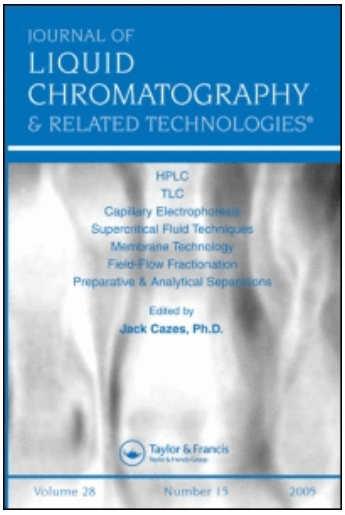

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SEPARATION AND DETERMINATION OF FIVE PENICILLINS BY REVERSED PHASE HPLC

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ABSTRACT

Rapid separation and quantitative determination of mixtures of amoxicillin, ampicillin, benzylpenicillin, cloxacillin and dicloxacillin was investigated by a reversed-phase high performance liquid chromatographic method with ultraviolet spectrometrical detection at 210 nm. A column Lichrosphere RP-18, 5μm particle size, was used as stationary phase, and mixture of methanol, acetonitrile and aqueous phosphate buffer as mobile phase. Improved retention times were achieved, and all the substances were eluted in 12 minutes. Baseline separation for the three of them, and similar sensitivity of all the five penicillins are the characteristics of the chromatogram. The regression data for the quantitative determination of each penicillin are also given in this work.

INTRODUCTION

Penicillins are widely used in veterinary practice as antibacterial drugs therapeutically as well as for the prevention of mastitis of small and large ruminative animals. As a result of this expanding use, these residues are usually detected in the milk of these animals. In laboratory practice the determination and monitoring of their concentration levels is of primary interest because they are of the most popular among other antibacterial drugs, such as tetracyclines, sulfonamides, e.t.c.

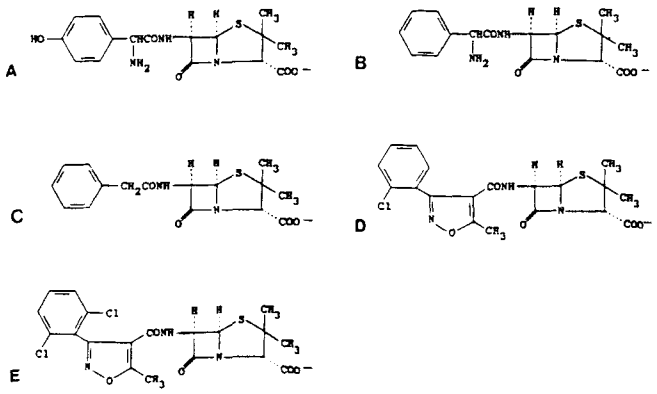

Figure 1. Formulas of the analysed penicillins. A. Amoxicillin, B. Ampicillin, C. Penicillin G, D. Cloxacillin and E. Dicloxacillin.

Penicillins are beta-lactam antibiotics, some of which have antimicrobial activity against both gram-positive and gram-negative organisms (1). Penicillins include bulky side chains attached to 6-aminopenicillinic acid.

The group of penicillins which was examined in this work contains amoxicillin, ampicillin, penicillin G (benzylpenicillin), cloxacillin and dicloxacillin, with the respective formulas given in Fig.1.

Many procedures for the determination of penicillins were reported in literature, applicable to electrophoresis (2), microbiological analysis (3), paper chromatography (PC) (4), thin layer chromatography (TLC) (5,6), gas-liquid chromatography (GLC) (7,8), and high performance liquid chromatography (HPLC) (9,10,11).

In this research a rapid reversed phase method was developed which does not need special accesories of liquid chromatography (derivatization pump (12), gradient elution programme or temperature development chamber). The method is easily applicable from not specially equiped laboratories and its performance characteristics are promissible for application in routine analysis of biological samples.

MATERIALS AND METHODS

The stock solutions of the five examined penicillins were prepared in double distilled water, and the standard solutions were prepared daily in a mixture of solvents similar to the mobile phase. Reagents obtained by SIGMA were anhydrous ampicillin and amoxicillin, sodium salts of cloxacillin, dicloxacillin and penicillin G (benzylpenicillin).

The conditions and parameters applied to the liquid chromatography were as follows:

Liquid Chromatography Pump: Gilson, Model 303.
Pressure: 1400 psi.
Loop volume: 20 μl.
Chromatographic column: Lichrosphere, RP-18, 5μm particle size, stainless steel, 250mmX4.0mm.
Mobile phase: Acetonitrile (chromatography grade)/methanol (chromatography grade)/ KH_2PO_4 aqueous solution 0.01M :(22-27-51, v/v/v), pH=4.5.
Flowrate: 0.5 ml/min.
Temperature: Ambient temperature (22°C).
Detector: UV-Vis, Gilson.
Wavelength: 210 nm.
Recorder sensitivity: 0.050 AUFS.

RESULTS AND DISCUSSION

1. Description of the Chromatograms.

The typical chromatogram from the column eluates of a mixture of the above penicillins are presented in Figure 2. The diagram corresponds to the concentration of 5 ppm in solution, the same for all the substances in the mixture.

From these it can be seen that the resolution between the five substances is good. This means that for the three finally eluted substances baseline separation was performed and for the two primary eluted substances the separation is also sufficient. The evaluation of the peak height in the chromatograms is carried out without specific problems.

The resolution factors of every penicillin relatively to the previous eluted penicillin are described in Table 1.

2. Retention Times.

The mean retention time from several runs (n=21) of mixtures of all the five penicillins in various concentrations are listed in the Table 1. Amoxicillin was chosen as an internal standard for the evaluation of the response factors of the other four penicillins. The evaluation of the response factors was performed for the peak height of the signal, as it is described below. The elution of the substances was performed in smaller retention times in comparison to other techniques (11,13), and for this reason there is no need of column oven for the elevation of column temperature. This is probably due to the higher ratio of acetonitrile in the elution mixture used, in comparison to literature reports. The lower viscosity of acetonitrile (0.345 cp, 25°C) than methanol (0.547 cp, 25°C) affects the eluotropic strength of the ternary mixture. Table 2

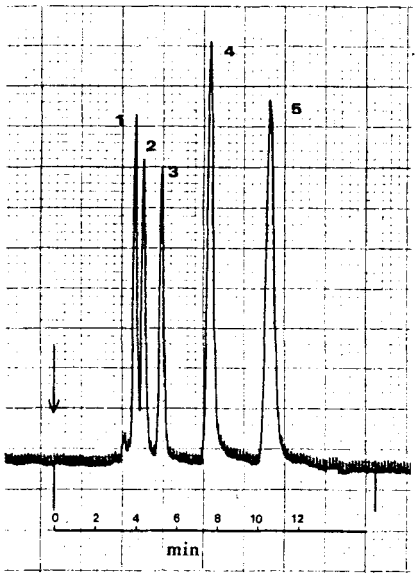

Figure 2. Typical chromatogram for the liquid chromatographic determination of 1. Amoxicillin, 2. Ampicillin, 3. Penicillin G, 4. Cloxacillin and 5. Dicloxacillin. Concentrations 5ppm in the mixture solution.

TABLE 1
Data about the retention times and resolution factors.

Substance	Mean Time (min)	Resolution Factors
amoxicillin	3.58	0.0
ampicillin	3.92	0.9
penicillin G	4.99	2.1
cloxacillin	7.47	3.2
dicloxacillin	10.64	3.2

TABLE 2

Comparison of procedures for the determination of penicillins.

Procedure	Briguglio(13)	Fletouris(11)	This work
stationary phase:			
column	ChromegabondC18	Partisil	Lichrosphere RP18
size	30cmX4.6mm	25cmX4.6mm	25cmX4.0mm
particles	10μm	5μm	5μm
temperature	22°C	40°C	22°C
mobile phase (% v/v):			
acetonitrile	19	38(+TBA)	22
methanol	11	0	27
phosphate aq.	70 (0.01M)	62 (0.02M)	51 (0.01M)
flowrate (ml/min)	1	1	0.5
characteristics:			
loop volume	20	20	20
wavelength nm	225	220	210
penicillins	9	5	5
resolution	good	very good	good
retention time (min):			
amoxicillin	3.6	-	3.58
ampicillin	4.0	-	3.92
penicillin G	6.2[+]	4.9[*]	4.99
cloxacillin	14.4[+]	9.5[*]	7.47
dicloxacillin	26.8[+]	12.5[*]	10.64

+: in reference 14, methicillin, penicillin V, oxacillin and nafcillin are separated in mean times.

*: estimation from figure 7 of reference 12.

summarises a comparison of the present procedure to other 2 procedures from the literature, and the results of retention times for some penicillins common in all the three procedures.

3. Regression analysis.

The calculation of the regression data for the analysis of the five substances was based on the chromatograms of 21 mixtures of them, in variable concentrations in the range 0.10 to 5.00 ppm. The summary of the regression data is given in Table 3.

TABLE 3

Regression data for the determination of penicillins in mixtures.

Substance	Regression equation	Corr.Coeff.	Error
amoxicillin	Y=5.7264+16.1833X	0.9980	2.2598
ampicillin	Y=5.2006+14.4845X	0.9969	2.4797
penicillin G	Y=4.1392+13.2445X	0.9853	4.9927
cloxacillin	Y=5.4161+19.7340X	0.9935	4.9112
dicloxacillin	Y=6.1512+16.5826X	0.9940	3.9890

TABLE 4

Response factors in the chromatograms of mixtures of amoxicillin, ampicillin, penicillin G, cloxacillin and dicloxacillin.

Substance	Mean	R.S.D.	Confidence Interval
amoxicillin (int.stnd)	1.000	-	-
ampicillin	1.126	3.897	1.096 - 1.157
penicillin G	1.236	8.051	1.166 - 1.306
cloxacillin	0.861	7.472	0.815 - 0.906
dicloxacillin	0.969	8.498	0.911 - 1.026

The estimation of the detection limits of the five substances in their mixtures was based in the equation $Y = Y_{bl} + 3s_{bl}$ and 6 blank determinations. The detection limits are between 0.03 and 0.05 ppm in the solution or 0.6 and 1.0 ng in the loop volume.

4. Response Factors.

As it is described previously, amoxicillin was chosen as internal standard for the evaluation of response factors of the other penicillins. The statistical evaluation was based on 21 chromatograms and the data are listed in Table 4.

The response factors of all the determined substances are ranged between 0.861 and 1.236, thus the chromatograms are consisting of peaks with similar heights. As a result of this, the sensitivity of them is also in the same order for the conditions used in this work, and any change of the attenuation would not cause the appearance or disappearance of a sole peak.

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