ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1992, p. 1276–1279
0066-4804/92/061276-04$02.00/0

Vol. 36, No. 6

High-Performance Liquid Chromatographic Method for Potency Determination of Amoxicillin in Commercial Preparations and for Stability Studies

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Received 28 October 1991/Accepted 8 April 1992

A reversed-phase column liquid chromatographic method was developed for the assay of amoxicillin and its preparations. The linear calibration range was 0.2 to 2.0 mg/ml (r = 0.9998), and recoveries were generally greater than 99%. The high-performance liquid chromatographic assay results were compared with those obtained from a microbiological assay of bulk drug substance and capsule, injection, and granule formulations containing amoxicillin and degraded amoxicillin. At the 99% confidence level, no significant intermethod differences were noted for the paired results. Commercial formulations were also analyzed, and the results obtained by the proposed method closely agreed with those found by the microbiological method. The results indicated that the proposed method is a suitable substitute for the microbiological method for assays and stability studies of amoxicillin preparations.

The present official assay method of the British Pharmacopoeia (8) for the analysis of potency of amoxicillin bulk drug is a mercury-nitrate titration. The *U.S. Code of Federal Regulations* (14), the United States Pharmacopeia (13), and the *Minimum Requirements for Antibiotic Products of Japan* (9) described three official methods for potency assay of amoxicillin preparations: a microbiological method, an iodometric assay, and an optical method. The regulations state that the results obtained from the microbiological method shall be conclusive.

However, there has recently been a move to replace expensive microbiological assays by chemical assays, e.g., high-performance liquid chromatography (HPLC). Several HPLC methods for the determination of amoxicillin in biological fluids have been reported (1, 2, 5–7, 11, 12, 15). Fewer methods have been reported for degraded compounds or impure amoxicillin (3, 4) and for the determination of amoxicillin in pharmaceutical samples (10).

In order to establish whether an HPLC method would be acceptable, it is important to determine whether it is robust enough for assaying samples kept under extreme conditions. Degradation in the sample should be equally reflected by microbiological and HPLC assays. This paper describes a comparison of a proposed HPLC method with a microbiological assay for the determination of amoxicillin in commercial formulations. Further, amoxicillin was kept at elevated temperatures as part of an accelerated degradation experiment and assayed by microbiological and HPLC methods.

MATERIALS AND METHODS

Instruments. A model 576 liquid chromatographic pump (Gasukuro Kogyo, Inc., Tokyo, Japan), a Gasukuro Kogyo model 502U spectrodetector, and a Gasukuro Kogyo model 12 chromatocorder were employed during the study. The mobile phase was pumped through a reversed-phase column (μBondapak C_{18}; inside diameter, 30 cm by 3.9 mm; particle size, 10 μm; Waters P/N 27324) with an isocratic flow rate of 1.5 ml/min. The detector was set at 254 nm. Chromatography was performed at room temperature. Injections of 20 μl of all solutions to be analyzed were made.

Reagents and materials. Methanol (liquid chromatography grade) was supplied by ALPS Chemical Co., Taipei, Taiwan. Glacial acetic acid (reagent grade) was supplied by E. Merck's Chemical Co., Darmstadt, Germany. 6-Aminopenicillanic acid was purchased from Sigma Chemical Co., St. Louis, Mo. Acetaminophen was a gift of Winthrop Laboratories Taiwan Branch Office, Sterling Products International Inc., Taipei, Taiwan. Amoxicillin was the National Laboratories of Foods and Drugs (Taipei, Taiwan, Republic of China) house standard. Different lot numbers of amoxicillin bulk drugs were kindly donated by Kingdom Pharmaceutical Co., Ltd., and Yuen Foong Chemical Co. Capsules, injections, and granules were obtained from commercial sources.

Mobile phase. The mobile phase was methanol–1.25% acetic acid (20:80, vol/vol). The mobile phase was filtered (0.45-μm-pore-size Millipore filter) and degassed with an ultrasonic bath prior to use.

Amoxicilloate preparation. Amoxicilloate was prepared as described by De Pourcq et al. (3). Amoxicillin trihydrate (100.0 mg) was dissolved in 20.0 ml of 0.1 N potassium hydroxide. After standing overnight, the solution was neutralized to pH 7.0 with 0.2 M potassium dihydrogen phosphate and diluted to 50.0 ml with water.

Internal-standard solution. Internal-standard acetaminophen (40.0 mg) was dissolved in 10.0 ml of methanol and diluted to 1.0 liter with water to form the internal-standard solution.

Amoxicillin standard solution. To form amoxicillin standard solution, internal-standard solution was added to an accurately weighed amount of amoxicillin sodium standard equivalent to a 50.0-mg potency of amoxicillin and the volume was brought up to 50.0 ml.

Sample preparations. To form sample preparations, internal-standard solution was added to an accurately weighed amount of bulk drugs, homogeneous capsule contents, or injection or granule formulations equivalent to a 50.0-mg

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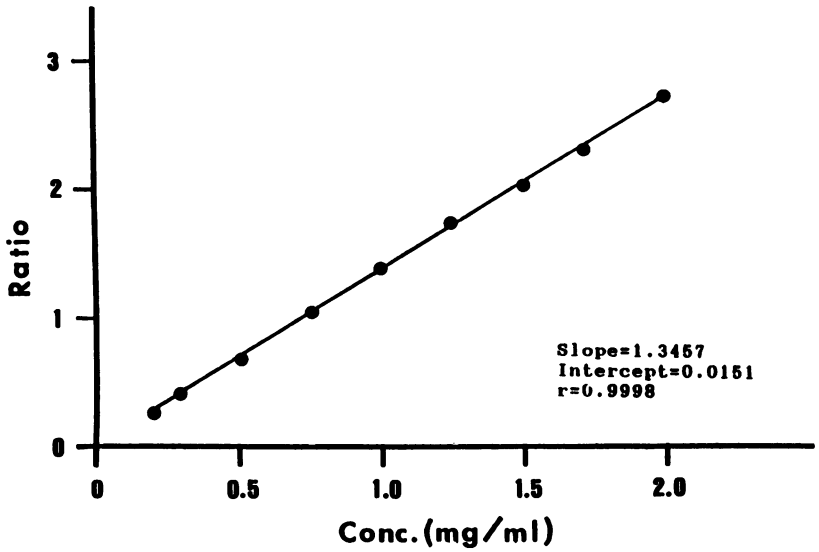

FIG. 1. Standard curve for amoxicillin. The points of the curve are at 0.2, 0.3, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mg/ml.

potency of amoxicillin and the volume was brought up to 50.0 ml.

Solution for linearity response. Nine concentrations of amoxicillin sodium, which ranged from 0.2 to 2.0 mg/ml, were prepared. Each concentration was chromatographed six times.

Solution for recovery studies. To an accurately weighed 50.0-mg potency of sample composites of commercial preparations were added different amounts of amoxicillin standard. Each solution was made up to 50.0 ml with internal-standard solution and was chromatographed in triplicate.

Microbiological assay procedure. *Bacillus subtilis* (Culture Collection and Research Center, Hsinchu, Taiwan) was used in the microbiological assay. According to the cup plate method, standards and test drugs were diluted to 1.0-mg/ml (potency) concentrated solution with distilled water and then diluted to 10.0 and 2.5 μg/ml with 1% phosphate buffer solution (pH 6.0) on the day of analysis. Five petri dishes with a 9.0-cm inside diameter were used for each sample. After incubation for 16 to 18 h, the inhibition zone diameter was measured by a zone analyzer (ZA-F; Toyo, Tokyo, Japan).

TABLE 1. Recovery of amoxicillin from various commercial composites

Formulation	mg added	mg found	% Recovered	Avg % recovered
Capsule				
250 mg	6.0	5.9	99.0	99.00
	11.4	11.4	99.8	
	15.8	15.7	99.2	
500 mg	6.0	6.0	99.6	99.75
	9.8	9.8	99.7	
	15.1	15.1	100.0	
Injection, 500 mg/vial	15.8	16.2	102.5	101.81
	32.2	32.6	101.1	
Granule, 125 mg/5 ml	3.1	3.2	102.0	100.94
	6.1	6.0	98.0	
	7.6	7.8	102.8	

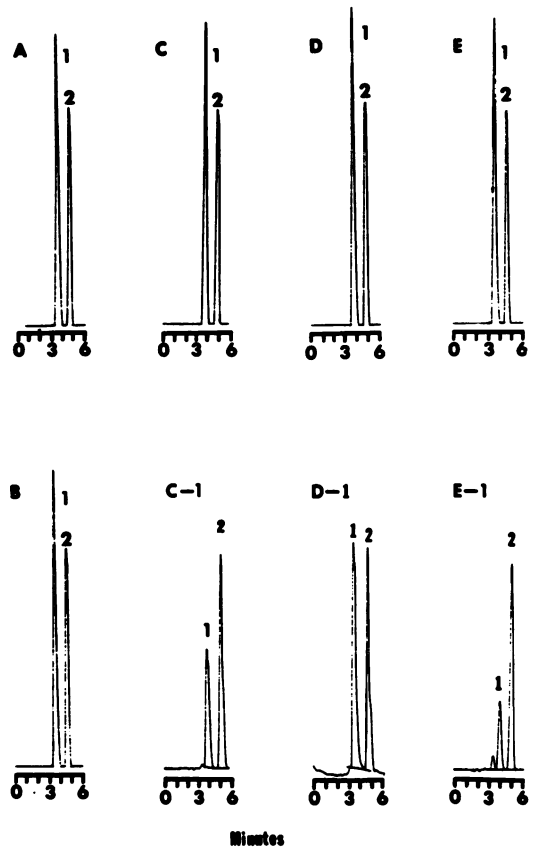

FIG. 2. Chromatograms of amoxicillin preparations. (A) House standard; (B) bulk drug substance; (C) 250-mg capsule; (C-1) degraded 250-mg capsule; (D) 250-mg-per-vial injection; (D-1) degraded 250-mg-per-vial injection; (E) granule; (E-1) degraded granule. Peaks: 1, amoxicillin (20 μg); 2, acetaminophen.

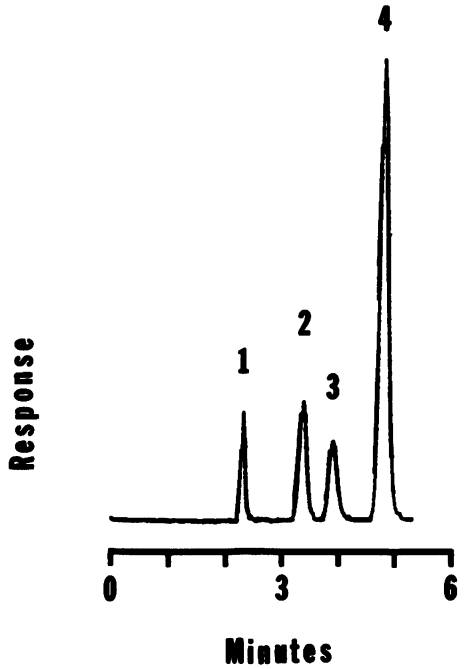

FIG. 3. Chromatogram of a mixture preparation. Peaks: 1, 6-aminopenicillanic acid; 2, amoxicilloates; 3, amoxicillin; 4, acetaminophen.

RESULTS AND DISCUSSION

The linearity of the peak-area ratio (amoxicillin versus internal standard) was verified by injection of nine solutions containing amoxicillin in a concentration range of 0.2 to 2.0 mg/ml. A straight line with a correlation coefficient of 0.9998 ($y = 0.0151 + 1.3457x$) was obtained, as shown in Fig. 1.

TABLE 2. Comparison of microbiological and HPLC assays for amoxicillin

Sample	Potency[a] in:	
	Microbiological assay	HPLC
Bulk drug		
House standard	847.1	847.1
USP standard	859.2	864.7
Brand A	945.5	978.6
Brand B	888.6	927.2
Dosage form, declared		
Brand A		
250 mg/capsule	97.8	98.3
500 mg/capsule	109.1	111.5
500 mg/vial	94.9	90.1
Brand B, 250 mg/capsule	88.6	84.3
Brand C, 500 mg/capsule	121.7	119.1
Brand D, 500 mg/capsule	115.2	112.7
Brand E, 500 mg/capsule	103.7	108.9
Brand F, 250 mg/vial	101.6	102.1
Brand G, 200-mg/g granules	115.7	115.0
Brand H, 100-mg/g granules	104.0	104.7

[a] The potency was determined as micrograms per milligram for bulk drug and as a percentage of the declared amount for dosage forms. Values for the microbiological assay are averages of five determinations; values for HPLC are averages of triplicate determinations.

TABLE 3. Comparison of percent potency of amoxicillin in capsule, injection, and granule formulations as determined by microbiological and HPLC methods

Formulation	% of declared concn in:	
	Microbiological method	HPLC
Capsule	100.8	99.3
	100.1	97.2
	98.9	99.8
	96.9	97.3
	95.5	96.8
	93.9	96.0
	57.2	62.6
	30.0	26.6
	27.1	31.6
	22.7	21.2
Injection	101.6	102.1
	94.9	90.1
	70.1	68.2
	65.6	63.5
	62.2	61.7
	54.7	51.3
	40.6	37.9
Granule	115.7	115.0
	104.0	104.7
	69.5	71.3
	59.7	62.7
	50.5	47.7
	26.6	28.3

Reproducibilities for both the within-day assay and the between-day assay were evaluated. The coefficients of variation, on the basis of peak-area ratios for six replicate injections in the within-day assay, were between 0.05 and 0.16% at the amount of 20 μg. The coefficient of variation in the between-day assay ($n = 5$) was 0.24% at the same amount.

The results of standard addition recovery studies of amoxicillin from sample composites of commercial preparations are shown in Table 1. The average recovery was greater than 99%. These data indicate that the proposed HPLC method is relatively unaffected by the sample matrix.

Typical chromatograms of the amoxicillin commercial dosage forms are shown in Fig. 2. The retention time was about 4.8 min for the internal standard and 3.8 min for amoxicillin. Excipients from commercial formulations did not interfere. Furthermore, the HPLC method can detect compounds related to amoxicillin, i.e., 6-aminopenicillanic acid and amoxicilloates, which were eluted prior to amoxicillin (Fig. 3).

When samples of capsule, injection, and granule formulations were heat degraded, the resulting mixtures yielded chromatograms containing additional peaks, none of which interfered with the interpretation and measurement of the chromatographic peaks for amoxicillin and acetaminophen, as shown in Fig. 2. Although only a little peak eluting just prior to amoxicillin was shown in Fig. 2, several additional peaks were found when the detection sensitivity was increased. To examine the purity of the amoxicillin peak in the degraded sample (stored at 150°C for 7 min), a UV photo diode array detector was used. The evaluation of chromatographic peak homogeneity was performed with absorbance ratios and a three-dimensional spectrochromatogram. The

results presented good confirmation of the amoxicillin peak identity (data not shown). In addition, a decrease in peak height (and/or peak area) with increase in temperature and time can be observed.

A number of samples of bulk drug substance and commercial preparations of eight brands were analyzed for amoxicillin content by HPLC. These samples were also assayed by the microbiological method. The results are shown in Table 2. A *t* test was applied to the data; analysis showed no significant difference at the 99% confidence level for any of the preparations when assayed by the microbiological or HPLC method.

A study was initiated to ascertain the suitability of the proposed method for stability studies. Samples of capsule, injection, and granule formulations were stored in temperature-controlled cabinets (ambient or 80 to 150°C). Samples were taken from the cabinets periodically for microbiological and HPLC assays. The assay values, expressed as a percentage of the level claimed, are given in Table 3. The paired values in Table 3 have correlation coefficients of 0.996 for capsule, 0.997 for injection, and 0.998 for granule dosage forms. Hence, no significant difference in the assay values obtained by the two analytical methods was found for degraded or nondegraded samples.

This study demonstrates the applicability of the proposed HPLC method for the potency determination of amoxicillin in bulk drug and capsule, injection, and granule formulations. The method can be successfully used for routine quality control and stability assays and offers advantages in speed, simplicity, and reliability.

ACKNOWLEDGMENTS

We thank Winthrop Laboratories Taiwan Branch Office, Sterling Products International Inc., Taipei, Taiwan; Kingdom Pharmaceutical Co., Ltd.; and Yuen Foong Chemical Co. for their generous gifts of samples and standards for the study. We also thank Yu-Tzer Lee and Hsiou Chuan Chung for their assistance in the preparation of the manuscript.

This work was supported by grant 80-44 from the Department of Health, Executive Yuan, Taipei, Taiwan, Republic of China.

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