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DETERMINATION OF DEGRADATION PRODUCTS AND IMPURITIES OF AMOXICILLIN CAPSULES USING TERNARY GRADIENT ELUTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A specific high-performance liquid chromatography method has been developed for the determination of amoxicillin penicilloic acids (PA), *p*-hydroxyphenylglycine (*p*-HPG), 6-aminopenicillanic acid (6-APA) and unidentified materials in amoxicillin capsules. The accuracy of the measurements was demonstrated by the standard addition technique on actual capsule samples. Within-day and between-day precision studies gave coefficients of variation of 3.15 and 1.70% for PA, 4.75 and 3.81% for *p*-HPG, and 5.59 and 32.23% for 6-APA, respectively.

Typical calibration and expanded linearity of response curves for these components show no curvature over the range of interest. The detection limits for PA, *p*-HPG and 6-APA are 0.7, 0.2 and 0.6 μg/ml of sample extract and are considered adequate for the intended use of the method. These levels correspond to 0.35, 0.10, 0.30 mg per 500-mg Amoxil® capsule, respectively.

INTRODUCTION

Amoxicillin is one of the amino-penicillin β-lactam antibiotics widely used in clinical chemotherapy. The quantitative determination of penicillins, their degradation products, and impurities is one of the more difficult problems in pharmaceutical analysis. This is because of the need to separate the parent compounds from their precursors, impurities, and various degradation products including polymers and that they are relatively unstable in aqueous solutions.

Several techniques for assaying penicillins have been reported in the literature. These include microbioassay, chemical assay[1,2], spectrophotometry[3–11], and chromatography[12–37]. Microbioassays and chemical assays of penicillins have been the most frequently used techniques for strength determination. It is well known that bioassay methods are non-specific, imprecise and often cannot be used to monitor

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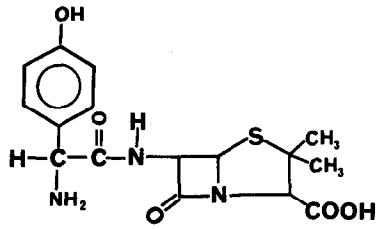
low levels of degradation products and impurities. The Code of Federal Regulations[38] states that the strength determinations obtained from the iodometric assay or microbioassay shall be conclusive.

Among various chromatographic techniques, high-performance liquid chromatography (HPLC), has the advantages of simplicity of sample preparation, excellent specificity, high precision and accuracy, as well as the necessary sensitivity in the separation and determination of these drugs, their various degradation products and impurities. Vree *et al.*[25] reported the HPLC analysis of amoxicillin in human body fluids, but did not monitor any of the degradation products or impurities.

Two recent papers[21,22] on the HPLC determination of amoxicillin in urine showed only one peak for amoxicillin penicilloic acid. On the other hand, four publications reported two peaks that corresponded to the penicilloic acid epimers of penicillin V[18], penicillin G[23,24] and ampicillin and amoxicillin[30]. In the course of our study on amoxicillin stability, two distinct peaks for amoxicillin penicilloic acids

I



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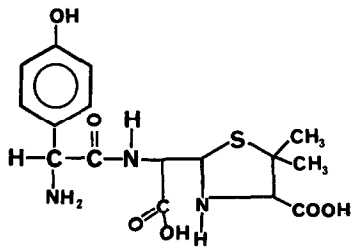

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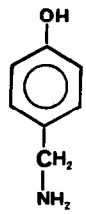

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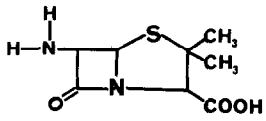

Scheme I. Structures of amoxicillin (I), amoxicillin penicilloic acids (II), *p*-hydroxyphenylglycine (III) and 6-aminopenicillanic acid (IV).

(α- and β-epimers) were also detected and their rate of epimerization in aqueous solutions reported[39].

The HPLC of 6-aminopenicillanic acid, the nucleus of amoxicillin and other penicillins, has been described by various researchers[18,40,41]. So far, only one HPLC separation or determination of *p*-hydroxyphenylglycine[37] has been reported and no report exists which comprehensively treats the quantitation of all these components found in amoxicillin dosage forms.

This paper describes a specific gradient elution HPLC method for simultaneous determination of amoxicillin penicilloic acids (α- and β-PA), *p*-hydroxyphenylglycine (*p*-HPG) and 6-aminopenicillanic acid (6-APA) in amoxicillin capsules (see Scheme I).

EXPERIMENTAL

Instrumentation

An automated HPLC system used in this study was assembled with the following modular components: a single-pump ternary solvent system (Model 5060, Varian, Walnut Creek, CA, U.S.A.), an auto-injection system (Model 725, Micromeritics, Norcross, GA, U.S.A.), a variable-wavelength UV detector (Model LC-75, Perkin-Elmer, Norwalk, CT, U.S.A. or Model SF757, Kratos, Ramsey, NJ, U.S.A.), and an integration system (Model HP-3356, Hewlett-Packard, Avondale, PA, U.S.A.). The following equipment was used for spectral examination of chromatographic peaks: HP-1040A high speed spectrophotometric detector, HP-85 personal computer, HP-8290 1M flexible disc drive, HP-7470A X-Y plotter, a syringe pump (ISCO Model 314, Instrumentation Specialties, Lincoln, NE, U.S.A.) and a manual loop injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.).

Reagents and materials

Reagent grade monobasic potassium phosphate (KH_2PO_4), dibasic potassium phosphate (K_2HPO_4) and potassium hydroxide (KOH) were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Acetonitrile and methanol, distilled-in-glass grade, were obtained form Burdick & Jackson (Muskegon, MI, U.S.A.) or Anachemia (Champlain, NY, U.S.A.). Amoxicillin penicilloic acid (β-form) was prepared according to a published procedure[42]. *p*-Hydroxyphenylglycine was prepared in-house and 6-aminopenicillanic acid was purchased from Aldrich (Milwaukee, WI, U.S.A.). Spherisorb-ODS (5 μm) bulk packing material was obtained from Phase Separations (Norwalk, CT, U.S.A.). Empty stainless-steel tubing, 316 grade, was purchased from Alltech (Deerfield, IL, U.S.A.) and Acrodisc 0.45-μm pore-size disposable filters were obtained from Gelman Sciences (Ann Arbor, MI, U.S.A.). Disposable syringes, 10-ml lock tip type, were available from American PharmeSeal (Glendale, CA, U.S.A.).

Preparations

Columns. Spherisorb-ODS (5 μm) columns (150 × 4.6 mm I.D.) were slurry-packed in-house as described previously[43].

Buffer solution. A liter of 0.05 *M* phosphate buffer solution (pH *ca.* 5.9) was prepared as follows: 10 ml of 0.5 *M* K_2HPO_4 (stock solution) and 90 ml of 0.5 *M* KH_2PO_4 (stock solution) were measured into a 1000-ml volumetric flask, which was

then filled with distilled water to the mark. This was thoroughly mixed and degassed in an ultrasonic bath (Branson). Either the 0.5 *M* stock solutions or the prepared mobile phase should be filtered through a 0.45-μm filter before use.

Standard mixture preparation. Three-level standard mixture preparations were prepared in water to contain *ca.* 3, 9 and 15 μg/ml for PA, 1, 3 and 5 μg/ml each for *p*-HPG and 6-APA. Sonication may be needed to help dissolving 6-APA in water. Both higher and lower concentrated standard mixture preparations should also be used to verify expanded linearity of response.

Sample preparation. Obtain average capsule fill weight for twenty capsules and grind contents to a fine powder with spex mill. Accurately weigh sample of powder equivalent to *ca.* 500 mg of amoxicillin, into a 500-ml volumetric flask. When the chromatographic system is ready, note the time, dissolve the sample, and dilute to volume with water. Place the flask in an ultrasonic bath for *ca.* 30 min, with occasional shaking. If necessary, add ice to the ultrasonic bath to keep the temperature below 30°C. Filter a portion of the sample solution through an Acrodisc 0.45-μm pore-size disposable filter prior to injection. Once in solution, samples should be assayed within 3 h to minimize the increase in PA due to hydrolysis. To minimize laboratory antibiotic contamination all unused samples and HPLC effluent should be treated with a 1% potassium hydroxide solution to destroy the β-lactam ring before disposal into chemical waste containers.

Preparation of equipment. The following conditions were used. Two 15-cm Spherisorb-ODS columns connected in series, flow-rate at 1.0 ml/min, detection wavelength at 220 nm and 0.2 a.u.f.s., and 50-μl loop injector and a run time of 50 min.

Gradient elution program. With a system capable of ternary gradient elution HPLC, the following time-programmed set-up was used:

Time (min)	0	5	25	30	35
%B/(A+B+C)	0	0	30	30	0
%C/(A+B+C)	0	0	10	10	0

Where A = phosphate buffer solution (0.05 *M*, pH *ca.* 5.9); B = methanol; C = acetonitrile.

Alternatively, the above ternary gradient could be replaced with a binary gradient system by premixing methanol and acetonitrile in the volume ratio of 3:1 and using the following modified program:

Time (min)	0	5	25	30	35
% B/(A+B)	0	0	40	40	0

where A = same as above; B = methanol–acetonitrile (3:1).

System suitability

The analytical columns were equilibrated with phosphate buffer solution until a relatively stable baseline was obtained at 220 nm and 0.2 a.u.f.s. Inject 50 μl of a

standard mixture preparation and the retention times of the PA, *p*-HPG, and 6-APA peaks must be between 1 and 12 min. If not, adjust the flow-rate between 0.7 and 1.2 ml/min.

Fig. 1 shows a typical chromatogram of a sample, and illustrates the resolution and baseline integration required for *p*-HPG,α- and β-PA, 6-APA, and other peaks. In order to obtain reasonably accurate quantitation of *p*-HPG and 6-APA, both the resolution of *p*-HPG and 6-APA from other peaks, and the integration parameters selected, are important.

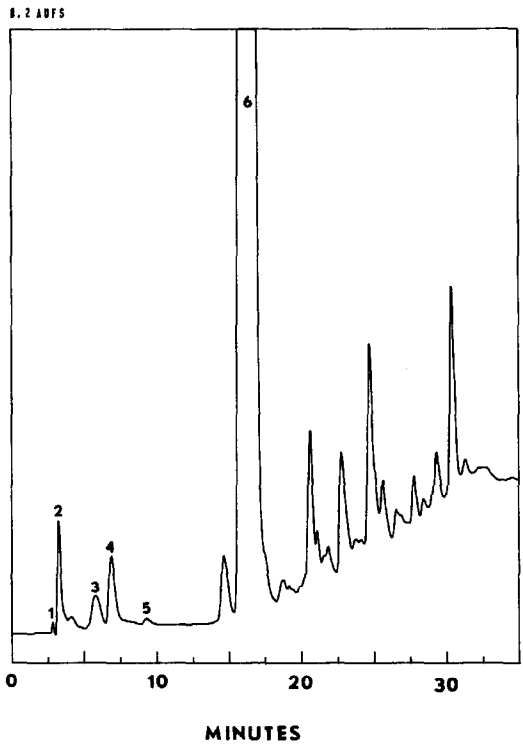

Fig. 1. Chromatogram of an amoxicillin capsule sample. Peak identification: 1 = solvent peak; 2 = *p*-HPG; 3 = α-PA; 4 = β-PA; 5 = 6-APA; 6 = amoxicillin. Note: all unlabeled peaks are unknown components.

The resolution between the α- and β-PA peaks can be partial, since the sum of the areas of the two peaks will be used in the quantitation of penicilloic acids in the sample.

CALCULATIONS

Calculate the least-squares linear regression line relating the areas of the standard peaks in the standard mixture chromatograms to their respective concentrations, for each of the standard compounds (for PA, use the sum of the α- and β-PA peak areas). The value of $s_{yx}\%$[44] must be not greater than 15%.

Obtain the amount of each of the components (PA, *p*-HPG, and 6-APA) in the capsules from the equation:

$$\text{milligrams of component per capsule} = \frac{(A_s - b)\ 500\ S\ W_a}{(m)\ (W_s)\ (1000)}$$

where A_s = area of the component peak in the sample preparation chromatogram (for PA, the sum of the areas of the α- and β-PA peaks); b = intercept of the calibration line; m = slope of calibration line (ml/μg); 500 = volume of sample preparation (ml); W_s = weight of sample (mg); W_a = average capsule fill weight (mg per capsule); 1000 = conversion factor (μg/mg); S = strength of standard, decimal.

Calculate the amount of unidentified materials (UM) from the equation:

$$\%\ \text{UM} = \frac{\text{UM}}{\text{NT}} \cdot 100\%$$

where UM = sum of peak areas of all unidentified materials; NT = net total peak area.

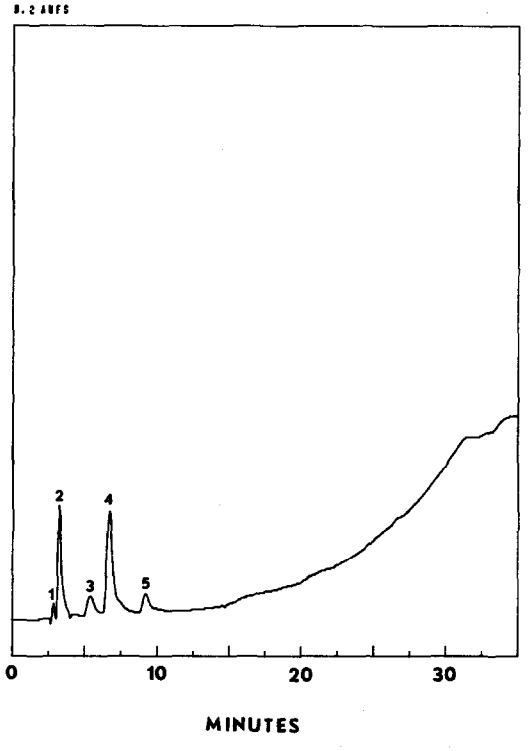

Fig. 2. Chromatogram of a standard mixture preparation. Peak identification: see Fig. 1.

RESULTS AND DISCUSSION

Method validation

It is standard practice for developers of HPLC methods to demonstrate method specificity, accuracy, precision, linearity and sensitivity for each drug component assayed.

Specificity

The specificity of an HPLC method derives primarily from the selectivity of the chromatographic system developed. The selectivity of this method has been demonstrated by the ability of the system to resolve various known degradation products, impurities, and unidentified materials from amoxicillin.

The detection wavelength at 220 nm, though non-selective, is preferred for the detection of all relevant components. Scheme I shows the structures of amoxicillin, its PA, *p*-HPG and 6-APA. Figs. 1 and 2 show typical sample and standard chromatograms obtained with this method. A placebo preparation for Amoxil® capsules (P/N#695 and #696) was also chromatographed under the same condition to support the specificity of the method (Fig. 3).

To demonstrate further the specificity of the method, the purity of the peaks of interest was examined with the HP-1040A high speed spectrophotometric diode

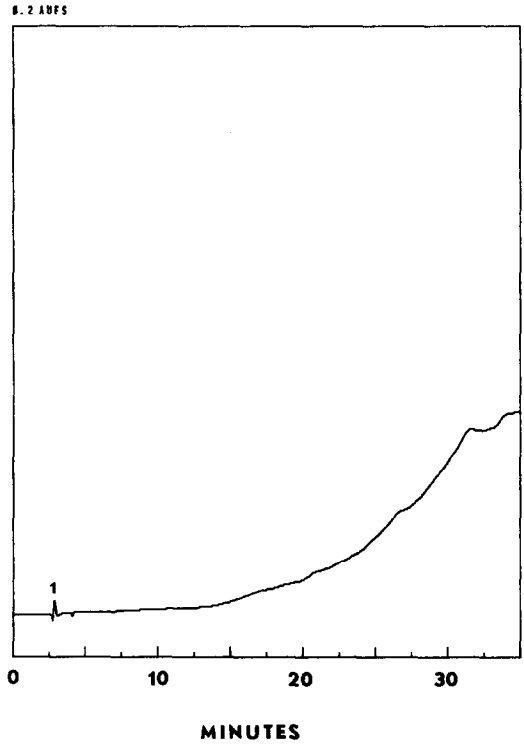

Fig. 3. Chromatogram of a placebo preparation of amoxicillin capsule. Peak identification: see Fig. 1.

array detector. Detection is based on a photodiode array which measures absorbance as a function of wavelength and time simultaneously. It processes these absorbance data at high speed to yield chromatograms and spectra which are then stored on a disc. Both chromatograms and spectra are acquired without having to interrupt the HPLC operation.

Fig. 4 shows four spectra each for the peak of α- and β-PA, *p*-HPG and 6-APA. In each case, the top spectrum (A) corresponds to a standard preparation, and the remaining three spectra (B–D) to the upslope, apex and downslope portions of the sample peak. Except for the tiny 6-APA peak in the sample (see Fig. 1), all other peaks examined yielded high quality spectra which helped confirm the homogeneity of the peaks detected. It should be noted that the spectra of α- and β-PA peaks look identical to each other and this evidence helped support the claim that these two peaks are epimers.

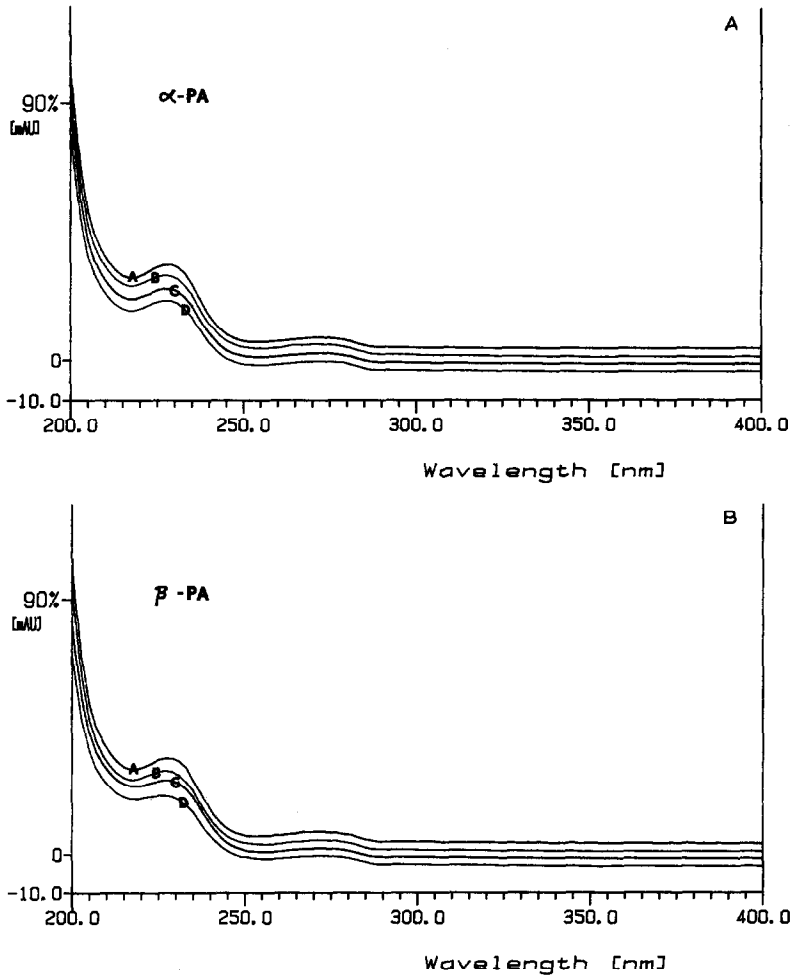

Fig. 4.

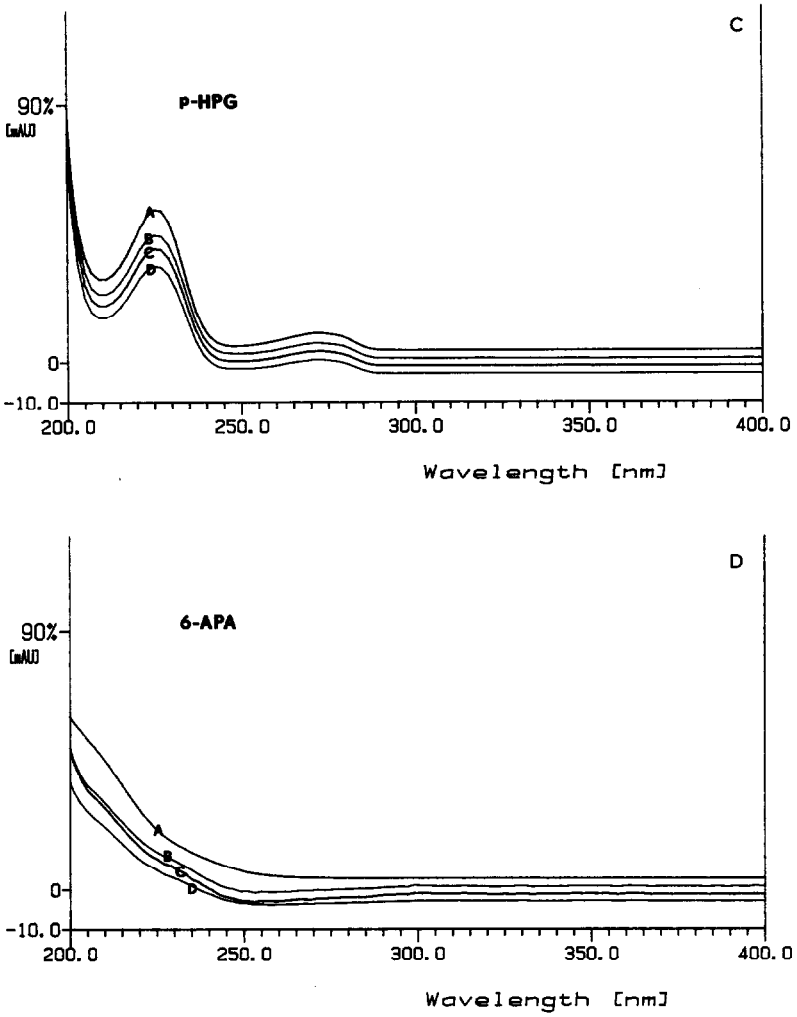

Fig. 4. Spectra of α-PA, β-PA, *p*-HPG and 6-APA. In each case, the top spectrum (A) is obtained at the peak apex of a standard preparation, and spectra B, C, and D at the upslope, apex and downslope of a sample preparation.

Accuracy

Since the amoxicillin raw material contains small amounts of synthetic impurities (*p*-HPG and 6-APA) and degradation products (α- and β-PA), it is not feasible to prepare a placebo for Amoxil capsule that is free from these components. Therefore, an actual sample (batch No. 1YDD) was used in a 3 × 4 matrix study. On each of the three days, four samples (one unspiked and three spiked at various levels) were assayed for PA, *p*-HPG and 6-APA. Data and results of the statistical analysis of accuracy are given in Table I.

Using only the data from the spiked samples, the relationship between found and input is adequately described by a linear regression since the quadratic coefficient

TABLE I

RECOVERY DATA BASED ON THREE-LEVEL SPIKING OF SAMPLES WITH PA, *p*-HPG AND 6-APA

Day	*Spike input (mg/cap)*			*Found (mg/cap)*		
	PA	*p-HPG*	*6-APA*	*PA*	*p-HPG*	*6-APA*
1	none	none	none	3.145	1.241	0.331
2	none	none	none	3.871	1.305	0.396
3	none	none	none	3.617	1.040	0.472
1	4.662	1.201	0.877	7.745	2.420	1.234
2	4.678	1.287	0.946	8.593	2.490	1.275
3	4.637	1.214	0.889	7.277	2.289	1.350
1	9.310	3.599	2.918	12.626	4.864	3.457
2	9.376	3.868	3.159	12.911	5.065	3.813
3	9.129	3.585	2.918	12.336	4.675	3.094
1	13.991	6.010	4.386	16.512	7.231	4.303
2	13.922	6.382	4.691	17.347	7.868	5.507
3	13.845	6.041	4.425	17.190	7.432	4.788
Quadratic regression of found on amount added (n = 9) (spiked samples only)						
				PA	*p-HPG*	*6-APA*
Test quadratic coefficient equals zero			$t =$	−0.64	1.43	0.03
Two-sided *p*-value				0.55	0.20	0.98
C.V. (%)				3.83	1.94	9.46
Covariance-linear regression of found on amount added (n = 12) (two groups: spiked vs. unspiked)						
Y-intercept of spiked samples				3.34	1.08	0.38
Mean of unspiked samples				3.54	1.19	0.40
Test for equality of *Y*-intercept and mean			$t =$	−0.45	−1.11	−0.08
Two-sided *p*-value				0.66	0.30	0.94
Statistical evaluation of recovery						
Linear regression of found on amount added (n = 12) (spiked and unspiked samples)						
Slope (95% confidence interval)				0.97 ±0.05	1.03 ±0.03	1.00 ±0.09
C.V. (%)				4.11	2.82	9.47

is not significantly different from zero (*p*-values are 0.55, 0.20 and 0.98 for PA, *p*-HPG, and 6-APA respectively).

The difference between the mean of the unspiked samples and the value predicted for unspiked samples (using linear regression of spiked data only) is not statistically significant (*p*-values are 0.66, 0.30, 0.94 for PA, *p*-HPG and 6-APA, respectively), therefore, recovery is evaluated using both the spiked data and unspiked data. The statistical evaluation of recovery indicates that 97, 103, and 100% of the amount added are recovered for PA, *p*-HPG and 6-APA, respectively.

An accuracy experiment was not performed for the unidentified materials, since their structures are unknown, but recoveries would be expected to be similar to the known recoveries for the other components discussed above.

Precision

One batch of Amoxil capsules (batch No. 1YDD) was assayed in replicate on three separate days. An estimate of within-day and between-day precision was made from these assay values. The results are shown in Table II. The sum of within-day and between-day variance estimates gave total C.V.s of 3.58, 6.09 and 32.71% for PA, *p*-HPG and 6-APA, respectively, which are considered acceptable.

It should be pointed out that these components are present in only minute amounts. Indeed, the amount of 6-APA present is just about at the detection limit of the method. Also, because of the known hydrolysis of amoxicillin to PA in solution, the level of PA found and hence the assay precision are influenced by the time between dissolution of sample and the injection of the sample solution into the HPLC column (see Fig. 5). The method specifies that the samples should be assayed as soon as possible to minimize this problem.

Sensitivity

With the operating conditions specified in the method, the sensitivities or de-

TABLE II

WITHIN DAY AND BETWEEN DAY PRECISION OF PA, *p*-HPG AND 6-APA

	Day	*Found (mg/cap)*	*Mean*	*S.D.*	*C.V. (%)*
(A) PA	1	3.39, 3.20, 3.32	3.30	0.10	2.91
	2	3.44, 3.51	3.47	0.05	1.42
	3	3.24, 3.49, 3.47, 3.51	3.43	0.13	3.68
		Overall	3.40	0.12	3.47
(B) *p*-HPG	1	1.33, 1.21, 1.15	1.23	0.09	7.45
	2	1.11, 1.12	1.11	0.01	0.63
	3	1.13, 1.18, 1.17, 1.14	1.15	0.02	2.06
		Overall	1.17	0.07	5.75
(C) 6-APA	1	0.49, 0.47, 0.50	0.49	0.01	3.14
	2	0.35, 0.30	0.32	0.03	10.88
	3	0.27, 0.30, 0.27, 0.27	0.28	0.01	5.40
		Overall	0.36	0.10	27.98

Variance estimates

Source	*C.V. (%)*		
	PA	*p-HPG*	*6-APA*
Within day	3.15	4.75	5.59
Between day	1.70	3.81	32.23
Total	3.58	6.09	32.71

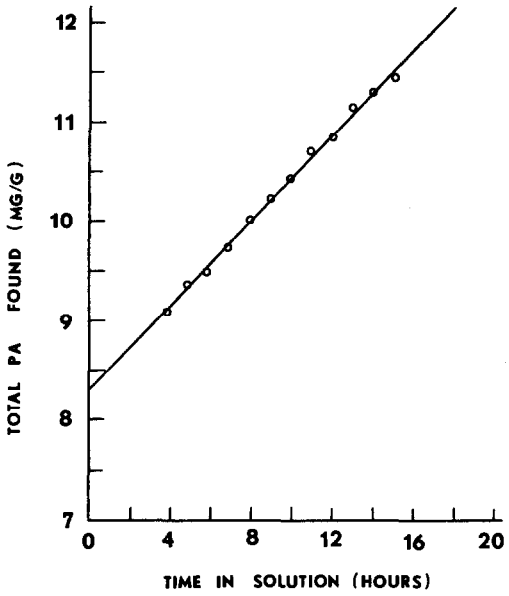

Fig. 5. A plot to show the increase of PA with time in aqueous solution. (Sample I.D. No. GAN-VH 30 month/RT sample).

tection limits were estimated to be *ca.* 0.7, 0.2 and 0.6 μg/ml for PA, *p*-HPG and 6-APA, respectively. These levels correspond 0.35, 0.10 and 0.30 mg of PA, *p*-HPG and 6-APA per 500-mg Amoxil capsule, respectively. This is based on a peak height 0.5% of full scale, which is considered adequate for the intended use of this method. Detection limits as calculated here are approximate and may vary with equipment used, age of the deuterium lamp, and actual settings.

Linearity

In the course of method development and actual use, the variability of the data about the regression line is assessed for each calibration curve obtained in terms of the standard deviation from the regression calculation, expressed as s_{yx}%[44]. Table III shows all s_{yx}% values obtained so far. An s_{yx}% value of 15% or less is considered acceptable for these components.

In addition, an expanded range of standards for each component was run to check the linearity of detector response. Solutions ranging from about 1.0 to 99.1 μg of PA, 1.2 to 73.0 μg of *p*-HPG and 1.8 to 61.4 μg of 6-APA per ml were tested. Statistical analysis of these data indicates that there is no curvature and that the intercept of the regression lines (area on concentrations) is not different from zero. The normal range of concentrations used in the assay are from *ca.* 3 to 15 μg of PA, 1–5 μg of *p*-HPG and 6-APA per millilitre. These concentrations (except that for 6-APA) are well within the range investigated for linearity.

This method has been used successfully for various formulations of Amoxil® capsules. With a slight change in the sample preparation steps this method was applied to raw materials and oral suspensions without any problems.

TABLE III

STANDARD DEVIATION OF STANDARD CURVES FOR PA, *p*-HPG, AND 6-APA

	s_{yx}%	*Number of points*
(A) PA	1.442	3
	0.830	3
	2.154	3
	5.245	3
	2.769	3
	3.059	3
	1.835	3
Mean	2.48	
(B) *p*-HPG	2.753	3
	0.655	3
	0.831	3
	2.964	3
	0.775	3
	5.780	3
	1.132	3
Mean	2.12	
(C) 6-APA	1.351	3
	4.114	3
	10.349	3
	7.910	3
	10.847	3
	4.030	3
Mean	6.43	

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