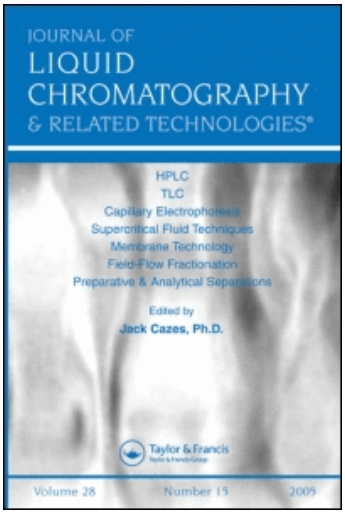


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Evaluation of LC Methods for the Separation of Amoxicillin and Its Related Substances

Zhu Yongxin[a]; E. Roets[a]; M. L. Moreno[b]; E. Porqueras[b]; J. Hoogmartens[a]
[a] Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, Leuven, (Belgium) [b] Centro National De Farmacobiologia, Instituto De Salud Carlos III, Madrid, (Spain)

To cite this Article Yongxin, Zhu , Roets, E. , Moreno, M. L. , Porqueras, E. and Hoogmartens, J.(1996) 'Evaluation of LC Methods for the Separation of Amoxicillin and Its Related Substances', Journal of Liquid Chromatography & Related Technologies, 19: 12, 1893 — 1908
To link to this Article: DOI: 10.1080/10826079608014013
URL: http://dx.doi.org/10.1080/10826079608014013

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1893–1908 (1996)

EVALUATION OF LC METHODS FOR THE SEPARATION OF AMOXICILLIN AND ITS RELATED SUBSTANCES

Zhu Yongxin[1], E. Roets[1], M. L. Moreno[2], E. Porqueras[2], J. Hoogmartens[1]

[1] Katholieke Universiteit Leuven
Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen
Faculteit Farmaceutische Wetenschappen
Van Evenstraat 4
3000 Leuven (Belgium)
[2] Centro National De Farmacobiologia
Instituto De Salud Carlos III
28220 Majadahonda, Madrid (Spain)

ABSTRACT

Five isocratic liquid chromatography (LC) methods have been examined for the separation of amoxicillin and its related substances on C_{18} or C_8 columns. The United States Pharmacopeia (USP) assay method gave better selectivity. Similar selectivity was obtained not only on C_{18} columns but also on C_8 and poly(styrene-divinylbenzene) columns. The good selectivity was confirmed by a second laboratory. A resolution test using cefadroxil was developed for the method performance. Based on the USP method, a gradient LC method was developed for the analysis of related substances in amoxicillin. This method has been proposed for the assay and purity control in the amoxicillin monographs of the European Pharmacopoeia and will be further examined in a collaborative study.

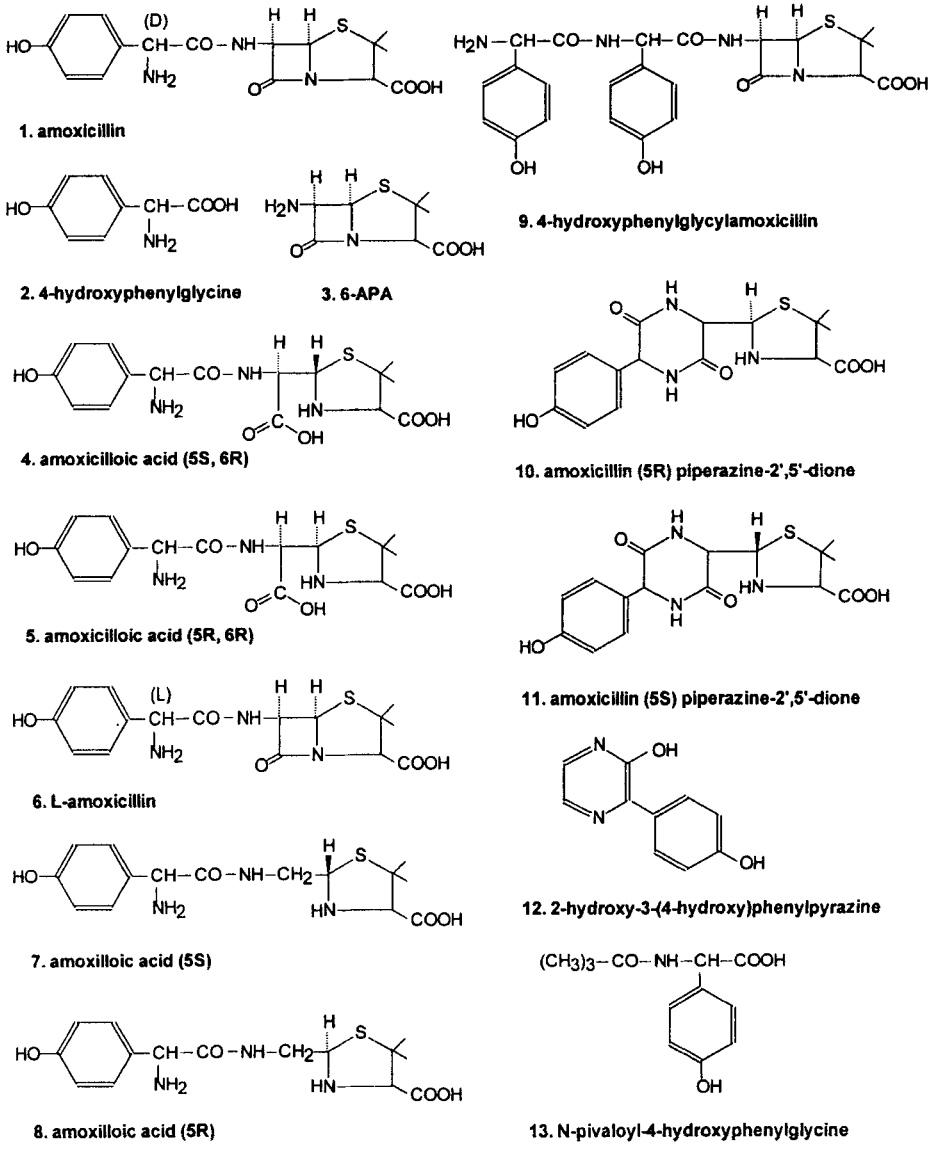
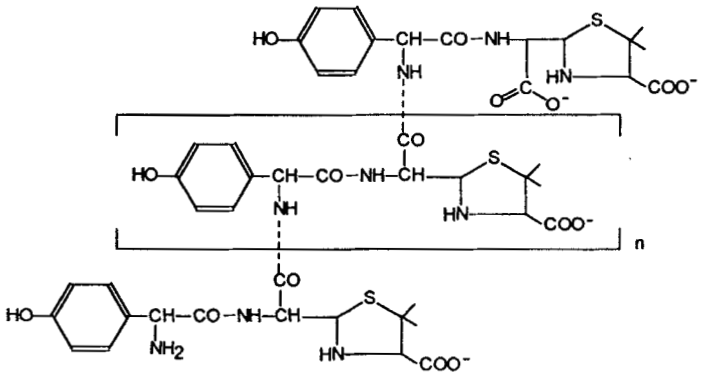
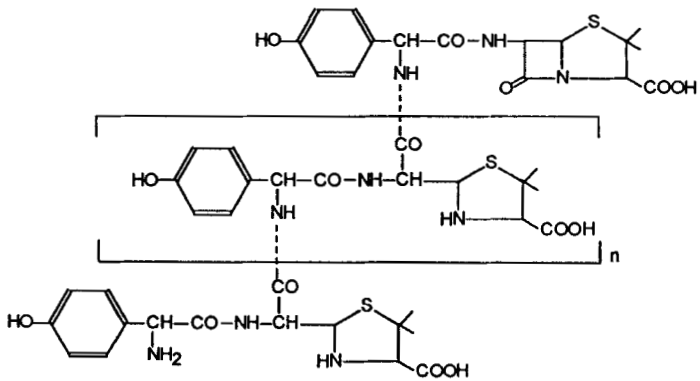


Figure 1. Structures of amoxicillin and its related substances.



14. dimeroates (n = 0)
trimeroates (n = 1)



15. dimer (n = 0)
trimer (n = 1)

Figure 1 (cont.). Structures of amoxicillin and its related substances.

INTRODUCTION

Amoxicillin is a semi-synthetic penicillin with activity against both gram-positive and gram-negative bacteria. It is available as injectable, capsule, and oral suspension. Amoxicillin may contain precursors, side products from the semi-synthesis and various degradation products including oligomers. Fig. 1

shows the structures of amoxicillin and a number of related substances, which were available. Some of the related substances were kindly donated by manufacturers, but most had to be prepared in the laboratory.

LC methods are quite often used for the analysis of amoxicillin. Some papers on amoxicillin describe the determination of amoxicillin in biological samples.[1-2] These methods highlight the separation of the antibiotic from the background of biological materials.

Some papers discuss the separation of amoxicillin from other penicillins or other drugs.[3-8] Some papers deal with the measurement of amoxicillin by using special detection techniques.[9-11]

A number of papers report the determination of amoxicillin in pharmaceuticals.[12-14] LC is widely used by manufacturers for assay of amoxicillin and is also prescribed by the USP.[15] However, the separation of amoxicillin and its related substances and the reproducibility of the selectivity on different columns have not been sufficiently discussed. This was mainly due to non availability of some related substances.

In the present study, the selectivity of five isocratic LC methods for assay has been examined. Two were taken from literature,[12,14] one from the USP[15] and the other two were made available by manufacturers of amoxicillin. Table 1 shows the LC conditions of the five methods. All use C_{18} stationary phases except method III, using C_8. The prescribed conditions were slightly adapted in our study, as shown.

The aim of the present study is to examine whether an existing assay method was sufficiently selective and whether it could be adapted in order to be suitable as a related substances test.

EXPERIMENTAL

Samples

Amoxicillin trihydrate and amoxicillin sodium are commercially available. Related substances originate from the semi-synthesis and from degradation. The structures of the available related substances are shown in Fig.1. D-4-Hydroxyphenylglycine (**2**) and 6-aminopenicillanic acid (6-APA, **3**) are the basic constituents of amoxicillin and are commercially available. L-amoxicillin (**6**), 4-hydroxyphenylglycylamoxicillin (**9**) and N-pivaloyl-4-hydroxyphenyl-glycine (**13**) can arise from the semi-synthesis of amoxicillin. Related

Table 1

LC Conditions for Five Isocratic LC Methods

Meth.	Source	Mobile Phase Prescribed	Column Temp. Prescribed	Flow Rate (mL/min) Prescribed	Detection* UV nm Prescribed	Column Temp. Used	Flow Rate (mL/min) Used
I	Mfr. 1	0.02 M phosphate buffer pH 5.0-CH_3OH (93:7)	40°C	1.5	230	40°C	1
II	Mfr. 2	0.1 M phosphate buffer pH 4.5-CH_3OH (95:5)	Ambient	---	230	30°C	1
III	LeBelle	0.05 M phosphate buffer pH 5.0-CH_3OH (94:6)	30°C	1	254	30°C	1
IV	Hsu	1.25 % acetic acid-CH_3OH (80:20)	Ambient	1.5	254	30°C	1
V	USP	0.05 M phosphate buffer pH 5.0-CH_3CN (96:4)	Ambient	1.5	230	30°C	1

*Wavelength used: 254 min.

substances **6** and **13** were obtained from Antibioticos and Biochemie S. A., Spain. The other related substances are decomposition products. Amoxicilloic acid (5S, 6R) (**4**) and amoxicilloic acid (5R, 6R) (**5**) were prepared as described by Munro.[16] The preparation of amoxilloic acid (5S) (**7**) and amoxilloic acid (5R) (**8**) will be described elsewhere. 2-Hydroxy-3-(4-hydroxyphenyl)pyrazine (**12**) was prepared in a similar way as described by Lebelle.[17] Amoxicillin (2R) piperazine-2,5-dione (**10**) and amoxicillin (2S) piperazine-2,5-dione (**11**) were prepared as described by Roets[18] and Haginaka.[19] 4-Hydroxyphenylglycylamoxicillin (**9**) was prepared in a similar way as described by Grant.[20] The oligomeroates (**14**) and oligomers (**15**) were prepared as described by Roets[18] and Bundgaard.[21]

Solvents and reagent

Acetonitrile (HPLC grade) was from Rathburn (Walkerburn, Scotland). Methanol (Roland, Brussels, Belgium) was distilled before use. Potassium dihydrogen phosphate, acetic acid were from Acros Chimica (Beerse, Belgium). Water was distilled twice.

Table 2

General Information on Columns

Columns	Stationary Phases	Particle Size (μm)
A, B*	Hypersil ODS (Shandon, Runcorn, UK)	5
C, D	Bio-Sil C_{18} (Bio-Rad, Nazareth, Belgium)	5
E, F*	Spherisorb ODS-1 (Phase Sep'n, Queensferry, UK)	10
G	RoSil C_8 (Alltech, Deerfield, IL, USA)	8
H	ChromSpher C_8 (Chrompack, Middleburg, Netherlands)	5
I, J	Zorbax C_8 (DuPont Co., Wilmington, DE, USA)	7
K	PRP-1 (Hamilton, Reno, NV, USA)	7-9
L, M*	PLRP-S (Polymer Laboratories, Church Stretton, UK)	8

* Columns used in laboratory B

LC Apparatus and Column

The equipment consisted of a L-6200 (Merck-Hitachi, Darmstadt, Germany), a Model CV-6-UHPa-N60 Valco injector (Houston, TX, USA), with a 20 μL loop or a 50 μL loop, a Model D 254 nm fixed-wavelength UV monitor (LDC/Milton Roy, Riviera Beach, FL, USA) and an integrator Model 3396 Series II (Hewlett-Packard, Avondale, PA). The columns (25 cm x 0.46 cm i.d.) used in this study are reported in Table 2. Most of the experiments are carried out in laboratory A using this equipment, but a number of experiments were repeated in laboratory B, to examine for reproducibility. The columns used in the laboratory B are identified with an asterisk. The equipment used in laboratory B was of similar quality.

Mobile Phase and Sample Preparation

The mobile phases were prepared as described in Table 1. For some columns, the amount of organic modifier was slightly adapted in order to obtain a similar retention time for amoxicillin.

In this study mobile phase was used as the solvent. For the selectivity study, the following concentrations were used: amoxicillin 1.2 mg/mL, **2** and **3**: 0.1 mg/mL, **4**, **5** and **6**: 0.2 mg/mL, **12**: 0.02 mg/mL, other related substances: 0.5 mg/mL, 20 μl of these solutions was injected.

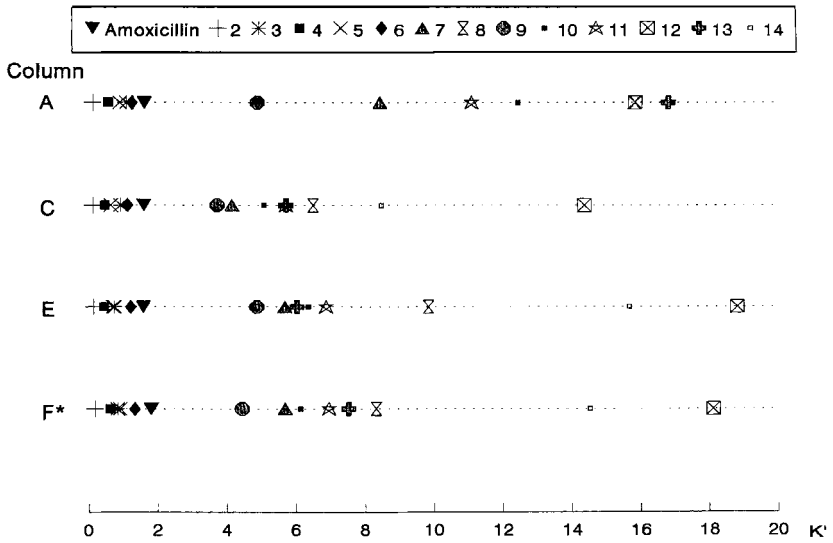


Figure 2. Capacity factors of amoxicillin and its related substances on C_{18} columns according to method 1.

RESULTS AND DISCUSSION

Selectivity Study

During preliminary work in laboratory A, three different C_{18} stationary phases (A, C, E) were used for the selectivity study of methods I, II, IV and V and three different C_8 stationary phases (G, H, I) were used for the selectivity study of method III. Some experiments were carried out using poly(styrene-divinylbenzene) stationary phases. The trimeroates (**14**, n = 1) and the oligomers (**15**) of amoxicillin were always eluted far after amoxicillin, therefore they are not shown in the results of the isocratic experiments. Results for other related substances with capacity factors (k') of more than 20 are not shown either. The experiments were repeated on one column in laboratory B.

For method I, the results are shown in Fig.2. The concentration of methanol in the mobile phase was adjusted for each column (A = 6, C = 5.5, E = 2, F* = 5) in order to obtain similar retention times for amoxicillin. Amoxicillin was completely separated from related substances on all four

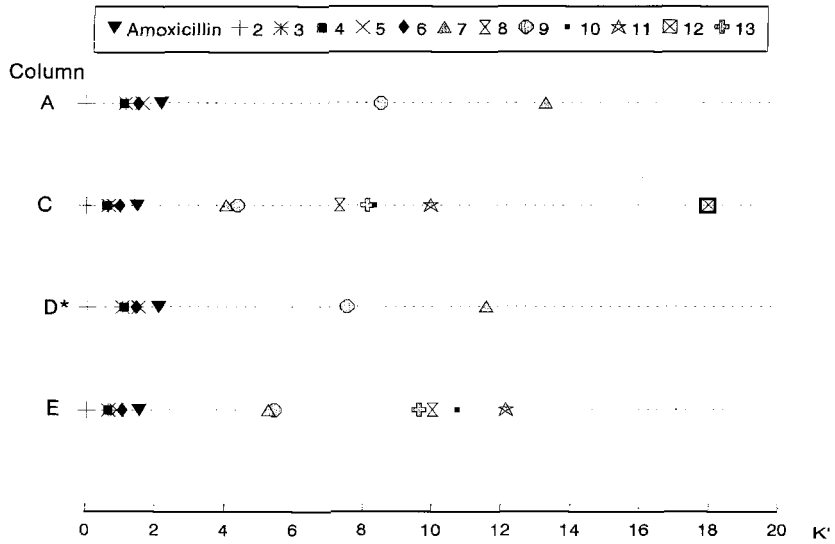


Figure 3. Capacity factors of amoxicillin and its relatied substances on C_{18} columns according to method 2.

columns. For method II, the results are shown in Fig.3. The concentration of methanol in the mobile phase was adjusted for each column (A = 5, C = 3.7, D* = 5.5, E = 3). Amoxicillin was completely separated from its related substances on the four columns.

Methods I and II are very similar, there are only small differences in the pH and the concentration of the buffer. For method III, the results are shown in Fig.4. The concentration of methanol in the mobile phase was adjusted for each column (G = 4, H = 2, I = 1.5, J* = 7.5). Amoxicillin was separated from its related substances on all four columns.

This method corresponds to method I, except that C_8 columns are used in method III. For method IV, the results are shown in Fig.5. The concentration of methanol in the mobile phase was adjusted for each column (A = 30, B* = 26, C = 10, E = 20). Amoxicillin was separated from its related substances on column E but not on columns A, B* and C.

In the figures above, it can be seen that for the same method, the sequence of related substances can be different on different columns, which is common for silica boned reverse phases.

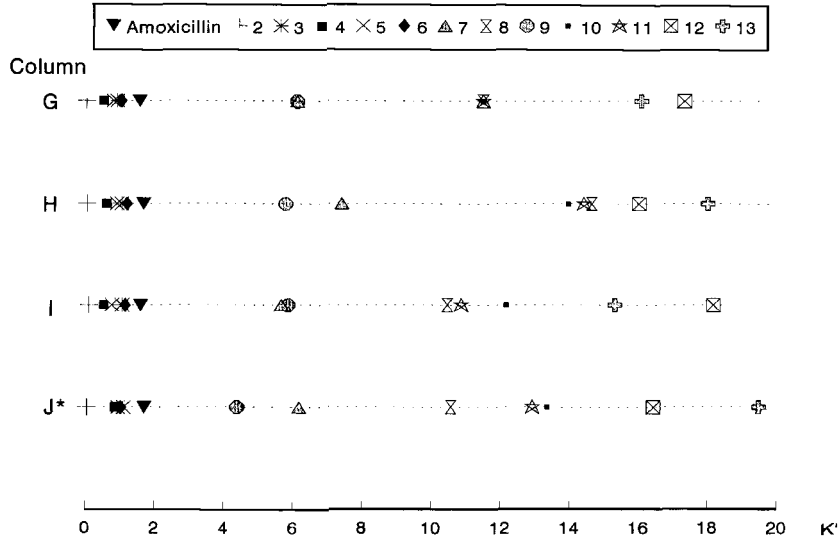


Figure 4. Capacity factors of amoxicillin and its related substances on C_8 columns according to method 3.

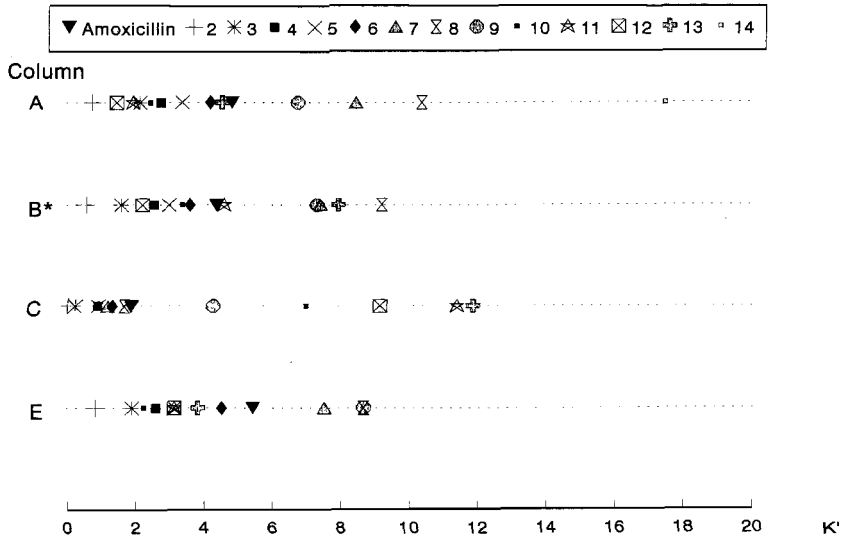


Figure 5. Capacity factors of amoxicillin and its related substances on C_{18} columns according to method 4.

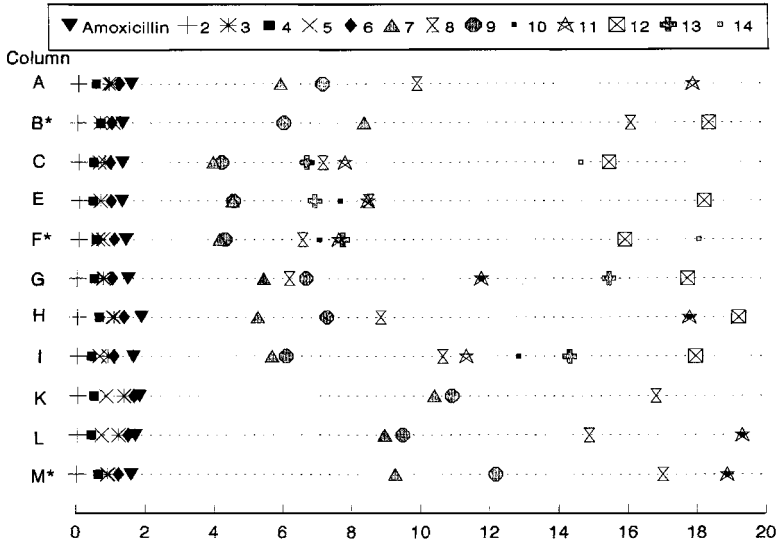


Figure 6. Capacity factors of amoxicillin and its related substances on different columns according to the USP method.

For the USP method, after preliminary work on three C_{18} columns in laboratory A it was clear that this method gave a satisfactory and repeatable selectivity. This was confirmed by laboratory B, using two C_{18} columns. The USP method is very similar to methods I and III, except that CH_3CN is used as the organic modifier instead of CH_3OH. As method V is a current official method, only this method was further examined with C_8 columns (G, H, I, J*) and even with poly(styrene-divinylbenzene) columns (K, L, M*) although methods I, II, III also gave satisfactory results. To improve the efficiency, columns K, L and M* were used at 50 °C instead of 30 °C as prescribed by the USP method. The results of the selectivity study are shown in Fig. 6, on all columns amoxicillin was separated from its related substances. The related substances were also reasonably well separated from each other but here also the sequence may be different on different columns.

The results on C_8 or C_{18} columns were similar. The separation from L-amoxicillin was somewhat less good on the polymer columns. Table 3 shows general information on method performance using the USP method. According to the USP monograph, the capacity factor of amoxicillin, k', must be between 1.1 and 2.8, the column efficiency not less than 1700 theoretical plates, the symmetry factor not more than 2.5. So all the parameters comply with the requirements of the USP method.

Table 3

General Information on Method Performance Following USP Method

Column	Content (%) of Acetonitrile in Mobile Phase	k′ Amoxicillin	S Amoxicillin	n Amoxicillin	Rs Amoxicillin Cefadroxil	α	α′
A	2.8	1.61	1.18	9690	9.2	1.68	1.28
B*	3.0	1.35	0.90	10240	7.7	1.73	1.31
C	2.0	1.34	1.25	3020	2.2	1.30	1.37
E	2.0	1.30	1.08	2160	2.5	1.42	1.33
F*	4.0	1.43	1.10	5670	3.5	1.29	1.31
G	2.0	1.50	1.10	4880	4.0	1.38	1.43
H	0.6	1.89	1.22	5830	5.7	1.56	1.36
I	1.0	1.66	1.19	4730	3.3	1.34	1.53
K	0.8	1.85	2.04	1710	4.9	1.91	1.09
L	0.8	1.73	1.50	3840	7.1	1.92	1.14
M*	0.8	1.60	1.30	2667	8.2	2.64	1.29

* Columns used in laboratory B

k′ = capacity factor; S = symmetry factor; n = number of theoretical plates; Rs = resolution.

$\alpha = k'_{\text{cefadroxil}} / k'_{\text{D-amoxicillin}}$; $\alpha' = k'_{\text{D-amoxicillin}} / k'_{\text{L-amoxicillin}}$

Resolution Test

A resolution test using cefadroxil was developed. The structure of cefadroxil is close to that of amoxicillin, therefore its chromatographic behaviour is related to that of amoxicillin. Cefadroxil has been used as an internal standard for the assay of amoxicillin.[2] The results for the resolution test are shown in Table 3. The resolution between cefadroxil and amoxicillin is more than 2.0 for all columns. It was preferred to use cefadroxil instead of L-amoxicillin, because the latter is not commercially available.

Related Substances Test

A related substances test which is based on the USP method was also developed. Considering the nature of the potential impurities, it is necessary to use gradient elution. The chromatographic procedure was carried out with mobile phase A: 0.05 M phosphate buffer pH 5.0-CH_3CN (99:1) and B: 0.05 M phosphate buffer pH 5.0-CH_3CN (80:20). A freshly prepared test solution with a concentration of 1.5 mg/mL was injected with a 50 µL loop. The elution was started isocratically with ratio A:B of 92:8. After 8 min., a linear gradient

Table 4

Capacity Factors of Amoxicillin and Its Related Substances on Three C_{18} Columns as Obtained by Gradient Elution

	k′ Columns		
	A	**C**	**E**
1	1.9	1.9	1.3
2	0.2	0.1	0.2
3	1.3	1.1	0.7
4	0.6	0.5	0.5
5	1.1	0.8	0.7
6	1.5	1.4	1.0
7	6.9	4.6	4.2
8	7.9	6.2	6.4
9	6.3	5.1	4.2
10	7.9	6.2	6.1
11	7.6	6.5	6.1
12	9.8	8.8	8.0
13	9.1	6.2	6.1
14 (n=0)	7.9	7.6	7.6
14 (n=1)	10.8	9.8	9.7
15 (n=0)	10.4	9.1	9.7
15 (n=1)	11.7	10.5	10.9

Mobile phase: A: 0.05 M phosphate buffer, pH 5.0-CH_3CN (99:1)
B: 0.05 M phosphate buffer, pH 5.0-CH_3CN (80:20)

Gradient elution: 0 to 8 min., isocratic elution with ratio A:B of 92:8; 8 to 22 min., a linear gradient elution to ratio A:B of 0:100; 30 to 45 min., isocratic elution with ratio A:B of 0:100; 45 to 60 min., isocratic elution with A:B of 92:8.

elution was started to reach a mobile phase ratio A:B of 0:100 over a period of 22 min. The chromatography was continued with mobile phase B during 15 min. Then the column was equilibrated with the originally chosen mobile phase during 15 min. The related substances test was examined using three

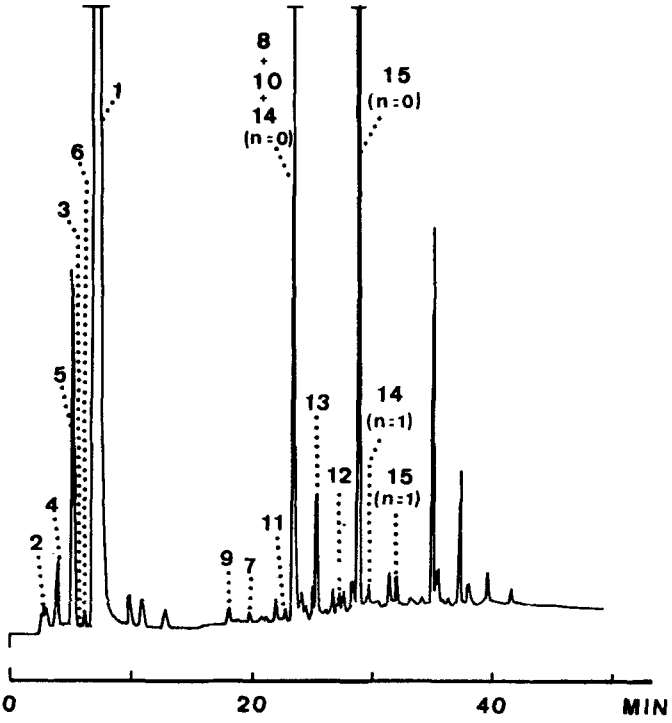


Figure 7. Typical chromatogram of amoxicillin sodium on Hypersil C_{18} (column A) with gradient elution. Mobile phase: A: 0.05 M phosphate buffer pH 5.0-CH_3CN (99:1), B: 0.05 M phosphate buffer pH 5.0-CH_3CN (80:20). Gradient elution: 0 to 8 min, isocratic elution with ratio A:B of 92:8; 8 to 22 min, a linear gradient elution to ratio A:B of 0:100; 30 to 45min, isocratic elution with ratio A:B of 0:100; 45 to 60 min, isocratic elution with A:B of 92:8.

C_{18} columns (A, C, E). The results are shown in Table 4. The results show the good selectivity of the gradient method for related substances. Column E gave a less good separation of related substances than columns A and C. Therefore it seems better to use only C_{18} columns with particle size of 5 μm. A typical chromatogram of an old sample of amoxicillin sodium obtained with column A is shown in Fig. 7.

Linearity, Repeatability and Stability

The quantitative aspects of this method have been examined. For linearity

amounts corresponding to 20, 30 or 40 μg of amoxicillin were analysed. The total number of analyses was 18. This yielded a calibration curve: $y = 557200x + 171000$, with y = peak area, x = amount injected in μg and with the correlation coefficient $r = 0.9999$ and the standard error of estimates $S_{y,x} = 46900$.

The repeatability was checked by analysing the same solution corresponding to 30 μg of amoxicillin six times. The relative standard deviation (RSD) for the peak area of amoxicillin was 0.15 %.

The stability at 22 °C of a solution (1.5 mg/mL) of amoxicillin trihydrate or amoxicillin sodium in the mobile phase A was examined. The solutions were injected every two hours over a period of 16 hours. No decrease of the peak area of amoxicillin was observed and the RSD values for the mean were 0.33 % (n = 8) and 0.25 % (n = 9) for the trihydrate and sodium salt, respectively. It was concluded that amoxicillin remained stable in the mobile phase A for at least 16 h.

Limit of Detection and Limit of Quantitation

For the related substances test, it was decided to inject 50 μL of a solution containing 1.5 mg of amoxicillin per mL. For this quantity, the limit of detection (LOD) was 0.02 % with a signal to noise ratio of 7. The limit of quantitation (LOQ) was 0.05 % (n = 6, RSD = 10 %).

CONCLUSION

It can be concluded that the USP method is one of the isocratic methods that is sufficiently and reproducibly selective to be used for the assay of amoxicillin. Cefadroxil may be used in the resolution test. The gradient elution method, based on the USP method, seems suitable as a related substances test.

The performance of the USP method and the related substances test derived from it will be further examined in a collaborative study.

ACKNOWLEDGEMENT

The National Fund for Scientific Research (Belgium) is acknowledged for financial support.

The authors thank Antibioticos and Biochemie S.A., Barcelona, Spain, for the gift of some of the related substances and I. Quintens for editorial assistance.

REFERENCES

1. T. L. Lee, L. D'Arconte, M. A. Brooks, J. Pharm. Sci., **68**, 454-458 (1979).

2. B. Charles, S. Chulavatnatol, Biomed. Chromatogr., **7**, 204-207 (1993).

3. T. Nakagawa, A. Shibukawa, T. Uno, J. Chromatogr., **239**, 695-706 (1982).

4. G. T. Briguglio, C. A. Lau-Cam, J. Assoc. Off. Anal. Chem., **67**, 228-231 (1984).

5. G. W. K. Fong, D. T. Martin, R. N. Johnson, B. T. Kho, J. Chromatogr., **298**, 459-472 (1984).

6. E. M. A. Moety, M. A. Abounassif, E. R. A. G. Kariem, N. A. Khattab, Talanta, **40**, 811-817 (1993)

7. W. A. Moats, L. Leskinen, J. Chromatogr., **386**, 79-86 (1987).

8. A. Aboul Khier, G. Blaschke, M. El Sadek, Anal. Lett., **17**, 1659-1666 (1984).

9. J. Carlqvist, D. Westerlund, J. Chromatogr., **164**, 373-381 (1979).

10. J. Carlqvist, D. Westerlund, J. Chromatogr., **344**, 285-296 (1985).

11. M. A. Brooks, M. R. Hackman, D. J. Mazzo, J. Chromatogr., **210**, 531-535 (1981).

12. M. J. LeBelle , W. L. Wilson and G. J. Lauriault, J. Chromatogr., **202**, 144-147 (1980).

13. P. de Pourcq, J. Hoebus, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Chromatogr., **321**, 441-449 (1985).

14. M.-C. Hsu, P.-W. Hsu, Antimicrob. Agents Chemother., **36**, 1276-1279 (1992).

15. **United States Pharmacopeia 23**, The United States Pharmacopeia Convention, Rockville, MD, USA (1995).

16. A. C. Munro, T. Kawai, Makromol. Chem., **67**, 1861-1865 (1978).

17. M. J. Lebelle, A. Vilim, W. L. Wilson, J. Pharm. Pharmacol., **31**, 441-443 (1979).

18. E. Roets, P. De Pourcq, S. Toppet, J. Hoogmartens, H. Vanderhaeghe, D. M. Williams, R. J. Smith, J. Chromatogr., **303**, 117-129 (1984).

19. J. Haginaka, J. Wakai, Chem. Pharm. Bull., **34**, 2239-2242 (1986).

20. N. H. Grant, H. E. Alburn, Chem. Abstr., **65**, P16976d (1966).

21. H. Bundgaard, C. Larsen, J. Chromatogr., **132**, 51-59 (1977).

Received December 7, 1995
Accepted December 20, 1995
Manuscript 4047