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SEPARATION OF THE SIDE-CHAIN DIASTEREOISOMERS OF PENICILLINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of the side-chain diastereoisomers of phenethicillin, propicillin, clometocillin, carbenicillin, ticarcillin, ampicillin, amoxicillin and azidocillin by high-performance liquid chromatography is described. For the first five compounds the diastereoisomer ratio has been determined in several samples, and for the first three products, these ratios are compared with those obtained by gas–liquid chromatography. The separations were carried out on a 25 cm × 4.6 mm I.D. column packed with Zorbax C_8, using mixtures of methanol–water–5% 0.2 *M* phosphate buffer pH 7.0 as the mobile phase. For carbenicillin and ticarcillin the epimerization of the diastereoisomers was followed by high-performance liquid chromatography.

INTRODUCTION

Commercial semisynthetic penicillins such as phenethicillin (I), propicillin (II), clometocillin (III), carbenicillin (IV) and ticarcillin (V) (see Fig. 1) are mixtures of two diastereoisomers since they are prepared by reaction of 6-aminopenicillanic acid (6-APA) with a racemic side-chain derivative. It is known that the antimicrobial activities of such diastereoisomers are not identical[1–4]. For I, II and III the difference in activity is small and the use of a pure diastereoisomer is not necessary[2–4]. The biological activities of the diastereoisomers of IV have been reported to be equivalent, although the similarity of the results was probably due to rapid isomerization[5]. Isomerization can also be supposed to occur in V, which is structurally closely related to IV. For some penicillins, such as ampicillin (VI), amoxicillin (VII) and azidocillin (VIII), the difference in activity is so important that only the more active diastereoisomer is prepared.

Several analytical procedures have been used to estimate the diastereoisomeric content in commercial penicillins. The isomer content of I is determined by a tedious microbiological assay according to the Code of Federal Regulations[6]. The British Pharmacopoeia 1973 prescribes optical rotation limits for I and II[7], but this procedure does not give precise information on the diastereoisomeric ratio. Proton (PMR) and ^{13}C (CMR) magnetic resonance spectroscopy can be used for the estima-

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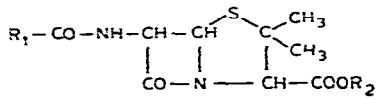


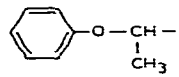
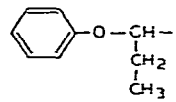
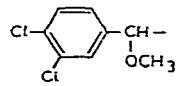
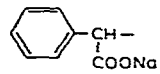
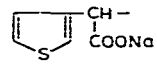
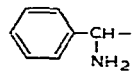
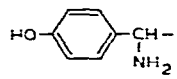
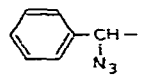
		R_1	R_2
I	Phenethicillin		K
II	Propicillin		K
III	Clometocillin		H
IV	Carbenicillin		Na
V	Ticarcillin		Na
VI	Ampicillin		H, Na
VII	Amoxicillin		H, Na
VIII	Azidocillin		K

Fig. 1. Penicillin structures.

tion of the diastereoisomeric ratio in I[8,9]. PMR allows good correlations in the region of 55–75% L-isomer, but leads to underestimations for higher contents. The CMR method is accurate over a wider range. Gas–liquid chromatography (GLC) has been described for I, II and III[1,10,11]. GLC separation of the diastereoisomers of α-hydroxybenzylpenicillin has also been described[12]. Volatile silyl derivatives or methyl esters have to be prepared for GLC. High-performance liquid chromatography (HPLC), where derivatization is not required, has been reported for the separation of the diastereoisomers of I and VI[13], of IV[10,14,15] and of V[14]. HPLC has also been described for the separation of the side-chain diastereoisomers of cefalexin[13], of 7-ureidoacetamidocephalosporins[16] and of moxalactam, which is a new cephalosporin with a carbenicillin-like α-carboxy-*p*-hydroxyphenylacetamido side-chain[17]. HPLC has also been proven useful in the separation of diastereoisomers of benzylpenicilloic

acid and of phenoxymethylpenicilloic acid[18–20]. In these products, stereoisomerism occurs in the penam part of the molecule.

In this paper we report the HPLC separation of the side-chain diastereoisomers of I–VIII using the same column (Zorbax C_8) and one mobile phase (methanol–water–5% 0.2 *M* phosphate buffer pH 7.0), the methanol content being changed from 5 to 50% depending on the penicillin examined. The diastereoisomeric content of several samples is reported and the data for I, II and III are compared with those obtained by GLC.

EXPERIMENTAL

Samples

Samples were of different origins: Bayer, Leverkusen, G.F.R.; Beecham, Heppignies, Belgium and Worthing, Great Britain; Pfizer, Brussels, Belgium; Smith-Kline-RIT, Genval, Belgium; Gist-Brocades NV, Delft, The Netherlands. Some reference samples were also available: L(−)-II and D(+)-II from Bayer, and L(+)-VII from Beecham; L(+)-III and D(−)-III by synthesis[2]; DL-VI by catalytic hydrogenation of DL-VIII[21–23]; DL-VII by mixing the pure diastereoisomers; DL-VIII by synthesis[21]. The prefixes D, L and DL used in this paper indicate only the stereochemistry of the side-chain.

Apparatus

The HPLC apparatus consisted of a Waters pump Model 6000A (Waters Assoc., Milford, MA, U.S.A.), a Valco injector Model CV-6-UPHa-N60 equipped with a 20-μl loop (Valco, Houston, TX, U.S.A.), a Waters detector Model 440 (254 nm) set at 0.05 a.u.f.s., a Pye Unicam integrator Model DP88 (Pye Unicam, Cambridge, Great Britain) and a Kipp & Zonen recorder Model BD40 (Kipp & Zonen, Delft, The Netherlands). A 25 cm × 4.6 mm I.D. column, packed with Zorbax C8 (Du Pont, Wilmington, DE, U.S.A.) was used for the analytical work. Preparative work was carried out on a 25 cm × 9 mm I.D. column, packed with the same material. The Varian Model 940 (Varian, Palo Alto, CA, U.S.A.) gas chromatograph was equipped with 5 ft. × 1/8 in. O.D. columns and an FID detector.

HPLC column packing procedure, reagents, mobile phases and operating conditions

Packing material (3.2 g) was suspended in 15 ml of carbon tetrachloride, sonicated for 3 min and packed into the column, fixed to a 10-cm precolumn, using a Haskel pump DSTV-122 (Haskel, Burbank, CA, U.S.A.). The inlet pressure was 5 bar and methanol–water (80:20) was used as the pressurizing liquid. All solvents were of reagent grade and were distilled in glass before use. The preparative column was packed in a similar manner. Methanol, >99% (Aldrich Europe, Beerse, Belgium), and distilled water were glass-distilled. Potassium monohydrogen phosphate and dihydrogen phosphate pro analysi (E. Merck, Darmstadt, G.F.R.) were used to prepare a 0.2 *M* buffer pH 7.0. The mobile phase consisted of methanol–water mixtures containing 5% (v/v) overall of phosphate buffer. The methanol content varied between 5 and 50% (v/v) depending on the penicillin examined. Mobile phases were degassed by sonication. The flow-rate was set at 1.0 ml/min and the paper speed at 5 mm/min. A 20-μl volume of mobile phase, containing 25–100 μg of sample, depend-

ing on the penicillin examined, was injected. The integrator was set following the manufacturers instructions. All separations were carried out at room temperature (about 20°C).

GLC operating conditions

The results reported were obtained on a column packed with 3% OV-17, the nitrogen flow-rate was 15 ml/min and the oven temperature between 210°C and 235°C, depending on the penicillin examined. The free acids of the penicillins were extracted into diethyl ether after acidification of an aqueous solution with phosphoric acid. Methyl esters of the penicillins were prepared by adding ethereal diazomethane. 2-μl volumes were injected. Other column packings, such as 3% OV-1, 1% OV-225 and 3% OV-225, were also tried.

RESULTS AND DISCUSSION

Separation of the diastereoisomers in penicillins I–V

Good separation of the side-chain diastereoisomers present in commercial penicillins I–V was obtained with Zorbax C8 reversed-phase packing material and water–methanol–5% 0.2 *M* phosphate buffer pH 7.0 mixtures as the mobile phase. Fig. 2 only shows the penicillin peaks. The diastereoisomeric penicilloic acids, which were present in some samples and were also separated, are not shown.

The chromatographic conditions are not very critical. After adaptation of the methanol–water ratio in the mobile phase, some of the samples were also successfully analysed on a LiChrosorb RP-8 column (E. Merck). Reversed-phase materials with longer chains such as C_{18} also can be used provided a higher amount of organic modifier is added to the mobile phase. Methanol can be replaced by the more expen-

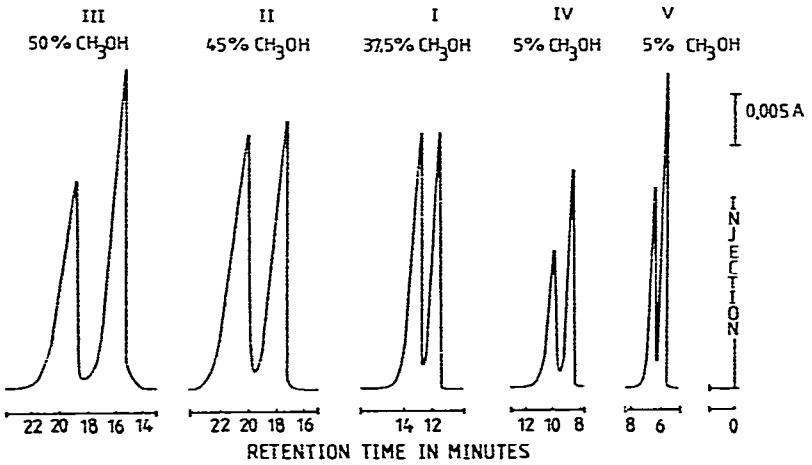


Fig. 2. Separation of the diastereoisomers of commercial penicillins on Zorbax C_8 with methanol–water–5% 0.2 *M* phosphate buffer pH 7.0 mixtures as the mobile phase, the methanol content being specified on the chromatograms. Column: 25 cm × 4.6 mm I.D. Flow-rate: 1 ml/min. Detection: 254 nm and 0.05 a.u.f.s.; paper speed, 5 mm/min. Identity of penicillins as in Fig. 1.

sive acetonitrile, but a lower concentration should be used. Small differences in pH of the mobile phase have little influence upon the separation.

Table I shows the diastereoisomer ratio of several samples of penicillins I–V, as determined by HPLC. For penicillins I–III the ratio was also determined by GLC. For these penicillins the D-isomer is eluted before the L-isomer, except in GLC of III, where the converse is true. No GLC results are reported for IV and V, since these penicillins were not stable under the conditions used.

Peak areas were measured and the diastereoisomer ratio was expressed as a percentage of the sum of the areas found for the two diastereoisomers. The value, $\bar{x}$, is the mean, calculated for the results of n injections of the same solution. Often the sample was analysed several times, on different days, by different operators and even on different columns of the same type. This explains why there is some variation between the standard deviations (S.D.) calculated for the different analyses.

The HPLC and GLC results obtained for I correspond quite well. Since the pure D- and L-isomers were not available, the identity of the GLC peaks was deduced from GLC literature results[1]. In HPLC the identity of the peaks was deduced by analogy with GLC results, based on peak areas, and confirmed by analogy with the closely related II, where the D-isomer is also eluted first. The results show that the isomer content can vary between batches supplied by different manufacturers. Sample I-F 1 only contains about 35% of the D-isomer, which is the less active[3]. This isomer has also been mentioned in the literature, probably erroneously, as the more active one[13].

The HPLC and GLC results for II also match very well. The L(−)-II and D-(+)-II isomers were available, which made peak identification easy. The results for sample II-A 3 are reproducible when the same solution is injected repeatedly, but for different solutions of the same sample very different diastereoisomer ratios are recorded. Since the ratio does not change when the solutions are kept for 1 day, it must be concluded that the sample is highly inhomogeneous. For samples II-E 1 and II-E 2 the manufacturer reports a content of respectively 46.2% and 45.9% of the D-isomer, as determined by HPLC, which is in good agreement with our results. For sample II-A 1 the manufacturer mentions a content of 46% of the D-isomer, which is higher than the 36% reported here. The method used by the manufacturer was not specified, but it probably was not a chromatographic one. It is observed that the ratios not only vary between batches from different manufacturers, but also for manufacturer A there is a variation between samples.

The availability of L(+)-III and D(−)-III isomers allowed peak identification. The elution order in HPLC and GLC is different, which must be kept in mind in the interpretation of the results, since all values are given for the first peak. The GLC and HPLC values do not match very well, probably due to some decomposition in GLC. Indeed, it was observed that the baseline was not reached between the solvent peak and the sample peaks, although the retention time was high. If it is accepted that during GLC the D-isomer is less stable, the difference in contents obtained in HPLC and GLC can be explained. Sample III-F 1 was obtained by synthesis in our laboratory[2].

The response of the FID detection system is not likely to be different for the diastereoisomers of the same penicillin. Since the GLC and HPLC results generally are in good agreement, it is also likely that the response from the UV detection is not

TABLE I

SEPARATION OF THE DIASTEREOISOMERS OF COMMERCIAL PENICILLINS

R_s = Resolution; t_R = retention time of second peak in min; $\bar{x}$ = relative amount of the diastereoisomer eluted first, in per cent; n = number of experiments carried out on the same solution; S.D. = standard deviation.

Penicillin	*Manufacturer and sample no.*	*HPLC*							*GLC*						
		% Methanol in mobile phase	*Isomer eluted first*	R_s	t_R	$\bar{x}$	n	*S.D.*	*Column temp. (°C)*	*Isomer eluted first*	R_s	t_R	$\bar{x}$	n	*S.D.*
I Phenethicillin	C 1	37.5	D	1.17	13	45.9	3	0.78	210	D	1.23	55	46.5	3	0.32
						46.3	5	0.35							
						44.6	3	0.30							
	F 1					33.3	3	0.25					34.8	3	0.15
						35.4	3	0.25							
						33.9	3	0.10							
	B 1					43.9	10	0.48							
	B 2					42.9	3	0.03							
II Propicillin	E 1	45.0	D	1.15	20.5	48.6	3	0.25	210	D	1.15	39	47.2	3	0.64
	E 2					46.3	4	0.19					47.2	3	0.91
						45.8	5	0.15							
						44.7	4	0.06							
	A 1					36.2	3	0.46					36.4	2	0.21
						35.7	4	1.58							
	A 2					47.1	4	1.16					46.7	3	0.15
						45.8	3	0.52							

	A 3					31.2	7	1.34					34.7	3	0.36
						23.8	3	0.55					47.3	2	0.70
						25.2	3	0.27							
						36.2	3	1.08							
						26.2	3	0.78							
						29.1	3	0.52							
						42.0	2	0.12							
III Clometocillin	F 1	50.0	D	1.62	19	55.7	4	0.12	235	L	2.16	75	49.9	7	1.25
						55.6	5	0.31					49.3	3	1.06
	D 1					57.5	7	0.97					48.5	6	0.85
						58.5	7	0.27					48.1	5	0.90
IV Carbenicillin	B 1	5.0	*	1.40	10	52.0	5	0.26							
	B 2					54.0	5	0.21							
	B 3					54.8	3	0.17							
						53.9	4	0.10							
V Ticarcillin	B 1	5.0	*	1.14	6.5	55.9	4	0.52							
						55.5	3	0.17							
	B 2					55.7	4	0.36							
						55.7	4	0.10							

* Stereochemistry of this isomer is unknown.

TABLE II

SEPARATION OF DIASTEREOISOMERS BY GAS–LIQUID CHROMATOGRAPHY

t_R = Retention time of second peak in min.

Penicillin	*Column packing and nitrogen flow-rate*				
	3% OV-17 15 ml/min	*3% OV-17 60 ml/min*	*3% OV-1 60 ml/min*	*1% OV-225 60 ml/min*	*3% OV-225 60 ml/min*
I Phenethicillin					
Oven temperature (°C)	210	210	200	202	258
R_s	1.23	1.22	0.96	1.22	1.20
t_R	55	37	20	27.5	32
II Propicillin					
Oven temperature (°C)	210	210	200	202	235
R_s	1.15	1.14	0.87	1.33	1.24
t_R	39	23	24	31	29
III Clometocillin					
Oven temperature (°C)	235	249	239	230	
R_s	2.16	2.30	2.22	3.1	
t_R	75	26	14	33	

very different. This was verified experimentally for the diastereoisomers of II and VII, no noticeable differences being observed in their UV spectra. The absorbances at 254 nm differed by less than 4%, which could also be due to small differences in the purity of the samples. Since peak areas, but not peak heights, were measured, the influence of the retention time on the diastereoisomer ratio was neglected.

The peaks of penicillins IV and V could not be identified since the pure isomers were not available. The diastereoisomer ratio does not vary between the samples examined. In the literature no precise information was found concerning the activity of the isomers and the rate of their isomerization[15]. Therefore it was decided to separate the diastereoisomers of IV and V on a semi-preparative scale and to follow their isomerization. The results will be discussed in the last part of this paper.

A sample of carfecillin, which is the phenyl ester of the side-chain carboxylic

TABLE III

SEPARATION OF COMMERCIAL PENICILLINS FROM THEIR SIDE-CHAIN DIASTEREOISOMERS

t_R = Retention time of second peak in min.

Penicillin	*% Methanol in mobile phase*	*Isomer eluted first*	R_s	t_R
VI Ampicillin	25	L	1.90	12
VII Amoxicillin	10	D	1.50	12.5
VIII Azidocillin	40	D	0.74	12

acid of IV, was also examined by HPLC. Although the methanol content and the pH of the mobile phase were varied, only one peak was observed in all chromatograms obtained. The presence of two diastereoisomers in the solution injected was confirmed by PMR spectrometry. Fresh solutions of carfecillin, on the contrary, contain only one isomer[24,25]. It is therefore concluded that the carfecillin diastereoisomers cannot be separated by the HPLC system described here. When carfecillin was analysed by GLC, decomposition, as already mentioned for IV, was observed.

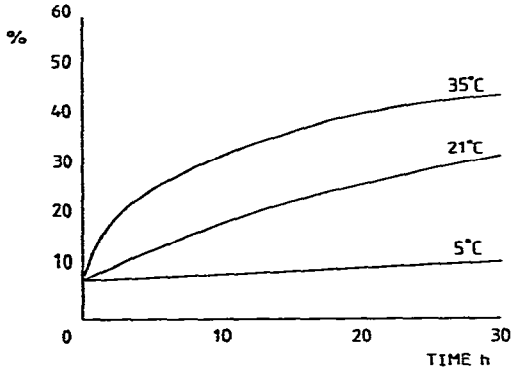
For GLC of penicillins I–III several chromatographic conditions were examined. The results are summarized in Table II. The chromatographic conditions are briefly mentioned as well as the resolution, R_s, and the retention time, t_R, for the second peak. The results reported in the first and second columns of Table II were obtained on different 3% OV-17 columns. III was not analyzed on the 3% OV-225 column since the retention time would have been too high. It can be concluded that, for the analysis of penicillins, HPLC is preferred to GLC. HPLC is faster, easier, since derivatization is not required, and more accurate, because the risk of decomposition during sample preparation is reduced.

Separation of side-chain diastereoisomers in penicillins VI–VIII

Although the commercial penicillins VI, VII and VIII are intended to contain only the more active D-isomer, a control of the stereochemical purity by HPLC seemed desirable. The results are summarized in Table III. The less active isomer was not detected in the commercial samples. It should be noted that for ampicillin the elution order is different: the L-isomer is eluted first. It is known from literature results[13] that the L-isomer of cefalexin is also eluted first; the same order of elution was observed in our laboratory for DL-cefadroxil, which was obtained by synthesis. These products are the cephalosporin analogues of VI and VII respectively.

Preparative separation of the carbenicillin and ticarcillin diastereoisomers and study of their isomerization

Preparative HPLC work was carried out under the same conditions as men-



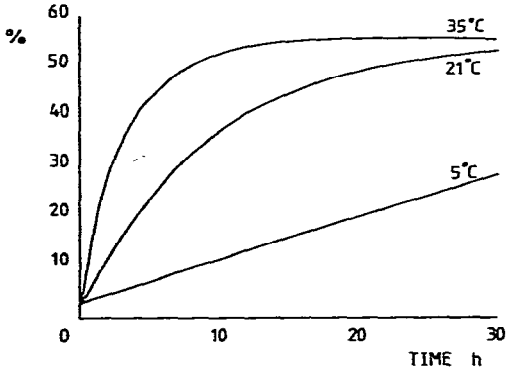
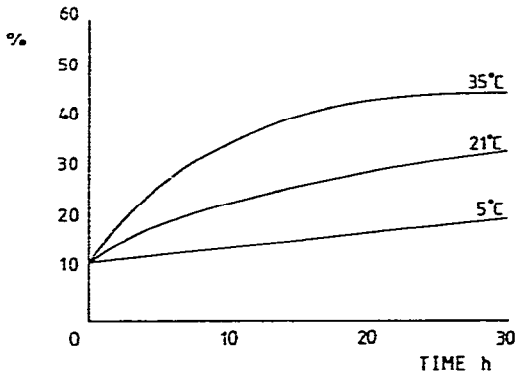


Fig. 3. Epimerization of carbenicillin IV-1 to IV-2 at three temperatures. Per cent of IV-2 is shown.

Fig. 4. Epimerization of carbenicillin IV-2 to IV-1 at three temperatures. Per cent of IV-1 is shown.



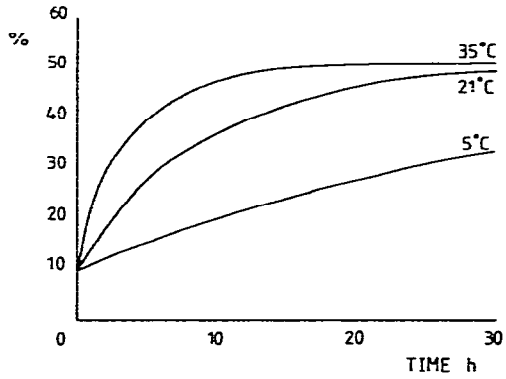


Fig. 5. Epimerization of ticarcillin V-1 to V-2 at three temperatures. Per cent of V-2 is shown.
Fig. 6. Epimerization of ticarcillin V-2 to V-1 at three temperatures. Per cent of V-1 is shown.

tioned for the analysis of IV and V, except that a 25 cm × 9 mm I.D. column was used and a flow-rate of 4 ml/min. 0.8-mg quantities were injected repeatedly and the fractions containing the diastereoisomers were collected and immediately cooled in ice–water. The solutions were concentrated at reduced pressure on a rotary evaporator, with the water-bath at room temperature and the cooling liquid at −30°C. In this way four aqueous solutions, buffered at pH 7.0 with phosphate from the mobile phase, were obtained: IV-1, IV-2 and V-1, V-2. The arabic numbers refer to the order of elution. The purity of the isomers was checked by HPLC immediately after concentration: IV-1 contained 7.6% of IV-2, and IV-2 2.8% of IV-1; V-1 contained 11.6% of V-2, and V-2 9.7% of V-1. The isomer content of the original solutions is reported in Table I. The four solutions were divided into three equal parts which were stored at 5°C, 21°C and 35°C respectively. The stability of the solutions was followed during 30 h on the analytical HPLC column. The results are shown in Figs. 3–6. It is clear that the first peaks, IV-1 and V-1, are more stable and therefore probably have the same stereochemistry. The influence of the temperature on the rate of isomerization is important. At 35°C, IV-1 and V-1 reach equilibrium after about 30 h, IV-2 and V-2 after about 15 h. This means that it is impossible to determine the activity of the isolated diastereoisomers by the usual microbiological method.

Recently[17] it was reported that the *R*-epimer of moxalactam, a cephalosporin with a 2-carboxy-2-(4-hydroxyphenyl)acetamido side-chain, is three times more active than the *S*-epimer; also that the excretion time of the two epimers is different. The side-chain of moxalactam only differs from that of carbenicillin by the presence of the 4-hydroxy group. Thus, it is possible that the activities of the two diastereoisomers of carbenicillin and ticarcillin are different, although it is not certain whether this would have a therapeutic significance.

The present results demonstrate that HPLC is an efficient and rapid method for the determination of the diastereoisomer ratio of penicillins having a chiral centre in the side-chain.

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REFERENCES

1 H. Vanderhaeghe, E. Evrard and M. Claesen, *Ber. XXIII Int. Kongr. der Pharmaz. Wissensch., Münster, Sept. 9–14, 1963*, 1964, p. 405.
2 H. Vanderhaeghe, P. Van Dijck, M. Claesen and P. De Somer, *Antimicrob. Agents Chemother.*, (1961) 581.
3 A. Gourevitch, G. A. Hunt and J. Lein, *Antibiotics Annual 1959–1960*, Medical Encyclopedia, New York, 1959–1960, p. 111.
4 E. Auhagen, Ch. Gloxhuber, G. Hecht, Th. Knott, E. Rauenbusch, J. Schawartz, J. Schmid, W. Scholtan and A. M. Walter, *Arzneim.-Forsch.*, 12 (1962) 751.
5 K. Butler, A. R. English, V. A. Ray and A. E. Timreck, *J. Infect. Dis.*, 122 (1970) Suppl. 1.
6 *Code of Federal Regulations*, Title 21440.82, U.S. Government Printing Office, Washington, DC, 1975.
7 *British Pharmacopoeia 1973*, H.M. Stationery Office, London, 1973.
8 W. L. Wilson, H. W. Avdovich, D. W. Hughes and G. W. Buchanan, *J. Pharm. Sci.*, 66 (1977) 1079.
9 K. Yamaoka, S. Narita, T. Nakagawa and T. Uno, *J. Chromatogr.*, 168 (1979) 187.
10 E. Evrard, M. Claesen and H. Vanderhaeghe, *Nature (London)*, 201 (1964) 1124.
11 Ch. Hishta, D. L. Mays and Michael Garofalo, *Anal. Chem.*, 43 (1971) 1530.
12 E. Roets, A. Vlietinck and H. Vanderhaeghe, *J. Antibiot.*, 30 (1977) 847.
13 F. Salto, *J. Chromatogr.*, 161 (1978) 379.
14 V. Das Gupta and K. R. Stewart, *J. Pharm. Sci.*, 69 (1980) 1264.
15 Ph. A. Twomey, *J. Pharm. Sci.*, 70 (1981) 824.
16 M. G. Young, *J. Chromatogr.*, 150 (1978) 221.
17 R. Konaka, K. Kuruma, R. Nishimura, Y. Kimura and T. Yoshida, *J. Chromatogr.*, 225 (1981) 169.
18 F. Nachtmann and K. Gstrein, *Int. J. Pharm.*, 7 (1980) 55.
19 F. Nachtmann, *Chromatographia*, 12 (1979) 380.
20 J. M. Blaha, A. M. Knevel and S. L. Hem, *J. Pharm. Sci.*, 64 (1975) 1384.
21 B. Ekström, A. Gomez-Revilla, R. Mollberg, H. Thelin and B. Sjöberg, *Acta Chem. Scand.*, 19 (1965) 281.
22 H. Vanderhaeghe, M. Claesen, P. Van Dijck and G. Parmentier, *Belg. Pat.*, 608,669 (1961).
23 H. Vanderhaeghe, M. Claesen, P. Van Dijck and G. Parmentier, *Brit. Pat.*, 918,169 (1963).
24 J. P. Clayton, M. Cole, S. W. Elson, K. D. Hardy, L. W. Mizen and R. Sutherland, *J. Med. Chem.*, 18 (1975) 172.
25 P. A. C. Gane, M. O. Boles and A. E. Bird, *Acta Cryst.*, B 38 (1982) 929.