High-Performance Liquid Chromatographic Analysis of Ampicillin

KIYOSHI TSUJI ˣ **and JOHN H. ROBERTSON**

Abstract □ A high-pressure liquid chromatographic method for the analysis of ampicillin is described. The method uses a 1-m long stainless steel column packed with anionic exchange resin, with a mobile phase of 0.02 *M* $NaNO_3$ in 0.01 *M* pH 9.15 borate buffer at a flow rate of 0.45 ml/min. The degradation products of ampicillin, penicillenic and penicilloic acids of ampicillin, can be separated and quantitated in less than 12 min of chromatographic time. The relative standard deviation for the analysis of ampicillin is less than 1%, and the method is sensitive to approximately 20 ng of ampicillin/sample injected. The method was applied to the analysis of various pharmaceutical preparations of ampicillin. It is also applicable, with a slight modification, for the analysis of penicillins G and V.

Keyphrases □ Ampicillin—high-pressure liquid chromatographic analysis □ Penicillins G and V—high-pressure liquid chromatographic analysis □ High-pressure liquid chromatography—analysis, ampicillin and penicillins G and V

Numerous chemical and physical methods and several automated methods are available for the determination of penicillins (1–13). Of these methods, the iodometric and hydroxylamine methods have been officially recognized by the Food and Drug Administration for "potency" determination (1). Although the speed of the automated iodometric and hydroxylamine methods (9–13) is fast, 20–60 determinations/hr, they are incapable of differentiating the intact penicillin molecules from the degradation compounds, since they determine the degraded penicillins, penicillanic and penicilloic acids.

Several GLC methods for penicillin have been reported (14–17); however, the methods determine penicillanic acid and have not been used for the analysis of ampicillin due mostly to difficulties in solubilization and derivatization of ampicillin. Chromatographic separation using a cross-linked dextran gel[1] column (18, 19) is slow and, to the knowledge of the authors, no high-performance liquid chromatographic (HPLC) method has been used to quantitate ampicillin, although an HPLC method was reported for the detection of ampicillin in nitrofurantoin (20) and for the determination of cephalosporin C (21).

EXPERIMENTAL

Apparatus—A modular liquid chromatograph equipped with a 254-nm UV monitor[2], a minipump[3], and a pulse dampener[4] were used.

An empty stainless steel column[5], 2.1 × 1000 mm, was first rinsed with tetrahydrofuran. Then the inside of the tubing was vigorously cleaned with a cotton string, presoaked with tetrahydrofuran, to remove loose metal particles. Chloroform was then drawn through the column, and the column was dried under a stream of dry nitrogen.

A 10-μm pore size stainless steel frit was fitted into the inlet end of the column, and a hex nut[6] with a stainless steel front, back lock ferrules, and cap[7] were attached to the column. Anionic exchange resin[8] was dry packed into the open end of the column by adding a small amount of the resin at a time and lightly tapping on the floor. After the column was tightly packed, a 2-μm pore size stainless steel frit was inserted into the outlet end of the column. The packed column was attached to a septumless injector valve[9] and the 254-nm UV monitor. The theoretical plates of the column, thus prepared, were approximately 555/m for the ampicillin peak.

Reagents—For the mobile phase, 0.02 *M* sodium nitrate in 0.01 *M* sodium borate, pH 9.15, was used.

Preparation of Ampicillin Standard Solution—The USP ampicillin reference standard was used "as is" at 867 μg/mg. Approximately 10 mg of the reference standard was accurately weighed[10] and placed in a 10-ml volumetric flask.

Prior to analysis, the standard was dissolved and diluted to the volume with the mobile phase.

Preparation of Samples—*Ampicillin Bulk Powder*—Approximately 10 mg of ampicillin bulk powder was accurately weighed and placed in a 10-ml volumetric flask. Prior to analysis, the sample was dissolved and diluted to volume with the mobile phase.

Hard-Filled Capsule—To minimize capsule weight variation, 10 capsules were emptied and their contents were accurately weighed. A portion of the powder equivalent to 1000 mg ampicillin was accurately weighed and placed in a 1000-ml volumetric flask. The contents were then diluted to volume with double-distilled water and shaken for 5 min to dissolve the ampicillin. About 25 ml of the suspension was placed into a 50-ml conical bottom centrifuge tube and then centrifuged at 2000 rpm for 20 min.

Sterile Injectables—Each vial containing 250 mg, 500 mg, or 1 g of product was reconstituted with the recommended volume of double-distilled water. An ultrasonicator[11] was used to dissolve each sample quickly.

From each of four 250-mg vials, 0.1 ml was removed with a 100-μl micropipet[12] with a disposable tip and placed in a 100-ml volumetric flask. The sample was then diluted to volume with the mobile phase.

From each of two 500-mg vials, 0.1 ml was removed and placed in a 100-ml volumetric flask. The sample was then diluted to volume with the mobile phase.

From one 1-g vial, 0.1 ml was removed and placed in a 100-ml volumetric flask. The sample was then diluted to volume with the mobile phase.

[1] Sephadex.
[2] Model 1285, Laboratory Data Control, Riviera Beach, Fla.
[3] Milton Roy, Laboratory Data Control, Riviera Beach, Fla.
[4] Model 709, Laboratory Data Control, Riviera Beach, Fla.
[5] DuPont, Wilmington, Del.
[6] No. 820349, DuPont.
[7] No. 201724, DuPont.
[8] VYDAC P150, AX, Applied Science Laboratories, Inc., State College, Pa.
[9] Micromeritics, Inc., Norcross, Ga.
[10] Cahn electrobalance, model G, Cahn Instrument Corp., Paramount, Calif.
[11] Ultrasonics, Inc., Plainview, Long Island, N.Y.
[12] Eppendorf, Brinkmann Instruments, Westbury, N.Y.

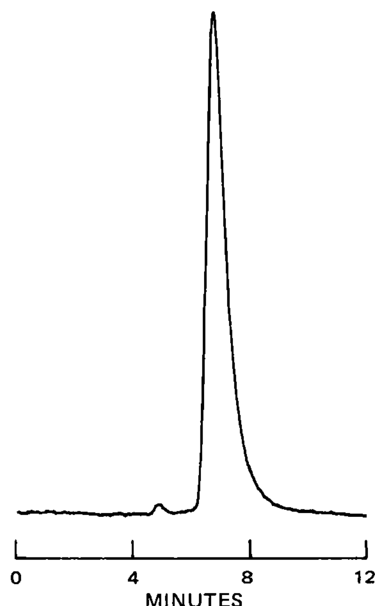

Figure 1—*HPLC analysis of ampicillin using a 1-m long anion-exchange column (room temperature) at a flow rate of 0.45 ml/min. Mobile phase was 0.02* M *$NaNO_3$ in 0.01* M *pH 9.15 borate buffer.*

Oral Formulation—Each bottle containing 125 or 250 mg of oral preparation was reconstituted with the recommended volume of double-distilled water. From one 125-mg bottle, 5 ml was pipetted into a 50-ml round-bottom centrifuge tube. Then 0.1 ml of concentrated hydrochloric acid was added and mixed, followed by 30 ml of chloroform. The centrifuge tube was capped and shaken vigorously for 5 min, using a reciprocal shaker[13], and then centrifuged for 2 min at 2000 rpm. Four milliliters of the upper aqueous layer was pipetted into a 100-ml volumetric flask and diluted to volume with 0.02 *M* pH 6.0 phosphate buffer.

From one 250-mg bottle, 5 ml was pipetted into a 50-ml round-bottom centrifuge tube. Then 0.1 ml of concentrated hydrochloric acid was added and mixed, followed by 30 ml of chloroform. The centrifuge tube was capped and shaken vigorously for 5 min, using the reciprocal shaker, and then centrifuged for 2 min at 2000 rpm. Two milliliters of the upper aqueous layer was pipetted into a 100-ml volumetric flask and diluted to volume with 0.02 *M* pH 6.0 phosphate buffer.

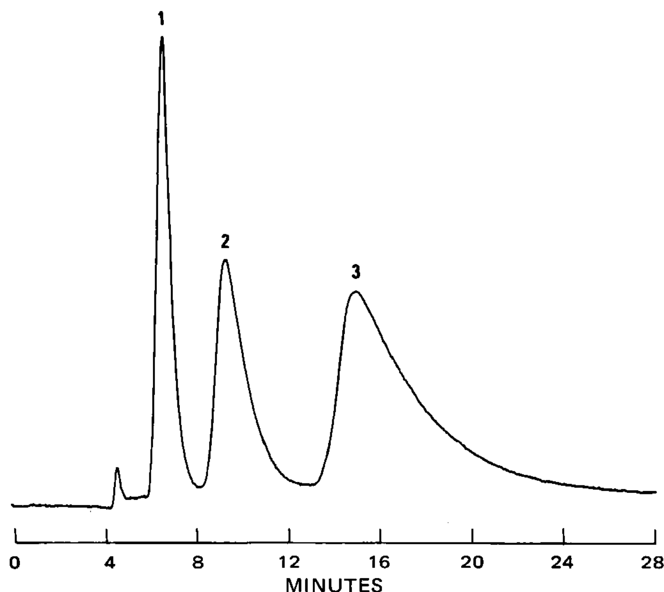

Figure 2—*HPLC separation of (1) ampicillin, (2) penicillin G, and (3) penicillin V using a 1-m long anion-exchange column (room temperature) at a flow rate of 0.45 ml/min. Mobile phase was 0.02* M *$NaNO_3$ in 0.01* M *pH 9.15 borate buffer.*

[13] Eberbach.

Table I—Precision of the HPLC Analysis for Ampicillin

Weight, mg	Peak Area	Area to Weight Ratio	Peak Height	Peak Height to Weight Ratio
10.036	289.3	28.83	25.15	2.506
10.000	280.7	28.07	25.30	2.530
10.062	304.1	30.22	26.00	2.584
9.976	279.2	27.99	25.25	2.531
10.110	283.6	28.05	25.45	2.517
10.034	284.3	28.33	25.70	2.561
10.158	280.0	27.56	25.75	2.535
10.000	294.7	29.47	25.70	2.570
10.032	283.0	28.21	25.60	2.562
10.000	263.2	26.32	25.80	2.580
10.036	280.4	27.94	25.65	2.556
10.120	285.7	28.23	25.65	2.535
	RSD	3.40%	*RSD*	0.98%

The pH 6.0 buffer must be used as a diluent for the oral formulation. The addition of the mobile phase precipitates incipients in the formulation, resulting in low recovery of ampicillin. However, pH 6.0 buffer may be substituted for the mobile phase as the diluent for all pharmaceutical formulations for better stability of ampicillin.

Chromatographic Conditions—The flow rate of the mobile phase was 0.45 ml/min, with a column pressure of about 34 atm (500 psi). The column temperature was ambient, and the chart speed was 0.6 cm (0.25 in.)/min. A 2.0-μl quantity of sample was quantitatively injected into the column, using a septumless injector valve at an electrometer range setting of 0.02 full scale.

RESULTS AND DISCUSSION

A typical chromatogram of ampicillin is shown in Fig. 1. The HPLC method takes less than 12 min of chromatographic time to analyze one sample.

Penicillins G and V can also be separated and quantitated by the HPLC method (Fig. 2). For the quantitation of penicillin G samples, a slight increase in the amount of sodium nitrate in the mobile phase to 0.03 *M* optimizes the retention time of penicillin G and speeds up the analysis. The relationship between the concentration of sodium nitrate and the retention time of various compounds was described by Nelson (22). Since procaine elutes much slower than penicillin G (relative retention of 11) and interferes with subsequent, multiple analysis of samples, procaine must be removed prior to analysis. For the quantitative analysis of penicillin V, the addition of methanol to 10% in the mobile phase speeds up the analysis.

Detection of Degradation Compounds—Commercially produced ampicillin powder was degraded in an alkaline solution for 1 week, and the degraded ampicillin sample was then chromatographed. As shown in Fig. 3, the HPLC method is capable of dif-

Table II—Analysis of Ampicillin Trihydrate Bulk Powder

Lot	Ampicillin, μg/ml HPLC	Ampicillin, μg/ml Iodometric
1	883	893
2	871	886
3	842	863
4	849	847
5	859	863
6	853	859
7	853	853
8	860	880
9	871	888
10	895	880

Table III—Analysis of Ampicillin Products

Label Content	Lot	Ampicillin, mg/Dosage Unit	
		HPLC	Iodometric
Sterile injectable			
250 mg	a	285, 281	277
	b	287, 287	285
500 mg	c	500, 480	527
	d	507, 502	556
1 g	e	943	1100
Hard-filled capsule			
250 mg	a	265	264
	b	244	246
	c	251	265
	d	246	256
500 mg	e	513	516
Oral formulation			
125 mg/5 ml	a	130	122
	b	124	121
	c	125	135
	d	124	122
250 mg/5 ml	e	257	259
	f	248	266
	g	248	262

ferentiating penicillenic and penicilloic acids of ampicillin from ampicillin. The presence of a small amount of penicillin G was also detected in this sample. These peaks were identified by matching the relative retention times with those of relatively pure penicillenic and penicilloic acids of ampicillin. Penicillenic acid was prepared by forming a mercuric mercaptide of ampicillin followed by decomposition with hydrogen sulfide (23). The purity of penicillenic acid thus prepared was approximately 84%. The penicilloic acid sample was obtained from a commercial source[14]. Since penicillanic acid has no UV absorption, its presence may not be detected by this method.

Since a slight degradation, less than 5%, can occur when ampicillin is left in the mobile phase for more than 1 hr, the ampicillin sample should be diluted in the mobile phase just prior to the analysis.

The minimum quantity of ampicillin detection was approximately 20 ng/sample when injected at an electrometer range set-

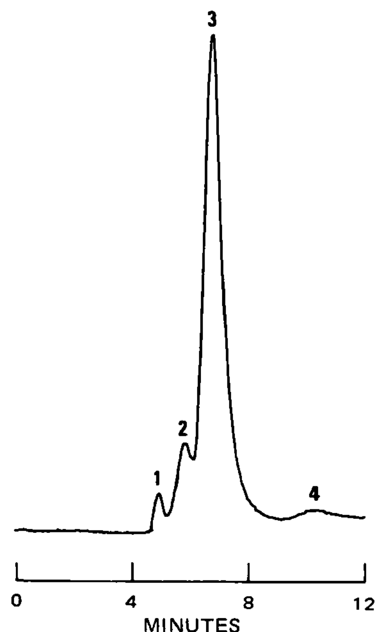

Figure 3—*HPLC analysis of ampicillin degraded in an alkaline solution indicating separation of (1) penicillenic acid, (2) penicilloic acid of ampicillin, (3) ampicillin, and (4) penicillin G.*

[14] Bristol Laboratories.

Table IV—Analysis of Ampicillin Trihydrate Bulk Powder Stored for 1 Month at 55°

Lot	Initial HPLC	Initial Iodometric	Initial Microbiological	Aged HPLC	Aged Iodometric	Aged Microbiological
	Ampicillin, μg/mg					
1	883	893	879, 863	823	862	830, 826
2	842	863	825, 894	770	840	777, 808
3	849	847	—	743	818	732, 757

ting of 0.01 full scale. Since the method is capable of detecting degradation compounds and since penicillenic acid, a degradation compound, was reported to have "allergenic equivalence" with penicillin G (24, 25), the HPLC method is of value for penicillin cross-contamination studies.

Quantitative Determination of Ampicillin—Various amounts of the USP ampicillin reference standard, ranging from 0.1 to 15 mg/ml, were prepared, and a 2.0-μl quantity of each was quantitatively injected into the column using a septumless injector valve. The calibration curve was linear (correlation coefficient of 1.000) over the entire range, with a linear regression of $y = 3.33x - 0.036$.

The precision of the HPLC method was determined by comparing 12 replicate preparations of the USP ampicillin reference standard at approximately 1.0 mg/ml. Table I indicates that the relative standard deviation of the method is 3.4% when the peak area to weight ratio is used for the calculation and less than 1% when the peak height to weight ratio is used. Thus, good precision can be obtained by use of the septumless injector valve without an internal standard.

Ten current lots of ampicillin trihydrate bulk powder were analyzed (Table II). The potency was calculated using the ampicillin reference standard at 867 μg/mg. The drug contents thus calculated were compared to those of the iodometric method. The values obtained by the HPLC method showed no statistically significant difference from those of the iodometric assay method.

Table III shows the results of analysis of pharmaceutical products containing ampicillin. The potencies calculated from the HPLC method showed no statistical difference from those of the iodometric method.

Three lots of ampicillin trihydrate powder were aged for 1 month at 55°. Data obtained by the HPLC method showed 6.8–12.5% reductions in potencies, indicating that the powders underwent degradation by the heat treatment (Table IV). As expected, no loss in potencies was detected by the iodometric method. Microbiological assay (1) values of these samples indicated degradation and agreed very well with those of the HPLC method.

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Determination of Major Impurity in Chlordiazepoxide Formulations and Drug Substance

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Abstract □ Procedures for quantitating the lactam impurity, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, which can be present in chlordiazepoxide formulations, is presented. The method consists of trapping chlordiazepoxide in sulfuric acid in kieselguhr, eluting the impurity with ether, and quantitating by UV spectrophotometry in absolute alcohol at 312 nm.

Keyphrases □ Chlordiazepoxide and chlordiazepoxide hydrochloride—UV analysis for lactam impurity (7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide) □ 7-Chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide—UV analysis in chlordiazepoxide and chlordiazepoxide hydrochloride □ UV spectrophotometry—analysis, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide in chlordiazepoxide formulations

Pharmacopeial monographs for the widely used tranquilizer chlordiazepoxide [7-chloro-2-(methylamino)-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide] and the hydrochloride salt include limit tests for the lactam (7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, I) and phenone (2-amino-5-chlorobenzophenone, II) impurities (1–3). These specifications allow a maximum of 0.1% of I and 0.01 (1, 2) or 0.05% (3) of II in the chlordiazepoxide drug substance. Monographs for capsules prepared with the hydrochloride salt permit a maximum of 3.0 and 0.1% of the two impurities (1, 3), respectively, while tablets prepared with chlordiazepoxide base may contain a maximum of 4% of I and 0.1% of II (2).

While limit tests are effective in monitoring impurities in chlordiazepoxide drug compounds and formulations, quality assurance procedures often require that the levels of impurities present in a drug substance or formulation be known precisely. This article describes methodology employing a "trap" column suitable for the quantitation of the impurity I present in the drug substance and formulations.

EXPERIMENTAL

Apparatus and Materials—Glass columns (2.5 × 20 cm), 0.2 *N* sulfuric acid, water-washed ether (ethyl), and absolute ethanol were used.

For purified kieselguhr, soxhlet extract kieselguhr[1] with methanol (ACS grade) for 24 hr. Thoroughly dry the support material and store in a well-closed bottle.

For purified absorbent cotton, soxhlet extract absorbent cotton with methanol (ACS grade) for 24 hr. Dry thoroughly and store in well-stoppered bottles.

For the standard solution of I, dissolve sufficient I in ether so that each milliliter contains 150 μg.

Procedure—Place a pledget of purified absorbent cotton at the bottom of the column. Then place purified kieselguhr (3 g), which has been triturated with 0.2 *N* sulfuric acid (3 ml), over the cotton. Tamp the layer lightly but evenly. Triturate a second 3-g portion of purified kieselguhr with 3 ml of 0.2 *N* sulfuric acid, add an accurately weighed portion of powdered tablet or capsule material or drug substance equivalent to about 25 mg of chlordiazepoxide (base or salt) or 5.0 ml of standard solution of I, and mix thoroughly. Transfer this material to the prepared column and tamp into a uniform second layer. Wipe the beaker and mixing rod with purified absorbent cotton and place the cotton on top of the prepared kieselguhr column.

Elute the column with water-washed ether. Collect at least 25 ml of eluate in a 50-ml volumetric flask and evaporate the eluate to dryness, employing a stream of dry nitrogen. Dissolve the resultant residue in ethanol and make to volume with this solvent. Measure the absorbances of standard and sample solutions at 312 nm against a similarly prepared blank containing no sample or standard.

Calculate the percent of I as follows:

$$\%\mathrm{I} = \frac{\text{weight of I in standard } (\mu g)}{} \times \frac{A_{spl}}{A_{std}} \times \frac{100}{\text{weight of chlordiazepoxide taken } (\mu g)} \quad \text{(Eq. 1)}$$

[1] E. M. Chemicals Inc., Elmsford, NY 10523