respectively. In summary, the HPLC method presented here is rapid, precise, and extremely suitable for the determination of phenylpropanolamine in serum and urine.

REFERENCES

(1) "Remington's Pharmaceutical Sciences," 15th ed., Mack Publishing Co., Easton, Pa., 1975, p. 820.

(2) L. Neelakantan and H. B. Kostenbauder, *J. Pharm. Sci.*, **65**, 740 (1976).

(3) L. M. Cummins and M. J. Fourier, *Anal. Lett.*, **2**, 403 (1969).

(4) H. Kinsun, M. A. Moulin, and E. C. Savini, *J. Pharm. Sci.*, **67**, 118 (1978).

(5) E. Appel, *Eur. J. Clin. Pharmacol.*, **8**, 161 (1975).

(6) T. L. Sprieck, *J. Pharm. Sci.*, **63**, 591 (1974).

(7) V. Das Gupta and A. G. Ghanekar, *J. Pharm. Sci.*, **66**, 895 (1977).

(8) T. R. Koziol, J. T. Jacob, and R. G. Achari, *J. Pharm. Sci.*, **68**, 1135 (1979).

(9) A. G. Ghanekar and V. Das Gupta, *J. Pharm. Sci.*, **67**, 873 (1978).

(10) "Over the Counter Cough/Cold Remedies," L. C. du Pont, Technical Report, E 32022.

(11) "Analysis of Pharmaceutical Products," Waters Associates, Publication N68, 1976, p. 11.

(12) M. Endo, H. Imamichi, M. Moriyasu, and Y. Hashimoto, *J. Chromatogr.*, **196**, 334 (1980).

(13) W. D. Mason and E. N. Amick, *J. Pharm. Sci.*, **70**, 707 (1981).

(14) J. E. Sinsheimer, I. G. During, and R. T. Williams, *Biochem. J.*, **136**, 763 (1973).

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support from the South African Council for Scientific and Industrial Research.

High-Performance Liquid Chromatographic Determination of Aspirin and Its Metabolites in Plasma and Urine

SHAMSUL K. BAKAR and SARFARAZ NIAZI ˣ

Received December 3, 1981, from the *Department of Pharmacodynamics, College of Pharmacy, University of Illinois Health Sciences Center, Chicago, IL 60612.* Accepted for publication February 25, 1982.

Abstract □ A simple quantitative method for the rapid determination of aspirin and its metabolites, salicylic acid, salicyluric acid, and gentisic acid, in plasma and urine using *o*-toluic and *o*-anisic acids, respectively, as internal standards was developed. Plasma proteins were precipitated by the addition of acetonitrile and, after centrifugation, the supernatant fluid was injected directly onto a reverse-phase column. The mobile phase consisted of an isocratic mixture of water, methanol, and glacial acetic acid (64:35:1, v/v/v) and the separated components were detected at 238 nm using a UV detector. Concentrations ≥0.5 μg/ml could be quantitated for aspirin or its metabolites in plasma. The peak heights and peak height ratios to the internal standard, *o*-toluic acid, were linear for the concentration range of 0.5–200 μg/ml. The aspirin metabolites in urine were isolated by extracting the acidified urine with ether and then reextracting the material into an aqueous buffer solution at pH 7.0. Twenty microliters of the buffer extract was directly injected onto the column. The separated components were detected and quantitated at 305 nm. Concentrations ≥5 μg/ml of salicyluric acid, salicylic acid, and gentisic acid could be determined accurately. The peak heights and peak height ratios to the internal standard, *o*-anisic acid, were found to be linear for the concentration range of 5–200 μg/ml in urine.

Keyphrases □ Salicylates—aspirin and metabolites, quantitation in urine and plasma, high-performance liquid chromatography □ Metabolites—of aspirin, quantitation in urine and plasma, high-performance liquid chromatography □ High-performance liquid chromatography—quantitation of aspirin and metabolites, urine and plasma

Aspirin is one of the most extensively used drugs. Recently, the importance of monitoring plasma levels of salicylates has been reviewed (1). A number of high-performance liquid chromatographic (HPLC) methods for the analysis of aspirin and its metabolites in plasma and urine have been reported (2–7). Few of the reported methods (3, 6) can determine aspirin in plasma, and they employ time-consuming extraction procedures. Since aspirin is rapidly hydrolyzed in blood and plasma (8), extraction procedures which delay the analysis of the sample will yield inaccurate results, underestimating the quantity of aspirin. Therefore, this work was initiated to develop a method for the rapid analysis of plasma samples for aspirin, salicylic acid, salicyluric acid, and gentisic acid, which does not require an extraction step.

The analysis of urine for the metabolites of aspirin using a simple extraction procedure is also desirable since urine usually contains a large number of acidic components (9, 10). Better results are obtained by eliminating as many undesirable components from the sample as possible before analysis. Injecting the urine onto a column without extraction (7, 11) shortens the useful life of the chromatography column.

EXPERIMENTAL

Materials—Aspirin USP[1], salicylic acid[1], salicyluric acid[1], gentisic acid[1], *o*-toluic acid[1], *o*-anisic acid[1], glacial acetic acid[2], anhydrous ether[3], acetonitrile[4], and methanol[4] were obtained commercially. The mobile phase consisted of water–methanol–glacial acetic acid (64:35:1, v/v/v), filtered and deaerated under reduced pressure.

Instrumentation—A dual-pump high-performance liquid chromatograph[5] equipped with a variable-wavelength UV detector[6], a 20-μl loop injector[7], and a reverse-phase microparticulate column[8] was used. The UV detector was connected to a linear recorder[9].

Stock Solutions—For the standard curves in plasma, aspirin, salicylic acid, salicyluric acid, and gentisic acid were singly dissolved in water–

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Mallinckrodt, St. Louis, Mo.
[4] Burdick and Jackson Laboratories, Muskegon, Mich.
[5] Perkin-Elmer Series 2 High-Pressure Liquid Chromatograph, Perkin-Elmer, Norwalk, Conn.
[6] Model LC-55 Variable Wavelength Detector, Perkin-Elmer, Norwalk, Conn.
[7] Model 7125 Rheodyne, Berkeley, Calif.
[8] μBondapak C_{18} column, Waters Associates, Milford, Mass.
[9] Linear Instruments, Model 261/MM, Irvine, Calif.

0022-3549/83/0900-1020$01.00/0

Table I—Peak Heights and Peak Height Ratios of Aspirin (III) and Its Metabolites Gentisic Acid (I), Salicyluric Acid (II), Salicylic Acid (IV), and the Internal Standard *o*-Toluic Acid (V) in Plasma

Concentration Added, μg/ml					Peak Height					Peak Height Ratio[a]			
I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV
0.5	0.5	0.5	0.5	10.0	3	5	3	3	41	0.07	0.12	0.12	0.07
1.25	1.25	1.25	1.25	10.0	7	12	4	6	42	0.17	0.29	0.10	0.14
2.5	2.5	2.5	2.5	10.0	12	20	7	10	46	0.26	0.43	0.15	0.22
5.0	5.0	5.0	5.0	10.0	24	37	15	25	43	0.56	0.86	0.35	0.58
12.5	12.5	12.5	12.5	10.0	60	89	42	59	44	1.40	2.02	0.95	1.34
25.0	25.0	25.0	25.0	100.0	123	171	87	120	450	2.72	3.80	1.93	2.67
50.0	50.0	50.0	50.0	100.0	246	353	184	255	441	5.58	8.00	4.17	5.78
100.0	100.0	100.0	100.0	100.0	480	670	360	460	436	11.01	15.37	8.26	10.55
200.0	200.0	200.0	200.0	100.0	970	1373	728	983	455	21.32	30.18	16.00	21.60

[a] Peak height of the drug/peak height of the internal standard.

Table II—Peak Heights and Peak Height Ratios of Gentisic Acid (I), Salicyluric Acid (II), Salicylic Acid (IV), and the Internal Standard *o*-Anisic Acid (VI) in Urine

Concentration Added, μg/ml				Peak Height				Peak Height Ratio[a]		
I	II	IV	VI	I	II	IV	VI	I	II	IV
6.0	6.0	6.0	10.0	10	16	9	23	0.43	0.70	0.39
10.0	10.0	10.0	10.0	19	21	14	21	0.90	1.00	0.67
20.0	20.0	20.0	10.0	39	45	33	21	1.86	2.14	1.57
40.0	40.0	40.0	100.0	91	100	79	225	3.57	3.92	3.10
80.0	80.0	80.0	100.0	174	185	150	242	7.19	7.64	6.20
120.0	120.0	120.0	100.0	276	272	219	237	11.65	11.48	9.24
160.0	160.0	160.0	100.0	362	363	301	228	15.88	15.92	13.20
200.0	200.0	200.0	100.0	455	451	372	235	19.36	19.19	15.83

[a] Peak height of the drug/peak height of the internal standard.

Table III—Regression Equations for Aspirin (III) and Its Metabolites Gentisic Acid (I), Salicyluric Acid (II), and Salicylic Acid (IV) in Plasma and Urine

Drug	Peak Height (y) *versus* Concentration (x)[a]	r^b	Peak Height Ratio (y) *versus* Concentration (x)[a]	r^b
		In plasma		
I	$y = 4.8410x + 0.4819$	0.9999	$y = 0.1070x + 0.0703$	0.9999
II	$y = 6.8261x + 2.4129$	0.9999	$y = 0.1509x + 0.1321$	0.9999
III	$y = 3.6457x - 1.9355$	0.9998	$y = 0.0806x - 0.0027$	0.9998
IV	$y = 4.8727x - 1.3624$	0.9995	$y = 0.1077x + 0.0251$	0.9997
		In urine		
I	$y = 2.2980x - 4.4384$	0.9998	$y = 0.0985x - 0.2269$	0.9994
II	$y = 2.2492x + 2.8104$	0.9996	$y = 0.0964x + 0.0873$	0.9996
IV	$y = 1.8771x - 2.1076$	0.9997	$y = 0.0805x - 0.1236$	0.9993

[a] Concentration in μg/ml. [b] Correlation coefficient.

acetonitrile (2:1) to produce solutions with final concentrations of 600 μg/ml. Due to the instability of aspirin in the solution, new solutions were prepared before each analysis. For the standard curves in urine, salicylic acid, salicyluric acid, and gentisic acid were dissolved together in water–acetonitrile (2:1) using a shaker[10] to yield a solution with a final concentration of 500 μg/ml for each of the components. The solution was kept refrigerated to avoid microbial degradation of the salicyluric and salicylic acids.

Internal Standards—The internal standard in plasma, *o*-toluic acid, was dissolved in water–acetonitrile (2:1) to yield solutions with concentrations of 50 and 500 μg/ml. For plasma samples containing 20–200 μg/ml of aspirin, salicylic, salicyluric, and gentisic acids, the concentration of the internal standard was 100 μg/ml; for plasma samples containing <20 μg/ml of the components, the internal standard concentration was 10 μg/ml.

The internal standard for urine, *o*-anisic acid, was dissolved in water to produce a solution with a concentration of 500 μg/ml. When the concentration of salicylic acid and its metabolites in urine was ≥40 μg/ml, the concentration of internal standard used was 100 μg/ml; when the salicylate concentration was <40 μg/ml in urine, the internal standard concentration was 20 μg/ml.

Standard Curves—*Plasma*—To 100 μl of rat plasma in a 500-μl polypropylene microcentrifuge tube[11], stock solution, the internal standard, water, and acetonitrile were added in such proportions that the final volume in each tube was 450 μl (300 μl of acetonitrile and 150 μl of a water–plasma mixture). The concentration of aspirin, salicylic acid, salicyluric acid, and gentisic acid ranged from 0.5 to 200 μg/ml in the water–plasma mixture. The internal standard concentration was 100 μg/ml in the mixture if the aspirin concentration was >20 μg/ml and 10 μg/ml if the aspirin concentration was ≤20 μg/ml.

The contents of the tube were vortexed[12] for 1 min and then centrifuged[13] for 5 min at 15,000 rpm. Twenty microliters of the supernatant was introduced directly onto the column at ambient temperature. The flow rate for the mobile phase was set at 2 ml/min (at 2800 psi), and the chart speed was 1 cm/min. The separated components were monitored at 238 nm, and the responses were recorded at 10 mV with proper attenuation of the recorder. The peak height and peak height ratio for each of the components in plasma were calculated.

For unknown samples, 50 μl of the plasma was precipitated with 100 μl of acetonitrile containing the internal standard in a 500-μl polypropylene microcentrifuge tube. This mixture was vortexed and centrifuged as described above for the standard curve. Twenty microliters was injected onto the column through the loop injector, and the peak height and peak height ratio of the separated components were calculated.

Urine—To 1 ml of urine contained in a 13-mm × 100-mm borosilicate glass culture tube with a polytef-lined screw cap, was added the salicylate

[10] Wrist Action Shaker, Burrell Corp., Pittsburgh, Pa.
[11] Eppendorf Micro Test Tube, Brinkmann Instruments Inc., Westbury, N.Y.
[12] Vortex Genie Mixer, Scientific Products, Evanston, Ill.
[13] Eppendorf Microcentrifuge, Model 5412, Brinkmann Instruments Inc., Westbury, N.Y.

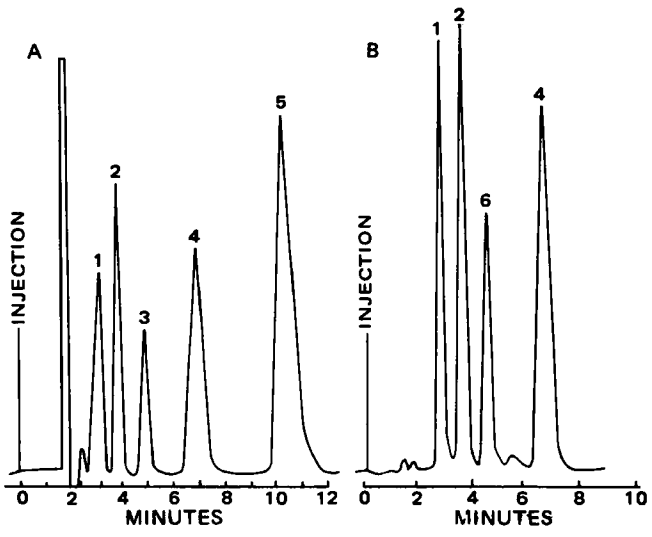

Figure 1—*Chromatograms of salicylic acid in blood (A) and urine (B). Key: (1) gentisic acid; (2) salicyluric acid; (3) aspirin; (4) salicylic acid; (5) o-toluic acid; (6) o-anisic acid. The concentrations for all components are 5 μg/ml in blood and 10 μg/ml in urine.*

mixture in aqueous solution to give concentrations ranging from 5 to 500 μg/ml. The concentration of *o*-anisic acid, the internal standard, was 100 μg/ml of urine when the salicylate concentration was ≥40 μg/ml and 10 μg/ml of urine if the salicylate concentration was <40 μg/ml in urine. One-half milliliter of 6 *N* HCl and 6 ml of anhydrous ether were also added, and the mixture was shaken for 15 min in a shaker and centrifuged for 10 min at 2000 rpm to separate the ether layer from the aqueous layer.

Five milliliters of the ether extract was transferred to another tube, and 1 ml of phosphate buffer (0.1 *M*, pH 7.0; prepared by dissolving 13.61 g of potassium dihydrogen phosphate and 2.38 g of NaOH pellets in water to make 1 liter) was added. The system was shaken for 15 min and then centrifuged at 2000 rpm for 10 min. The ether layer was aspirated, and 20 μl of the aqueous buffer solution was directly injected onto the column under conditions identical to those described earlier for plasma, except that the eluate was monitored at 305 nm.

For the unknown urine samples, 1 ml of urine was acidified with 0.5 ml of 6 *N* HCl. The rest of the procedure was the same as that described above.

Accuracy and Precision Studies—Both the plasma and urine were spiked with aspirin and its metabolites (salicylic acid, salicyluric acid, and gentisic acid) to yield mixtures with concentrations ranging from 25 to 200 μg/ml. Five samples from each of these concentrations were processed appropriately and analyzed as described earlier for the preparation of the standard curves in plasma and urine.

RESULTS AND DISCUSSION

Standard Curves in Plasma and Urine—Figure 1 shows a typical chromatogram of aspirin and its metabolites in spiked plasma and urine. The components were well separated both in plasma and urine. The peaks were identified by their retention times, determined for each component by injecting the compounds individually. Additional identifications were made by injecting different concentrations of the same compound and observing the proportional changes in the peak height and peak height ratio. The retention time for each component was found to be constant at all concentrations, and the values for gentisic acid, salicyluric acid, aspirin, *o*-anisic acid, salicylic acid, and *o*-toluic acid were 3.0, 3.7, 4.8, 5.0, 7.1, and 10.2 min, respectively.

Tables I and II list the peak heights and peak height ratios from the analysis of aspirin and its metabolites in plasma and urine, respectively. Both peak heights and peak height ratios were linear for the concentration range studied in plasma (0.5–200 μg/ml) and in urine (6–200 μg/ml). This was evident from the correlation coefficients (*r*) obtained for the regression line (Table III). The high values of *r* confirmed that there was an excellent linearity between the salicylate concentration and the peak height and peak height ratio, both in plasma and urine.

Recovery Studies in Plasma and Urine—Table IV shows the percent recovery of aspirin and its metabolites in plasma. The percent recoveries are similar at different concentrations. The greatest recovery was of aspirin (99%), followed by salicyluric acid (94%), salicylic acid (91%), and gentisic acid (62%).

It has been reported that salicylic acid and its metabolites, salicyluric acid and gentisic acid, are completely recovered in the aqueous acetonitrile phase following precipitation of plasma with an equal volume of acetonitrile (5). It has also been observed that one volume of acetonitrile is not sufficient to completely precipitate the protein in plasma (12). Preliminary experiments showed that two volumes of acetonitrile, rather than three, were sufficient to precipitate the proteins in plasma. This reduced the dilution of the components in plasma, thus increasing the sensitivity of the analytical method.

Ideally, the entire amount of the salicylates should be present in the plasma–acetonitrile supernatant. But the fact that not all the components were recovered completely from the plasma suggests that there was some kind of binding of the salicylates, especially gentisic acid (~38%), with the precipitated proteins.

The recovery studies with urine samples at different concentrations showed that all the components were equally extracted both from urine and water. When chloroform was used instead of ether, aspirin and salicylic acid were extracted almost completely, but the extraction of gentisic acid and salicyluric acid was poor (<50%). The overall recoveries from the urine using the proposed procedure were calculated to be 97, 95, 92, and 91% for gentisic, salicyluric, *o*-anisic, and salicylic acids, respectively.

Accuracy and Precision Studies in Plasma and Urine—The method proposed here eliminates the necessity of evaporating the organic phase, which is time consuming and involves a number of steps which may result in large variations in the analytical data (3, 6). Since the method described does not involve extraction steps, the variation in the data is considerably decreased; this is reflected by the data in Tables V and VI for plasma and urine, respectively. The low coefficients of variation (*CV*) and the narrow range in each of the concentrations studied suggest that the analytical method is precise and accurate.

This method has little dilution effect since 1 ml of the urine was finally extracted into 1 ml of buffer. In this respect, the method for urine analysis was more sensitive than that for plasma, since the plasma was diluted twice with acetonitrile.

Using this method, some sensitivity is lost because of the selection of 305 nm as the detection wavelength. At this wavelength, the absorptivity of different salicylate metabolites has been found to be less than at 238 nm. However, the latter wavelength cannot be used for urine samples because of numerous endogenous acidic components present in the urine (9, 10) that interfere with the analysis of the salicylate metabolites. At 305 nm the peaks are well separated and the metabolites are detected with sufficient sensitivity.

The proposed method could also be adapted for smaller urine samples (≤100 μl) or urine samples containing submicrogram quantities of aspirin metabolites simply by evaporating the 5 ml of ether extract and dissolving the residue in ~50 μl of the mobile phase.

Selection of Internal Standards—*o*-Anisic acid was used as the in-

Table IV—Recovery Study of Aspirin (III) and Its Metabolites Gentisic Acid (I), Salicyluric Acid (II), and Salicylic Acid (IV) in Plasma and Water

Concentration, μg/ml		Peak Height I	II	III	IV
48.78	Plasma	480	690	360	460
	Water	791	743	365	505
	Recovery, %	60.7	92.9	98.6	91.1
24.39	Plasma	246	353	184	237
	Water	395	376	185	258
	Recovery, %	62.3	93.9	99.5	91.9
12.195	Plasma	123	171	89	120
	Water	197	186	92	136
	Recovery, %	62.5	91.9	96.7	88.2
6.098	Plasma	60	89	42	59
	Water	98	93	41	65
	Recovery, %	61.2	95.7	102.4	90.8
2.439	Plasma	25	37	15	25
	Water	41	39	15	28
	Recovery, %	61.0	94.9	100.0	89.3
1.22	Plasma	13	20	8	13
	Water	21	21	8	14
	Recovery, %	61.9	95.2	100.0	92.9
	Average recovery, %	61.6	94.1	99.5	90.7
	SD	0.7	1.5	1.9	1.7
	CV	1.1	1.6	1.9	1.9

Table V—Accuracy and Precision Studies of the Analysis of Aspirin and Its Metabolites in Plasma

Compound	Concentration Added, μg/ml	Concentration Found[a], μg/ml		
		Mean + *SD*	Range	*CV*, %
Aspirin	25.0	25.02 + 1.04	23.91–26.26	4.2
	50.0	49.98 + 2.25	47.72–53.31	4.5
	100.0	101.47 + 2.53	98.95–104.16	2.5
Salicylic acid	25.0	24.66 + 0.80	24.50–26.70	3.1
	50.0	49.69 + 1.68	49.21–51.33	3.3
	100.0	100.12 + 2.19	97.32–102.68	2.2
Salicyluric acid	25.0	24.66 + 0.80	24.50–26.70	3.1
	50.0	49.69 + 1.68	49.21–51.33	3.3
	100.0	100.12 + 2.19	97.32–102.68	2.2
Gentisic acid	25.0	25.15 + 0.57	24.48–25.79	2.2
	50.0	49.63 + 2.20	46.16–51.58	4.4
	100.0	101.27 + 2.40	98.49–102.88	2.4

[a] $n = 5$.

Table VI—Accuracy and Precision Studies of the Analysis of Aspirin Metabolites in Urine

Compound	Concentration Added, μg/ml	Concentration Found[a], μg/ml		
		Mean + *SD*	Range	*CV*, %
Salicylic acid	40.0	41.37 + 1.14	39.80–42.66	2.9
	120.0	119.55 + 4.05	115.97–125.29	3.4
	200.00	200.23 + 3.49	196.48–204.80	1.8
Salicyluric acid	40.0	39.19 + 0.41	37.20–40.73	3.3
	120.0	120.23 + 1.51	117.49–123.30	2.0
	200.0	200.40 + 4.31	193.14–207.25	2.6
Gentisic acid	40.0	40.6 + 1.27	38.74–41.79	3.4
	120.0	119.54 + 2.30	116.70–122.80	2.0
	200.0	201.04 + 4.54	196.36–205.43	2.3

[a] $n = 5$.

ternal standard in urine. This compound has been used previously as an internal standard in plasma samples (5), but not in urine samples which had been processed by an extraction procedure. However, *o*-anisic acid could not be used for the analysis of aspirin because of the close proximity of their retention times (4.8 and 5.0 min for aspirin and *o*-anisic acid, respectively). This problem was solved by selecting *o*-toluic acid, which has a retention time of 10.2 min, well beyond the peak of salicylic acid. Its absorptivity at 237 nm is comparable with salicylic acid. However, in urine, *o*-anisic acid was used as an internal standard instead of *o*-toluic acid because of the long retention time of the latter.

In HPLC analyses of aspirin metabolites, the peaks for gentisic acid and salicyluric acid frequently overlap (7). In the present study, the mobile phase developed for the analysis was very efficient in separating the peaks for gentisic and salicyluric acids and in preventing the tailing of the salicylic acid peak.

REFERENCES

(1) M. Mandelli and G. Tognani, *Clin. Pharmacokinet.*, **5**, 424 (1980).

(2) C. P. Terweij-Groen, T. Vahlkamp, and J. C. Kraak, *J. Chromatogr.*, **145**, 115 (1978).

(3) G. W. Peng, M. A. F. Gadalla, V. Smith, A. Peng, and W. Chiou, *J. Pharm. Sci.*, **67**, 710 (1978).

(4) I. Bekersky, H. G. Boxenbaum, M. H. Whitson, C. V. Puglisi, R. Pocilinko, and S. A. Kaplan, *Anal. Lett.*, **10**, 539 (1977).

(5) B. E. Cham, D. Johns, F. Bochner, D. M. Imhoff, and M. Rowland, *Clin. Chem.*, **25**, 1420 (1979).

(6) L. I. Harrison, M. L. Funk, and R. E. Ober, *J. Pharm. Sci.*, **69**, 1268 (1980).

(7) D. L. Maulding and J. F. Young, *J. Pharm. Sci.*, **69**, 1224 (1980).

(8) A. M. Morgan and A. B. Truitt, Jr., *J. Pharm. Sci.*, **54**, 1640 (1965).

(9) M. Molnar and C. Horvath, *J. Chromatogr.*, **143**, 391 (1977).

(10) L. D. Mell, Jr., in "Biological/Medical Applications of Liquid Chromatography," vol. 10, G. H. Hawk, Ed., Dekker, New York, N.Y., 1977, pp. 619–636.

(11) B. E. Cham, F. Bochner, D. M. Imhoff, D. Johns, and M. Rowland, *Clin. Chem.*, **26**, 111 (1980).

(12) M. Bernardo, *Clin. Chem.*, **25**, 1861 (1979).