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High-Pressure Liquid Chromatographic Determination of Amoxicillin in Urine

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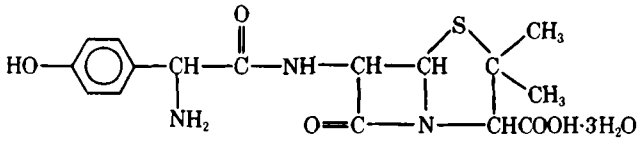
Abstract □ A rapid and specific high-pressure liquid chromatographic (HPLC) assay was developed for the simultaneous determination of amoxicillin and its penicilloic acid metabolite in urine. The two compounds, assayed directly in urine or after dilution with water–methanol (85:15), are separated by reversed-phase chromatography and quantitated spectrofluorometrically following postcolumn derivatization with fluorescamine. Linear calibration curves were measured in the ranges of 25–250 and 50–400 ng injected for amoxicillin and the penicilloic acid metabolite, respectively. The sensitivity limit of the assay is 2.5–5.0 μg/ml of urine for amoxicillin and the penicilloic acid metabolite. Urine samples (0–8 hr) taken from six subjects following single 250-mg po doses and assayed by HPLC showed ranges of cumulative percent of the dose excreted as amoxicillin and the penicilloic acid metabolite (reported as amoxicillin equivalents) of 50.2–68.0 and 21.6–30.0%, respectively. An excellent correlation (r = 0.985) was demonstrated for the measurement of amoxicillin concentrations by the HPLC and microbiological assays.

Keyphrases □ Amoxicillin—high-pressure liquid chromatographic determination in urine □ Antibacterials—amoxicillin and penicilloic acid metabolite, high-pressure liquid chromatographic determination in urine □ High-pressure liquid chromatography—analysis, amoxicillin and penicilloic acid metabolite in urine

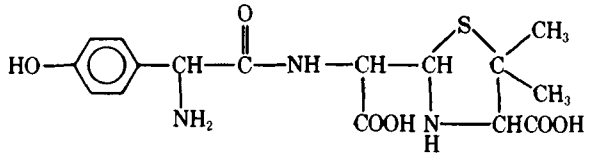
Amoxicillin[1] (I) [D-(−)-α-amino-*p*-hydroxybenzylpenicillin trihydrate], synthesized from 6-aminopenicillanic acid, is an orally absorbed, acid-stable, semisynthetic, broad-spectrum antimicrobial agent (1–3). Studies on its biotransformation demonstrate that it is excreted in urine intact and as the penicilloic acid of amoxicillin (II) [6-D-(−)-α-amino-*p*-hydroxyphenylpenicilloic acid] (4, 5).

BACKGROUND

Routine determinations of I in biological fluids are usually performed by microbiological assay using one of a variety of sensitive bacterial strains



I



II

(6). Since the metabolic cleavage of the β-lactam ring to II inactivates this compound toward microbiological activity, the microbiological assays are specific for I in the presence of II. The analysis of II has been reported using a separate aliquot of the sample by iodometric titration (7) and by TLC, followed by formation of an ammonium–molybdate complex and spectrophotometry (5).

Spectrofluorometric methods are capable of measuring ampicillin (8–10) and amoxicillin (11–14) in serum, plasma, or urine following therapeutic doses. The spectrofluorometric assays are based on the formation of strongly fluorescent products by heating either in the presence of formaldehyde (8, 11–13) or uranylacetate in acid solution (10) or at room temperature in the presence of mercuric chloride in neutral solution (9, 14). Jusko (8) postulated that the fluorescent product formed is a 3,6-disubstituted diketopiperazine and demonstrated that ampicillin levels measured by the spectrofluorometric assay were appreciably higher than those measured in identical samples by a microbiological assay. This result was attributed to the formation of the identical fluorophore by both the penicilloic acid and the penicillin.

Thus, the spectrofluorometric methods described using formaldehyde (8, 11–13) or uranylacetate (10) are "total" assays, which measure the sum of the penicillin and penicilloic acid. The spectrofluorometric methods using mercuric chloride described for ampicillin (9) and amoxicillin (14) differ from the other methods employing formaldehyde (8, 11–13) in that

[1] Larotid, Hoffmann-La Roche Inc., Nutley, NJ 07110.

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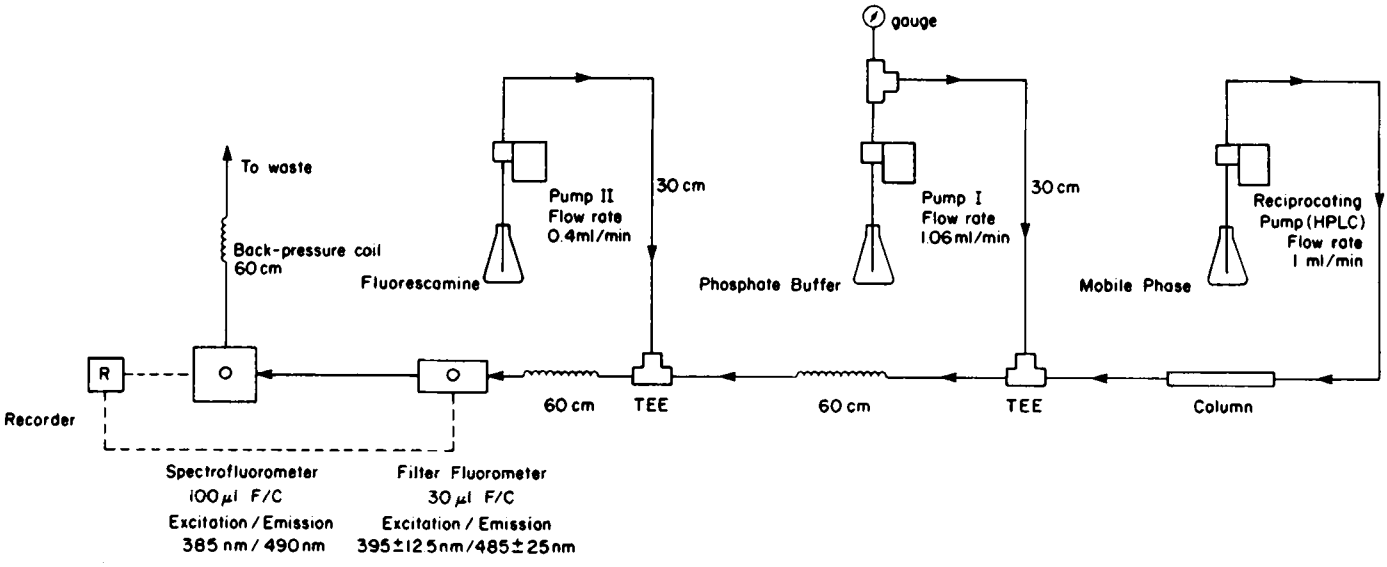

Figure 1—*Schematic diagram of equipment used for HPLC determination of I and II.*

only the penicilloic acid forms the fluorophore. Two aliquots of the same sample are required for the measurement of the penicillin and its penicilloic acid (9, 14). The first sample is required for the direct measurement of the "free" penicilloic acid fraction. The second sample is used to measure "total" penicilloic acid after alkaline hydrolysis of the penicillin to the penicilloic acid. The measurement of free penicillin is based on the difference between the "total" and "free" penicilloic acid fractions.

The aim of the present study was to develop a routine chemical assay capable of the simultaneous and specific measurement of both I and II without multiple assays and differential measurement. High-pressure liquid chromatography (HPLC) using spectrophotometric detection was recently described for the analysis of penicillins in pure forms (15–17). Utilization of HPLC separation followed by postcolumn derivatization with fluorescamine is a facile and reproducible procedure and has been used successfully for the determination of peptides (18) and kanamycin (19). The method described permits the rapid, simultaneous measurement of I and II in urine without extraction and with minimal sample cleanup. The method was successfully applied following the administration of therapeutic doses of amoxicillin and can be used in biopharmaceutical and pharmacokinetic studies.

EXPERIMENTAL

Column—The 30-cm × 4-mm i.d. stainless steel column contained octadecyltrichlorosilane chemically bonded (10% by weight) to 10-μm silica gel[2]. The column generated 10,000 plates/m.

Instrumental Parameters—The HPLC separation was performed using the column together with a reciprocating piston pump[3] and loop injector[4]. The isocratic mobile phase was a mixture of water–methanol–acetic acid (85:15:0.5) at a column head pressure of 1.6×10^3 psi and a constant flow rate of 1.0 ml/min. Two pumps[5] were used to add the 0.2 *M* phosphate buffer (pH 11.4) and fluorescamine reagent at flow rates of 1.06 and 0.4 ml/min, respectively. All connections from the effluent to the detectors were made with 1.5-mm o.d. × 0.3-mm i.d.[6] polytef[7] tubing, with the exception of the tubing from the buffer reagent and fluorescamine reservoir which was 3.0 mm o.d. × 1.5 mm i.d.[8]. All mixing tees, tube end fittings, couples, and gauges were obtained from a commercial source[9] (Fig. 1). The total flow rate through the system was 2.46 ml/min.

Fluorometric measurement was made with a filter fluorometer[10] using a polytef tube flowcell[11] of 32 μl and interference filters at 395 ± 12.5 and 485 ± 25 nm for excitation and emission, respectively. The excitation source was a 4-w fluorescent lamp[12], and detection was made with a nine-stage photomultiplier[13] powered by a regulated 700-v dc power supply[10]. In series with this detector was a spectrofluorometer[14] with a rectangular 0.1-ml quartz flowcell[15]. This instrument contains a 150-w xenon lamp[16] for excitation at 385 nm and measures the resultant emission at 490 nm using a photomultiplier tube[17]. A back-pressure coil (approximately 60 cm of polytef tubing) was attached to the exit line of the second detector to eliminate the formation of air bubbles in the flowcell. The filter fluorometer and spectrofluorometer were connected to a recorder[18] with input voltages of 1 v and 10 mv, respectively, with a chart speed of 2.54 cm/2 min.

A schematic diagram of the instrumentation used for determination of I and II is shown in Fig. 1. Under the described conditions, the retention times of the fluorescamine derivatives of I and II was 7.0 and 5.2 min, respectively (Fig. 2*a*). The injection of 250 ng of I and 400 ng of II/10 μl yielded peaks of nearly full-scale response at an attenuation of ×32 using the filter fluorometric detector. The injection of the same amounts of I and II using the spectrofluorometer with the selector at 1 and sensitivity at 7 yielded peaks of nearly full-scale deflection.

Reagents—All inorganic reagents were analytical grade and were prepared in deionized, distilled water.

Stock Buffer Solution—Phosphate buffer (1.0 *M*, pH 11.4) was prepared by mixing 530 ml of 1 *M* potassium phosphate dibasic (trihydrate)[19] and 470 ml of saturated tribasic sodium phosphate (Na_3PO_4)[19]. The mixture was adjusted to pH 11.4 with 1 *M* potassium phosphate monobasic ($KH_2PO_4 \cdot 3H_2O$) or saturated tribasic sodium phosphate.

Reagent Buffer Solution—The stock buffer solution was diluted with deionized, distilled water to yield 0.2 *M* phosphate buffer (pH 11.4). This solution was pumped into the effluent stream by Pump I at a rate of 1.06 ml/min (Fig. 1) to adjust the pH of the effluent of the liquid chromatographic column to 8–8.5 for optimum yield of the fluorescamine derivative.

Fluorescamine Reagent—A solution containing 20 mg of fluorescamine[20]/100 ml of acetone[21] was prepared. The reagent was pumped into the effluent stream by Pump II (Fig. 1) at a rate of 0.4 ml/min.

Mobile Phase—The mobile phase for HPLC separation was a mixture

[2] No. 27324, μBondapak C_{18}, Waters Associates, Milford, MA 01757.
[3] Model 6000, Waters Associates, Milford, MA 01757.
[4] Model U6K, Waters Associates, Milford, MA 01757.
[5] Model 396-31, Part 19-60006-001, Milton Roy mini-pump (16–160 ml/hr), Laboratory Data Control, Englewood Cliffs, NJ 07636.
[6] No. 200-34, Rainin Instrument Co., Fort Lee, NJ 07024.
[7] Teflon, du Pont.
[8] No. 200-32, Rainin Instrument Co., Fort Lee, NJ 07024.
[9] Tees No. 200-22, tube end fittings No. 200-00, couples No. 200-16, and gauges (0–1000 psi) No. 904-01, Rainin Instrument Co., Fort Lee, NJ 07024.
[10] Models 500-D and 500-C fluorometer detectors, Cambridge Bioengineering Inc., Cambridge, MA 02142.
[11] Cambridge Bioengineering Inc., Cambridge, MA 02142. (An 8-μl flowcell with filter accessory recently became available.)
[12] Cambridge lamp, F4T5/212/60°.
[13] RCA IP28 or equivalent.
[14] Model 204, Perkin-Elmer Corp., Norwalk, CT 06856.
[15] No. 22-0275, Perkin-Elmer Corp., Norwalk, CT 06856. (A 10-μl flowcell accessory kit; No. 010-0137, recently became available.)
[16] No. 350-1646 (OSR AM), Perkin-Elmer Corp., Norwalk, CT 06856.
[17] RCA R212 or equivalent.
[18] Speedomax, XL, 625 series dual-pen recorder, Leeds and Northrup, North Wales, PA 19454.
[19] Mallinckrodt Inc., St. Louis, MO 63160.
[20] Fluram, Hoffmann–La Roche Inc., Nutley, NJ 07110.
[21] Spectrograde, Eastman Chemical Co.

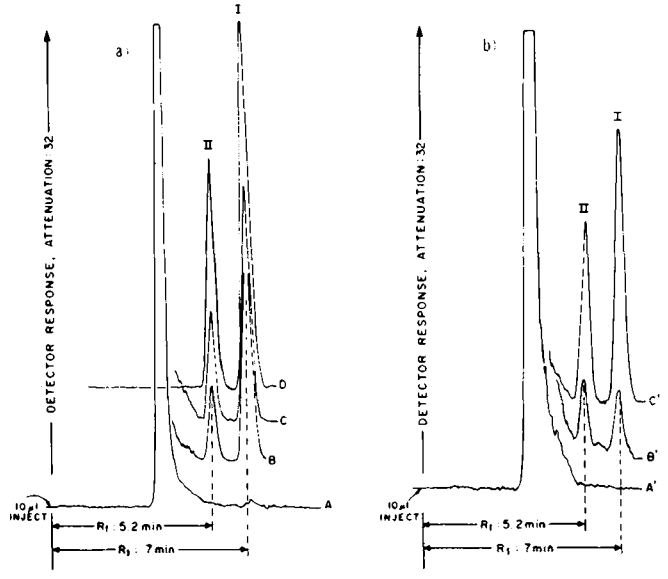

Figure 2—*Chromatograms of HPLC assay using a filter fluorometric detector. Key:* (a) *A, diluted control urine; B, human subject diluted urine after an oral dose; C, diluted control urine containing added standards; D, authentic standards;* (b) *A′, control urine; B′, human subject urine after an oral dose; and C′, control urine containing added standards.*

of water–methanol[22]–acetic acid[23] (85:15:0.5), which was deaerated in an ultrasonic bath prior to use.

Standard Solutions—First, 11.78 mg of I, 6-[(*R*)-2-amino-2-(*p*-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic acid trihydrate ($C_{16}H_{19}O_5N_3S{\cdot}3H_2O$, mol. wt. 419.45 corrected for 84.9% potency), was weighed out into a 10-ml volumetric flask and dissolved in deionized, distilled water. This stock solution contained 1.0 mg of I/ml. Then 11.15 mg of II, (α *R*, 2*R*, 4*S*)-α-[(*R*)-2-amino-2-(*p*-hydroxyphenyl)acetamido]-4-carboxy-5,5-dimethyl-2-thiazolidineacetic acid disodium salt ($C_{16}H_{19}O_5N_3Na_2S$, mol. wt. 427.38), equivalent to 10.00 mg of the free acid of II, was weighed out into a 10-ml volumetric flask and dissolved in deionized, distilled water. This stock solution contained 1.0 mg of II/ml.

These stock solutions were used to prepare the following six mixed standard solutions (A–F) containing 25, 50, 100, 150, 200, and 250 µg of I/ml and 50, 100, 150, 200, 300, and 400 µg of II/ml, respectively, by dilution with water–methanol (85:15). The mixed standards (A–F) were diluted further (1:10) with water–methanol (85:15) to yield a series of mixed working solutions containing 2.5, 5, 10, 15, 20, or 25 µg of I/ml and 5, 10, 15, 20, 30, or 40 µg of II/ml, respectively. A 10-µl aliquot of these mixed working solutions equivalent to 25, 50, 100, 150, 200, or 250 ng of I and 50, 100, 150, 200, 300, or 400 ng of II was injected onto the HPLC column to establish an external calibration curve of peak height *versus* concentration injected of I and II.

One-milliliter aliquots of Standards A–F were added directly to 1 ml of control urine and diluted to 10 ml with water–methanol (85:15) as internal standards for the construction of an internal standard curve. The internal standard calibration curve of peak height *versus* concentration injected was used to determine the concentration of the unknowns by interpolation.

All external standards and internal standards of I and II should be prepared fresh daily.

Urine Analysis—A 5-ml urine sample was placed into a 15-ml conical tube and centrifuged to spin down the suspended materials. A 1-ml aliquot of this supernate was transferred to another 15-ml centrifuge tube, and 9 ml of water–methanol (85:15) was added. A 10-µl aliquot of this supernate was injected into the chromatograph (Fig. 2*a*). For higher sensitivity, a 10-µl aliquot of the supernate could be assayed directly by HPLC (Fig. 2*b*).

Upon completion of a day's analysis, it was necessary to flush the phosphate reagent line with distilled water and the fluorescamine reagent line with acetone to avoid plugging.

Calculations—Quantitation was based on a linear relationship between peak heights of the fluorescamine derivatives and the concentrations of material added to the control urines. The peak heights of I and II in the respective internal standards and unknowns were determined. The absolute amount injected in the unknowns was obtained by interpolation from the internal standard curve. These values were corrected for the appropriate dilution and calculated as micrograms per milliliter of urine.

Precision and Sensitivity Limit—The efficiencies of postcolumn derivatization of I and II to their respective fluorescent derivatives from a urine sample as compared to a water (control) sample were 92.2 ± 5.6 and 100.4 ± 3.3% (*SD*), respectively. These calculations were based on the analysis of 10-µl aliquots of mixtures of 1 ml of urine or water to which were added 1 ml of Solutions A–F and 8 ml of water–methanol (85:15). Experiments demonstrated that the efficiencies of fluorophore formation of I and II were essentially identical using the direct injection of the urine supernate.

The sensitivity limits of detection were 25.0 and 50.0 µg/ml of urine for I and II, respectively, using the 10:1 dilution with water–methanol (85:15). Sensitivity limits of 2.5 and 5.0 µg/ml of urine for I and II, respectively, were obtained without dilution of the urine supernate. The specificity of the assay against endogenous interferences was demonstrated by the absence of peaks with retention volumes similar to I and II in the chromatograms of the control urines.

RESULTS AND DISCUSSION

The original goal of this work was to develop a rapid, straightforward HPLC assay suitable for the simultaneous analysis of I and II in both plasma and urine. Studies on the extractability of these compounds showed that, due to their amphoteric nature, recoveries were very poor. Therefore, investigations were undertaken to assay these compounds after derivatization in the biological specimen and extraction of the derivatives or to assay the biological specimens directly.

The first approach met with partial success using the technique of fluorophore formation (8) with minor modifications. The assay required the formation of a protein-free filtrate of plasma with metaphosphoric acid and heating to 70° for 15 min. The filtrate was washed twice with 2 × 10-ml portions of ether and then made strongly acidic with concentrated hydrochloric acid. This solution was heated (100°) in the presence of formaldehyde to yield an extractable yellow fluorophore, which was quantitated by HPLC using filter fluorometric detection with a low pressure mercury lamp excitation source (excitation of 365 and emission of >451 nm). Analysis was performed using a silica gel[24] column with an

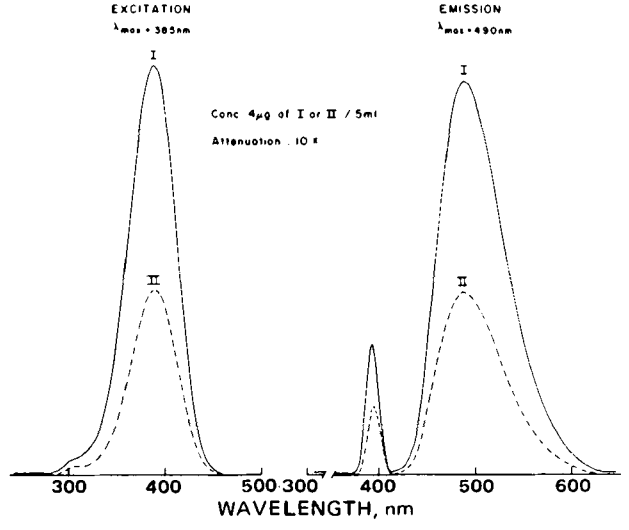

Figure 3—*Uncorrected excitation–emission spectra of a solution containing 4 µg of I and II, 0.4 ml of 1 M phosphate buffer (pH 8), and 0.2 ml of 20 mg fluorescamine/100 ml of acetone, diluted to 5 ml with water–methanol (85:15).*

[22] Fisher Scientific, Springfield, NJ 07081.
[23] J. T. Baker Chemical Co., Phillipsburg, NJ 08865.
[24] A 0.25-m × 4.6-mm i.d. Partisil silica gel 10-µm stainless steel column, Whatman, Clifton, NJ 07014.

Table I—Comparison of the HPLC Assay Using a Filter Fluorometer and a Spectrofluorometer as Detectors

Compound I, μg/ml			Compound II, μg/ml		
Filter Fluorometer (a)[a]	Spectrofluorometer (b)[a]	Ratio (a/b)	Filter Fluorometer (a)[a]	Spectrofluorometer (b)[a]	Ratio (a/b)
136.5	133.4	1.02	57.8	46.0	1.25
217.5	210.3	1.03	80.6	86.0	0.94
145.7	159.1	0.92	64.4	58.6	1.09
213.5	221.7	0.96	71.5	71.4	1.00
270.9	283.4	0.96	118.7	120.0	0.99
118.0	129.3	0.99	50.8	50.0	0.92
r = 0.989			r = 0.982		
Mean ± *SD*		0.98 ± 0.04			1.03 ± 0.11

[a] Filter fluorometer attenuation at ×32; spectrofluorometer selector at 1 and sensitivity at 7.

Table II—Urinary Excretion of I and II over 0–8 hr following Single 250-mg po Doses in Six Human Subjects

	Microbiological Assay—Excreted as Unchanged I		HPLC Assay[a]					
			Excreted as Unchanged I		Excreted as II[b]			
Subject	μg/ml (a)	% of Dose Recovered	μg/ml (b)	% of Dose Recovered	μg/ml	% of Dose Recovered	Total Dose Recovered, %	Ratio (a/b)
1	135.0	54.0	136.5	54.6	57.8	23.1	77.7	0.99
2	222.6	63.2	217.5	61.8	80.6	22.9	84.7	1.02
3	158.2	73.8	145.7	68.0	64.4	30.0	98.1	1.08
4	223.3	69.2	213.5	66.2	71.5	22.2	88.3	1.05
5	264.5	59.6	270.9	61.0	118.7	26.7	87.7	0.98
6	100.9	44.3	118.0	50.2	50.8	21.6	71.8	0.85
Range		44.3–73.8		50.2–68.0		21.6–30.0		
Mean		60.7		60.3		24.4		
Mean ± *SD*								1.00 ± 0.07

[a] Using filter fluorometer. [b] Reported as I equivalent. Factor = (419.45/383.38) = 1.09.

isocratic mobile phase of methylene chloride–methanol (95:5) at a flow rate of 1.6 ml/min on a commercially available liquid chromatograph.

Under these conditions, both I and II yielded the identical derivative (R_t 5.6 min) with a limit of sensitivity of 200 ng/ml of plasma. Comparison of the fluorescent properties of the yellow derivative of I and II (excitation/emission maxima: 363/452 nm) with those of the colorless authentic standard of the 3,6-disubstituted diketopiperazine (excitation/emission maxima: 280/305 nm) demonstrated that the 3,6-disubstituted diketopiperazine was not produced upon heating I and II in acid in the presence of formaldehyde, as suggested by Jusko (8). When the 3,6-disubstituted diketopiperazine itself was reacted under the conditions described, it produced a compound with the identical fluorescent properties as the derivative of I and II. The chemical structure was recently identified as 2-hydroxy-3-phenyl-6-methylpyrazine (20).

The assay was applied to the analysis of I in plasma and urine of a dog following a 500-mg dose of a commercial formulation[1]. The levels obtained were compared to those measured by the microbiological assay using *Staphylococcus aureus* (ATCC 6538P) as the test organism. Over a 6-hr period, plasma levels measured by the HPLC tecnhique were as much as 15 times higher than those measured by the microbiological assay. The large difference was attributed to the formation of the identical fluorophore by II, the nonmicrobiologically active metabolite of I. The possibility of other unidentified inactive metabolites of I yielding the same fluorophore cannot be discounted. Thus, in plasma, this procedure is only useful for the measurement of total levels of amoxicillin (I) and its metabolites. Due to the lack of specificity, this approach was discontinued. Modification of the reaction conditions for this procedure (8) as well as that of Miyazaki *et al.* (9, 14) did not yield two derivatives that were separable by HPLC for quantitation. Attempts to form fluorescamine derivatives prior to HPLC of plasma samples were also unsuccessful due to formation of multiple products upon derivatization.

HPLC separation of I and II followed by a highly specific reaction with fluorescamine under alkaline condition permitted the direct determination of these compounds in urine. The limitation of this procedure is its inability to measure levels of I and II in the protein-free filtrates of plasma due to a lack of sensitivity.

The fluorescamine derivatives of I and II were examined by spectrofluorometry prior to HPLC analysis to establish reaction conditions to maximize the yield of the fluorophore. The optimum reaction pH was 8–8.5 with excitation and emission maxima for the fluorescamine derivatives at 385 and 490 nm, respectively (Fig. 3). The quantum yield of fluorescence for II was approximately half that of I and was probably related to the structural differences. A concentration of 20 mg of fluorescamine/100 ml of acetone was determined to be optimal for postcolumn derivatization. A concentration of 10 mg/100 ml of acetone resulted in a 50% loss in response, while concentrations greater than 20 mg/100 ml resulted in precipitation of the reagent in the polytef tubing.

The main criteria for the choice of the detector were its high energy output for excitation at approximately 390 nm and its selectivity for monitoring the fluorescence emission at approximately 490 nm. Both a filter fluorometer[12] and a spectrofluorometer[13] were evaluated as postcolumn detectors for fluorescence measurement. The data indicated that both instruments gave comparable results (r = 0.989 and r = 0.982) for I and II, respectively (21) (Table I). Since the two instruments evaluated met both these criteria, either can be employed for pharmacokinetic studies.

Urinary Excretion in Humans—The HPLC assay was applied to the measurement of the urinary excretion of I in six human subjects following administration of a single oral 250-mg dose. Urine samples were collected prior to administration (control) and for 8 hr thereafter. Levels of unchanged I and II were determined by HPLC. The percent of dose recovered as I within the 8-hr period ranged from 50 to 68% of the ad-

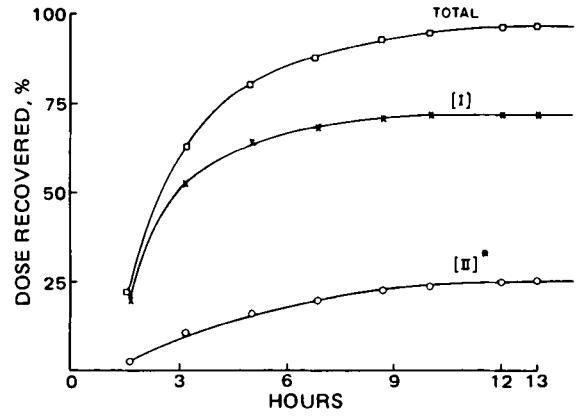

Figure 4—*Percent of dose of I and II recovered in the urine at intervals over 13.00 hr from a human subject following a single 250-mg po dose (* = reported as I equivalent).*

Table III—HPLC Measurement [a] of the Urinary Excretion of I and II at Various Time Intervals in One Human Subject following a 250-mg po Dose

Hours at Conclusion of Excretion Interval	Unchanged I			II [b]			Total Dose Accumulated, %
	Total Excreted, mg	% of Dose Recovered	% of Dose Accumulated	Total Excreted, mg	% of Dose Recovered	% of Dose Accumulated	
1.58	48.6	19.4	19.4	6.2	2.5	2.5	21.9
3.17	82.6	33.0	52.4	20.4	8.2	10.7	63.1
5.00	29.1	11.7	64.1	14.1	5.6	16.3	80.4
6.83	10.4	4.2	68.3	8.5	3.4	19.7	88.0
8.67	5.9	2.4	70.7	7.1	2.8	22.5	93.2
10.00	1.6	0.6	71.3	3.0	1.2	23.7	95.0
12.00	1.1	0.4	71.7	2.8	1.1	24.8	96.5
13.00	0.4	0.1	71.8	1.0	0.4	25.2	97.1
21.50	n.m. [c]			n.m. [c]			
24.50	n.m. [c]			n.m. [c]			

[a] Using filter fluorometer. [b] Reported as I equivalent. [c] Not measurable.

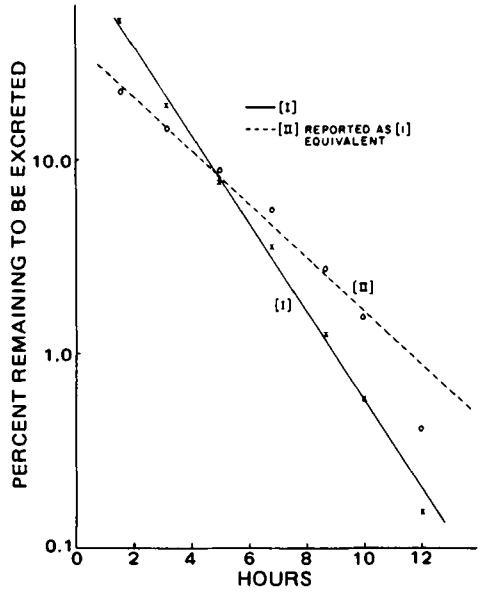

Figure 5—*Plot of the percent of dose remaining to be excreted as I and II in the urine of a human subject following a single 250-mg po dose.*

ministered dose (Table II). The recoveries of II, calculated as equivalents of I, excreted within the same period ranged from 22 to 30%. The I levels also were measured by the microbiological assay using *S. aureus* (ATCC 6538P) as a test organism (22) (Table II). An excellent correlation (r = 0.985) (21) was obtained for the determination of I by HPLC and bioassay, demonstrating that the two procedures were equivalent. These data are in good agreement with previously reported (4) recoveries of 72 ± 15 and 19 ± 12.6% for I and II, respectively, in a 12-hr period following a single 500-mg po dose.

The urinary excretion of I and II also was measured in another subject following a single 250-mg po dose. Ten urine samples were collected at intervals over 24.5-hr, the volume voided was measured, and the specimens were assayed by HPLC only. Approximately 100% of the drug was recovered in the urine as I and II (Table III and Fig. 4). A semilogarithmic plot of percent of intact I remaining to be excreted *versus* time (Fig. 5) yielded a linear plot with a slope equivalent to a half-life for I of 1.3 hr. The half-life value for I calculated for this subject was in good agreement with half-life values reported in the literature ($t_{1/2}$ = 1.1–3.2 hr) (23). The penicilloic acid metabolite (II) also exhibited linear elimination characteristics (Fig. 5). These findings demonstrate the utility of this method for clinical pharmacokinetic evaluation.

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