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PREPARATIVE LOW-PRESSURE CHROMATOGRAPHY OF ANTIBIOTICS ON A COLUMN OF DIOL-BONDED SILICA GEL

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SUMMARY

Several schemes are presented which illustrate the general utility of column chromatography on diol-bonded silica gel for the purification of various antibiotics. The antibiotics include rosarimicin, rosarimicin dimethylacetal, everninomicin D, Juvenimicin A_4, coloradocin and the benzanthrins. A set of partition coefficients, determined in different two-phase solvent systems, for a given antibiotic or antibiotic complex, can be used in selecting appropriate solvent systems for this chromatography as well as for semi-preparative counter-current chromatography on the Ito coil planet centrifuge.

INTRODUCTION

In most cases, submerged fermentations produce only trace amounts of antibiotics (1–10 mg/10–12 g of total solute). Therefore, multi-step purification schemes are required to isolate these trace components. To insure maximum recoveries, it is important, whenever possible, to use gentle chromatographic techniques such as liquid chromatography (LC) on "low interactive" packings and counter-current chromatography (CCC). Within the past few years, there has been a resurgence in the use of CCC due to the commercialization of high-performance counter-current chromatographs[1]. One example, the Ito coil planet centrifuge (CPC)[2,3], has been extremely useful for the purification of natural products on a semi-preparative scale[4,5]. The sample capacity of the CPC is generally less than 500 mg. Therefore, it was decided to investigate the use of LC with CCC solvent systems (many developed for the CPC) as a preparative method for the purification of antibiotics.

The success of LC greatly depends upon the nature of the column. Various problems are associated with the use of silica gel, Celite and polysaccharides (*e.g.*, cellulose), all of which are classical supports of the stationary phase in partition systems. Silica gel forms a stable stationary phase with most solvent systems, but it is a strong adsorbent and may participate[6] in the separation process to the extent that chromatographic behavior and recovery of samples are affected. On the other hand, Celite and various polysaccharides are such weak adsorbents that they often bind stationary phases only weakly[7]. Sephadex® LH-20 (hydroxypropyl-bonded Se-

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phadex® G-25) has been widely used in liquid–liquid partition chromatography[8,9]. The major disadvantage of LH-20 is its unpredictable swelling in mixed solvents. The observation that the surface of diol-bonded silica gel (a 1,2-dihydroxypropyl-bonded support) resembles that of LH-20, prompted us to test it. Successful use of diol-bonded silica gel in biochemistry as a hydrophilic "low-interactive" support for affinity chromatography[10,11] and size-exclusion chromatography[12–14] provided an even greater impetus for us to explore it in preparative LC. Moreover, there has been at least one report on the advantages of using diol-bonded silica gel in the high-performance liquid chromatographic (HPLC) analysis of sterols[15].

Five schemes are presented in our paper to demonstrate the key role of LC on diol-bonded silica gel (diol-LC) in the purification of several different types of antibiotics. In each case, preparative diol-LC is shown as complementary to semi-preparative CCC. The purpose of our paper is to focus on the results of the diol-LC experiments and deal only peripherally with the other aspects of the purification schemes.

EXPERIMENTAL

Apparatus and equipment

Standard open glass columns were fed by gravity. Heavy walled glass columns with threaded upper and lower (PTFE screened) end plates (Key Scientific, Mount Prospect, IL, U.S.A.) were used for low-pressure chromatography. An empty column serving as the solvent reservoir was connected through PTFE tubing to the column containing the packing. The system was pressurized with air up to a maximum of 30 p.s.i. Fractions were collected using either an FC-220 collector (Gilson, Middleton, WI, U.S.A.) or a Micro Fractionator (Gilson).

The Ito coil planet centrifuge (PC, Potomac, MD, U.S.A.) with a 325-ml coil and accessories (as set up in our laboratory) has been described[4]. The Chromatotron[16] Model 7924T (Harrison Research, Palo Alto, CA, U.S.A.) operated with a rotor having a 1-mm layer of silica gel (Kieselgel 60 PF_{254} gipshaltig, EM Reagents), was used for preparative thin-layer chromatography (TLC).

Materials

Solvents were of either AR or HPLC grade. Diol-bonded silica gel and octadecyl-bonded (C_{18}) silica gel were 40 μm Sepralyte® (Analytichem, Harbor City, CA, U.S.A.). Sources of other packings were: Sephadex® LH-20 (Pharmacia, Piscataway, NJ, U.S.A.), Amberlite® XAD-7 and XAD-2 (Rohm & Haas, Philadelphia, PA, U.S.A.). XAD-2 was boiled repeatedly in methanol before it was used.

Procedures

Diol-bonded silica gel (150 g) in methanol was slurried into a column, 60 × 2.5 cm I.D., or, for larger samples, 60 × 5.0 cm I.D. (410 g of support), and equilibrated in 3 to 4 bed volumes of the less polar phase of a two-phase system. Ideally, the sample was applied in the less polar phase; if its solubility was inadequate, it was dissolved in a minimal volume of methanol or another appropriate solvent, and diluted with the less polar phase before it was applied to the column. Elution was carried out, first, with 2 to 3 bed volumes of the less polar phase; if the compound(s)

of interest failed to be eluted, chromatography was continued by eluting with 2 to 3 bed volumes of the more polar phase. Operating pressures were varied from 1–10 p.s.i. depending upon the desired flow-rate. The progress of the separation could be monitored with a suitable detector. However, for the examples in this paper, a disc agar diffusion assay[17] was carried out on the collected fractions. After several runs, the diol packing became colored. To remove these impurities it was necessary to wash the packing, first with several bed volumes of methanol and then with 0.1% acetic acid in methanol or 0.01% triethylamine in methanol. If it was necessary to use both an acid and a base treatment, these were separated by a methanol wash.

Partition coefficients, approximated by the agar diffusion method[4], were the basis for selection of two-phase solvent systems for LC or CPC purification of the antibiotics described in this paper. Partition coefficients were calculated as follows: for LC solvent systems, it was percent activity in more polar phase/percent activity in less polar phase; for CPC solvent systems it was percent activity in stationary phase/percent activity in mobile phase. A compilation of useful two-phase solvent systems listed in descending order of their ability to resolve polar compounds and ascending order to resolve less polar compounds is shown in Table I. Many of these systems are less than ideal for the CPC because the phases separate too slowly and form emulsions[18].

Antibiotic recoveries were monitored in activity units. The total antibiotic activity in the starting broth was assigned 100 activity units. This value was related to a zone of inhibition in an agar diffusion assay, for a given volume of broth. Antibiotic purity at the final stage was estimated by making a comparison between the ^{1}H nuclear magnetic resonance (^{1}H NMR) integrals of the various signals attributed to the antibiotic and the corresponding ^{1}H NMR integrals of the signals attributed to the impurities. All of the antibiotics were fully characterized by spectral means. Spectral data can be found in the references as indicated in the purification schemes below.

Purification schemes

(1) Separation and purification of aurodox. Supernatant broth [21 l, 100 activity units (u)] was applied to a gravity-fed (*g*-fed) column of XAD-7 and 100 u (10 g) was eluted with methanol. This material was triturated with the lower phase of solvent system 20 (Table I). Evaporation of the solvent gave an oily solid (100 u, 5 g). This was applied to a *g*-fed column of LH-20 in methanol and 100 u (1.5 g) was eluted from the column. This sample was purified further in two successive low-pressure diol-LC runs on 150 g of packing. The first with solvent system 19 afforded 75 u (200 mg) which was eluted in the lower phase and the second with solvent system 10 afforded 75 u (60 mg) which also was eluted in the lower phase. Final purification in the CPC (solvent system 19A) gave two fractions of aurodox[19] (Fig. 1) totaling 75 u: one 39 mg (80% pure by ^{1}H NMR) and the other 7 mg (>95% pure by ^{1}H NMR).

(2) Separation and purification of antibiotic complex OSA 68. Supernatant broth (25.5 l, 100 u) adjusted to pH 6.7 was extracted with *n*-butyl alcohol. Evaporation of the solvent left an oily residue (100 u, 6.6 g). The residue was separated into two activity bands, 75 u (2.0 g) and 25 u (2.6 g), on a *g*-fed column of LH-20 in methanol. The 75-u band was applied to a *g*-fed column of LH-20 equilibrated in the lower phase of solvent system 19 and 60 u (700 mg) was eluted in the same phase.

TABLE I

EXAMPLES OF TWO-PHASE SOLVENT SYSTEMS FOR LC AND CCC IN APPROXIMATE ORDER OF DECREASING POLARITY

Solvent system No.	*Composition*
1	*n*-Butyl alcohol–acetic acid–water (4:1:5)*
2	*n*-Butyl alcohol–*n*-propyl alcohol–water (2:1:3)*
3	*n*-Butyl alcohol–0.1 *M* ammonium acetate, pH 6.8 (1:1)
4	*n*-Butyl alcohol–0.1 *M* sodium chloride (1:1)
5	*n*-Butyl alcohol–water (1:1)*
6	*n*-Butyl alcohol–ethyl acetate–acetic acid–water (2:2:1:5)
7	*n*-Butyl alcohol–hexane–ethanol–acetic acid–water (3:2:1:1:5)*
8	Ethyl acetate–isopropyl alcohol–1.0 *M* potassium phosphate buffer, pH 7 (3:1:4)*,**
9	Ethyl acetate–chloroform–methanol–water (4:2:2:1)
10	Chloroform–methanol–water (1:1:1)**
11	Chloroform–isopropyl alcohol–methanol–water (5:1:6:4)
12	Chloroform–*n*-propyl alcohol–methanol–water (45:6:60:40)**
13	Benzene–ethanol–water (1:1:1)*
14	Benzene–chloroform–ethyl acetate–methanol–water (2:45:3:60:40)
15	Benzene–chloroform–methanol–water (5:5:7:2)
16	Tetrachloromethane–chloroform–methanol–water (5:5:8:2)***
17	Tetrachloromethane–methanol–water (5:4:1)
18	Hexane–ethyl acetate–methanol–water (18:42:30:30)***
19	Hexane–1,2-dichloroethane–chloroform–methanol–water (1:1:1:2:1)**
20	Hexane–methanol (2:1)
21	Hexane–ethyl acetate–nitromethane–methanol (8:2:2:3)

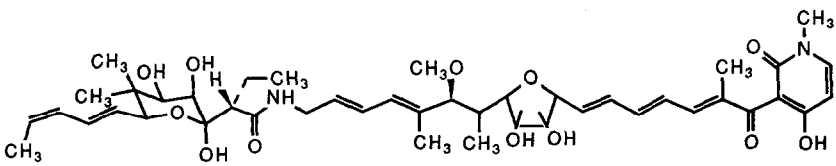
* Less than ideal for CCC.

** Modified systems: 8A (1:1:2), 10A (5:10:6), 10B (7:13:8), 12A (45:2:60:40), 12B (5:1:6:4), 19A (1:1:1:1:1).

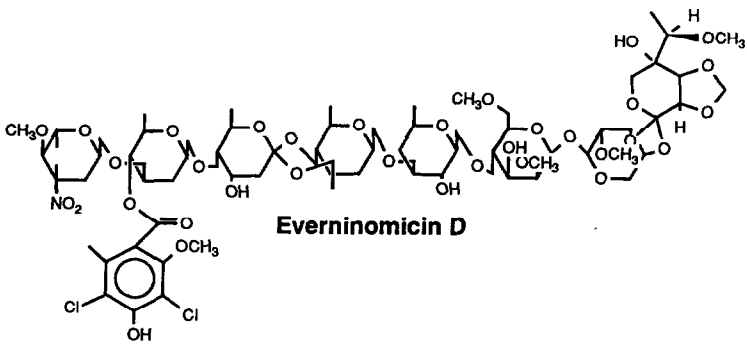
*** For modified systems see ref. 4.

Further purification of this sample by low-pressure diol-LC (150 g of packing) in solvent system 17 gave 45 u (80 mg) which was eluted in the upper phase. This was separated in the CPC (solvent system 10) into two bands 25 u (8 mg) and 20 u (3 mg). The 20-u band (85% pure by ^{1}H NMR) was everninomicin D[20] (Fig. 1). The 25-u band was resolved into two bioactive components in the chromatotron by eluting with trichloromethane–methanol (3:1). The faster moving component (90% pure by ^{1}H NMR) was rosarimicin dimethylacetal[21] (2 mg) and the slower one 90% pure by ^{1}H NMR) was rosarimicin[22] (4 mg); see Fig. 1.

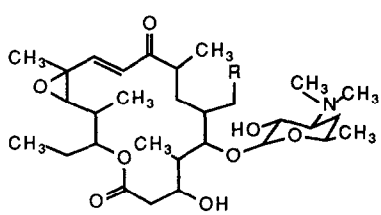
(3) Separation and purification of antibiotic complex OSA 89. Supernatant broth (17.2 l, 100 u) adjusted to pH 7 was applied to a *g*-fed column of XAD-2 and 100 u (11.6 g) was eluted with methanol. This sample was applied to a *g*-fed column of XAD-7 and 100 u (4.5 g) was eluted with a step gradient (in 25% increments) going from 100% water to 100% methanol. Chromatography of the 4.5 g sample (above) on LH-20 in methanol gave 80 u (960 mg) which was purified further on an LH-20 column equilibrated in the lower phase of solvent system 19 and 80 u (220 mg) was eluted in the same phase. Purification of this sample by low-pressure diol-LC (150 g of packing) in solvent system 17 gave 80 u (22 mg) which was eluted in



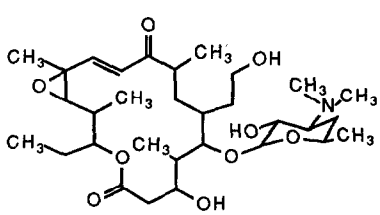
Aurodox



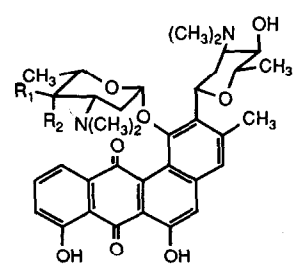
Everninomicin D



	R
Rosarimicin	CHO
Rosarimicin Dimethylacetal	CH(OCH3)OCH3



Juvenimicin A_4



	R_1	R_2
Benzanthrin A	H	OH
Benzanthrin B	OH	H

Fig. 1. Structures of antibiotics.

the upper phase. Final purification took place in the CPC in a system specifically developed for macrolide separations[23] [heptane–benzene–acetone–isopropyl alcohol–0.01 *M* phosphate buffer, pH 7 (25:50:15:10:25)]. Two activity bands (both 90% pure by ^{1}H NMR) were obtained; one was rosarimicin[22] (20 u, 1.5 mg) and the other was juvenimicin A_4[22] (40 u, 3.9 mg); see Fig. 1.

(4) Separation and purification of coloradocin. Supernatant broth (11.5 l, 100 u) adjusted to pH 4 was applied to a *g*-fed column packed with XAD-2 and 100 u (15.8 g) was eluted with methanol. This sample was chromatographed in two successive runs on a column of LH-20 in methanol giving 100 u (3.4 g). Further purification was carried out in two successive low-pressure diol-LC steps. The first, in solvent system 16 (on 410 g of packing), afforded 100 u (1.5 g) which was eluted in the lower phase. The second in solvent system 17 (on 150 g of packing) afforded 75 u (370 mg) which was eluted in the later fractions of lower phase and beginning fractions of upper phase. Chromatography of this sample in the CPC (solvent system 10) gave 75 u (167 mg). Final purification was accomplished with a low-pressure C_{18} column (40 μm packing) in a step gradient (25% increments) going from 100% water to 100% methanol to give 40 u (88 mg) of coloradocin (90% pure by ^{1}H NMR). The structural elucidation of coloradocin, a new antibiotic, will be presented elsewhere[24].

(5) Separation and purification of benzanthrins A and B. Supernatant broth (73 l, 100 u) adjusted to pH 8.5 was extracted with dichloromethane. Evaporation of the solvent left a dark red residue (100 u, 7.7 g). The residue was separated into two activity bands on a *g*-fed column of LH-20 in methanol. One band (60 u, 4.7 g) was found to be enriched in benzanthrin A and the other (20 u, 1.3 g) was found to be enriched in benzanthrin B. Each was purified further by low-pressure diol-LC in solvent system 16 followed by elution with 0.01% triethylamine in methanol. The larger sample on 410 g of packing gave 50 u (620 mg) and the smaller sample on 150 g of packing gave 20 u (232 mg). Final purification of the 50-u sample in the CPC with solvent system 16 (modified to 4:1:4:1) gave 214 mg of benzanthrin A[25] (Fig. 1) and 10 mg of benzanthrin B[25] (Fig. 1) totaling 40 u. Final purification of the 20-u sample in the CPC as above gave 53 mg of benzanthrin A and 33 mg of benzanthrin B totaling 20 u. Purity (^{1}H NMR) of the final benzanthrin A samples was >95% and for the benzanthrin B samples 80–90%.

RESULTS AND DISCUSSION

Diol-LC on the 1.5 g sample which was eluted from LH-20 (purification scheme 1), demonstrates the utility of this method for the purification of aurodox. In solvent system 19 the antibiotic activity (partition coefficient = 0.25) greatly favored the less polar phase. In this system, aurodox exhibited low retention on the column while the impurities were more highly retained. This resulted in about an eight-fold purification (calculated from sample weight reduction) and only a 25% loss of aurodox (calculated from loss of activity units). Unless a certain selectivity was desired, as in the above example, it was found useful to choose a solvent system in which the antibiotic activity had a partition coefficient of 1 to 1.5. On this basis, solvent system 10 was chosen for the second diol-LC experiment (purification scheme 1). This solvent system achieved an additional three-fold purification with no loss of antibiotic.

Purification schemes 2 and 3 involved the use of solvent system 17 in the diol-LC step for the purification of two antibiotic complexes containing macrolides. In the case of complex OSA 68, a nine-fold purification was achieved. A ten-fold purification was achieved with solvent system 17 in the case of OSA 89 complex. Elution of the antibiotics in the diol-LC step in each scheme required changing to the more polar phase. Selection of solvent system 17, which gave partition coefficients of 4 and 3 for OSA 68 and 89, respectively, was intended to achieve greater selectivity, in each case, than what resulted with the prior LH-20 columns in which early elution of the antibiotics occurred in the less polar phase of solvent system 19. It is noteworthy that system 19 is one of the few organic-aqueous systems which can be used with LH-20, without significant expansion of the bed, in the course of the elution from less polar to more polar phase.

Purification scheme 4 summarizes the steps in the isolation and purification of the antibiotic coloradocin. Two preparative diol-LC columns were employed in succession to give approximately a nine-fold overall purification and only a 25% loss of activity. The antibiotic activity had a partition coefficient of 0.125 in the first system. The lower (less polar) phase of this system eluted the antibiotic early, leaving colored impurities retained on the column. A partition coefficient of 2 was determined for antibiotic activity in the second system. Coloradocin was recovered in the later stages of lower (less polar) phase elution and early stages of upper (more polar) phase elution.

Purification scheme 5 has two parallel diol-LC steps (one on a benzanthrin A enriched fraction and the other on one enriched in benzanthrin B). In both cases initial recovery of activity units was low, even after elution with several bed volumes of the upper (more polar) phase. Good recovery was obtained only after elution with 0.01% triethylamine in methanol (column wash conditions). This result was unexpected, as the partition coefficient for the antibiotic activity was close to unity in solvent system 16. The benzanthrins each have two strongly basic dimethylamino groups which may have interacted with exposed silanol groups on the support. However, this effect was not seen with the macrolide antibiotics which possess one similar basic group. The diol-bonded silica gel used in purification scheme 5 had been the support for several previous chromatographies. This may have resulted in the loss of some of the bonded phase. In spite of this, about an eight-fold purification was achieved in the two parallel runs.

CONCLUSION

It is likely that CCC will play a greater role in solving the ever increasing number of difficult purification problems which are commonplace in the field of biotechnology and the pharmaceutical industry. The ease in which solvent systems can be selected, the large number of solvent systems available (the list in Table I is by no means comprehensive), and the excellent recoveries of valuable natural products makes this technique attractive. However, it has been limited by a smaller sample capacity and certain restrictions on choices of solvent systems (depending upon the CCC method). The latter problem has been addressed by the use of a prototype, temperature controlled, horizontal flow-through CPC[26]; the former requires expensive custom-made counter-current chromatographs (with up to kg capacities) such as the one offered by Sanki Instruments.

Diol-LC using CCC solvent systems represents an alternative method, limited only by column size, for the purification of larger samples. Although only five examples using diol-LC are described in our paper, we since have solved, in a similar manner, several other purification problems. The value of this hydrophilic but "low-interactive", pressurizable packing, already has been demonstrated in size-exclusion and affinity chromatography. An added value may come from its potential application to process scale LC.

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REFERENCES

1 N. B. Mandava, Y. Ito and W. D. Conway, *Am. Lab. (Fairfield, Conn.)*, Oct. (1982) 62 (part 1); Nov. (1982) 48 (part 2).
2 Y. Ito, *J. Chromatogr.*, 192 (1980) 75.
3 Y. Ito, *Anal. Biochem.*, 102 (1980) 150.
4 G. M. Brill, J. B. McAlpine and J. E. Hochlowski, *J. Liq. Chromatogr.*, 8 (1985) 2259.
5 W. D. Conway and Y. Ito, *LC, Liq. Chromatogr. HPLC Mag.*, 2 (1984) 368.
6 J. P. Crombeen, S. Heemstra and J. C. Kraak, *J. Chromatogr.*, 282 (1983) 95.
7 J. J. Kirkland, *Modern Practices of Liquid Chromatography*, Wiley, New York, 1971, p. 183.
8 Pamphlet: Sephadex® LH-20, Pharmacia Fine Chemicals, Uppsala, Sweden, 1978, p. 12.
9 J. V. Jizba, V. Přikrylová and H. Lipavská, *J. Chromatogr.*, 329 (1985) 193.
10 R. R. Walters, *J. Chromatogr.*, 249 (1982) 19.
11 R. R. Walters, in I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition, Proceedings of the 5th International Symposium, Orlando*, Academic Press, New York, 1983, p. 261.
12 P. Roumeliotis and K. K. Unger, *J. Chromatogr.*, 185 (1979) 445.
13 D. P. Herman, *Diss. Abstr. Int. B*, 4 (1983) 3967 (12, Pt. 1).
14 R. W. Stout and J. J. DeStefano, *J. Chromatogr.*, 326 (1985) 63.
15 E. H. J. M. Jansen, H. Van Blitterswijk, P. W. Zoontjes, R. Both-Miedema and R. W. Stephany, *J. Chromatogr.*, 347 (1985) 375.
16 F. Orsini and L. Verotta, *J. Chromatogr.*, 349 (1985) 69.
17 K. Kavanagh, *Analytical Microbiology*, Academic Press, New York, Vol. I, 1963, p. 72; Vol. II, 1972, p. 292.
18 Y. Ito and W. D. Conway, *J. Chromatogr.*, 301 (1984) 405.
19 H. Maehr, M. Leach, T. H. Williams and J. F. Blount, *Can. J. Chem.*, 58 (1980) 501.
20 A. K. Ganguly, O. Z. Sarre, D. Greeves and J. Morton, *J. Am. Chem. Soc.*, 97 (1975) 1982.
21 H. Reimann, R. S. Jaret, M. M. Nafissi-Varchi, A. K. Ganguly and O. Sarre, *Ger. Pat.*, DE 2607459 (1976).
22 A. Kinumaki, K.-I. Herada, T. Suzuki, M. Suzuki and T. Okuda, *J. Antibiotics*, 30)1977) 450.
23 J. B. McAlpine, L. Katz, D. N. Whittern, D. P. Brown, J. S. Tuan and K. D. Grebner, *Program and Abstracts of the 26th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 29, 1986, New Orleans*, p. 138.
24 R. R. Rasmussen, M. H. Scherr, J. B. McAlpine, D. N. Whittern, A. M. Buko, H. J. R. Weintraub and E. T. Olejniczak, *Program and Abstracts of the 26th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 29, 1986, New Orleans*, p. 264.
25 R. Rasmussen, M. Nuss, M. Scherr, S. Mueller, L. Mitscher and J. McAlpine, *Program and Abstracts of the 25th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 29, 1985, Minneapolis*, p. 799; *J. Antibiotics*. in press.
26 M. Knight, Y. Ito, J. L. Sandlin and A. M. Kask, *J. Liq. Chromatogr.*, 9 (1986) 791.