Submicrogram Assay for Scopolamine in Plasma and Urine

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Abstract □ A GLC–mass spectrometric method for scopolamine, sensitive to 50 pg/ml for a 4-ml plasma or urine sample, was developed. The method used a deuterated internal standard to minimize variability in absolute recovery in the extraction procedure. Scopoline and deuterated scopoline were formed from the base-catalyzed hydrolysis of scopolamine and the internal standard and were analyzed as the heptafluorobutyrates, using a GLC–mass spectrometric system by monitoring the *m/e* 138 and 141 fragments, respectively.

Keyphrases □ Scopolamine—submicrogram assay in plasma and urine, heptafluorobutyryl derivatives □ GLC–mass spectrometry—analysis, scopolamine in plasma and urine

Scopolamine, an anticholinergic drug, is a tropane alkaloid. The existing methods of assaying scopolamine in biological fluids, such as GLC (1) and acid-dye techniques (2, 3), lack sensitivity when therapeutic doses are administered. The most definitive study on scopolamine and its metabolites in biological fluids and tissues involved the use of radioactively labeled scopolamine in mice, rats, guinea pigs, and marmosets (4). This method was unsuitable for humans.

It was decided to develop a sensitive assay, using a stable isotope-substituted, internal standard in conjunction with GLC–mass spectrometry, that could be used to assay scopolamine in biological fluids after administration of an antinauseant dose. The method involved monitoring a fragment, produced under electron-impact ionization, of the heptafluorobutyryl derivative of scopoline (I). Scopoline was formed from the base-catalyzed hydrolysis of scopolamine.

Walle and Ehrsson (5) previously made the heptafluorobutyryl derivative of tropine, a hydrolysis product of atropine, in a study of the electron-capture responses of amino and alcoholic compounds. Blake *et al.* (6) recently used the diheptafluorobutyryl and dipentafluoropropionyl derivatives of 2-hydroxymethyltropine, formed through lithium aluminum hydride reduction of cocaine, to monitor cocaine indirectly in horse urine by electron-capture detection.

The pharmacokinetics of amphetamine and phentermine have been investigated by GLC–mass spectrometry, using trifluoroacetyl derivatives and deuterium-substituted analogs as internal standards (7). A similar approach was developed for homovanillic acid in cerebrospinal fluid, using the pentafluoropropionyl derivatives of the methyl ester of homovanillic acid (8). Homovanillic acid was assayed both by GLC–mass spectrometry, with the trideuterated methyl ester as an internal standard, and by electron-capture detection, with a homolog of homovanillic acid as the internal standard. As has been pointed out previously, some fluorinated acyl derivatives are readily adapted to both GLC–mass spectrometry and electron-capture detection studies because of favorable chromatographic properties in the subnanogram range (9).

The heptafluorobutyryl derivative of scopoline was used not for its sensitivity toward electron-capture detection but because it chromatographed well at the picogram level and showed a favorable fragmentation pattern under electron-impact ionization. The use of the deuterated internal standard for selective ion monitoring negated the necessity of finding an internal standard with similar partitioning properties, but with different retention times, which would have been required if electron-capture detection was used. Also, the use of "windows" (selective ion monitoring) in the GLC–mass spectrometric method greatly reduced the problem of biological interferences as compared to electron-capture detection.

EXPERIMENTAL

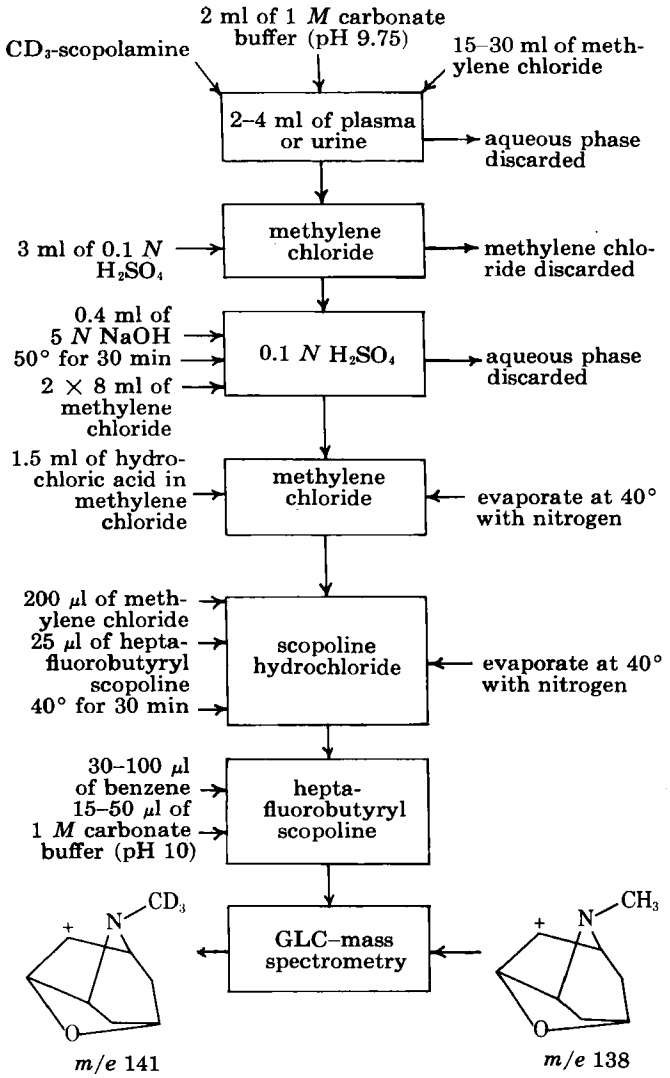
Materials—A GLC quadrupole mass filter system[1], in conjunction with an automatic peak selector[1], was used in the assay. An electron energy of 30 ev and an ionization current of 0.5–1.0 mamp were employed. The automatic peak selector was wired so that only two channels were used, maximizing the signal-to-noise ratio. The *m/e* 138 fragment of I and the *m/e* 141 fragment of the heptafluorobutyryl derivative of trideuteromethylscopoline (II) were monitored.

The sampling time was 10 msec. A preamp filter of 300 amu/sec and 30 μF capacitance for the output signal for each channel were used. It was sometimes necessary to use between 10 and 30 mv bucking for the 141 channel as compared to the 138 channel when small amounts of internal standard were used because of the inherent background within the system.

Glass columns, 1.8 m (6 ft), 2 mm i.d., were silanized and packed with either 3% OV-17 on Gas Chrom Q[2] (100–120) or 1% OV-225 on Gas Chrom Q[2] (100–120). Plasma samples were chromatographed on 3% OV-17 at 95° at a flow rate of 30 ml/min, while urine samples were chromatographed on 1% OV-225 under similar conditions. The retention time of I was 2.5 min on the former column and 2.2 min on the latter column. OV-225 invariably resolved peaks, which occasionally occurred in urine samples, from I that were not resolved with OV-17. Also, a reduction in column temperature could be used to improve resolution of I from peaks endogenous to the urine samples if blanks suggested the possibility of interferences. However, this was rarely necessary. Plasma samples were chromatographed on 3% OV-17 since there were no interfering peaks and this column bled less than did the OV-225.

Cylindrical separators (30 or 60 ml), with Teflon valves and stoppers, and 13-ml centrifuge tubes were used. The glassware was silanized with 5% dimethyldichlorosilane[2] in pyridine for 4 hr. The glassware was then washed with toluene, methanol, and methylene chloride and dried at 80°. The glassware was used up to 15 times before resilanization. After being used in an assay, the glassware was washed with laboratory detergent, methanol, and methylene chloride. The cylindrical separators and centrifuge tubes, together with the Teflon stoppers, were shaken vigorously with methanol

[1] Model 1015 Finnigan Corp., Sunnyvale, Calif.
[2] Applied Science Laboratories, Inc., State College, Pa.



Scheme I—Flow diagram of extraction procedure

and methylene chloride and dried just prior to performing an assay.

Synthesis of (−)-Scopolamine-(N-CD_3) Hydrobromide Hydrate—The deuterated compound was synthesized by the method of Schmidt *et al.* (11) who synthesized (−)-scopolamine-(N-$^{14}CH_3$) hydrobromide hydrate. Deuterated methyl iodide of 99.5 atom % isotopic purity[3] was used. The deuterated preparation gave a single peak by GLC, whose retention time was the same as scopolamine. The percent of the CH_3 moiety relative to the CD_3 was approximately 1%, as indicated by GLC–mass spectrometry.

Extraction Procedure—(−)-Scopolamine-(N-CD_3) hydrobromide hydrate, the internal standard, was added to 2–4 ml of plasma or urine. To the biological sample, contained in 30- or 60-ml cylindrical separators, were added 2 ml of 1 *M* carbonate buffer (pH 9.75) and 15–30 ml of methylene chloride[4]. If 4 ml of plasma was used, a large volume (30 ml) of methylene chloride was used to prevent emulsion formation. This was not a problem with urine. Also, the volume of organic solvent could be reduced in plasma extractions if 5% isopropanol[4] in methylene chloride was used. The inclusion of 5% isopropanol produced no extraneous peaks in the chromatograms.

The cylindrical separators were shaken vigorously in a horizontal position in a shaker[5] for 20 min and were then centrifuged at

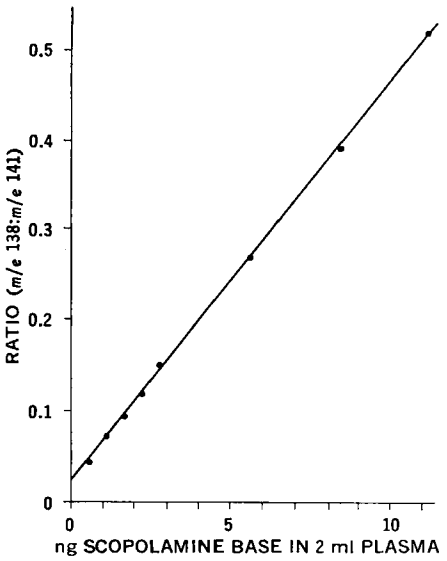


Figure 1—*Standard curve of scopolamine in plasma; 24.1 ng of internal standard, CD_3-scopolamine hydrobromide hydrate, was used.*

2000 rpm; the organic layer was removed through the Teflon valve into a 30-ml cylindrical separator containing 3 ml of 0.1 *N* H_2SO_4. These separators were then vigorously shaken for 10 min and centrifuged at 1500 rpm, and the organic layer was removed and discarded through the Teflon valve. To the 3 ml of 0.1 *N* H_2SO_4 was added 0.4 ml of 5 *N* NaOH, and the tubes were heated in a convection oven at 50–60° for 30 min.

After cooling, the alkaline aqueous phase was extracted with 8 ml of methylene chloride, the tubes were centrifuged at 1000 rpm, and the organic layer was released into a 13-ml centrifuge tube. Methylene chloride (1.5 ml), into which hydrogen chloride gas was bubbled, was added to this organic extract and the contents were vortexed. The contents were evaporated at 40° under a gentle stream of nitrogen[6] to approximately 1 ml. The aqueous alkaline phase was again extracted with 8 ml of methylene chloride and the organic phase was added to the 1 ml of the first extract in the 13-ml centrifuge tubes. After adding 1.5 ml of methylene chloride containing hydrogen chloride and vortexing, the contents were evaporated to dryness.

Care was taken to discontinue evaporation when the centrifuge tubes appeared dry. To the "invisible" residue was added 200 µl of methylene chloride plus 25 µl of heptafluorobutyric anhydride[7]. The centrifuge tubes were capped with Teflon stoppers and the contents were heated at 40° in a water bath. The centrifuge tubes were immersed to the 1-ml graduation so that the methylene chloride condensed in the upper part of the centrifuge tube and created a washing action. After 30 min the centrifuge tubes were spun briefly at 1500 rpm to concentrate the methylene chloride in the bottom of the tube. Methylene chloride and excess derivatizing reagent were removed under a gentle stream of nitrogen at 40°. The evaporation interval was 5 min.

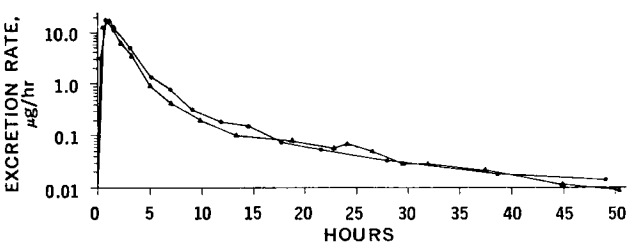


Figure 2—*Urinary excretion profiles following oral doses of scopolamine. Key: ▲, Subject 1, 906 µg (base); and ●, Subject 2, 800 µg (base).*

[3] ICN Isotope and Nuclear Division, Cleveland, Ohio.
[4] Nanograde solvents, Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Eberbach Corp., Ann Arbor, Mich.
[6] N-Evap, Organomation Associates, Shrewsbury, Mass.
[7] Pierce Chemical Co., Rockford, Ill.

Table I—Parameters for Nonlinear Regression Fit of Urinary Excretion Data

	Six-Parameter Equation					
	A	*B*	*C*	*a*	*b*	*c*
Subject 1	35.5	0.29	116	0.72	0.068	4.5
Subject 2	30.1	0.28	50.7	0.58	0.066	3.1
	Four-Parameter Equation					
	A		*B*	*a*	*b*	
Subject 1	34.4		0.29	0.71	0.068	
Subject 2	26.9		0.27	0.56	0.065	

To the residue, 30–100 μl of benzene, followed by 15–50 μl of 1 *M* carbonate buffer (pH 10), was added. The contents were vortexed and then centrifuged at 2000 rpm. Up to 10 μl of the benzene layer was injected. The contents of the centrifuge tubes were maintained at 10° in a refrigerated centrifuge to minimize loss of the benzene layer due to evaporation. See Scheme I for a flow diagram of the extraction procedure.

Standard Curve and Sample Analysis—A three-point standard curve was constructed for each series of analyses because of significant slope and intercept variations of standard curves from day to day. A statistical analysis of this variation is presented in the discussion. Scopolamine hydrobromide[8] was added to either plasma or urine in amounts ranging from 2 to 30 ng when, for example, 30 ng of internal standard was employed. If smaller or larger quantities of the internal standard were employed, the quantities of scopolamine added were adjusted so that the ratios would span the range from about 0.1 to 1.0.

Quantities of CD_3-scopolamine hydrobromide hydrate between 2 and 30 ng were added to 2–4 ml of plasma or urine, depending on the level of drug expected in the biological fluids. If a ratio of less than 0.05 was expected for a given sample, the amount of internal standard was reduced to increase the ratio. However, the same standard curve, constructed with a different quantity of internal standard, could be used to obtain the amount of scopolamine in the sample. The ratio was used to obtain the quantity of scopolamine present in the biological sample from the standard curve. This ratio was multiplied by the ratio of the internal standard in the sample to the internal standard used to construct the standard curve (Fig. 1). This technique was similar to that employed by Garrett and Hunt in the analysis of tetrahydrocannabinol (10).

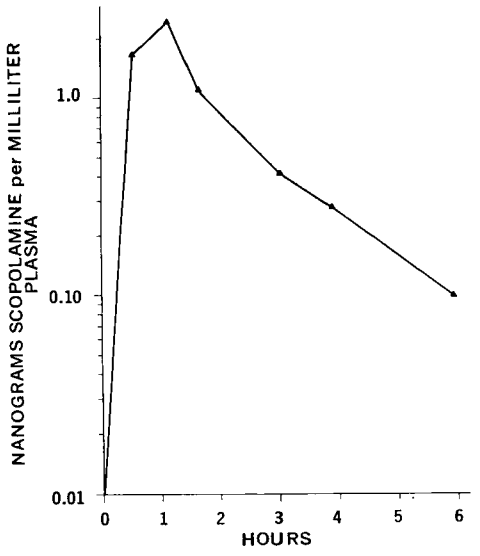


Figure 3—*Plasma levels following oral dose of scopolamine for Subject 1, 906 μg (base).*

[8] USP Reference Standard.

Table II—Major Mass Fragments of Heptafluorobutyryl CH_3-Scopoline and Heptafluorobutyryl CD_3-Scopoline

Heptafluorobutyryl CH_3-Scopoline		Heptafluorobutyryl CD_3-Scopoline	
m/e	% of *m/e* 81	*m/e*	% of *m/e* 81
81	100	81	100
138	86.1	141	81.2
94	20.8	97	22.2
96	11.3	99	10.9
108	6.3	111	4.9
110	5.4	113	5.0
154	3.1	157	3.0
169	2.5	169	1.8
351	0.4	354	0.35
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RESULTS

The urinary excretion profiles for two subjects and the plasma levels of scopolamine for one of these subjects following oral administration of scopolamine are presented in Figs. 2 and 3, respectively. Figure 4 shows typical chromatograms obtained in the analysis of urine samples. The instantaneous clearance for scopolamine can be calculated from the curves and is close to 120 ml/min, indicating that glomerular filtration is the prime mode of clearance. The total amount of scopolamine excreted from both subjects was 4–5% of the orally administered dose.

The urinary excretion curves were fitted by a nonlinear regression program to the equation $dD_E/dt = Ae^{-at} + Be^{-bt} - Ce^{-ct}$ for each subject (Table I). Since there was a scarcity of data points prior to the maximum excretion rate, since there was the possibility of a lag time, and since *c*, the rate constant for absorption, was significantly larger than *a*, it was decided to fit the curves to equation $dD_E/dt = Ae^{-at} + Be^{-bt}$, neglecting points prior to the maximum (Table I). Thus, the parameters for the *a* and *b* phases of the

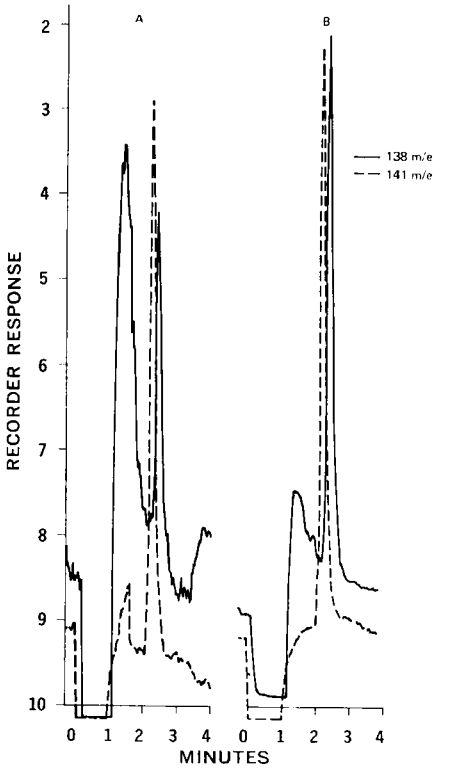

Figure 4—*Chromatograms of heptafluorobutyryl scopoline and heptafluorobutyryl CD_3-scopoline obtained from typical urine samples. Key: A, 0.192 ng/ml scopolamine (calculated) and 6.78 ng of internal standard; and B, 1.97 ng/ml scopolamine (calculated) and 13.56 ng of internal standard.*

excretion curves were not greatly affected by inclusion or neglect of the first-order absorption term. The parameters are functions of the rate constants for a two-compartment open model.

DISCUSSION

When scopolamine was derivatized with heptafluorobutyric anhydride and chromatographed, there was a tendency for the molecule to undergo pyrolytic elimination of heptafluorobutyric acid in the injection port. The presence of aposcopolamine was confirmed with GLC–mass spectrometry by a strong *m/e* 285 molecular ion. Therefore, it was decided to use a more indirect method, namely, the hydrolysis of scopolamine to scopoline and its conversion to a heptafluorobutyryl derivative (I), since it was more stable when chromatographed.

Compound I had excellent chromatographic properties in the 50–100-pg range. A quadrupole mass filter was used as a detector. The major mass fragments are listed in Table II. The most abundant fragment, retaining the three deuterium atoms at 30 ev, was *m/e* 141. Therefore, the *m/e* 138 and 141 fragments, representative of I and II, respectively, were monitored simultaneously. It was necessary to derivatize the free OH group in scopoline to prevent adsorption on the column. Furthermore, the ionization of the heptafluorobutyryl scopoline derivative (I) resulted in significantly less fragmentation than the ionization of scopoline under similar conditions.

Compound I had a tendency to undergo hydrolysis. However, 75% of the acylated derivative remained after 20 hr if benzene and carbonate buffer were added to the residue. The derivative was less stable in benzene in the absence of carbonate and markedly less stable in methylene chloride. When the hydrolysis of I was followed using a flame-ionization detector, a gradual increase in scopoline could be detected. Possibly, the carbonate liberated the free amine from a complex of I and heptafluorobutyric acid which was more susceptible to hydrolysis.

Absolute recoveries were investigated by adding 10 ng of scopolamine to 2 ml of plasma and conducting the extraction procedure. The recoveries, when measured against a standard prepared by derivatizing a known quantity of scopoline, ranged between 80 and 95%. The use of the deuterated internal standard removed this variability. However, high absolute recoveries were still desirable since the concentrations of scopolamine in the biological fluids were generally very small.

As mentioned previously, the method was somewhat indirect. The major metabolite of scopolamine was shown to be scopolamine glucuronide in animal studies (4), which was not extracted in the assay procedure. Scopine and aposcopolamine, minor metabolites, interfere with measurement of scopolamine. However, the method was developed to evaluate various dosage forms in human subjects rather than to elucidate the metabolism of the drug. A more refined method to discern the metabolites is now under investigation.

The average residual sum of squares for 13 regression lines for 13 three-point standard curves was 6.05×10^{-4}. The difference between the sum of squares of the 39 data points about a single regression line and the above-mentioned sum of squares was 6.23×10^{-3}. The ratio between the two variances was 5.58. The value from F tables yielded F (24, 13, 0.99) = 3.59. Thus, there was strong indication of significant variation in the standard curves.

The average value of the slopes, when expressed as the ratio of the *m/e* 138 to 141 responses times the ratio of the amount of deuterated scopolamine to scopolamine, was 1.03 ± 3.2% *RSD*. The average value and relative standard deviation of the intercepts were 0.0167 and ±55%, respectively. The large variation in the intercept was largely due to the degree of resolution between the *m/e* 138 and 139 peaks. The much smaller variation in the slope was probably due to the accuracy in focusing on the *m/e* 138 and 141 fragments.

Sjöguist and Änggård (8) noted similar problems in the assay for homovanillic acid. Therefore, it was decided to run a three-point standard curve each time a series of biological samples was to be analyzed. To test the precision of the assay, five aliquots of a urine sample were analyzed on different days, each with its own standard curve. The average value and relative standard deviation were 254 pg/ml and 2.5%, respectively. The variations of the slope and intercept during an analytical run were negligible.

The response ratio was determined for seven 4-ml aliquots of urine. The concentration of scopolamine in this urine, from a patient who had taken an oral dose of scopolamine, was 144 pg/ml. The average value of the response ratio was 0.105 ± 2.9% *RSD*. Thus, the consistency in the addition of the internal standard and the extraction procedure was very good.

A statistical analysis of the precision in determining the response ratios was recently presented in a manufacturer's application bulletin (12). Large amounts of internal standard relative to the endogenous compound can decrease the precision when the internal standard contributes to the *m/e* fragment monitored for the endogenous compound. For this reason, the amount of CD_3-scopolamine added to samples was chosen to yield response ratios between 0.1 and 2.0. If a particular sample had a response ratio significantly different from this range, it was usually rerun with an appropriately adjusted amount of internal standard.

CONCLUSION

The experiments demonstrate the utility of selective ion monitoring in conjunction with stable isotopes as internal standards in pharmacokinetic studies. The requirements in developing sensitive assays using this technique are that: *(a)* the compound or a derivative chromatograph well at the subnanogram level, and *(b)* the compound or derivative fragment under electron-impact ionization in a manner to produce a few fragments representing a significant portion of the total ionization. By using a GLC–mass spectrometric method for scopolamine, the urinary excretion rates for the unconjugated drug, which represented 4–5% of an oral dose of less than 1 mg, were followed over five orders of magnitude.

REFERENCES

(1) H. Kolb and P. W. Patt, *Arzneim.-Forsch.*, **15**, 924(1965).

(2) J. Delaville, *Ann. Biol. Clin.*, **20**, 479(1963).

(3) Z. I. ElDaravoy and S. L. Tompsett, *Analyst*, **81**, 601(1956).

(4) G. Werner and K.-H. Schmidt, *Hoppe-Selyer's Z. Physiol. Chem.*, **349**, 471(1968).

(5) T. Walle and H. Ehrsson, *Acta Pharm. Suecica*, **7**, 389(1970).

(6) J. W. Blake, R. S. Ray, J. S. Noonan, and P. W. Murdick, *Anal. Chem.*, **46**, 288(1974).

(7) A. K. Cho, B. J. Hodshon, B. Lindeke, and G. T. Miwa, *J. Pharm. Sci.*, **62**, 1491(1973).

(8) B. Sjöguist and E. Änggård, *Anal. Chem.*, **44**, 2297(1972).

(9) E. Änggård and A. Hankey, *Acta Chem. Scand.*, **23**, 3110(1969).

(10) E. R. Garrett and C. A. Hunt, *J. Pharm. Sci.*, **62**, 1221(1973).

(11) H.-L. Schmidt, G. Werner, and G. Kumpe, *Justus Liebigs Ann. Chem.*, **688**, 288(1965).

(12) R. C. Murphy, *Finnigan Spectra*, **3** (1973).

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