Journal of Chromatography, 236 (1982) 469–480
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,446

QUANTITATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF AMPICILLIN*

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(First received August 27th, 1981; revised manuscript received October 8th, 1981)

SUMMARY

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for the potency determination of the trihydrate and anhydrous forms of ampicillin and its sodium salt. An acid phosphate-acetonitrile mobile phase was used with caffeine as an internal standard. Efficiencies exceeding 15,000 plates per meter (or reduced plate height of 13) were obtained with precision normally better than 2% and a good linear range of detection (0.7–36 μg) at 254 nm. Good statistical correlations were shown when the results obtained from the RP-HPLC method were compared to those from the official methods.

INTRODUCTION

Ampicillin (D(−)-α-aminobenzylpenicillin) (CAS Registry 69-53-4 for the anhydrous, 7177-48-2 for the trihydrate, and 69-52-3 for the sodium salt), derived from 6-aminopenicillanic acid (6-APA), is an antibiotic used therapeutically on a wide scale because of its relatively rapid systemic absorption, its low toxicity, and its biological activity against a broad spectrum of Gram-positive and several Gram-negative pathogens. The assay of ampicillin currently consists of the following official[1] procedures: (a) microbiological agar diffusion, (b) iodine titration, (c) hydroxylamine colorimetry, (d) non-aqueous acid titration, (e) non-aqueous base titration.

The microbiological assay reflects the end use of the drug, but the method is slow and tedious and lacks precision and specificity. The chemical assays require available functional groups in order to react quantitatively. But these assays are limited by the fact that they depend on reactions that may also occur with other components of the drug substance, which interfere. Consequently, the adoption of a concordance test (difference between two non-aqueous titrations) proved relatively successful except in cases when the molecule did not retain its integrity. Clearly a different analytical approach offering greater specificity became a necessity.

* Presented at the *American Pharmaceutical Association, Academy of Pharmaceutical Sciences, San Antonio, TX, U.S.A., November 1980.*

High-performance liquid chromatography (HPLC) offered the best potential for quantitating the intact principle of the drug after separating and differentiating it from undesirable substances such as oligomers, degradation products, and residual contaminants, such as dimethylaniline, which is currently monitored by gas chromatography[2].

Column chromatographic techniques with Sephadex G-25[3,4], ion-exchange[5], and reversed-phase (RP) HPLC[6–8] have been described for the analysis of ampicillin in concurrent polymerization and degradation processes. Bracey[9] used a strong anion-exchange column to isolate and detect ampicillin as a contaminant in nitrofurantoin. A similar type of column was used by Tsuji and Robertson[10] to develop an assay procedure for the antibiotic. Subsequently, qualitative RP-HPLC of ampicillin in dosage forms was promoted for commercial purposes[11] to separate different penicillins[12,13], to determine partition coefficients of penicillins[14], and to analyze antibiotic susceptibility disks[15]. It proved to be an effective method for the analysis of biological materials by direct detection at 225 nm[16] or by post-column derivatization[17], and for separating diastereoisomers[18]. Because none of these references provided a satisfactory analytical method applicable to regulatory use, this study was undertaken to develop a more specific and reliable method for the potency determination of the trihydrate and anhydrous forms of ampicillin and its sodium salt.

EXPERIMENTAL

Apparatus

Investigations were carried out at various times with three different sytems: (1) a S-P Model 3500 liquid chromatograph with a Model 8200 UV detector (Spectra-Physics, Santa Clara, CA, U.S.A.) and a 20-μl loop injector (Valco, Houston, TX, U.S.A.), (2) a Model 995 liquid chromatograph with detector (Tracor, Austin, TX, U.S.A.) and an injector (Rheodyne, Cotati, CA, U.S.A.), (3) a Model 6000A solvent delivery system equipped with a U6K septumless valve injector and a Model 440 UV detector (Waters Assoc., Milford, MA, U.S.A.). These systems were connected to a strip chart recorder and an electronic integrator such as an H-P 7100 (Hewlett-Packard Co., Palo Alto, CA, U.S.A.) and an Autolab Minigrator (Spectra-Physics), respectively.

Columns

Except for the special Pyrocarbon column[19], the only satisfactory columns were the reversed-phase types such as the octadecyl silane (ODS) bonded silicas like Spherisorb S5-ODS (Phase Separations, Queensferry, Great Britain), Partisil ODS-2 (Whatman, Clifton, NJ, U.S.A.), and Partisil PXS-525 ODS (Whatman), μBondapak C_{18} (Waters) or a well-capped C-2 silica such as Zorbax TMS (DuPont Instruments, Wilmington, DE, U.S.A.) that exhibit some degree of polarity. The columns used for quantitations were Partisil PXS-525 ODS (25 cm × 4.6 mm I.D.) and Zorbax TMS (15 cm × 4.9 mm I.D.). A guard column (10 cm × 4.6 mm I.D.) gravity-packed with an ODS bonded silica of larger size (40–60 μm) was connected to the inlet of the analytical column. Columns were evaluated for performance and suitability as suggested by Bristow and Knox[20] with a program described and modified for the APL programming language to suit our needs[21,22]. The column was conditioned to equilib-

rium by the passage of about 10 to 15 void volumes of eluent, followed by about 75 μl of sample injected in 15-μl portions.

Chemicals and reagents

The ampicillin standard, labeled at 864 μg/mg with 13.52% moisture, was obtained from the United States Pharmacopeia (USP). Caffeine (USP) was the internal standard. Acetic acid and potassium dihydrogen phosphate were reagent grade. Acetonitrile was "distilled in glass" quality (Burdick & Jackson, Muskegon, MI, U.S.A.). All the water used was purified by the Milli-Q-Reagent-Grade water system (Millipore, Bedford, MA, U.S.A.).

Solutions

Stock solutions. Stock solutions of acetic acid and of potassium dihydrogen phosphate, each 1.0 *M*, were prepared with water.

Diluent. A 10.0-ml aliquot of 1.0 *M* phosphate stock solution was combined with 1.0 ml of 1.0 *M* acetic acid stock solution and diluted to 1.00 liter with water (pH about 4.1). This diluent was used to dissolve all samples as well as the internal standard.

Mobile phase. The eluent was prepared by adding 10.0 ml of 1.0 *M* phosphate stock solution and 1.0 ml of 1.0 *M* acetic acid stock solution to about 500 ml of water; then 80 ml of acetonitrile was added and the solution was diluted to 1.00 liter with water while mixing. This solution was degassed by passage through a 0.5-μm filter and ultrasonicated for at least 2 min before use. As an added precaution, helium was allowed to sparge perceptibly through a metal filter into this mobile phase during the analysis to ensure maximum elimination of dissolved air.

Internal standard. Approximately 20 mg of caffeine was dissolved in 100 ml of diluent.

Standard solution. An amount of the working ampicillin standard equivalent to about 25 mg on the dried basis was accurately weighed into a 25-ml volumetric flask. After 1.0 ml of the internal standard was added, the flask was filled to volume with diluent and the solution was mixed. The solution was further shaken and ultrasonicated until it became totally clear. Samples of ampicillins were treated in the identical manner as the standard. It is important that standards and samples be dissolved just before analysis.

Conditions for quantitation

Instrument system 3 as described under *Apparatus* was used for quantitation with operating temperature ambient and flow-rate 1.5 ml/min. The detector sensitivity was controlled by the attenuator of the electronic integrator to produce peak heights greater than 50% f.s.d. The chart speed was normally set at 2.54 mm/min (0.1 in./min) for assays, but for computation of performance parameters, chromatograms were obtained at 25.4 mm/min to improve the accuracy of manual measurements.

Assay and calculations

Carefully measured 15.0-μl aliquots of standard and sample solutions were injected sequentially into the chromatograph. Quantitation was achieved by normal-

izing the peak height (and/or area) with the internal standard and comparing to that of a reference standard material according to the following equation:

$$[P_x/(C_x \times I_x)/P_s/(C_s \times I_s)] \times 100 = \% \text{ purity}$$

where P is peak height (or area) of ampicillin, C is concentration of solution corrected for moisture content, I is peak height (or area) of the internal standard, and x and s refer to analyte sample and reference standard material, respectively.

All graphs were plotted and all calculations and data reduction were performed through the APL time-sharing system of the IBM 370/168.

RESULTS AND DISCUSSION

The sodium salt must be dissolved in the acid phosphate diluent, but good quantitation was obtained for the trihydrate and anhydrous materials by dissolving them in water only. Inasmuch as ampicillin is least prone to hydrolysis in its zwitterionic form, a slightly acid aqueous system with a pH near its isoelectric point will maximize its stability during the course of the analysis, even though its solubility is minimal at that pH[23]. Therefore an ampicillin concentration of about 1 mg/ml was selected, but as noted previously, the samples should be dissolved just before injection.

Calibration

Varying amounts of loop-injected ampicillin (*i.e.*, same volume of solutions of different concentrations) were plotted against peak heights and corrected for recorder sensitivity for the first calibration. A rectilinear relationship with a coefficient of correlation of 0.9998 and a 0.70% relative standard error was obtained by regression analysis for a 50-fold dilution of the amount of drug injected (0.7–36 μg); the upper limit of this range is well below the loading capacity of the column. Further examination of 22 values of the calibration factor (peak height per unit concentration) plotted against concentration (different quantities in the same volume) showed a constant value with a coefficient of variation of 4.7% with the largest deviations occurring at the lower concentrations.

Another calibration plot based on injections of aliquots of different volumes of the same solution was obtained with the U6K injector. Volumes (2.5–20 μl) injected by syringe and plotted against peak areas (or heights) yielded the straight line shown in Fig. 1. For an injection of 0.4 μg, the extrapolated peak height is about 5 mm, which is more than twice the signal-to-noise ratio at that sensitivity. However, a plot of these volumes against the calibration factor (area/μl) proves that the apparent linearity in Fig. 1 is misleading. The calibration factor, which decreased by about 30% as volume increased over an 8-fold volume change, is shown in Fig. 2. This discrepancy clearly demonstrates the advisability of using an internal standard and maintaining a constant syringe volume from injection to injection, particularly with non-loop injectors.

System performance

Fig. 3 is a typical chromatogram of a freshly dissolved sample of ampicillin

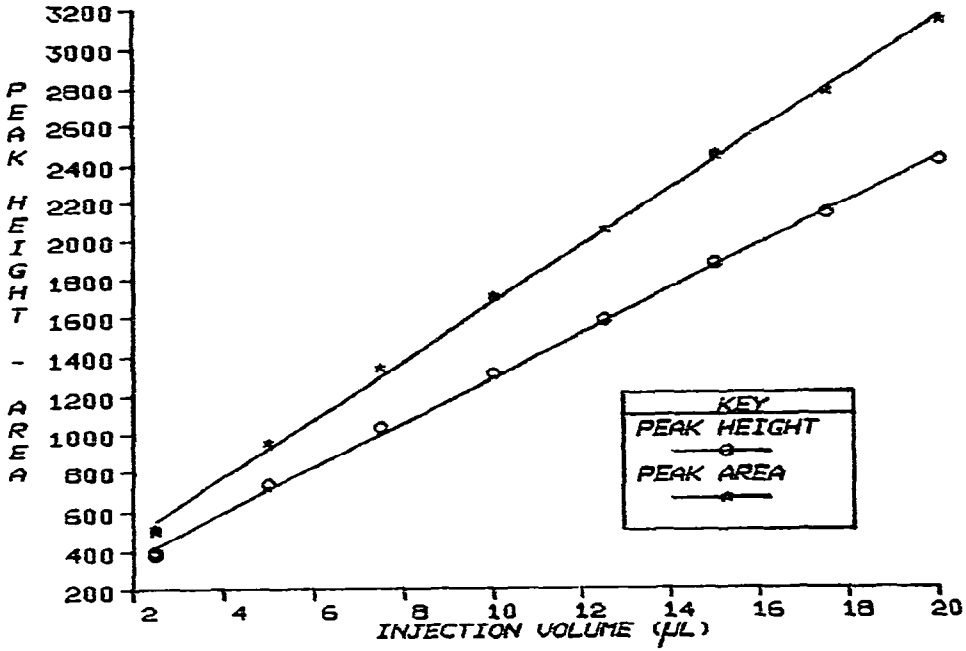

Fig. 1. Calibration of ampicillin syringe and U6K injections.

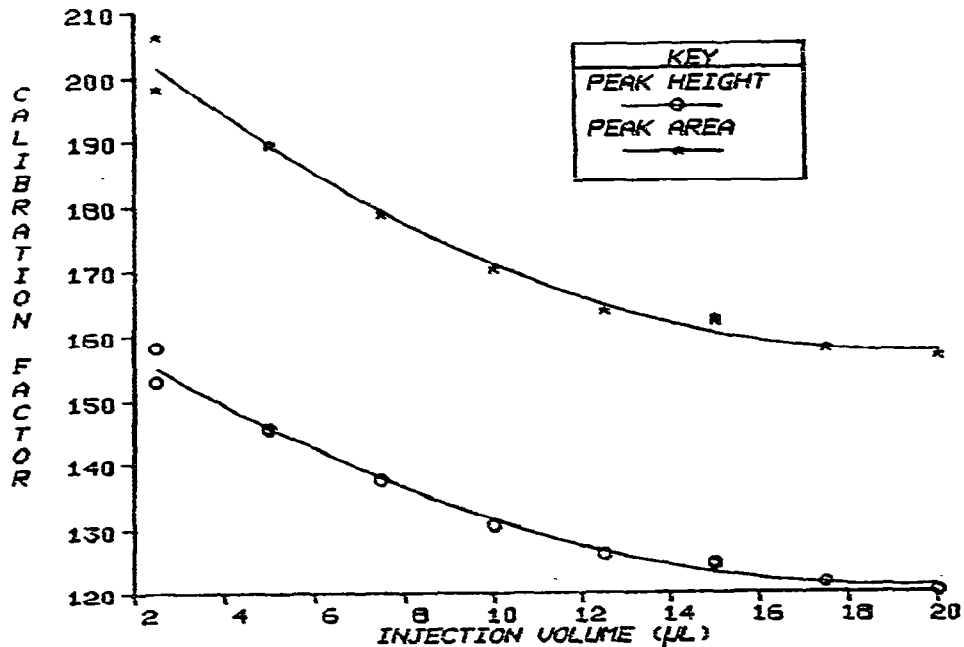

Fig. 2. Constancy of the calibration factor in syringe and U6K injections.

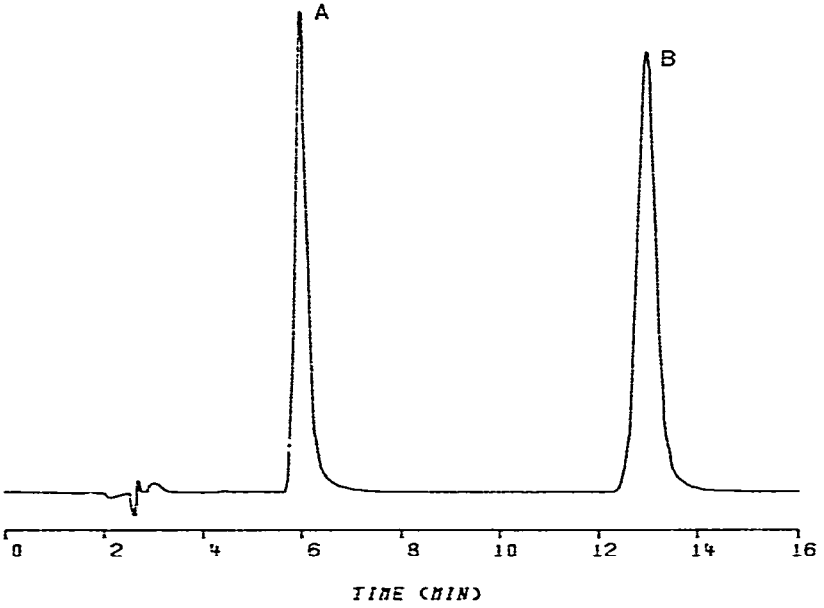

Fig. 3. Chromatogram of freshly dissolved (A) ampicillin and (B) caffeine on Partisil PXS-525 ODS.

used for potency measurement, whereas the chromatogram in Fig. 4 is that of a solution that has incurred a slight degradation just by standing for several days. A chromatogram obtained at higher sensitivity (Fig. 5) shows peaks of minor components.

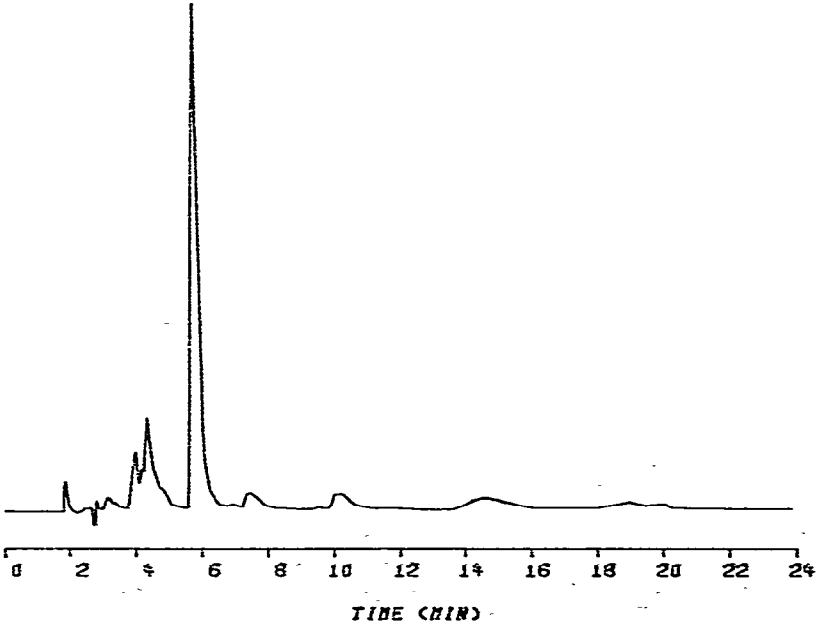

Fig. 4. Chromatogram of slightly degraded ampicillin solution on Partisil PXS-525 ODS.

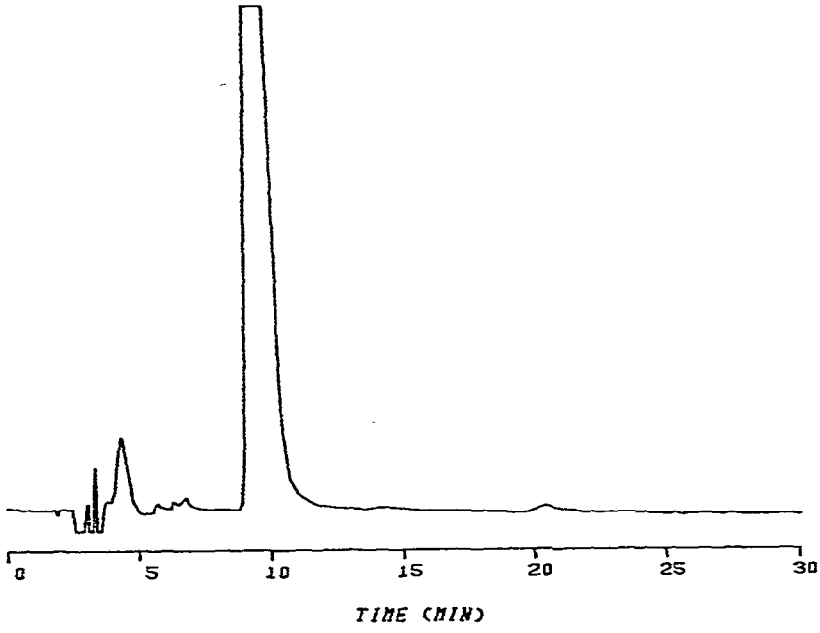

Fig. 5. Chromatogram of freshly dissolved ampicillin at higher sensitivity on Partisil PXS-525 ODS.

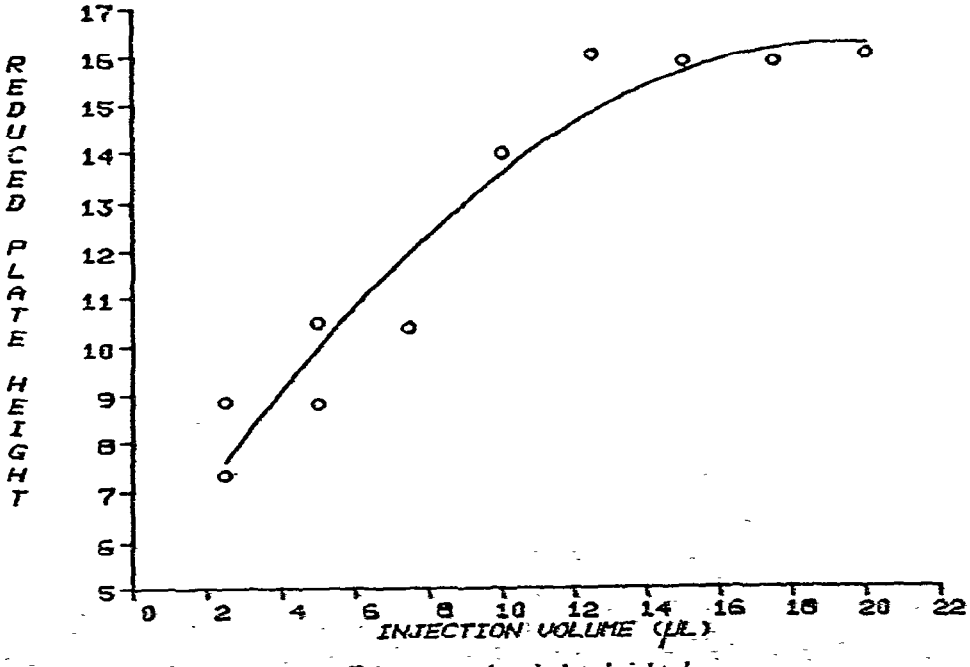

Fig. 6. Effect of sample size on efficiency as reduced plate height, h.

A 15-cm Zorbax TMS column typically exhibits efficiencies, n, of 4230 and 11,640 plates or reduced plate heights, h_r, of 9.8 and 3.6, and capacity factors (k) of 0.82 and 1.78 for ampicillin and caffeine, respectively, with a selectivity of 2.16, a retention ratio of 1.5, and a resolution of 8.90 between the two peaks. The shape of the chromatographic peak tends to skew and to shift from the center of gravity with increasing concentration as evidenced by a slight increase in the retention volume and a corresponding increase in the capacity factor. Although greater efficiencies are attained with smaller amounts of injected solutes, (Fig. 6), compromises are necessarily made with respect to quantity injected and flow-rates.

Quantitative analysis

Table I summarizes the results of a number of assays on Partisil PXS-525 ODS of several lots of ampicillin representing a variety of manufacturers. Results were calculated from electronic area integration and from concurrent peak height measurement; Table I gives the combined averages. Values calculated from peak heights correlate well with values calculated from peak areas with a coefficient of variation of 1.1%. This is a better correlation than that derived from several replicate results obtained from different weights of the same sample.

TABLE I

RESULTS OF AMPICILLIN ASSAYS (OVERALL) ON PARTISIL PXS-525 ODS

S.D. = Standard deviation; C.V. = coefficient of variation.

Sample	*Number of assays*	*Mean potency (%)*	*Variance (%)*	*S.D. (%)*	
1	4	104.65	0.96	0.98	0.93
2	6	99.95	1.10	1.05	1.05
3	4	96.44	0.11	0.32	0.34
4	5	99.84	0.03	0.16	0.16
5	5	103.37	0.50	0.71	0.69
6	5	102.89	0.51	0.71	0.69
7	4	101.01	0.39	0.62	0.62
8	5	102.62	0.49	0.70	0.69
9	4	99.11	0.08	0.28	0.28
10	9	95.07	1.98	1.41	1.48
11	4	99.72	0.15	0.39	0.39
12	4	103.08	0.01	0.12	0.12
13	5	101.98	0.36	0.60	0.59
14	4	100.53	0.54	0.73	0.73
15	4	100.78	0.19	0.44	0.43
16	4	101.94	0.40	0.63	0.62
17	4	94.97	0.03	0.16	0.17
18	8	95.73	2.11	1.45	1.52
19	4	93.42	1.16	1.08	1.15
20	14	100.27	1.93	1.39	1.39
All	106	99.63	10.10	3.18	3.19
95% Confidence interval of the mean = 99.02–100.25					
Peak areas	56	99.61	10.88	3.30	3.31
95% Confidence interval of the mean = 98.73–100.50					
Peak heights	50	99.66	99.43	3.07	3.08
95% Confidence interval of the mean = 98.78–100.53					

Improved quantitative results were obtained when the system included the guard column.

A correction for loss on drying (LOD) was applied to the initial weighing of all samples, but inaccuracies in this LOD naturally affected the assay results in a like manner.

Good quantiative results, especially with the loop injector, were obtained by direct comparison with an external standard, but the use of an internal standard with a good electronic data system significantly improved precision. The internal standard was used in this analysis solely to enhance the reliability of quantitation. Caffeine was the internal standard selected because it is well resolved from the peaks of interest, is stable, possesses good spectral properties, and is readily available in high purity.

The low absorptivity of the solute accounts for the relative lack of sensitivity at 254 nm, which is indicated by the need to inject upwards of 15 μg into the chromatograph. Nonetheless, this method is amply adequate for pharmaceutical bulk analysis and could easily serve as a stability-indicating method, although the amount injected could be readily reduced 10-fold with a likely increase in efficiency and symmetry. Should greater sensitivity be required, techniques can be devised to magnify the absorptivity of the solute by derivatization of the functional groups with highly chromophoric reagents[17].

Qualitative analysis and impurities

Pyrocarbon packing material is an experimental support prepared by Colin *et al.*[19] by controlled pyrolysis of an organic substance onto a silica substrate. A column packed with this material yielded a chromatogram with good peak separation, but the elution required a mobile phase containing 50% methanol, indicating a retention mechanism more akin to normal-phase than to reversed-phase separation.

The penicillins appear to be easily separated by reversed-phase chromatography. Table II shows the relative retentions on Spherisorb S5-ODS of several penicillins with the same solvent system as described above for the analysis of ampicillin. Results are also shown for a larger ratio of acetonitrile to phosphate buffer which was used to attain more practical retention times.

Yet with the mobile phase described above, Zorbax ODS, which has a very high carbon content and little or no residual silanol content, could not separate ampicillin from one of its impurities, seen as a shoulder on the main peak.

The identity of the active principle is presumed when the retention of the unknown is identical to that of a reference standard under identical experimental conditions. For confirmation of its identity, the substance under the major peak was collected, freeze-dried, and identified by IR and proton NMR spectroscopy.

Different approaches, such as neutron activation analysis and gas–liquid chromatography of residual dimethylaniline[2], have been attempted in an effort to characterize foreign substances in ampicillin. However, the amount of dimethylaniline found was insufficient to account for the total content of halogens (*i.e.*, mainly chlorides); it is likely that the excess was due to the presence of ampicillin hydrochloride. These past efforts have resulted in reducing the total content of undesirable substances in the drug product. The presence of impurities in these samples has been a subject of intense concern for many years[3–8]. Two reactants in the synthesis and likely contaminants in bulk samples of ampicillin, namely, 6-APA and phenylglycine, were

TABLE II

RETENTION OF PENICILLINS ON SPHERISORB S5-ODS

Compound	*k*	
	*10% Acetonitrile**	*35% Acetonitrile*
Ampicillin	1.64	NR**
Amoxycillin	0.19	NR**
Potassium penicillin G	6.41	0.76
Procaine penicillin G	–	0.95
Penicillin V	14.7	1.08
Sodium oxacillin	26.2	1.42
Sodium nafcillin	–	2.26

* Essentially same solvent system as described in the HPLC method.
** NR = Not retained.

injected into the chromatographic column and were essentially unretained. Other minor peaks that were observed have not been characterized fully in our laboratories.

Previous studies in other laboratories have clearly demonstrated the presence and character of several contaminants in ampicillin[7,8,10,23,24]. Penicillenic and penicilloic acids with a trace of penicillin G were detected in an alkaline solution of ampicillin[10]. In addition to α-aminobenzyl penicilloic acid, which was not retained on the column, Bundgaard[5–8] detected, identified, and quantitated oligomers which varied in size from dimers to octamers. The formation of these oligomers is promoted in alkaline media through auto-aminolytic degradation, but it should be noted that those

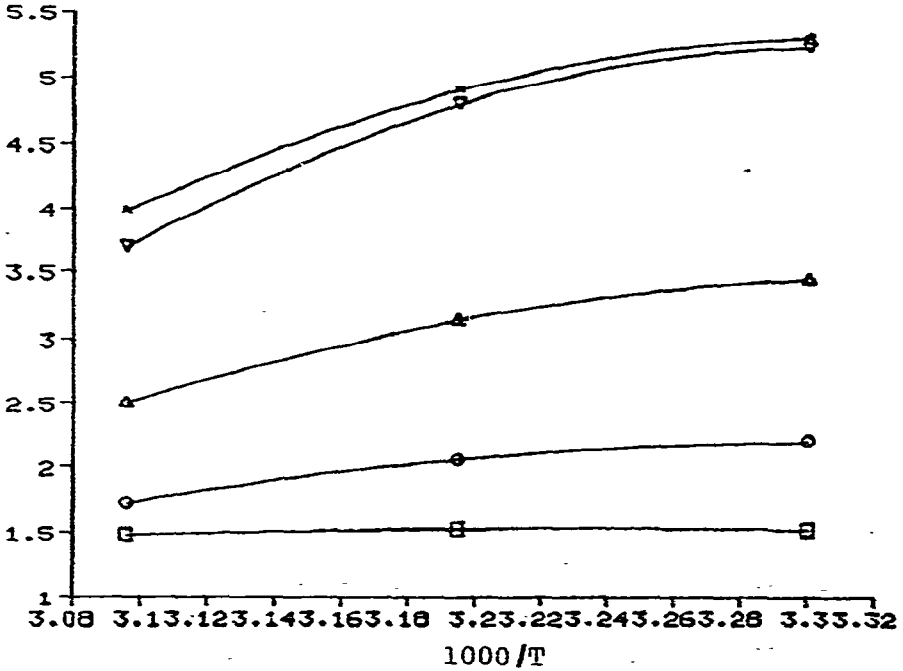

Fig. 7. Effect of temperature, T in °K, on capacity factors, k_1 and k_2, respectively (△ and ▽); selectivity, α (○); efficiency, n in plates/1000 (★); and pressure drop, P in bars/1000 (□).

solutions for chromatography were prepared in high concentrations at pH levels well above 7 in citrate and borate buffers[7,8], which had been described as deleterious to stability[23]. On the other hand, a strongly acidic solution used in a fluorometric assay yielded a fluorophor, 2-hydroxy-3-phenylpyrazine, as an ampicillin degradation product[24].

Temperature effect

Because the role of temperature has recently attracted more attention as an important parameter in HPLC[19], it was briefly investigated with a Spherisorb S5-ODS column with 12% methanol in 0.5 *M* sodium dihydrogen phosphate as the mobile phase. A significant decrease in the pressure drop and capacity factor for ampicillin and an impurity peak were observed when the temperature was increased as shown in Fig. 7, but no beneficial effects on efficiency and selectivity were noted.

TABLE III

COMPARISON OF TWO ASSAY METHODS FOR AMPICILLIN
σD = Standard deviation of difference.

Sample	*Potency (%)*	
	Hydroxylamine	*HPLC*
1	99.30	104.65
2	103.30	99.95
3	97.60	96.44
4	101.80	99.84
5	100.40	103.37
6	98.50	102.89
7	97.40	101.01
8	102.00	102.62
9	96.10	99.11
10	98.00	95.07
11	98.90	99.72
12	102.60	103.08
13	100.40	101.98
14	100.50	100.53
15	101.00	100.78
16	101.10	101.94
17	99.80	94.97
18	97.00	95.73
19	96.40	93.42
20	100.00	100.27
Number of samples	20	20
Maximum	103.30	104.65
Minimum	96.10	93.42
Range	7.20	11.23
Mean	99.60	99.87
S.D.	2.08	3.18
Median	99.90	100.40
C.V. (%)	2.09	3.18
95% Confidence limit of the mean:		
Lower limit	98.63	98.38
Upper limit	100.58	101.36
	$\sigma D = 2.72$	
	$t = 0.43$	

A statistical difference *is not* detected at the 95% confidence level.

Low viscosity is inherently crucial in HPLC, but in this system the energy expended to reduce the viscosity of the mobile phase is undesirable because it adversely affects the stability of the analyte. Evidently, it is important to maintain a constant temperature during the analysis for best results.

Validation of results

A comparison of results obtained by HPLC and the official methods by the paired *t*-test indicates that a significant statistical difference exists between the amine titration and each of the other methods, including HPLC. The presence of small amounts of ampicillin hydrochloride could certainly be a contributory interference in the amine titration. On the other hand, the HPLC, acid titration, hydroxylamine titration, and colorimetric assay methods revealed no such difference that could be ascribed to methodology. Statistical data for the hydroxylamine titration and HPLC methods are given in Table III to illustrate this.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge support from the various staff members of the National Center for Antibiotic Analysis, most notably Thomas G. Alexander, and from Richard Venable, Division of Drug Chemistry, for assistance with computer graphics. I also wish to acknowledge the gift of Pyrocarbon from Professor G. Guiochon, as well as valued discussions with the staff of the Laboratoire de Chimie Analytique Physique, École Polytechnique, Palaiseau, 91128 France, particularly Henri Colin, José Carlos Diez-Masa, Theresa Czajkowska, and Irena Miedziak.

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