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SEPARATION OF AMPICILLIN ESTERS AND THEIR DIASTEREOISOMERS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

Bacampicillin, talampicillin and pivampicillin can be separated by thin-layer chromatography on silanized silica gel with ethanol–water–2 *M* ammonium acetate (50:40:10) as the mobile phase. The diastereoisomers of bacampicillin and talampicillin can be separated by use of high-performance liquid chromatography. Good results are obtained with a Zorbax C_8 column (25 cm × 0.46 cm) and ethanol–water–0.2 *M* phosphate buffer pH 7.0 (40:55:5) as the mobile phase. The *R*:*S* ratio of bacampicillin samples varies between about 1:2 and 1:3, and for talampicillin samples *R*:*S* is always about 1:1.

INTRODUCTION

Several esters of ampicillin have been described as pro-drugs to improve oral absorption. Pivampicillin (PIV) was the first ester to be introduced clinically[1,2]. Other similar esters, talampicillin (TAL)[3,4] and bacampicillin (BAC)[5], were subsequently described. Their structures are shown in Fig. 1. The ester groups of TAL and BAC contain an asymmetric carbon atom and therefore the commercial products may consist of a mixture of epimers.

In this paper the separation of TAL, BAC and PIV by thin-layer chromatography (TLC) is described. The epimers of TAL and BAC were separated by high-performance liquid chromatography (HPLC) and the epimer ratio was determined. The separation of BAC diastereoisomers by HPLC has recently been described but no details about the quality of the separation were given[6]. The separation of the two diastereoisomers of ampicillin and of other penicillins, epimeric in the side chain, has been reported[7].

EXPERIMENTAL

Ampicillin (AMP), hydrochloride salts of ampicillin esters (TAL, BAC, PIV) and talampicillin napsylate were obtained from Astra (Södertalje, Sweden), Beecham (Heppignies, Belgium), Belphar (Brussels, Belgium), Continental Pharma (Brussels,

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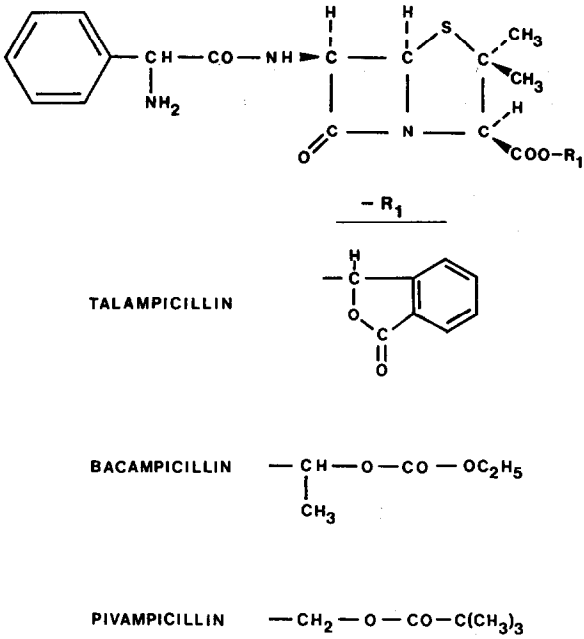

Fig. 1. Structures of the ampicillin esters.

Belgium), Leo Pharmaceutical Products (Hälsingborg, Denmark), Pfizer (Brussels, Belgium) and Upjohn (Puurs, Belgium).

Reagents and solvents

Chemicals for the preparation of buffer solutions were of *pro analysi* quality (E. Merck, Darmstadt, F.R.G.). Ethanol (96%, v/v) was of commercial grade. Acetonitrile for HPLC was obtained from Rathburn Chemicals (Walkerburn, U.K.). Water was distilled from glass apparatus. Other solvents were of >99% purity (Janssen Chimica, Beerse, Belgium) and were used as such except for tetrahydrofuran, which was distilled to remove the stabilizer, after confirmation of the absence of peroxides.

TLC

Precoated silanized silica gel plates, Fertigplatten Kieselgel 60 F_{254} silanisiert, were obtained from E. Merck. Laboratory-made silanized silica gel plates were prepared using a suspension of 35 g of Kieselgel 60 HF_{254} silanisiert (E. Merck) in 60 ml of water–methanol (2:1). After initial drying at room temperature for several hours, the layers were dried further at 50°C overnight. All layers used were 0.25 mm thick.

The mobile phases used are given in Table I. They comprised one or two organic modifiers, water and buffer solution. The organic modifiers used were acetone, acetonitrile, ethanol, *tert.*-butanol, dimethylformamide, dimethyl sulphoxide, methanol and tetrahydrofuran. A 2 *M* solution of ammonium acetate was used as the buffer solution; when a pH value is indicated, it was achieved with glacial acetic acid.

Sample solutions (10 mg/ml) were prepared in methanol–water (1:1) (AMP, TAL · HCl, BAC · HCl) or in methanol–water (3:1) (PIV · HCl) and 1-μl aliquots were applied to the plates. The plates were developed over a distance of *ca.* 15 cm in filter-paper-lined chromatographic tanks, which had been saturated for at least 1 h. They were dried in a stream of hot air and placed in a tank saturated with iodine vapour for detection.

HPLC

The pump unit consisted of a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) equipped with a pulse dampener and a manometer as described[8]. The HPLC apparatus further comprised a Valco injector, Model CV-6-UHPa-N60, equipped with a 10-μl loop (Valco, Houston, TX, U.S.A.), an Altex UV detector, Model 153 (254 nm), equipped with an 8-μl flow cell (Altex, Berkeley, CA, U.S.A.), a Kipp recorder, Model BD40 (Kipp & Zonen, Delft, The Netherlands) and a Pye Unicam integrator Model DP88 (Pye Unicam, Cambridge, U.K.).

The columns (25 cm × 0.46 cm I.D.) were packed as described[7] with μBondapak C_{18}, 10 μm (Waters Assoc., Milford, MA, U.S.A.), Hypersil ODS, 5 μm (Shandon, Cheshire, U.K.), Spherisorb S5 C_8, 5 μm (Phase Sep, Norwalk, CT, U.S.A.) and Zorbax C_8, 7 μm (Du Pont, Wilmington, DE, U.S.A.). The columns were kept at 25°C by means of a glass jacket, as described[9].

All the mobile phases contained one organic modifier and 5% (v/v) 0.2 *M* phosphate buffer pH 7.0, the volume being made up with water. The amount of organic modifier varied with the column used and is specified in the tables. The organic modifiers were acetonitrile, ethanol, *tert.*-butanol, methanol, 2-propanol and tetrahydrofuran. The mobile phases were degassed by sonication. The flow-rate was set at 1.0 ml/min and the chart speed at 5 mm/min. Sample solutions were prepared as described for TLC and 100-μg aliquots were injected. The detector was set at 0.16 a.u.f.s.

RESULTS AND DISCUSSION

In Table I the R_F values obtained on silanized silica gel are reported. The values are the means of the results obtained from several chromatograms. Values obtained with laboratory-made plates are given in parentheses. The mobile phases, previously described for TLC of penicillins[10] or cephalosporins[11], are not suitable since chromatography of the less polar esters requires an increased content of organic modifier. Although the R_F values obtained with mobile phases A, B and C suggest separation of PIV, BAC, TAL and AMP, the separation is insufficient due to tailing. This could be reduced by the addition of acetonitrile (mobile phases D and E) but the separation of BAC and TAL was still not complete. The use of mobile phase F, containing ethanol, allows complete separation of the three esters. The addition of acetone to the mobile phase (G) does not improve the separation. In order to examine the influence of the buffer pH, the 2 *M* ammonium acetate solution was adjusted to pH 4.2, 5.2 and 6.0 (mobile phases H, I and J). No improvement is observed at these pH values and the spots show tailing.

Although the separation obtained with mobile phase F is very satisfactory, the analysis time is rather long, *i.e.*, about 4.5 h with precoated plates and about 8 h with

TABLE I

$R_F \cdot 100$ VALUES FOR AMPICILLIN AND ESTERS CHROMATOGRAPHED ON SILANIZED SILICA GEL

PIV = Pivampicillin; BAC = bacampicillin; TAL = talampicillin; AMP = ampicillin. Values obtained with laboratory plates are given in parentheses. A 2 *M* solution of ammonium acetate was used as the buffer solution; when a pH value is indicated this was achieved with glacial acetic acid.

Mobile phase	*Sample*			
	PIV	*BAC*	*TAL*	*AMP*
A Methanol–water–buffer (60:30:10)	36 (33)	40 (36)	44 (41)	73 (65)
B Methanol–water–buffer (60:35:5)	35 (39)	38 (44)	44 (49)	75 (65)
C Methanol–water–buffer (65:30:5)	42 (41)	46 (44)	50 (47)	76 (66)
D Methanol–acetonitrile–water–buffer (40:15:35:10)	34 (29)	41 (34)	45 (37)	78 (70)
E Methanol–acetonitrile–water–buffer (45:15:30:10)	42 (37)	45 (44)	49 (47)	78 (75)
F Ethanol–water–buffer (50:40:10)	33 (29)	38 (33)	44 (39)	75 (65)
G Ethanol–acetone–water–buffer (50:5:35:10)	44 (31)	48 (36)	53 (40)	78 (63)
H Ethanol–water–buffer pH 4.2 (50:40:10)	48 (45)	53 (48)	58 (54)	74 (67)
I Ethanol–water–buffer pH 5.2 (50:40:10)	39 (48)	43 (51)	48 (59)	76 (67)
J Ethanol–water–buffer pH 6.0 (50:40:10)	57 (51)	61 (56)	64 (63)	77 (66)
K Acetonitrile–water–buffer (30:60:10)	6	8	7	77
L Acetonitrile–water–buffer (40:50:10)	15	25	19	63
M Tetrahydrofuran–water–buffer (30:60:10)	6	11	9	91
N Tetrahydrofuran–water–buffer (40:50:10)	18	28	27	92
O *tert.*-Butanol–water–buffer (35:55:10)	37	40	43	79
P Acetone–water–buffer (40:50:10)	5	8	8	32
Q Acetone–water–buffer (45:45:10)	13	18	21	89
R Dimethyl sulphoxide–water–buffer (40:50:10)	*	*	*	*
S Dimethylformamide–water–buffer (40:50:10)	*	*	*	*

* Streaking from the start point.

laboratory-made plates. In order to reduce the analysis time a number of other organic modifiers was examined (mobile phases K–S). All these separations are very poor except that with *tert.*-butanol (mobile phase O), but even this separation is inferior to that obtained with mobile phase F and the development time is even longer

(about 6.5 h). Therefore mobile phase F is considered as the best choice. Generally the separation pattern obtained with the laboratory-made plates is comparable with that obtained with the precoated plates, but differences in R_F values are observed.

None of the TLC systems used (Table I) shows any separation of the diastereoisomers of BAC and TAL. Therefore, the separation of these diastereoisomers was attempted with a system similar to that formerly described for the separation of side-chain diastereoisomers of penicillins[7]. This involves the use of a Zorbax C_8 column and methanol–water–0.2 *M* phosphate buffer pH 7.0 as the mobile phase. The separation of BAC epimers is easily achieved but the TAL epimers, although separated, appear superposed on a background peak of strongly absorbing impurities and therefore cannot be determined quantitatively. Other organic modifiers were examined and the results are shown in Table II. For completeness the results for PIV are also mentioned. Acetonitrile and tetrahydrofuran only allow a poor separation of TAL epimers and no separation of BAC epimers. Ethanol gives a nice separation of both TAL and BAC epimers. Typical chromatograms are shown in Fig. 2. Other alcohols such as 2-propanol and *tert.*-butanol also separate all the epimers, but the results are inferior. Therefore the use of ethanol is preferred.

TABLE II

RETENTION TIMES OF AMPICILLIN ESTERS ON ZORBAX C_8

The mobile phases comprised organic modifier–water–0.2 *M* phosphate buffer pH 7.0 [*x*:(95 − *x*):5].

Organic modifier	*x*	*Retention time (min)*				
		TAL		*BAC*		*PIV*
Methanol	60	14.7*	16.3*	23.6	25.4	41.4
Methanol	65	8.5*	9.2*	12.7	13.4	20.5
Acetonitrile	40	17.3	18.5	20.8	20.8	32.6
Tetrahydrofuran	35	18.0	19.6	20.0	20.0	33.4
Ethanol	40	18.7	21.4	29.9	32.2	61.7
2-Propanol	28	18.2	21.0	27.6	30.6	62.0
tert.-Butanol	23	15.6	18.2	23.8	25.8	55.2

* Product peaks are superposed on a background peak of strongly absorbing impurities.

The separation of BAC epimers with 2-propanol at pH 4.0 has been described[6]. By carrying out this method with a μBondapak C_{18} column, as described, the BAC epimers were separated but the peaks were much broader than with the system described here. A similar picture was obtained with the TAL epimers. On Zorbax C_8 the epimers were not separated at all with this mobile phase.

The ampicillin esters were analysed on several stationary phases with two mobile phases containing different amounts of ethanol. The results are reported in Table III. The three esters can easily be separated on all the columns. The diastereoisomers can also be separated on all the columns but the resolution on Zorbax C_8 is better. For some separations the resolution is not good enough to calculate a resolution value following the instructions of the European Pharmacopoeia[12].

TABLE III

COMPARISON OF THE SEPARATION OF AMPICILLIN ESTERS ON DIFFERENT REVERSED-PHASE COLUMNS

The mobile phases comprised ethanol–water–0.2 *M* phosphate buffer pH [*x*:(95 − *x*):5]. ND = Not determined due to insufficient separation.

Column	*Peak*	*Mobile phase with 40% ethanol*							*Mobile phase with 42.5% ethanol*						
		Retention time (min)			*Resolution*		*Symmetry factor*		*Retention time (min)*			*Resolution*		*Symmetry factor*	
		TAL	*BAC*	*PIV*	*TAL*	*BAC*	*TAL*	*BAC*	*TAL*	*BAC*	*PIV*	*TAL*	*BAC*	*TAL*	*BAC*
μBondapak C_{18}, 10 μm	1	12.5	16.7	31.7	1.06	ND	1.00	ND	9.9	12.9	22.9	0.89	ND	1.00	ND
	2	13.9	18.2				1.04	1.05	10.9	13.9				1.00	1.00
Hypersil C_{18}, 5 μm	1	11.9	19.3	41.2	1.37	ND	1.22	ND	10.1	15.7	30.9	1.24	ND	1.27	ND
	2	13.7	21.0				1.25	1.20	11.5	16.9				1.31	1.13
Spherisorb C_8, 5 μm	1	14.7	19.6	38.9	1.21	1.86	1.13	1.06	12.8	17.1	31.1	0.97	1.63	1.21	1.00
	2	15.9	21.7				1.06	1.05	13.7	18.8				1.14	1.10
Zorbax C_8, 7 μm	1	18.7	29.9	61.7	1.98	1.48	1.10	1.15	12.4	23.3	43.7	1.56	1.25	1.13	1.13
	2	21.4	32.2				1.09	1.07	13.9	25.2				1.11	1.08

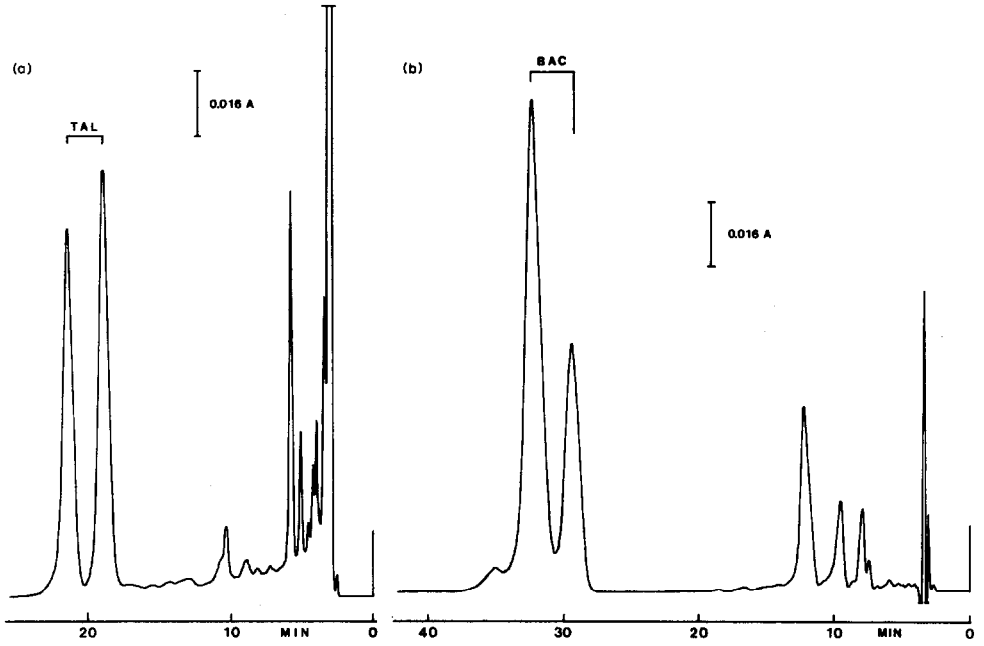

Fig. 2. High-performance liquid chromatograms of (a) talampicillin hydrochloride and (b) bacampicillin hydrochloride on Zorbax C_8 (25 cm × 0.46 cm) with ethanol–water–0.2 *M* phosphate buffer pH 7.0 (40:55:5) as the mobile phase. Detection: 254 nm, 0.16 a.u.f.s. Amount injected: 100 μg. Old samples were chosen to demonstrate the selectivity of the chromatographic system.

TABLE IV

EPIMER RATIO IN COMMERCIAL SAMPLES OF AMPICILLIN ESTERS

X = Relative amount of the epimer eluted first; *n* = number of experiments; S.D. = standard deviation.

Ester salt	*Manufacturer and sample no.*	*Isomer eluted first*	*X*	*n*	*S.D.*
Bacampicillin hydrochloride	A_1	*R*	31.7	4	0.6
	A_2		26.5	2	0.5
	B		30.3	2	0.6
	C		36.5	2	0.2
Talampicillin hydrochloride	D_1	Unknown	51.5	2	0.2
	D_2		53.1	2	0.8
	D_3		51.9	2	0.2
	D_4		54.4	2	0.1
	E_1		52.7	3	1.1
	E_2		52.1	4	2.4
	E_3		51.9	2	0.2
	E_4		52.4	3	1.5
	F		48.9	3	0.4
Talampicillin napsylate	G		51.8	5	1.4

The Zorbax column and ethanol as the organic modifier were used to analyse a number of samples. The results are shown in Table IV. Since the pure *S* epimer of BAC was kindly provided by Astra the first peak eluted could be identified as the *R* epimer. The peaks of TAL were not identified since a pure isomer was not available. For TAL the *R*:*S* ratio was always about 1:1 for the samples examined. These results confirm the data obtained by NMR spectroscopy[4]. It should be noted that the rates of hydrolysis of the TAL epimers by blood are very similar[4]. For BAC the *R*:*S* ratio varies between 1:3 and 1:2. No information is available concerning the ratio of the contents of the two diastereoisomers in commercial samples of this penicillin, although some physical properties have been discussed[5,6,13]. The oral absorption of TAL and BAC by humans has been compared with that of ampicillin and amoxicillin[14,15].

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REFERENCES

1 W. von Daehne, E. Frederiksen, E. Gundersen, F. Lund, P. Morch, H. J. Petersen, K. Roholt, R. Tybring and W. O. Godtfredsen, *J. Med. Chem.*, 13 (1970) 607.
2 W. von Daehne, W. O. Godtfredsen, K. Roholt and L. Tybring, *Antimicrob. Agents Chemother.*, 4 (1971) 431.
3 Y. Shiobara, A. Tachibana, H. Sasaki, T. Watanabe and T. Sado, *J. Antibiot.*, 27 (1974) 665.
4 J. P. Clayton, M. Cole, S. W. Elson, H. Ferres, J. C. Hanson, L. W. Mizen and R. Sutherland, *J. Med. Chem.*, 19 (1976) 1385.
5 N. O. Bodin, B. Ekström, H. Forsgren, L. P. Jalar, L. Magni, C. H. Ramsay and B. Sjöberg, *Antimicrob. Agents Chemother.*, 8 (1975) 518.
6 H. Nyqvist and M. Nicklasson, *Acta Pharm. Suec.*, 22 (1985) 215.
7 J. Hoogmartens, E. Roets, G. Janssen and H. Vanderhaeghe, *J. Chromatogr.*, 244 (1982) 299.
8 I. Wouters, S. Hendrickx, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 291 (1984) 59.
9 P. De Pourcq, J. Hoebus, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 321 (1985) 441.
10 S. Hendrickx, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.*, 291 (1984) 211.
11 J. H. Hoogmartens, E. E. Roets and H. J. Vanderhaeghe, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 173.
12 *European Pharmacopoeia*, Maissonneuve, Sainte Ruffine, 2nd ed., 1982.
13 H. Nyqvist and M. Nicklasson, *Acta Pharm. Suec.*, 22 (1985) 229.
14 L. Verbist, *Antimicrob. Agents Chemother.*, 10 (1976) 173.
15 J. Sjövall, L. Magni and T. Bergan, *Antimicrob. Agents Chemother.*, 13 (1978) 90.