillary was tapped in a vial containing 5 μm dry silica particles to fill a ca. 0.5 mm length into the capillary. An Archer Torch Model B (Radio Shack, New Haven, CT, USA) microtorch fueled with butane was used to make a retaining frit at the end of the column by sintering the silica particles at elevated temperature. A 5% (w/v) slurry of the functionalized bead III was made in methanol and sonicated for 30 min. Then, the capillary was connected to a cylindrical stainless steel reservoir (30×4.7 mm) which was filled with the slurry and connected to a Constametric III metering pump (Thermo Separation Products, San Jose, CA, USA). Methanol was employed as the packing solvent and the flow-rate was set at 0.4 ml/min. Once the front of the packings reached 200 mm, the flow-rate of the packing solvent was turned down to zero. After reaching near atmospheric pressure the column was disconnected from the reservoir and washed at the same flow-rate with methanol for 1 to 2 h then deionized water for 2 h. Then another retaining frit was prepared by placing a

thin layer of 5 μm silica on the top of the polymeric beads which was then sintered with butane flame while keeping water pumped through. A 3–4 mm long detection window was also formed during sintering. The column was stabilized for another 2 h and the flow-rate was turned to zero to gradually release the pressure in the column.

2.5. Capillary electrochromatography: apparatus and procedure

The experiments were conducted using a Model MDQ P/ACE system capillary electrophoresis unit equipped with a P/ACE system MDQ UV detector (Beckman, Fullerton, CA, USA). A Model 6588-12U IBM personal computer with Beckman MDQ capillary electrophoresis software version 2.2. and Windows 95 (Microsoft, Redmond, WA, USA) was used to control the instrument and to acquire and process the data.

For separation of macrolide antibiotics, 30 m*M* aqueous phosphate buffer, pH 8, containing acetonitrile and ethanol of various concentrations was used. The microlides were dissolved in MeOH–water (1:1) to obtain a solution containing 1 mg/ml of each component. Concentration of erythromycin containing various impurities in MeOH–water (1:1) was 3 mg/ml. The samples were injected at −5 kV for 2 s. The EOF marker, DMSO, at a concentration of 2 μl/ml in water, was injected at −2 kV for 1 s. Between runs the column was rinsed with acetonitrile for 10 min followed with the running mobile phase for 40 min at 100 p.s.i. inlet pressure (1 p.s.i.=6894.76 Pa). Then with both ends pressurized at 100 p.s.i. the column was equilibrated electrokinetically at the operating voltage for 10 min. All samples were detected at 206 nm. All experiments were carried out at 25°C unless otherwise specified.

3. Results and discussion

3.1. Characterization of the polymeric microspheres

The poly(GMA–DVB) beads were examined by scanning electron micrography (SEM). In Fig. 2 a typical micrograph is depicted. The picture shows that the beads have a narrow particle size distribution with an average diameter of 3 μm. The particle size distribution shown in Fig. 3 was evaluated as 3±0.1 μm by measuring the diameter of about 84 particles.

The specific surface area and the mean pore diameter of the microspheres were determined from the data on nitrogen adsorption–desorption measurements according to the BET method. The specific area was found to be 1.82 m^2/g by the BET plot and as seen in Fig. 4 the internal pore size distribution is bimodal with maxima at 300 and 800 Å.

3.2. CEC of macrolide antibiotics with the cationic column

3.2.1. Design of the stationary phase

Macrolides are complex carbohydrates and important antibiotics. They are produced by the actinomycetes fungi species *Streptomyces*. Of the macrolide antibiotics currently in clinical use, erythromycin and its derivatives are the most widely prescribed. The common skeleton is a 12–16-membered lactone ring with attached neutral and/or amino sugars. The structures of these compounds which have molecular masss in the range of 689 to 842 are shown in Fig. 5. These compounds are most commonly isolated and purified by reversed-phase HPLC [31,32]. A disadvantage of HPLC in dealing with such basic molecules on silica-based reversed-phases is the strong tailing of the peaks due to interaction with residual silanols on the stationary phase. Numerous efforts have been made to mask the silanol groups and thus reduce peak tailing and to develop a suitable method for the analysis of erythromycin and its derivatives. These include the use of mobile phase additives and new stationary phases based on organic polymeric support instead of silica based sorbents [33,34].

In the present work we set forth to employ the dispersion polymerization method to prepare organo–polymeric microspheric support and to investigate the feasibility of using such support to separate the macrolide molecules by CEC. The poly(GMA–DVB) beads were functionalized to obtain a cationic chromatographic surface with octadecyl chains. Fig. 1 shows the chemistry employed in the functionalization of the microspherical polymeric support with *N*-methyloctadecylamine. The resulting tertiary

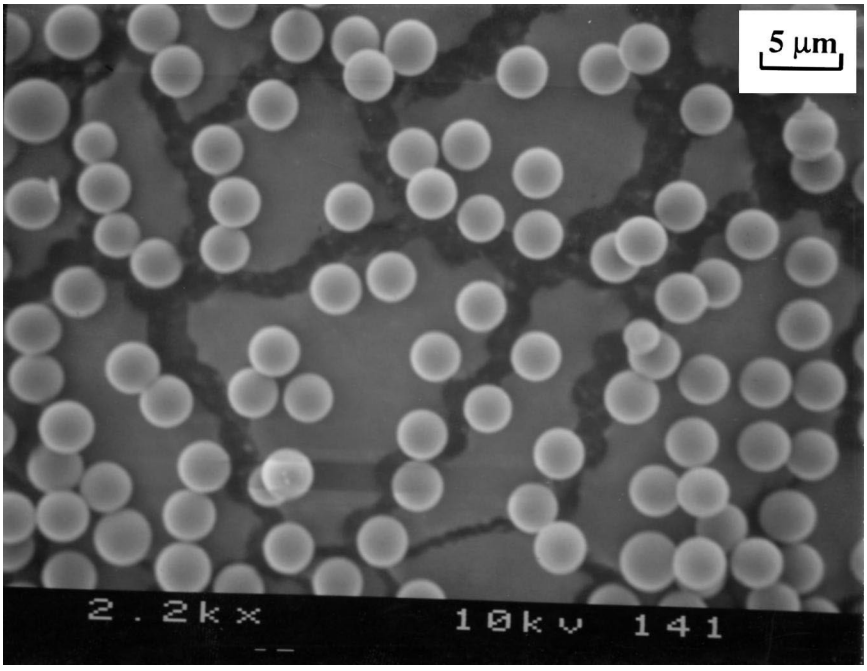


Fig. 2. Scanning electron micrograph of the monodispersed poly(GMA–DVB) beads.

amino groups were further treated with iodomethane to form quaternary ammonium groups. The positively charged surface of the stationary phase can generate strong EOF over the entire pH range upon applying electric field onto the column. The above treatment reduced adverse electrostatic effect and the concomitant peak tailing that was often seen with silica based columns. The octadecyl chains were linked to the support surface to provide the hydrophobic retentive sites for the reversible chromatographic binding of the macrolides to the stationary phase in the chromatographic process.

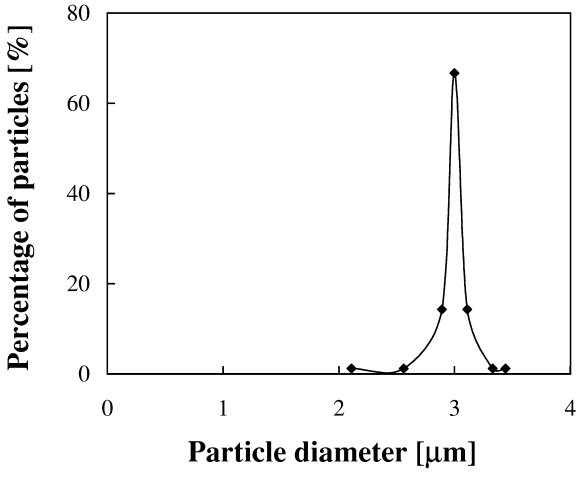


Fig. 3. Partcle size distribution of the monodispersed poly(GMA–DVB) beads obtained by measuring the diameters of 84 microspheres from the SEM picture in Fig. 2.

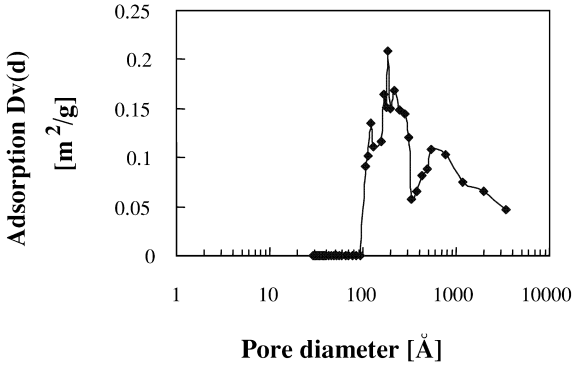


Fig. 4. Pore volume distribution of the poly(GMA–DVB) microspheres measured by the adsorption using the BET method at −196°C.

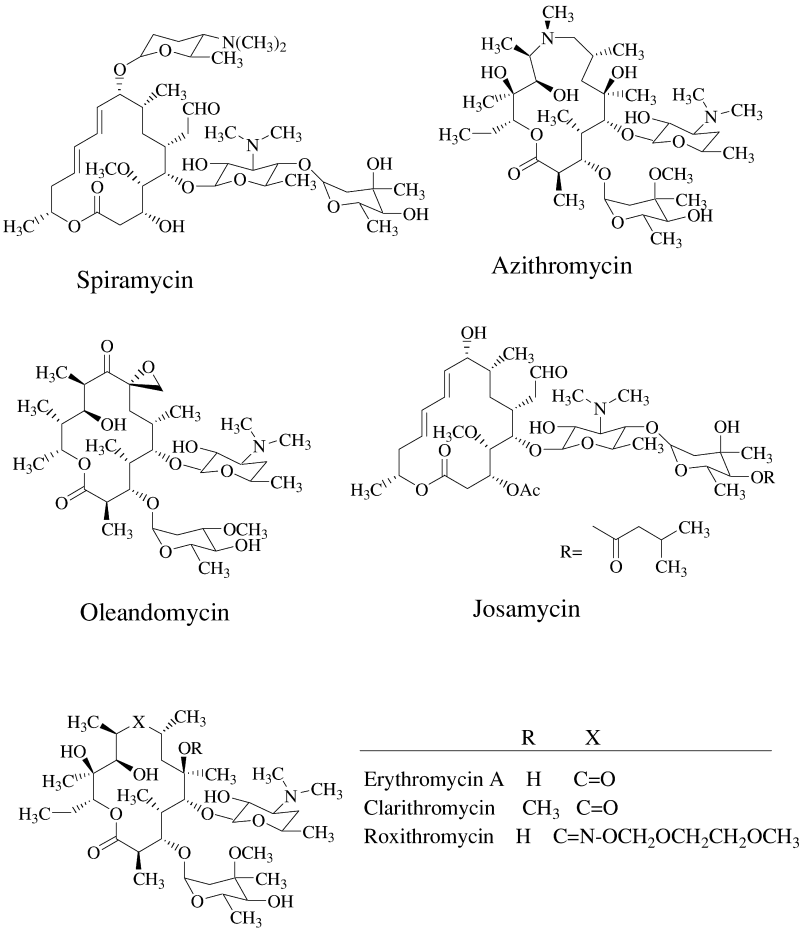


Fig. 5. Chemical structure of erythromycin and its derivatives.

3.2.2. CEC separation of erythromycin and its semi-synthetic derivatives

Since erythromycin is unstable in acidic solutions relatively high pH was used in our study. A mixture of seven closely related macrolides, whose structures are shown in Fig. 5, was subjected to CEC with the cationic column carrying octyldecyl chains and quaternary ammonium groups (SAX-CEC). The mobile phase contained 45% acetonitrile (v/v) and 10% EtOH (v/v) in 30 m*M* phosphate buffer, pH 8.0. The applied voltage was −15 kV. Sensitive detection of these macrolides presents a challenge as they lack significant chromphores. Therefore detection setting at 206 nm was selected at which most solutes have some UV absorbance. The resulting electrochromatogram is depicted in Fig. 6. The column efficiency of the 20/30 cm long column was in the range of 38 000 to 48 000 theoretical plates.

3.2.3. Separation of erythromycin impurities by CEC

The analysis of related impurities in pharmaceutical samples presents a formidable challenge because a large number of closely related minor components has often to be separated in the presence of the parent compound at relatively high concentration. Therefore, the determination of drug related impurities is another area where the potential of CEC can be demonstrated in practical applications. For instance, CEC can be used to verify impurity data obtained by HPLC. Disagreement or good agreement

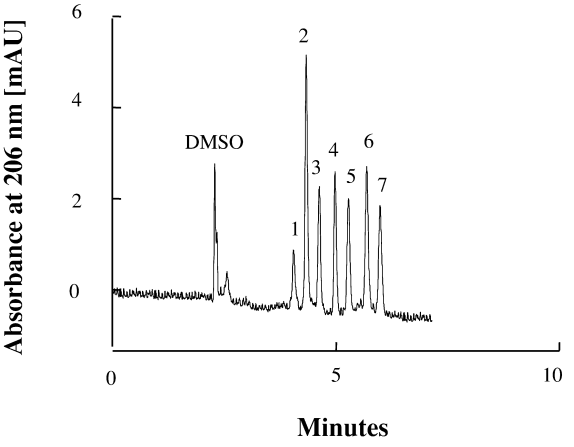


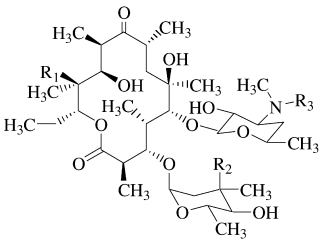
Fig. 6. Isocratic CEC of seven macrolides. Cationic column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, 45% acetonitrile (v/v) and 10% EtOH (v/v) in 30 m*M* phosphate buffer, pH 8.0; applied voltage, −15 kV; detection, 206 nm. Sample: (1) spiramycin, (2) erythromycin, (3) oleandomycin, (4) josamycin, (5) azithromycin, (6) clarithromycin, (7) roxithromycin. Mobility of EOF measured with DMSO, $\mu_{eof}=3.05\ 10^{-8}$ $m^2\ s^{-1}\ V^{-1}$.

between the results obtained by HPLC and CEC will provide very useful information for further work.

A commercial sample of crude erythromycin A was subjected to CEC with the SAX-CEC column. The chemical structures of the components are shown in Fig. 7. In the CEC experiment the mobile phase was 25% (v/v) acetonitrile and 25% (v/v) ethanol in 30 m*M* phosphate buffer, pH 8.0. Fig. 8 shows the chromatogram under these conditions. It can be seen from Fig. 8 that several impurities were baseline separated from the major component, erythromycin A. The impurities were identified using authentic standards as erythromycins B and C, *N*-demethylerythromycin A and erythromycin enol ether.

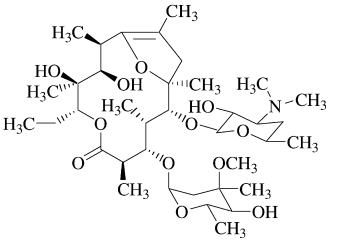
4. Conclusions

The results of this study demonstrate that the porous monodisperse poly(GMA–DVB) micro-



Erythromycin

	R_1	R_2	R_3
Erythromycin A	OH	OCH_3	CH_3
Erythromycin B	H	OCH_3	CH_3
Erythromycin C	OH	OH	CH_3
N-demethyl erythromycin A	OH	OCH_3	H



Erythromycin A enol ether

Fig. 7. Chemical structure of erythromycin A and its impurities.

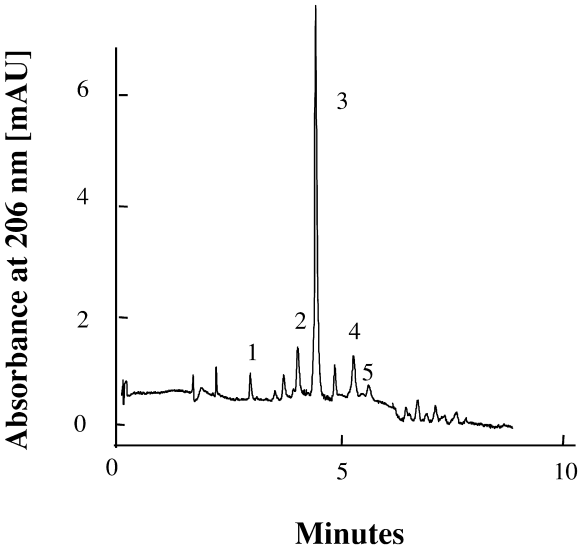


Fig. 8. Isocratic CEC of erythromycin A and its impurities. Cationic column, 30 cm (effective length 20 cm)×50 μm I.D.; mobile phase, 25% (v/v) acetonitrile and 25% (v/v) ethanol in 30 m*M* phosphate buffer, pH 8.0; applied voltage, −15 kV; detection, 206 nm. Sample: (1) *N*-demethylerythrymycin A, (2) erythromycin C, (3) erythromycin A, (4) erythromycin B, (5) erythromycin enol ether. Mobility of EOF measured with DMSO, $\mu_{eof} = 3.33\ 10^{-8}\ m^2\ s^{-1}\ V^{-1}$.

spheres are well suited for the separation of macrolide antibiotics by isocratic CEC with high efficiency and resolution. The chemistry in introducing quaternary ammonium groups to the chromatographic surface has proven to be effective in reducing the adverse electrostatic interactions between the macrolides and the stationary phase that are usually observed with silica-based packings. The results have shown that the introduction of novel stationary phases, which are tailor-made to the particular separation problems, can be advantageous and most likely necessary for the further development and applications of CEC.

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References

[1] H. Yamamoto, J. Baumann, F. Erni, J. Chromatogr. 593 (1992) 313.
[2] H. Rebscher, U. Pyell, Chromatographia 38 (1994) 737.
[3] N.W. Smith, M.B. Evans, Chromatographia 38 (1994) 649.
[4] M.M. Dittman, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[5] C. Fujimoto, Y. Fujise, E. Matsuzawa, Anal. Chem. 68 (1996) 2753.
[6] G. Choudhary, Cs. Horváth, J. Chromatogr. A 781 (1997) 161.
[7] C.G. Huber, G. Choudhary, Cs. Horváth, Anal. Chem. 69 (1997) 4429.
[8] A. Nahum, Cs. Horváth, J. Chromatogr. 203 (1981) 53.
[9] K.E. Bij, Cs. Horváth, W.R. Melander, A. Nahum, J. Chromatogr. 203 (1981) 65.
[10] D.P. Lee, J. Chromatogr. Sci. 20 (1982) 203.
[11] D. MacBlane, N. Kitagawa, J.R. Benson, Am. Lab. 2 (1987) 134.
[12] Y.-F. Maa, Cs. Horváth, J. Chromatogr. 445 (1988) 71.
[13] Y.-F. Maa, F.D. Antia, Z.E. Rassi, Cs. Horváth, J. Chromatogr. 452 (1988) 331.
[14] Y.-F. Maa, S.-C. Lin, Cs. Horváth, U.-C. Yang, D. Crothers, J. Chromatogr. 508 (1990) 61.
[15] J.C. Moore, J. Polym. Sci. A. 2 (1964) 835.
[16] T. Ellingsen, O. Aune, J. Ugestad, S. Hagen, J. Chromatogr. 535 (1990) 147.
[17] L.I. Kulin, P. Flodin, T. Ellingsen, J. Ugelstad, J. Chromatogr. 514 (1990) 1.
[18] K. Hosoya, J.M.J. Fréchet, J. Liq. Chromatogr. 16 (1993) 353.
[19] E.C. Peters, M. Petro, F. Švec, J.M.J. Fréchet, Anal. Chem. 70 (1998) 2288.
[20] I. Gusev, X. Huang, Cs. Horváth, J. Chromatogr. A 855 (1999) 273.
[21] S. Zhang, X. Huang, J. Zhang, Cs. Horváth, J. Chromatogr. A 887 (2000) 465.
[22] S. Zhang, J. Zhang, Cs. Horváth, J. Chromatogr. A 914 (2001) 189.
[23] Y. Liu, D.J. Pietrzyk, Anal. Chem. 72 (2000) 5930.
[24] K.E.J. Barrett, Br. Polym. J. 5 (1973) 259.
[25] A. Rembaum, R.S.K. Yen, D. Kempner, J. Ugelstad, J. Immunol. Methods 52 (1982) 341.
[26] J.W. Vanderhoff, M.S. El-Aasser, F.J. Micale, E.D. Sudol, C.M. Tseng, A. Silvanowicz, D.M. Kornfeld, F.A. Vicente, J. Dispos. Sci. Technol. 5 (1984) 231.
[27] S. Margel, E. Weisel, J. Polym. Sci. Polym. Chem. Ed. 22 (1984) 145.

[28] C.K. Ober, M.L. Hair, J. Polym. Sci. Polym. Chem. Ed. 25 (1987) 1395.
[29] R. Hu, V. L Dimonie, E.D. Sudol, M.S. El-Aasser, J. Appl. Polym. Sci. 55 (1995) 1411.
[30] J. Zhang, X. Huang, S. Zhang, Cs. Horváth, Anal. Chem. 72 (2000) 3022.
[31] K. Tsuji, J. Goetz, J. Chromatogr. 147 (1978) 359.
[32] T. Cachet, F. Quintens, E. Roets, J. Hoogmartens, J. Liq. Chromatogr. 12 (1989) 2171.
[33] J. Paesen, E. Roets, J. Hoogmartens, Chromatographia 32 (1991) 162.
[34] J. Paesen, D.H. Calam, J.H.M. Miller, G. Raiola, A. Rozanski, B. Silver, J. Hoogmartens, J. Liq. Chromatogr. 16 (1993) 1529.