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COMPARISON OF HPLC AND SOME OFFICIAL TEST METHODS FOR DIFFERENT PENICILLINS *

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SUMMARY

The selectivity, reproducibility and demand of time of some pharmacopoeia-methods and reversed-phase HPLC were compared for the 3 penicillins, phenoxymethylpenicillin potassium, benzylpenicillin sodium and ampicillin trihydrate. The selectivity of the official methods is not satisfactory, and only a combination of these methods guarantees the recognition of the most probable impurities. Superior is the HPLC; this technique is highly selective and one of the fastest and most precise methods.

INTRODUCTION

For the determination of the β-lactam antibiotics, phenoxymethylpenicillin (penicillin V) potassium, benzylpenicillin (penicillin G) sodium and ampicillin trihydrate, different methods are described in the literature. Most used for serial analysis are chemical methods that can be applied for many different β-lactams: the iodometric method (Alicino, 1946) and the hydroxylamine-method (Ford, 1947; Boxer et al., 1949). Both techniques have been taken up into different pharmacopoeia. Another chemical method, similar in its principle to the iodometry, is the determination of penicillins with mercury(II) solution (Karlberg et al., 1976) which is included in the drafts of the next edition of the European Pharmacopoeia for some penicillins. Another possibility is the spectrophotometrical measurement at 325–345 nm of penicillinic acid mercuric mercaptides formed in the presence of mercuric chloride and imidazole (Bundgaard et al., 1972; Bundgaard, 1974, 1977). This method is used for ampicillin in the British Pharmacopoeia 1973, Addendum 1978. Besides this, a lot of other chemical and physical methods are in existence, a summary of which was given by Hughes et al., 1976.

* This paper is dedicated to Dr. H. Kuntscher on the occasion of his 65th birthday.

The development of HPLC brought about an important improvement in the analysis of penicillins. By modern UV-detectors a sensitive detection is possible, high efficiency columns guarantee good separations within a short time. At first, ion exchangers were used as stationary phases (Blaha et al., 1975; Tsuji et al., 1975), and then reversed-phase materials brought further improvements (Hartmann et al., 1976; Vree et al., 1978; Nachtmann, 1979; Vadino et al., 1979).

Little literature exists about a comparison of the different methods. Roksvaag et al. (1979) describe an investigation, in which the iodometric and a HPLC-method on anion exchangers are compared for penicillin G sodium. In stability tests no significant difference between both the techniques was found. A comparison of the determination of ampicillin, amoxycillin and cefuroxime in rabbits by reversed-phase HPLC and a microbiological assay is described by Hekster et al. (1979). An excellent correlation was found. As advantages of HPLC the authors cite its speed and specificity. A systematical investigation including some pharmacopoeia-methods has not been published up to now. In this paper an evaluation of reversed-phase HPLC in comparison to the most important official test methods for some penicillins shall be made.

EXPERIMENTAL

Materials and methods

All model mixtures were made with test substances of Biochemie, Kundl, Austria. The purity of the β-lactam antibiotics was: penicillin V potassium 99.8%, penicillin G sodium 99.7%, ampicillin trihydrate 99.2%. All added by-products had a chromatographic purity better than 95%. The polymers of ampicillin consisted of a mixture of at least 20 substances with a molecular weight greater than that of ampicillin. The official tests were carried out with reagents corresponding to the requirements of the pharmacopoeia. For HPLC tests LiChrosorb RP 8, particle size 10 μm (Merck, Darmstadt, G.F.R.) was used as stationary phase, analytical grade solvents (Merck) for the mobile phase.

Instruments

The HPLC determinations were performed using a high pressure pump Altex 110 A in connection with a Perkin Elmer LC 55 spectrophotometric detector at 220 nm. The injection system was a Rheodyne 7120 loop injector, the injection volume was 20 μl. All described separations were carried out with a steel column, 250 × 3.2 mm i.d. The column was placed in a thermostatted water bath at 50 ± 0.5°C. This procedure is a simple way for applying higher temperatures, and thermostatting the pumping or injection system proved to be unnecessary. Peak integrations and calculations of the data were done with a laboratory data system 3352 (Hewlett Packard). For the measurement of the diameter of the inhibition zones in the microbiological tests a petriscope (Cambridge Instruments, England) coupled to a Quantimet 720 (Cambridge Instruments, England) was used.

RESULTS AND DISCUSSION

The test methods were compared in respect to their selectivity, reproducibility and time of analysis. The selectivity was checked by analyzing mixtures, where known

amounts of by-products were added to the antibiotics. The reproducibility is expressed as relative standard deviation of 8 determinations of the substances. The given time of analysis is only realistic in the case of the test series.

Penicillin V potassium

For the test of the selectivity, phenoxymethylpenicilloic acid (penicilloic acid V), 4-hydroxy-penicillin V (p-OH-V) and penicillin G sodium were added to penicillin V potassium. The following methods were checked: (a) microbiological test according to Code of Federal Register, No. 436.105; (b) iodometrical test according to Code of Federal Register, No. 436.204; (c) spectrophotometrical test according to Code of Federal Register, No. 440.73(6); (d) spectrophotometrical test according to British Pharmacopoeia 1973, p. 362; (e) mercury-titration according to draft European Pharmacopoeia; and (f) HPLC.

Excellent HPLC-separations of penicillins are possible with small particle-reversed phases. As mobile phases, mixtures of methanol with phosphate buffer at pH = 7.0 proved to be well suited. At elevated temperatures the analysis time can be decreased without losses in separation efficiency (Nachtmann, 1979). The optimum for a selective determination of penicillin V was a solvent system consisting of phosphate buffer pH 7/methanol (60 : 40) at 50°C column temperature. All added by-products are separated from penicillin V (Fig. 1), which is the basis of the high selectivity of this method.

The comparison of the 6 test methods is summarized in Table 1. With HPLC it is possible to get accurate results in any of the tested cases. The microbiological method gives

TABLE 1

COMPARISON OF 6 TEST METHODS FOR THE DETERMINATION OF PENICILLIN V POTASSIUM (PV)

Mixture	Theory (%)	Results in %					
		A	B	C	D	E	F
PV [a]	100	100	100	100	100	100	100
PV + 2% penicilloic acid V	98.0	97.5	98.4	100.7	98.0	98.1	98.9
+ 4% penicilloic acid V	96.0	96.5	95.8	100.1	98.9	96.5	96.1
+ 6% penicilloic acid V	94.0	91.5	92.9	100.8	99.4	94.6	94.5
+ 2% *p*-OH-penicillin V	98.0	98.5	101.1	100.8	98.7	100.7	97.0
+ 4% *p*-OH-penicillin V	96.0	97.0	100.6	98.9	96.7	100.1	95.3
+ 6% *p*-OH-penicillin V	94.0	96.5	100.5	97.8	95.1	98.9	94.1
+ 2% penicillin G sodium	98.0	99.0	100.3	97.7	95.4	99.5	97.7
+ 4% penicillin G sodium	96.0	101.5	100.8	95.5	90.2	100.0	95.4
+ 6% penicillin G sodium	94.0	99.5	100.6	93.4	86.0	100.8	94.4
Relative S.D. (n = 8)		2.4%	1.5%	0.4%	1.3%	0.5%	0.6%
Time/analysis (min)		75	20	10	60	60	20 15 [b]

[a] Penicillin V potassium was used as standard with 100%.

[b] For automated injection.

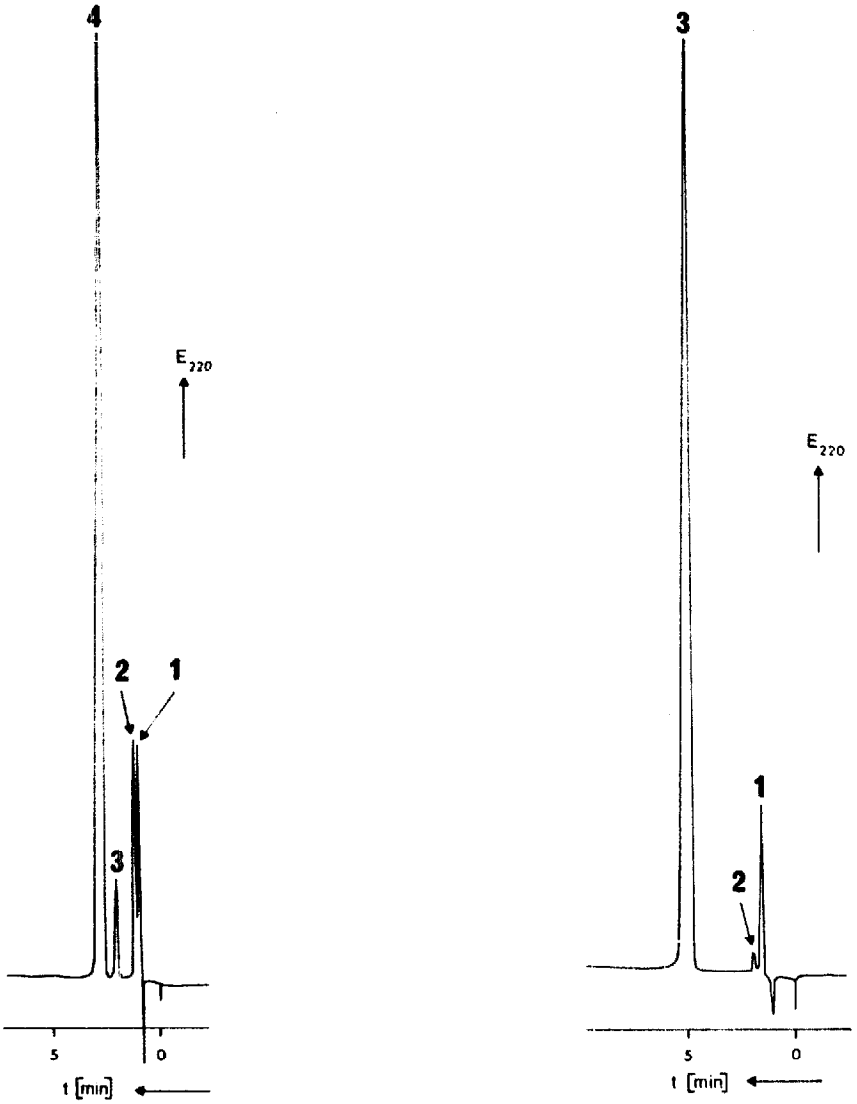

Fig. 1. Separation of penicillin V potassium and some impurities. 1, penicilloic acid V; 2, penicillin G; 3, *p*-hydroxy-penicillin V; and 4, penicillin V. Column, LiChrosorb RP 8, 10 μm, 250 × 3.2 mm i.d.; solvent, phosphate buffer (0.015 M) pH 7/methanol (60 : 40); temperature, 50°C; injection volume, 20 μl; flow rate, 1.5 ml/min.

Fig. 2. Separation of penicillin G sodium and some impurities. 1, (+)-α-benzylpenicilloic acid; 2, (3)-β-benzylpenicilloic acid; and 3, penicillin G. Solvent, phosphate buffer (0.015 M) pH 7/methanol (70 : 30); other conditions: see Fig. 1.

only accurate results, if the contaminant is biologically inactive, contaminations of penicillin V with other penicillins are therefore not detectable. The selectivity of the iodometric method and the mercury titration are equal: the presence of degradation products does not cause wrong results, but this is the case if other penicillins are present. Using the spectrophotometric tests the opposite is true: penicillin V is simulated by a degradation product, penicilloic acid V.

TABLE 2

COMPARISON OF 4 METHODS FOR THE DETERMINATION OF PENICILLIN G SODIUM (PG)

Mixture	Theory (%)	Results in %			
		A	B	C	D
PG [a]	100	100	100	100	100
PG + 2% penicilloic acid G	98.0	97.0	98.6	97.0	98.7
+ 4% penicilloic acid G	96.0	95.0	96.4	95.9	96.4
+ 6% penicilloic acid G	94.0	94.2	93.9	94.1	93.9
+ 2% penicillin V potassium	98.0	100.4	99.6	99.5	98.2
+ 4% penicillin V potassium	96.0	99.7	99.6	99.1	96.8
+ 6% penicillin V potassium	94.0	101.0	99.6	100.0	94.2
Relative S.D. (n = 8)		2.0%	1.5%	0.5%	0.6%
Time/analysis (min)		75	20	60	25 15 [b]

[a] Penicillin G sodium was used as standard with 100%.
[b] For automated injection.

The most precise methods with a relative standard deviation of 0.5% are the spectrophotometric determination (CFR), the mercury titration and the HPLC. The iodometric and spectrophotometric method (BP 73) resulted in relative standard deviations of 1.5%, the microbiological method 2.4%.

The greatest sample output with an analysis time of 10 min can be realized spectrophotometrically (CFR). The 2-fold time (20 min) is necessary for iodometry and HPLC, while with automated HPLC even a shorter time is possible. For the necessary titration and the spectrophotometric determination according to BP 73 60 min are needed, and the longest time (75 min) is necessary for the microbiological test.

Penicillin G sodium

The products penicilloic acid G and penicillin V potassium were mixed with penicillin G sodium in order to check the selectivity of the following methods: (a) microbiological test according to Code of Federal Register, No. 436.105; (b) iodometrical test according to Code of Federal Register, No. 436.204; (c) mercury titration according to draft European Pharmacopoeia; and (d) HPLC.

For HPLC nearly the same conditions as described for penicillin V can be used. The polarity of the mobile phase must be increased, the solvent system phosphate buffer pH 7/methanol (70 : 30) proved to be optimal. As shown in Fig. 2, the impurities are completely separated from penicillin G. For penicillin V no peak is visible in the chromatogram, and its elution time is about 15 min. Therefore accurate values can be expected from HPLC for all mixtures. The comparison of the methods is given in Table 2. The results are practically identical to those obtained for penicillin V potassium.

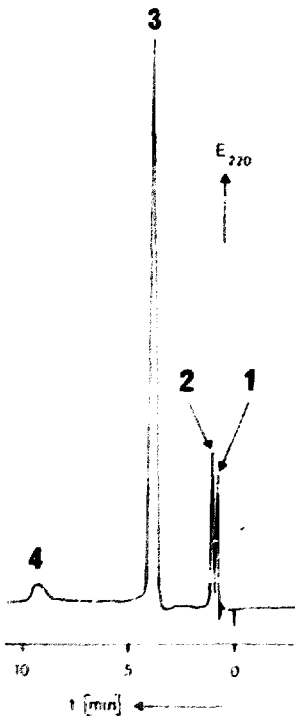

Fig. 3. Separation of ampicillin trihydrate and some impurities. 1, 6-APA; 2, penicilloic acid of ampicillin; 3, ampicillin; 4, penicillin G. Solvent, phosphate buffer (0.015 M) pH 7/methanol (80 : 20); other conditions: see Fig. 1.

Ampicillin trihydrate

Three different impurities were added to the standard substance ampicillin trihydrate. These impurities were: (i) mixture of ampicillin polymers; (ii) 6-aminopenicillanic acid (6-APA); and (iii) penicillin G sodium. The following methods were checked: (a) microbiological test according to Code of Federal Register, No. 436.105; (b) iodometrical test according to Code of Federal Register, No. 436.204; (c) colorimetry using imidazol/mercury according to British Pharmacopoeia 73/addendum 1978 (p. 4); (d) mercury titration according to draft European Pharmacopoeia; and (e) HPLC.

The same column as described for penicillin V potassium and penicillin G sodium can be used for HPLC-determination of ampicillin trihydrate. As ampicillin has a higher polarity, the water content of the mobile phase must be increased. The solvent system phosphate buffer pH 7/methanol (80 : 20) at 50°C gave good results. A separation of ampicillin with the described impurities is shown in Fig. 3. The polymers are not eluted from the column under the described conditions, 6-APA and penicillin G are well separated from ampicillin.

A summary of the comparison of the 5 test methods is given in Table 3. HPLC is the only one of the tested techniques that produced accurate results in all tested cases. The microbiological test was selective except for the mixtures with penicillin G sodium. The colorimetry (BP 73, addendum 1978) gave accurate results, but if ampicillin was contaminated with its degradation products (penicilloic acid, polymers) the presence of

TABLE 3

COMPARISON OF 5 TEST METHODS FOR THE DETERMINATION OF AMPICILLIN TRIHYDRATE (AMP)

Mixture	Theory (%)	Results in %				
		A	B	C	D	E
Amp [a]	100	100	100	100	100	100
Amp + 2% penicilloic acid	98.0	96.8	99.0	98.4	99.0	98.5
+ 4% penicilloic acid	96.0	95.5	96.0	97.6	97.3	96.1
+ 6% penicilloic acid	94.0	92.9	95.7	94.7	95.5	93.3
+ 2% polymers	98.0	98.7	99.2	100.2	98.0	97.6
+ 4% polymers	96.0	97.8	97.6	94.4	96.6	95.3
+ 6% polymers	94.0	93.3	96.6	92.3	96.0	94.2
+ 2% 6-APA	98.0	97.5	99.3	102.4	103.0	97.7
+ 4% 6-APA	96.0	96.5	100.5	103.7	104.4	95.8
+ 6% 6-APA	94.0	93.5	103.6	103.8	105.7	93.5
+ 2% penicillin G sodium	98.0	102.5	100.7	100.6	100.4	98.2
+ 4% penicillin G sodium	96.0	98.0	100.7	99.9	101.3	95.8
+ 6% penicillin G sodium	94.0	98.0	100.5	98.4	101.4	94.8
Relative S.D. (n = 8)		2.2%	1.5%	0.5%	0.5%	1.2%
Time/analysis (min)		75	20	40	60	20 15 [b]

[a] Ampicillin trihydrate was used as standard with 100%.
[b] For automated injection.

6-APA caused results higher than 100%. Therefore it is possible to find the theoretical value if 6-APA and degradation products are present in a sample of ampicillin trihydrate. Iodometry and mercury titration are very similar to the colorimetric test in respect to their selectivity.

The highest precision with a relative standard deviation of 0.5% can be reached by the mercury titration and colorimetry. HPLC and iodometry are equal at 1.5%, for the microbiological technique and the relative standard deviation was 2.2%.

The fastest methods for the determination of ampicillin are iodometry and HPLC, which is in agreement with the results for the two other tested penicillins. For the colorimetry the two-fold time, for the mercury titration the 3-fold time, and for the microbiological test about the 4-fold time of analysis is necessary.

CONCLUSIONS

For the 3 tested penicillins analogous results were gained. Iodometry (CFR) and mercury titration (draft Ph.Eur.) are equivalent. The selectivity is identical, the higher precision of the mercury titration must be paid for by a higher time per analysis. A combination of different official test methods is necessary to assure the quality of penicillins. The

only method that was able to produce accurate values in all tested cases was the HPLC. These methods have been routinely used for the quality control of the described penicillins without problems for more than one year.

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