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QUANTITATIVE DETERMINATION OF AMOXICILLIN AND ITS DECOMPOSITION PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Amoxicillin, amoxicilloates, amoxicillin oligomers and amoxicillin piperazine-2,5-dione are separated by reversed-phase (C_8) high-performance liquid chromatography with gradient elution. Quantitative results are reported for a number of samples. Amoxicillin trihydrate samples mostly contain amoxicilloate as the main impurity. Samples of the sodium salt also contain the piperazine-2,5-dione and the dimer. Higher oligomers such as the trimer and tetramer were not present in significant amounts. Several samples were also analysed by a mercurimetric titration method.

INTRODUCTION

A number of papers on high performance liquid chromatography (HPLC) of amoxicillin describe the determination of the antibiotic in biological samples[1–9]. The influence of crown ether addition to the mobile phase on the retention of amoxicillin was investigated[8] and amperometric detection was reported to be useful[9]. The systems described are generally less suitable for the determination of impurities, such as oligomers, since the main concern of the method is the separation of the antibiotic from the background of biological origin.

HPLC was also used to separate the side-chain diastereoisomers of amoxicillin[10] and the C5-epimers of amoxilloic acid[11,12]. A comparison between mercury–imidazole colorimetric assay and HPLC determination of amoxicillin has been published[13]. The separation of amoxicillin and amoxicillin dimer by HPLC was also reported[14]. HPLC was used to study the stability of sodium amoxicillin in intravenous infusion fluids[15]. In the latter paper, the identity of the degradation products was not investigated. While our work nearly reached completion the determination of the more polar, faster eluting impurities of amoxicillin has been described. The majority of the impurities, eluted after amoxicillin, was not determined nor identified[16].

Recently we reported on the preparation and structure elucidation of amoxi-

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cillin oligomers[17]. In this paper we describe the separation by HPLC of amoxicillin, amoxicillin oligomers and other impurities. Quantitative results are shown for a number of samples.

EXPERIMENTAL

Samples

Samples were kindly donated by: Astra, Södertälje, Sweden; Beecham, Heppignies, Belgium; Bristol-Myers, Syracuse, NY, U.S.A.; Gist-brocades, Delft, The Netherlands and also by M. J. Lebelle, Drug Research Laboratories, Department of Health and Welfare, Canada.

Chemicals

Reagent-grade methanol (Janssen Chimica, Beerse, Belgium) is distilled before use. Water is bidistilled. Other reagents are of pro analysi quality (Merck, Darmstadt, F.R.G.).

HPLC apparatus; operating conditions

The HPLC apparatus consists of a Varian LC 4200 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.), equipped with a Waters U6K injector (Waters Assoc., Milford, MA, U.S.A.), a Pye Unicam LC 3 UV detector set at 274 nm and 0.08 a.u.f.s. (Pye Unicam, Cambridge, U.K.), a Kipp & Zonen BD 40 recorder (Kipp & Zonen, Delft, The Netherlands) and a Pye Unicam DP88 integrator. The 25 cm × 0.46 cm I.D. column, packed with Zorbax C_8, 7 μm (Du Pont, Wilmington, DE, U.S.A.) is kept at 30°C by means of a home-made glass water jacket, connected to a circulating water bath. The packing procedure is the same as that previously described before[10]. Drilled-out reducing unions (Swagelok, Solon, OH, U.S.A.) are used as the end-fittings for the stainless-steel column. PTFE ferrules are used in order to protect end-fittings and frits against rapid wear. Each end of the column is fixed in the glass jacket (18 mm I.D.) by means of an O-ring (Parker Seal Company, Culver City, CA, U.S.A.) part no. N 674-70-2-205, fitted between the reducing union and the nut. Since the ring does not glide easily into the glass jacket, it is enveloped with PTFE tape. This enhances both jacket life and impermeability. The column is protected by a pre-column (4 cm × 0.46 cm I.D.) fitted between the pump and the injector and packed with LiChrosorb RP-2, 10 μm (Merck, Darmstadt, F.R.G.). Gradient elution is performed with mobile phases A and B, consisting of methanol–0.2 *M* potassium phosphate buffer (pH 7.0)–water (5:5:90, v/v) for A and (50:5:45, v/v) for B. The gradient is programmed as follows: 5% of mobile phase B for 5 min, increazing at a rate of 2% of B per min to 65% of B, decreazing at a rate of 8% of B per min to the original concentration. The flow-rate is set at 1 ml/min, the paper speed at 5 mm/min and 25-μl volumes are injected.

Sample preparation, calibration curves and reproducibility

Internal standard solution (IS): 70 mg of sulfadimidine are dissolved in mobile phase A, and the solution is diluted to 1000 ml with the same solvent.

Sample preparation. Each sample (200 mg) is weighed into a 20-ml volumetric flask. Amoxicillin trihydrate samples are dissolved in 8 ml of 0.2 *M* phosphate buffer

(pH 11.0). Samples of the sodium salt are dissolved in mobile phase A. Immediately 10.0 ml of IS is added and the volume is made up to 20.0 ml with mobile phase A. The sample is injected on to the column within 1 min after preparation.

Calibration curves. y = ratio peak area/IS area; x = concentration of the solution injected in mg/20 ml. A correction factor (1.060) is introduced for the difference in molecular mass when results for a sodium salt are calculated from a calibration curve obtained with an acid, and vice versa.

A sample of amoxicillin trihydrate, found to be very pure as determined by mercurimetric titration and by HPLC, is used to prepare a calibration curve. The amount weighed is corrected for the water content and for the peak area in the chromatogram corresponding to impurities (1.3%), which were all expressed as amoxicillin. Solutions are prepared as described above for the samples. $y = 0.0831x - 0.0196$; $r = 0.999$.

Amoxicilloate is formed in solution by dissolving 100.0 mg of amoxicillin trihydrate in 20 ml of 0.1 *N* potassium hydroxide. After standing overnight, the solution is neutralised to pH 7.0 with 0.2 *M* potassium dihydrogen phosphate and diluted to 50.0 ml with water. To aliquots of this solution, 10 ml of IS are added and the solution is diluted to 20.0 ml with mobile phase A. On injection of these solutions two peaks are obtained with a difference in retention time of 10 sec. This is due to formation of diastereoisomers of the amoxicilloate by epimerisation at the C-5 position[11,12]. Since the ratio of the peaks is time dependent, the sum of the peaks is used for calculations. $y = 0.0953x + 0.0163$; $r = 0.997$.

Amoxicillin dimer is obtained as previously described[17]. An aliquot containing 20.0 mg is dissolved in mobile phase A, and the volume is made up to 20.0 ml with the same solvent. To aliquots of this solution IS and mobile phase are further added as described for the amoxicilloate. $y = 0.061x + 0.0064$; $r = 0.999$. Amoxicillin piperazine-2,5-dione is obtained as previously described[17]. Dilutions are prepared as described for the dimer. $y = 0.105x + 0.0024$; $r = 0.999$.

Peaks, to which no particular structure could be assigned, are calculated as amoxicillin.

Reproducibility. A sample was analysed 7 times. Mean values for the content in % (m/m) and coefficients of variation (v) are: sodium amoxicillin, 79%, $v = 0.75$; sodium amoxicilloates, 2.6%, $v = 3.1$; sodium piperazine-2,5-dione, 1.3%, $v = 10$; sodium amoxicillin dimer, 5.0%, $v = 2.8$.

Mercurimetric titration

Samples are also titrated by the mercurimetric method, which has been adopted in the European Pharmacopoeia. Degradation products and total penicillins are titrated separately, and the amoxicillin content is obtained from these results by subtraction. The end-point is determined potentiometrically by using a mercurous sulphate reference electrode and a platinum indicator electrode.

Degradation products. To 0.250 g (m, in g) are added 25 ml of buffer solution (pH 9.0) and 0.5 ml of acetic anhydride, and the mixture is stirred for 3 min. To this soln. is added 10 ml of acetate buffer solution (pH 4.6), and the whole is immediately titrated with 0.02 *M* mercuric nitrate (n, in ml). The percentage content of degradation products (D) as $C_{16}H_{19}N_3O_5S$ is calculated from the expression $0.7308\ n/m$.

Amoxicillin. To 50.0 mg (m_1 in g) are added 10 ml of buffer solution (pH 9.0)

TABLE I

ANALYSIS OF SAMPLES OF AMOXICILLIN TRIHYDRATE

ND = Not determined due to limited amount of sample; NA = no structure is assigned to the small peaks. Figures in parentheses, below a result, indicate the number of experiments. Absence of this figure indicates that the analysis was not repeated.

Sample	*Manufacturer*	*Water content (%, m/m)*		*High-performance liquid chromatography (%, m/m)*				*Mercurimetric titration (%, m/m)*	
		*LOD**	*KF***	*Amoxicillin*	*Amoxicilloic acids*	*Piperazine-2,5-dione*	*Other peaks*	*Degradation products (D)*	*Amoxicillin*
British Pharmacopoeia Standard		ND	ND	84.9 (2)	1.6	NA	1.3	1.6	85.4 (2)
10	A	12.7 (2)	13.0 (4)	86.9 (2)	0.3	NA	1.1	0.5	84.2 (3)
11	B	13.2	13.2	84.3 (2)	0.3	NA	1.2	0.7	85.1 (4)
12	B	12.8	13.2	84.0 (2)	0.3	NA	1.5	0.7	84.3 (4)
13	B	13.0	13.4	84.0 (2)	0.4	NA	1.2	0.8	84.8 (3)
14	C	ND	ND	78.1 (2)	0.4	0.6	2.8	4.8	79.2
15	C	ND	ND	81.9 (2)	0.5	0.6	2.0	4.6	78.9 (2)
16	C	ND	ND	82.4 (2)	1.1	0.5	1.7	4.3	80.2 (2)
17	C	ND	ND	81.4 (2)	0.6	0.7	3.1	3.9	80.2 (2)

* Loss on drying *in vacuo* at *ca.* 25 Pa, 60°C, over P_2O_5, during 3 h.

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and 0.2 ml of acetic anhydride, and the mixture is stirred for 3 min. To this solution is added 10.0 ml of 1 N sodium hydroxide, and, after 15 min, 10.0 ml of 1 *N* nitric acid and 20 ml of acetate buffer solution (pH 4.6) are added. The solution is slowly titrated with 0.02 *M* mercuric nitrate (n_1, in ml), so that the titration takes *ca.* 15 min. Ignoring any preliminary inflection on the titration curve, the percentage content of $C_{16}H_{19}N_3O_4S$ is calculated from the expression

$$\frac{0.7308\ n_1}{m_1} - D$$

For the calculation of results for sodium salts ($C_{16}H_{18}N_3NaO_5S$) the factor 0.7748 is used instead of 0.7308.

Acetate buffer solution (pH 4.6) is prepared by dissolving 5.4 g of sodium acetate trihydrate in 50 ml of water, adding 2.4 g of glacial acetic acid and diluting to 100.0 ml with water. Buffer solution (pH 9.0) is prepared by dissolving 6.20 g of boric acid in 500 ml of water, adjusting to pH 9.0 with 1 *N* sodium hydroxide and diluting to 1000 ml with water.

RESULTS AND DISCUSSION

A typical chromatogram obtained with sodium amoxicillin is shown in Fig. 1. Amoxicillin is well separated from amoxicilloates, oligomers and piperazine-2,5-dione. The preparation and structure of these impurities has been discussed in detail elsewhere[17]. Although amoxicilloate is eluted very rapidly, the presence of two peaks,

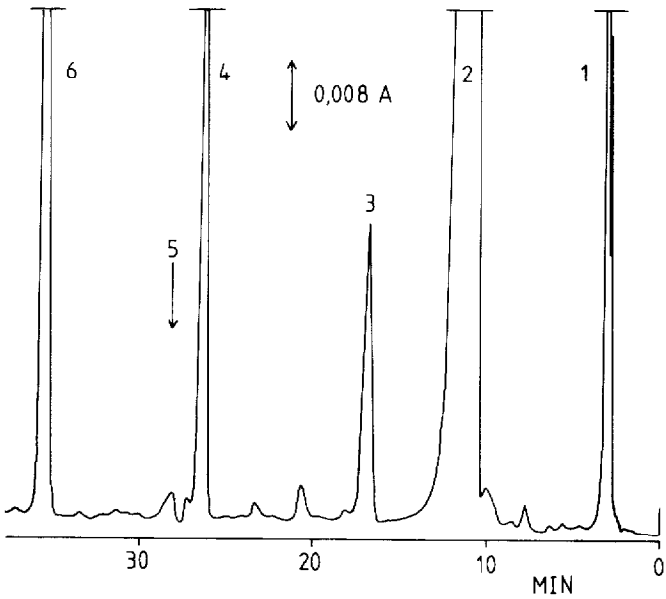

Fig. 1. Typical chromatogram of sodium amoxicillin. See Experimental for chromatographic conditions. Peaks: 1 = amoxicilloates (5%, m/m); 2 = amoxicillin; 3 = amoxicillin piperazine-2,5-dione (2%, *m/m*); 4 = amoxicillin dimer (5%, *m/m*); 5 = amoxicillin trimer (<0.25%, *m/m*); 6 = internal standard.

corresponding to two epimers, is visible. The epimerisation of amoxicilloate has been discussed recently[11,12].

The separation by HPLC of impurities present in sodium amoxicillin has been described before, but the presence of piperazine-2,5-dione was not mentioned, neither was the trimer available as a reference product[14]. The piperazine-2,5-dione was probably not separated from amoxicillin by that method since the dimer was eluted very soon after amoxicillin. The isocratic method described by Lebelle *et al.*[13] does not seem to be suitable for quantitation of the oligomers since the elution of amoxicillin itself needs 15–20 min. Under such conditions, relatively small amounts of more strongly retained products, such as oligomers, will not be detectable.

Results obtained with a series of samples of amoxicillin trihydrate are reported in Table I. The correlation between HPLC and the mercurimetric method is quite good, the mean of the differences for the amoxicillin content being *ca.* 1.2%, which is within experimental error. The samples proved to be rather pure in so far that most of the time the smaller peaks were not assigned to a particular structure (impurity), but were summed up as "other peaks" and were calculated as amoxicillin. The lower purity of samples 14–17, all from the same manufacturer, is confirmed by both HPLC and titration results. The impurities present behave mainly as structures with an opened β-lactam ring since they are titrated as degradation products. This

TABLE II

ANALYSIS OF SAMPLES OF AMOXICILLIN TRIHYDRATE

Figures in parentheses, below a result, indicate the number of experiments. Absence of this figure indicates that the analysis was not repeated. Samples donated by M. J. Lebelle.

Sample	*High-performance chromatography (%, m/m)*				*Chemical assay (%, m/m)*		
	Lebelle[13] *Amoxicillin*	*This laboratory*			*Imidazole-mercury colorimetry (Lebelle) Amoxicillin*	*Mercurimetric titration*	
		Amoxicillin	*Amoxicilloic acid*	*Other peaks*		*Degradation products (D)*	*Amoxicillin*
2A	80.6	80.0	0.5	2.9	79.9	3.4	81.2 (2)
2B	80.6	78.2	0.6	2.6	82.4	3.4	81.4 (2)
3A	80.6	83.0	0.2	1.6	82.5	3.3	80.7 (2)
3B	83.0	80.9 (2)	0.2	1.9	83.9	2.3	83.0 (2)
4A	85.2	85.0 (2)	1.0	1.8	85.0	1.4	84.7 (2)
4B	84.2	81.6 (3)	0.8	2.2	84.8	2.2	84.2 (2)
4C	81.9	80.9 (2)	0.4	2.1	81.6	3.4	82.9 (2)
5	83.3	83.1 (2)	0.2	2.2	83.6	2.4	84.6 (2)
6	81.5	82.5 (2)	0.6	1.7	82.9	3.4	82.9 (2)

TABLE III

ANALYSIS OF SAMPLES OF SODIUM AMOXICILLIN

ND = Not determined due to lack of sample; NM = not measurable (< 0.25%). Figures in parentheses, below a result, indicate the number of experiments. Absence of this figure indicates that the analysis was not repeated.

Sample	*Water content** (%, m/m)	*High-performance liquid chromatography (%, m/m)*						*Mercurimetric titration (%, m/m)*	
		Sodium amoxicillin	*Amoxicilloates*	*Piperazine-2,5-dione*	*Dimer*	*Trimer*	*Other peaks*	*Degradation products (D)*	*Sodium amoxicillin*
20	3.3 (2)	79.1 (2)	5.7	1.6	5.2	NM	1.5	8.0	85.8 (3)
21	3.3 (2)	78.9 (2)	2.4	1.0	4.2	NM	0.8	10.7	81.2 (2)
22	3.3	79.0 (7)	2.6	1.3	5.0	NM	1.1	10.9	81.2 (2)
23	3.5	80.2	1.6	1.5	4.9	NM	2.9	ND	ND
24	3.2	87.5 (2)	2.4	1.3	5.3	NM	0.7	4.7	88.5
25	3.5	82.8 (2)	1.4	1.7	4.4	NM	1.0	7.9	80.5
26	7.8	61.2 (2)	4.2	3.4	15.3	1.5	2.3	12.8	69.2

* Loss on drying *in vacuo* at about 25 Pa, 60°C, over P_2O_5, during 3 h.

is reflected in the difference between HPLC amoxicilloate values and results for degradation products obtained by titration. When purified amoxicillin dimer is titrated under the conditions mentioned above, 53% of degradation products and 97% of total penicillins are found. Amoxicillin piperazine-2,5-dione is titrated as corresponding to *ca.* 75% of degradation products, which means that during mercurimetric titration it does not fully behave as amoxicilloic acid.

Table II shows results obtained with a number of samples of amoxicillin trihydrate, which were previously analysed by Lebelle *et al.*[13]. The correlation with the results of Lebelle *et al.* is quite good. For the chemical assays the mean of the differences is $< 1\%$, for the HPLC assays this value is situated between 1 and 2%. The mean of the differences between our HPLC and mercurimetric titration results is also 1–2%. All these values are within experimental error.

Table III shows results obtained with a number of samples of sodium amoxicillin. It should be emphasised that the analyses were performed in a period when the production of sodium salts was still in development. For the sodium salts the mean of differences between HPLC and chemical assay of amoxicillin is $> 3\%$, the latter method giving higher results. This is explained by the higher content of oligomer impurities, which contribute to the total penicillins value since they contain a β-lactam ring. When half of the percentage found for the dimer is added to the HPLC result for amoxicillin, the mean of the differences with the titration results is reduced to between 1 and 2%, which is within experimental error. It is observed that sodium salts contain five to ten times more amoxicilloates than amoxicillin trihydrate. Piperazine-2,5-dione contents of 1–2% are to be considered as common, as well as a dimer content of 4–5%. Peaks corresponding to trimer normally represent less than 0.25% and are therefore summed up with "other peaks". Sample 26, which contains an excess of water, compared with the other sodium salts, is also much richer in oligomers, while the amoxicilloate content is not excessively high. It is believed that, due to the higher moisture content, small amounts of sodium amoxicillin can dissolve and react to form oligomers, which recrystallise. This sample is the only one to contain an appreciable amount of trimer. These results show how much the stability of sodium amoxicillin is dependent on moisture content.

It can be concluded that, by the HPLC method described here, it is possible to separate amoxicillin from its major impurities. For samples of amoxicillin trihydrate, the HPLC results correspond well with those of mercurimetric titration. In the case of sodium salts, however, the amoxicillin content is overestimated by the latter method owing to the effect of oligomers (mainly dimers).

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