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Detection of Amphetamine and Methamphetamine-Type Materials in Pharmaceutical and Biological Fluids by Fluorometric Labeling

The increase in illegal use of phenylethylamine-type drugs has produced concurrent investigations for facile analytical methods to detect and identify such drugs, particularly amphetamine and methamphetamine. Two of the most sensitive and rapid methods widely used are thin-layer and gas-liquid chromatography [*1–7*]. These methods have the main advantage of being able to separate different types of phenylethylamine compounds in a single preparation. They also have the disadvantages of lengthy preparation procedures for general screening purposes.

Fluorescent labeling reagents for the detection of amine-containing compounds have been used during the past few years. Fluorescamine, 4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione(1), has been used for the analysis of amino acids, pesticides, proteins, and primary amines [*8*], whereas NBD-Cl, 7-chloro-4-nitro benzo-2-oxo-1,3-diazole, has been shown to react with amine compounds to yield derivatives that are highly fluorescent [*9*]. Gupta [*10*] reports the use of NBD-Cl to detect primary and secondary amines of forensic interest as well as the major metabolite of methadone, enamine. Monforte [*11*] also reports this reaction as well as the reaction with the tertiary amine propoxyphene. Dansyl-Cl, 5-dimethylamino-1-naptha-lensulfonyl-chloride, has found use in the quantitative analysis of free amine groups in peptides [*12*]. None of the references contains any report of attempts to isolate and chemically characterize the fluorescent NBD-Cl derivatives.

This report outlines a procedure by which amphetamine and methamphetamine-type compounds can be detected in drug preparations and urine by synthesizing fluorescent derivatives of these compounds with NBD-Cl and then separating them by thin-layer chromatography.

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Experimental

Reagents

All chemicals in this project were of reagent grade. The NBD-Cl was obtained from Pierce Chemical, Rockford, Ill. Amphetamine and methamphetamine were obtained from Aldrich Chemical Co., Milwaukee, Wisc.

Synthesis Procedure

The reaction between NBD-Cl and phenylethylamine-type compounds in which the amine group is constituted as the primary or secondary structure was assumed to proceed as indicated in Fig. 1. Hence, it was concluded that the reaction medium should contain

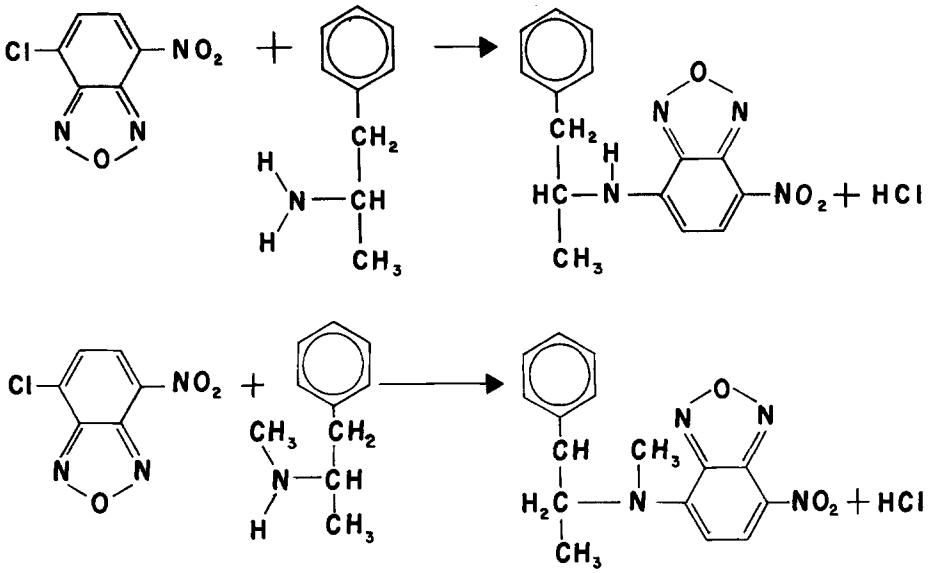

FIG. 1—*Reaction scheme for amphetamine and methamphetamine.*

buffer substances to keep the hydrogen chloride concentration low to obtain a good yield of product. The possibility of a hydrolysis reaction at the chlorine position of the NBD-Cl molecule was foreseen if a highly basic medium was used; hence, 0.1*N* sodium carbonate solution was chosen as buffer. Dioxane was chosen as a suitable solvent for both NBD-Cl and the amphetamines.

Standard solutions of amphetamine, methamphetamine, and NBD-Cl in dioxane were prepared, each at a concentration of 5 mg/ml. A typical synthesis involved mixing 2 ml NBD-Cl, 1 ml amphetamine solution (or methamphetamine), and 10 ml 0.1*N* $NaHCO_3$. This mixture was stirred for 1 h at room temperature. At the end of this period the reaction mixture exhibited intense fluorescence, clearly visible without an ultraviolet lamp. The reaction mixture was then evaporated to dryness in a Rotovac drier with steam. A solid residue resulted from the methamphetamine reaction, whereas a dark brown oily residue resulted from the amphetamine reaction.

The solid residue from the methamphetamine reaction was transferred to a 20-cm column packed with activated Fluorisil® and eluted, first with 100 ml hexane, then

by a second elution with 125 ml benzene. The column was then eluted with 2000 ml of ethyl ether to remove the adsorbed derivative. The ether eluate was evaporated to dryness at room temperature, yielding dark brown crystals mixed with a wax-like substance. The residue was redissolved in chloroform, poured through a fresh, activated Fluorisil® column, and eluted successively with 50 ml hexane, 100 ml benzene, and 500 ml ethyl ether. The ether fraction was evaporated at room temperature, yielding dark brown needle-shaped crystals as the residue. The oily residue from the amphetamine reaction was dissolved in ethyl ether, and Nuchar® charcoal was added. After filtration, the ether fraction was concentrated to a few millilitres by evaporation. Benzene (25 ml) was added, and the remaining ether was evaporated. The benzene portion was poured through an activated Fluorisil® column and eluted with 60 ml hexane, 150 ml benzene, and 160 ml ethyl ether, respectively. The ether portion was evaporated to dryness at room temperature, yielding a pale yellow wax-like residue. All attempts to obtain the NBD-amphetamine derivative as a crystalline solid met with failure.

Spectroscopic analyses were performed on the purified derivatives. These included fluorescence (Fig. 2), infrared, nuclear magnetic resonance (Fig. 3), and visible-ultraviolet (Table 1) analyses. In addition, the methamphetamine derivative was felt to be sufficiently pure (melting point, 160 to 161 °C) to obtain an elemental analysis (Table 2). The results of these analyses have led the authors to conclude that the reaction between NBD-Cl and the amphetamines does yield the products indicated by the equations of Fig. 1.

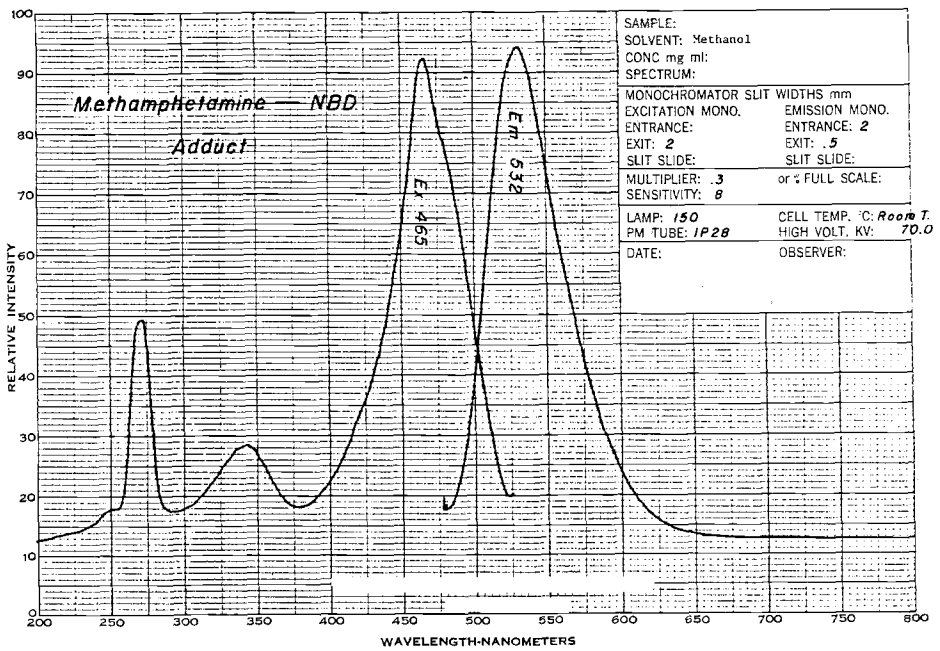

FIG. 2—*Fluorescence spectrum of NBD adduct.*

Attempts to correlate spectrofluorometric data (excitation; emission) with nature of the amine group were not successful for two reasons: (1) the reaction mixture turns dark and becomes optically opaque and (2) after the reaction mixture was purified both adducts (amphetamine, methamphetamine) exhibited the same fluorescence maxima (Fig. 2).

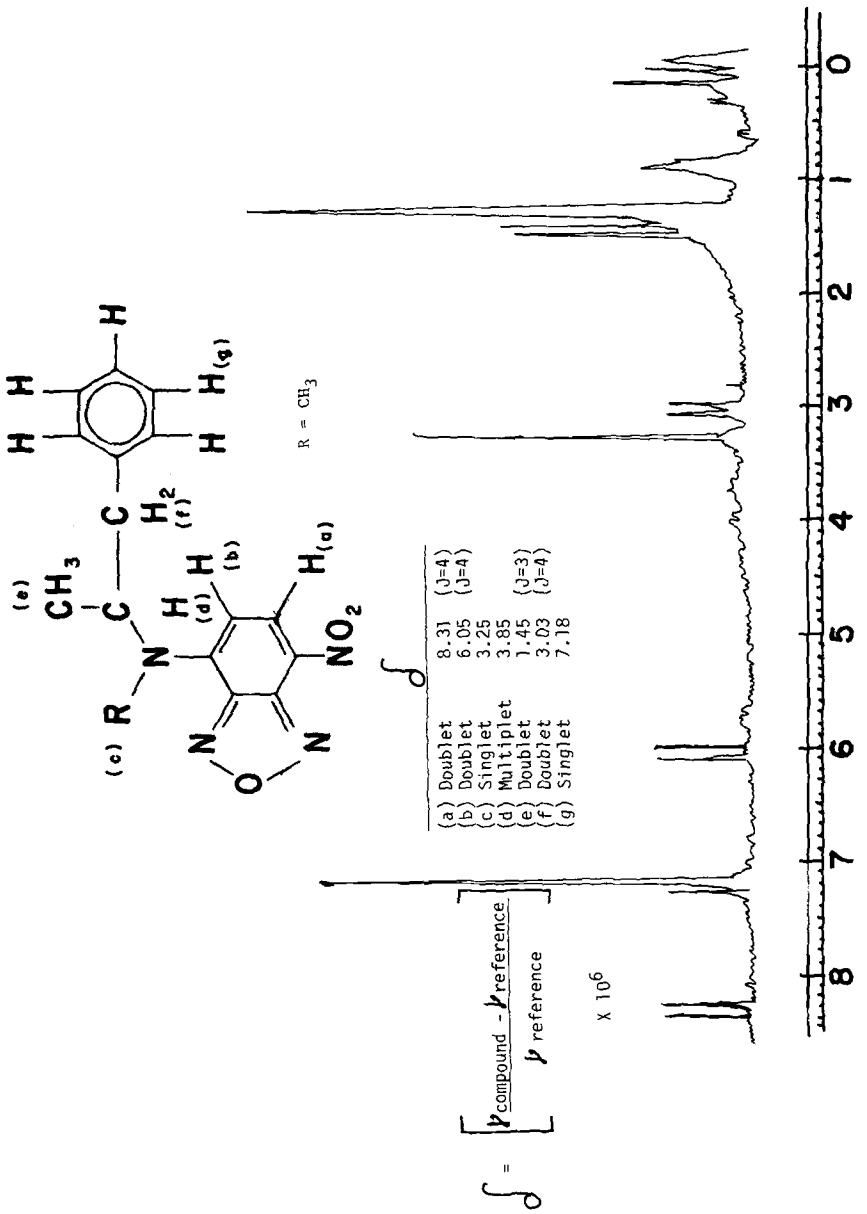

FIG. 3—*Nuclear magnetic resonance spectrum of methamphetamine adduct.*

TABLE 1—*Results of ultraviolet spectrophotometric analysis.*

Compound	Maximum (Methanol), nM
NBD-Cl	260, 340
Amphetamine	257.5
Methamphetamine	257.5
NBD-Amphetamine	260, 325, 455
NBD-Methamphetamine	340, 375

TABLE 2—*Results of elemental analysis of the NBD-methamphetamine adduct.*

	C, %	H, %	N, %	O, %
Observed	58.56	5.30	15.86	20.07
Calculated	61.5	5.2	17.9	15.4

Thin-Layer Chromatography—Tablet Analysis

Several solvent systems for thin-layer chromatography (TLC) plate development were investigated; the best separation results were obtained with solvent systems of pentane-ethyl ether in a volume ratio of 1 to 1 and methanol-carbon tetrachloride in a volume ratio of 1 to 20. Two silica gel TLC plates were spotted with a portion of an unknown sample, after preparation, and one plate was developed in pentane-ethyl ether and the other in methanol-carbon tetrachloride. Each plate was, of course, also spotted with portions of NBD-amphetamine derivative and NBD-methamphetamine derivative, the two being easily separated on the plate (Figs. 4 and 5). The procedure for tablet or capsule analysis is outlined in the following steps.

1. Crush about 100 mg of the tablet or capsule.
2. Dissolve in 10 ml of 0.1*N* $NaHCO_3$.
3. Add 1 ml of 0.4*M* NBD-Cl in dioxane.
4. Allow a reaction time of 1 h at room temperature or 15 min on a steam bath.
5. Extract the solution twice with 20 ml of chloroform and dry the extracts with anhydrous sodium sulfate or phase filter paper.
6. Evaporate the extract to dryness and redissolve in a minimum amount of acetone.
7. Spot 5 to 10 μl of the acetone solution onto two silica gel TLC plates which have been previously spotted with the two NBD-amphetamine derivatives.
8. Develop one plate in the pentane-ethyl ether solvent and the other in the methanol-carbon tetrachloride solvent.
9. View under an ultraviolet lamp.

Thin-Layer Chromatography—Urine Analysis

Amphetamine and methamphetamine in urine have been detected and identified using the same general procedure.

1. Make a 30-ml sample of urine basic (pH 7 to 8) with $NaHCO_3$, if necessary.
2. Add 2 ml of 0.4*M* NBD-Cl in dioxane reagent and allow a reaction time of 1 h at room temperature or 15 min on a steam bath.
3. Extract the solution twice with 20 ml of chloroform and dry with anhydrous Na_2SO_4 or phase filter paper.

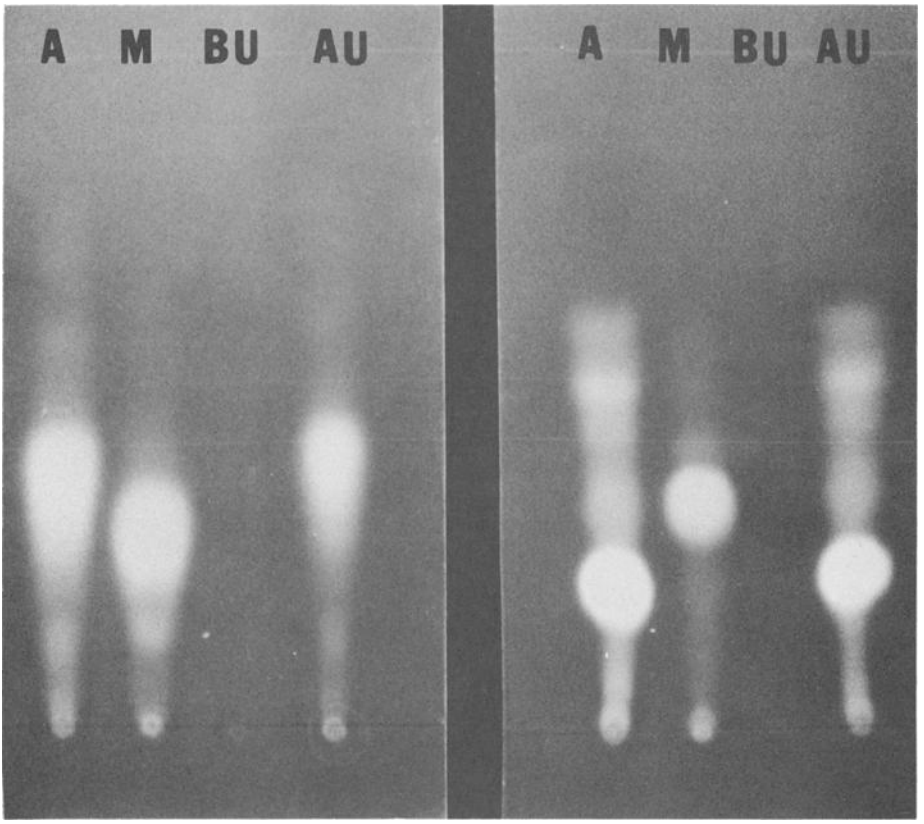

FIG. 4—*Thin-layer chromatograms on silica gel G;* (left) *Solvent 1 and* (right) *Solvent 2. A = NBD-amphetamine derivative; M = NBD-methamphetamine derivative; BU = blank urine; and AU = urine spiked with 1 μg/ml amphetamine.*

4. Evaporate the chloroform extract to dryness and redissolve with 5 ml of dichloroethane.

5. Pass the dichloroethane fraction through an activated Fluorisil® column (0.5 by 10.0 cm) and collect the eluate. Pass 15 to 20 ml of dichloroethane and collect and combine the two elutants.

6. Evaporate the eluate to dryness and redissolve with a few drops of dichloroethane. Spot (20 μl) on silica gel TLC plates with standards. Develop the plates in the two solvent systems and view with ultraviolet lamp (Fig. 5).

Interfering Substances

The possibility of other substances yielding identical R_f values to the amphetamine and methamphetamine derivatives with this procedure was investigated. More than 500 pharmaceutical and illicit preparations have been tested to date, and no false positives have been observed as long as both solvent systems were used.

A number of commonly available drugs were obtained in relatively pure form from manufacturers and tested with this procedure (Table 3). The column titled "Derivative" indicates whether or not reaction with NBD-Cl produced a fluorescent substance. The NBD-derivative of 4-methoxyamphetamine gave an R_f value very close to that of the

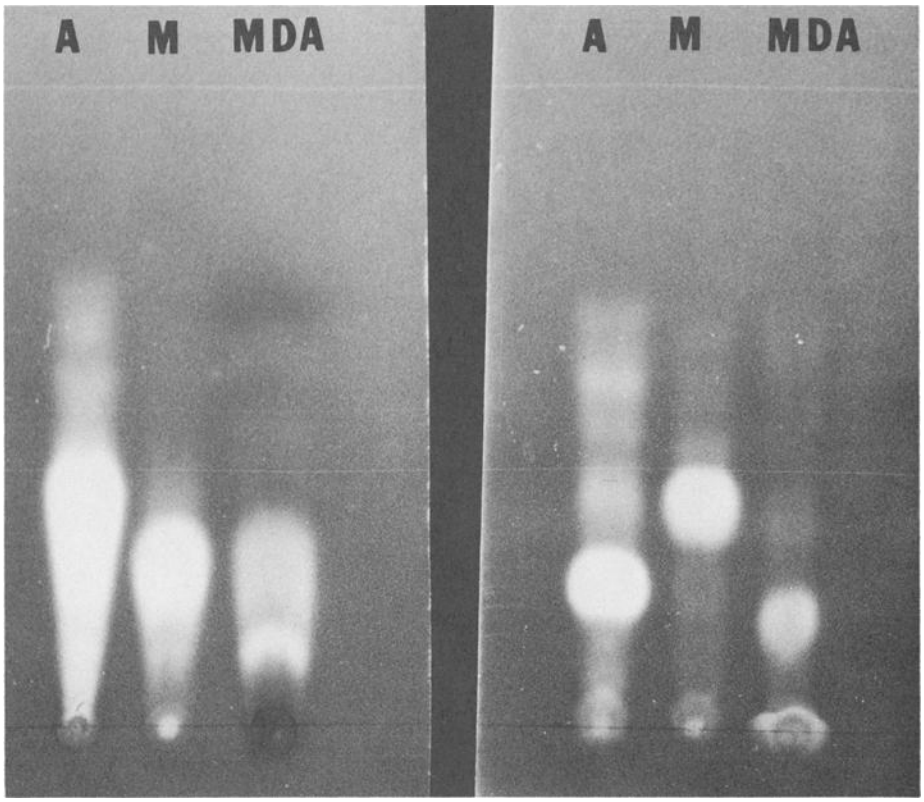

FIG. 5—*Thin-layer chromatograms on silica gel G;* (left) *Solvent 1 and* (right) *Solvent 2. A = NBD-amphetamine derivative; M = NBD-methamphetamine derivative; MDA = NBD-methylenedioxyamphetamine derivative.*

NBD-methamphetamine derivative using the pentane-ethyl ether solvent, but it could be distinguished with the methanol-carbon tetrachloride solvent. Similar results were obtained with 3,4-methylenedioxyamphetamine (Fig. 5).

Conclusions

The procedure described in this report has a number of advantages worth considering. The sample preparation is not long and complex, and amphetamine and methamphetamine can be detected in very small amounts: as low as 3.4 ng/ml of extract for tablet analysis and 0.1 μg/ml for urine analysis. Furthermore, NBD-Cl apparently does not react with tertiary amines or indole-type nitrogen compounds under the conditions used in this work. Another advantage is the relative absence of interference in the TLC phase of the analysis. Although any primary or secondary amine will probably yield a fluorescent derivative with NBD-Cl, the TLC analysis with the two-solvent system provides a technique to resolve a possible false positive result.

The procedure can be used for general screening of suspected amphetamine-type

TABLE 3—*Data generated by thin-layer chromatography for selected drugs.*

Compound	Derivative	Relative Mobility to Amphetamine	
		Solvent 1	Solvent 2
1. Phenmetrazine	yes	0.69	1.50
2. Methylphenidate	yes	0.88	1.39
3. Phendimetrazine	no	...	...
4. Butabarbital	no	...	...
5. Dimethyltryptamine	no	...	...
6. Meperidine	no	...	...
7. Reserpine	no	...	...
8. Phencyclidine	no	...	...
9. Mescaline	yes	0.25	0.35
10. 3,4-Methylenedioxyamphetamine	yes	0.78	0.80
11. Lysergic acid diethylamide	no	...	...
12. Caffeine	no	...	...
13. Amphetamine sulfate	yes	1.0	1.0
14. *N*-Propylamphetamine	yes	1.20	1.86
15. Ephedrine	yes	0.57	0.31
16. Ethylnorephinephrine	yes	0.0	0.81
17. Meprobamate	no	...	...
18. Methadone	no	...	...
19. Levo-amphetamine alginate	yes	1.0	1.0
20. Dextroamphetamine tannate	yes	1.0	1.0
21. Amphetamine hydrochloride	yes	1.0	1.0
22. Amphetamine thyroid	yes	1.0	1.0
23. 4-Methoxyamphetamine	yes	0.74	1.0
24. Amtriptyline	yes	0.64	1.72
25. Aspirin	no	...	...
26. Chloropheneramine	yes	0.49	0.86
27. Diazepam	no	...	...
28. Phentermine	yes	1.21	1.97
29. Phenylpropanolamine	yes	0.01	0.01
30. Methamphetamine	yes	...	...
31. Propoxyphene	no	...	...

preparations. A color screening test can be used on a given preparation and if positive results are obtained, the substance can then be treated with NBD-Cl reagent. If a fluorescent product is obtained, then the suspicion of an amphetamine-type compound is reinforced and the analyst can proceed with a more detailed analysis. At present more than 500 different samples have been run. This procedure is presently being used routinely for amphetamine analysis in "street drugs" in at least four laboratories in the United States.

Summary

A rapid and sensitive method for detecting amphetamine and methamphetamine in drug preparations and biological fluids has been developed. Amphetamine and methamphetamine in pharmaceutical and clandestine drug preparations can be easily screened from other contaminating drugs and readily identified by their fluorescence, with subsequent separation accomplished by TLC. The same general procedure can also be used to detect amphetamine and methamphetamine in human urine at concentrations of 0.1 μg/ml.

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References

[1] Bruce, R. and Maynard, W. Jr., *Analytical Chemistry,* Vol. 41, No. 7, 1969.
[2] Beckett, A., Tucker, G., and Moffat, A. C., *Journal of Pharmacy and Pharmacology,* Vol. 19, 1967, p. 273.
[3] Sunshine, I., Fike, W., and Handesman, H., *Journal of Forensic Sciences,* Vol. 11, 1966, p. 428.
[4] Beckett, A. and Rowland, M., *Journal of Pharmacy and Pharmacology,* Vol. 17, 1965, p. 59.
[5] Brochmann-Hanssen, G. and Svendsen, A. B., *Journal of Pharmaceutical Sciences,* Vol. 51, 1962, p. 393.
[6] Goldbaum, L., *Progress in Chemical Toxicology,* Vol. 2, 1965, p. 221.
[7] Parker, K. D., Fontan, C. R., and Kirk, P. L., *Analytical Chemistry,* Vol. 34, 1962, p. 1345.
[8] Stein, S., Bohlen, P., Imai, K., Stone, J., and Udenfriend, S., *Fluorescence News,* Vol. 7, No. 2, 1973.
[9] "Regis Lab Notes," No. 10, June 1971, Regis Chemical Co., Morton Grove, Ill.
[10] Gupta, R. N., Chittim, B. G., and Keane, P. M., *Journal of Chromatographic Science,* Vol. 12, 1974, p. 67.
[11] Monforte, J., Bath, R. J., and Sunshine, I., *Clinical Chemistry,* Vol. 18, 1972, p. 1329.
[12] Reisch, J., Kommert, H. J., and Clasing, D., *Pharmazeutische Zeitung,* Vol. 115, 1970.

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