Reaction of *N*,3,3-Trimethyl-1-phenyl-1-indanpropylamine Hydrochloride with 7-Chloro-4-nitrobenzofurazan and Its Application to Spectrophotofluorometric Assay

RAMAZAN TURDIU, MELVIN H. PENNER[x], and LESTER CHAFETZ

Abstract □ The reaction of *N*,3,3-trimethyl-1-phenyl-1-indanpropylamine hydrochloride, a secondary amine, with 7-chloro-4-nitrobenzofurazan yielded a well-defined, crystalline, fluorescent derivative which was isolated and characterized. A sensitive spectrophotofluorometric method was developed based on this reaction and was applied to the assay of capsules and compatibility samples.

Keyphrases □ *N*,3,3-Trimethyl-1- phenyl-1-indanpropylamine hydrochloride—reaction with 7-chloro-4-nitrobenzofurazan, isolation and characterization of derivative, application to spectrophotofluorometry □ 7-Chloro-4-nitrobenzofurazan—used as reagent for spectrophotofluorometric analysis of *N*,3,3-trimethyl-1-phenyl-1-indanpropylamine hydrochloride, isolation and characterization of derivative □ Spectrophotofluorometry—reaction of *N*,3,3-trimethyl-1-phenyl-1-indanpropylamine hydrochloride with 7-chloro-4-nitrobenzofurazan

N,3,3-Trimethyl-1-phenyl-1-indanpropylamine hydrochloride (I) is currently being investigated for its antisecretory and antidepressant properties. A reliable and sensitive method was necessary for the quantitation of this drug formulated with a variety of excipients and at concentrations encountered during compatibility studies, stability testing, and clinical evaluations.

It was immediately apparent that the measurement of its UV absorption lacked sufficient sensitivity. The spectrum showed only benzenoid absorption and λ_{max} = 272 nm (ϵ = 1300). A useful "handle" for the analysis of this compound proved to be the secondary amine function. No fluorescence could be detected when this amine was allowed to react with 1,2-naphthoquinone-4-sulfonic acid according to the procedure of Pesez and Bartos (1).

The reaction of 7-chloro-4-nitrobenzofurazan (II) with amino acids and primary and secondary amines to yield highly fluorescent derivatives which were stable in aqueous solution was described (2) (Scheme I). Unlike fluorogenic reagents, such as dansyl chloride, commonly employed with amines, 7-chloro-4-nitrobenzofurazan does not fluoresce and therefore the blanks are low. In addition, the resulting derivatives are excited by visible light at 470 nm and fluoresce at 510 nm.

Since the first paper (2), several reports appeared describing the utility of this reagent; however, most have been primarily of a qualitative nature and precise details regarding reaction conditions are lacking. Two-dimensional TLC on silica gel plates was used to separate the benzofurazan derivatives of a number of phenethylamine drugs (3) in which the derivatives of primary amines were yellow, having an absorption maximum in methanol of 447–464 nm, while the reaction products with secondary amines were reddish with a λ_{max} in methanol of 470–480 nm. The same technique was applied to the detection of methamphetamine and its metabolite, 1-phenyl-2-aminopropane, in urine (4). The structures of the aminobenzoxadiazoles of 11 phenethylamine drugs were characterized by mass spectrometry (5). This fluorogenic reagent has also been utilized for the detection of sulfonamides (6), alkaloids and other nitrogen-containing drugs (7), and thiols (8).

Recently, *N*-methyl- and *N*,*N*-dimethylcarbamate insecticides were determined quantitatively (9) with 7-chloro-4-nitrobenzofurazan. After hydrolysis of the carbamates in 0.1 *M* sodium bicarbonate, the liberated alkyl amines were reacted with the fluorogenic reagent to yield aminobenzofurazans separable by TLC. Quantitation was achieved *via* fluorometric scanning of the developed chromatoplates.

Study of the reaction of 7-chloro-4-nitrobenzofurazan with I led to a sensitive and reproducible fluorometric assay. Reaction conditions have been optimized so that essentially complete derivatization occurs.

EXPERIMENTAL[1]

Isolation and Characterization of Benzofurazan Derivative of I—The reaction was first performed on a milligram scale to isolate and characterize the product obtained. Compound I, 16.5 mg, was dissolved in 0.6 ml methanol–water (1:2) and to this was added 1.5 ml of a 1.0% solution of the reagent in methanol. Upon the addition of 0.1 ml 1 *N* sodium hydroxide to the clear yellow solution, a red flocky precipitate began to emerge, and the reaction mixture was allowed to stand overnight at room temperature. The precipitate was washed with a small amount of methanol and recrystallized from chloroform–methanol (1:2).

Pure material was isolated by preparative TLC, employing cyclohexane–ethyl acetate (1:1) as the solvent system. The intensely fluorescent main zone appearing at R_f 0.47 was eluted with methanol, the solution volume was concentrated, and the product was allowed to crystallize. After drying in an Abderhalden apparatus at 100° for 6 hr, a bright-red crystalline solid was obtained, mp 175–176°; λ_{max} CH_3OH: 480 (log ϵ 4.41) and 340 (3.91) nm. A molecular weight of 456 was obtained by mass spectrometry; the fragmentation pattern showing other significant peaks at *m/e* 426, 207, 191, 129, 114, 91, and 77 was consistent with the proposed structure. The purity of the derivative was verified by TLC in an

[1] A Farrand Mark 1 spectrophotofluorometer equipped with a 150-w xenon arc lamp, two grating monochromators, a 1P28 photomultiplier, and a solid-state photometer was used to record all fluorescence measurements. Melting points were taken in a capillary tube on a Thomas-Hoover melting-point apparatus and are uncorrected. Spectral data were obtained with a Cary model 14 recording spectrophotometer. TLC used chromatoplates obtained from Analtech, Inc., coated with 0.25-mm layers of silica gel GF. The plates were activated before use by heating at 105° for 30 min. The reagent, 7-chloro-4-nitrobenzofurazan, was synthesized according to a reaction scheme described previously (2).

Table I—Spectral Data for the Aminobenzofurazan in Various Solvents

Solvent	Absorption Maximum, nm	ϵ	Excitation Maximum, nm	Fluorescence Maximum, nm	Fluorescence Intensity[a]
Cyclohexane	453 325	21,400 8,000	465	490	55
Chloroform	472 338	25,200 8,000	470	510	85
Methanol	480 340	25,700 8,200	470	525	5

[a] Fluorescence intensities measured with an instrument range setting of 3- and 10-nm slits.

alternate solvent system consisting of chloroform–methanol–concentrated ammonia (95:5:3), where a single fluorescent zone at R_f 0.78 was detected.

Anal.—Calc. for $C_{27}H_{28}N_4O_3$: C, 71.03; H, 6.18; N, 12.27. Found: C, 70.68, 70.70; H, 6.20, 6.13; N, 12.06, 12.27.

Assay of I in Solid Samples—*7-Chloro-4-nitrobenzofurazan Solution*—Prepare a fresh 0.25% solution of the reagent in methanol daily.

Sodium Acetate Buffer—Dissolve 2.5 g of reagent grade sodium acetate in 50 ml alcohol USP.

Standard Preparation—Dissolve about 8 mg of I standard, accurately weighed, in 50.0 ml methanol. Make a 10:50 dilution with methanol.

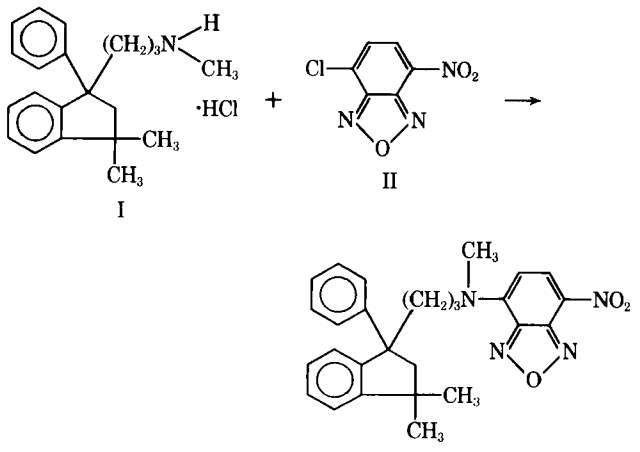
Sample Preparation—Carefully transfer the equivalent of about 8 mg of active, accurately weighed, into a 50-ml glass-stoppered centrifuge tube. Extract the solids with 2 × 25 ml methanol, shaking each time for about 10 min. Centrifuge and decant the supernatant liquid into a 50-ml volumetric flask, filtering through a small pledget of glass wool. Dilute to volume with methanol. Make a 10:50 dilution with methanol.

For those samples containing ascorbic acid or oxone, which interfere with the derivative formation, presumably by destroying the reagent, use the following extraction procedure. Transfer the equivalent of approximately 8 mg of I, accurately weighed, into a 125-ml separator containing 25 ml water and add 2.5 ml of 1 *N* sodium hydroxide. Extract for 2 min with 3 × 25 ml of chloroform. Wash the combined chloroform extracts with 10 ml water, filter through sodium sulfate into a 100-ml volumetric flask, and dilute to volume with chloroform. Withdraw 2.0-ml aliquots of each chloroform extract, transfer to a 15-ml glass-stoppered centrifuge tube, add 0.5 ml 0.1 *N* methanolic hydrochloric acid, and evaporate to dryness with the aid of a gentle stream of nitrogen. Redissolve the residue with 5.0 ml methanol.

Procedure—Pipet 1 ml of each sample and standard solution and 1 ml of methanol (to serve as a reagent blank) into separate, appropriately labeled, 10-ml volumetric flasks. To each in turn, add 0.5 ml sodium acetate buffer and 0.2 ml fluorogenic reagent. Mix, stopper well, and place in a 70° water bath for 45 min. Remove the flasks from the bath and pipet 1 ml acetic acid into each. Dilute to volume with methanol. Further dilute 3.0 ml of each sample, standard, and blank solution to 50.0 ml with chloroform. Determine the fluorescence of each sample (F_U), standard (F_S), and blank (F_B) preparation. Use a suitable sensitivity setting with an excitation wavelength of 470 nm, a fluorescence wavelength of 510 nm, and 10-nm slits. For the assay of the dosage form:

$$\text{milligrams active ingredient per capsule} = \frac{F_U - F_B}{F_S - F_B} \times \frac{A}{B} \times \frac{C}{4} \qquad \text{(Eq. 1)}$$

where F_U, F_S, and F_B are as defined previously; A is the standard weight in milligrams; B is the sample weight in milligrams; and C is the average net capsule weight in milligrams.



Scheme I

RESULTS AND DISCUSSION

Effect of Solvent on Spectral Characteristics of Benzofurazan Derivative—The wavelength of maximum absorption of the derivative is dependent on the polarity of the solvent. As the polarity of the solvent increases, the peak shifts to longer wavelength due to ionization of the nitro group, and the absorption increases. Since the absorption spectrum of the derivative showed a marked solvent effect, the fluorescence spectrum was checked for shifts in emission wavelength and intensity differences due to different solvents (Table I).

Linearity of Fluorescence with Concentration—The sensitivity of the reaction is illustrated by the data shown in Fig. 1, which relates fluorescence intensity to the concentration of I. A linear relationship was obtained over the concentration range of 0–50 μg of amine in the reaction mixture. After dilution, this corresponds to 0–0.3 μg of amine/ml of the final solution read. Each point on the curve represents the average fluorometric response of duplicate reaction mixtures. Readings were taken with a sensitivity range setting of the photometer of 10.

Optimum Reaction Conditions—Although the reaction employed for the isolation and characterization of the derivative involved the use of sodium hydroxide, the resulting solution pH was not suitable for quantitative purposes. At this pH, high blanks were observed, apparently due to decomposition of the reagent. It was also ascertained that the amine derivative was much less stable at this pH than when sodium acetate was utilized for pH control. The solution must be sufficiently alkaline to convert the amine hydrochloride to the free base since the salt gives little or no reaction, as indicated by the very low fluorescence response.

The optimum amounts of sodium acetate and fluorogenic reagent were arrived at experimentally by varying each of these two

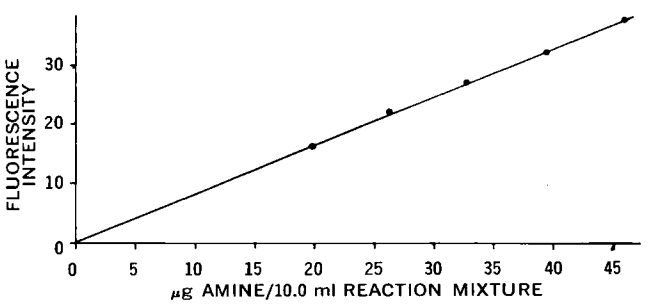


Figure 1—*Fluorescence intensity produced by the reaction of scalar amounts of amine with 7-chloro-4-nitrobenzofurazan in sodium acetate-buffered solution at 70° for 45 min.*

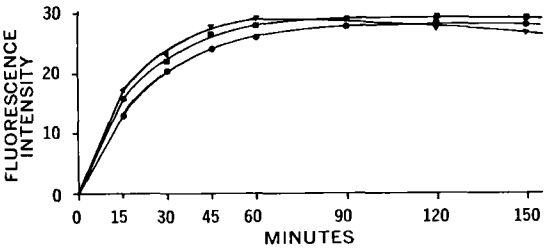


Figure 2—*Optimization of sodium acetate content. Fluorescence intensity was monitored up to 2.5 hr as a function of the amount of sodium acetate (5%): —▼—, 0.2 ml; —■—, 0.5 ml; and —●—, 1 ml. Concentration of amine = 30 μg in all cases.*

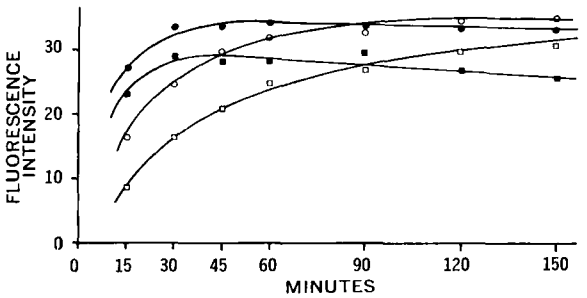


Figure 3—*Variation of fluorescence intensity as a function of the amount of reagent: —□—, 0.1 mg; —○—, 0.2 mg; —●—, 0.5 mg; and —■—, 1.0 mg. Amount of amine = 30 μg in all cases.*

variables until the maximum fluorescence was obtained. The readings obtained over a 2.5-hr reaction period, employing 0.2, 0.5, and 1.0 ml of a 5% sodium acetate solution, are plotted in Fig. 2. The use of 0.5 ml of buffer resulted in an apparent pH of 7.4 and was chosen as the optimum amount since it resulted in the highest and most reproducible response. Four simultaneous reaction mixtures, each containing 30 μg of amine and 0.5 ml sodium acetate solution but varying amounts of reagent, were reacted at 70° up to 2.5 hr. The fluorescence measured was plotted against time to produce the family of curves presented in Fig. 3. The reaction mixture containing 0.5 mg reagent produced the maximum response after approximately 30 min and remained relatively constant. Although virtually complete derivatization has occurred in the final procedure after 45 min at 70°, the addition of 1 ml of acetic acid was found to be a convenient means of halting the reaction. This simple expedient resulted in more reproducible readings.

Assay of I in Compatibility Samples—Weighed amounts of active ingredient were admixed with tartaric acid, sodium carbonate, oxone, and ascorbic acid. These additives were chosen as representative acid, base, oxidizing, and reducing agents to warn of potential solid-solid incompatibilities in the dosage form. The solids were triturated in a glass mortar in a 1:1 weight ratio and assayed. The results of these assays and recovery experiments performed without grinding are presented in Table II. Quantitative recovery of the amine was only obtained when the triturated samples were transferred with the aid of solvent since adsorption of the active ingredient on the glass surface apparently occurred.

Table II—Assay of Compatibility Samples

Additive	Percent Recovery of Active Ingredient
Tartaric acid	95.6, 93.5, 88.2, 88.4[a]
	100.1, 99.9, 99.9, 100.6[b]
	100.5, 99.0[c]
Sodium carbonate	93.8, 95.2[a]
	99.8, 101.7[c]
Lactose	89.7, 88.7, 87.4, 89.7[a]
	99.5, 98.6, 99.3, 99.1[b]
	98.7, 99.1[c]
Ascorbic acid	90.3, 91.4[a]
	98.2, 98.4, 99.6[b]
	101.2, 104.2[c]
Oxone	86.3, 88.2[a]
	98.3, 102.1[b]
	98.3, 99.8[c]

[a] Solids were triturated in a glass mortar and a weighed portion was assayed. [b] Solids were triturated in a glass mortar, and the entire sample was quantitatively transferred with solvent. [c] Recovery of known amounts of active ingredient mixed with additives without grinding, and the entire sample was quantitatively transferred with solvent.

Table III—Assay Values of Capsule Formulations

Declared, mg/capsule	100.0	25.0	5.0	1.0
Found, mg/capsule	101.9	24.1	5.0	0.99
	100.7	25.1	5.3	1.0
	99.3	24.5	5.1	1.0
	98.6	24.7	5.1	1.0
	100.4	24.5	5.0	
	99.0			
	98.2			
	100.0			
	99.6			
Average, mg/capsule	99.7	24.6	5.1	1.0
Relative standard deviation, %	1.2	1.5	2.4	0.5

The addition of a small amount of methanolic hydrochloric acid to the chloroform extracts of the ascorbic acid and oxone compatibility samples was employed to convert the free amine base extracted to the hydrochloride salt. This was necessary since the free base is somewhat volatile, and low recoveries even of extracted standards were obtained after evaporation of the chloroform to dryness.

Quantitation of Active Ingredient in Capsule Formulations—Four capsule formulations were prepared for dose finding studies at labeled concentrations of 100, 25, 5, and 1 mg active ingredient/capsule. The capsules were assayed *via* direct methanolic extraction (Table III). Pooling the results of all 23 capsule assays resulted in an average recovery of 99.9% with a relative standard deviation of 1.9%.

SUMMARY AND CONCLUSION

The reaction of I with 7-chloro-4-nitrobenzofurazan was carefully investigated and resulted in the isolation and characterization of the expected derivative. Upon optimizing the reaction conditions, whereby essentially complete derivatization takes place and a highly fluorescent species is formed, a sensitive reproducible analytical method was developed for the quantification of the active ingredient in a variety of solid mixtures.

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[x] To whom inquiries should be directed.

Relationship of Composition of Nonaqueous Binary Solvent Systems and Dielectric Constant

A. K. AMIRJAHED* and MARTIN I. BLAKE[x]

Abstract □ Five nonaqueous binary solvent systems are described. Each system is composed of a polar and a nonpolar component, both of which are inert and do not exhibit leveling effects. The dielectric constant of each system is related to the composition of the polar component expressed as mole fraction, percent weight by weight and volume by volume. Consequently, it is possible to prepare a solvent mixture with a specific dielectric constant within the range of values of dielectric constant provided by the system. The formulas relating dielectric constant to the particular expression of the composition of the polar component are reported.

Keyphrases □ Nonaqueous binary solvent systems—relationship between composition of polar component and dielectric constant, equation □ Dielectric constant of nonaqueous binary solvent system—relationship to composition of polar component, equation □ Solvent systems, nonaqueous binary—relationship between composition of polar component and dielectric constant, equation

The literature on nonaqueous titrimetry has expanded considerably over the last 3 decades. Solvents and their merits, solvent–solute interactions, titrants and their advantages, electrode systems, and different combinations of electrodes have been studied extensively, and these and similar topics were reviewed (1). The scope of nonaqueous solvent chemistry, the role of the solvent in chemical reactions, and related topics were discussed (2), and a thorough treatment of the electronic theory of acids and bases, acidic and basic radicals, neutralization, displacement, and acid and base catalysis was presented (3). Kolthoff and Bruckenstein (4) considered acid–base strength and equilibria in nonaqueous solutions.

Excellent reviews of the literature on nonaqueous titrimetry (5–8) and on its use in pharmacy (9) were presented. Theory, titrants and their standardization, solvents, and applications in general and in pharmaceutical analysis were covered (9). In all, wherever nonaqueous solvents are discussed the importance of the dielectric constant of the solvent is also emphasized and qualitative inferences are usually given. However, a rigid quantitative relationship is seldom indicated. In fact, in potentiometric titrations in nonaqueous media as well as any other scientific study where such media are employed and where the dielectric constant of the medium is of importance, a binary solvent system that is well defined in terms of the dielectric constant and the corresponding composition should be useful.

THEORETICAL

When assuming an ideal mixture of two solvents, one nonpolar (referred to as Solvent 1) and the other polar (referred to as Solvent 2) where there is no change in the volume of the two solvents upon mixing and the effect of electrical forces of surrounding molecules on each other is negligible, the following terms can be defined:

M_1 = gram molecular weight of Solvent 1
M_2 = gram molecular weight of Solvent 2
n_1 = moles of Solvent 1
n_2 = moles of Solvent 2
X_1 = mole fraction of Solvent 1 in the mixture of the two solvents
X_2 = mole fraction of Solvent 2 in the mixture of the two solvents
$\bar{V}_1$ = molar volume of pure Solvent 1 = M_1/ρ_1, in which ρ designates density
$\bar{V}_2$ = molar volume of pure Solvent 2 = M_2/ρ_2, in which ρ designates density
$\bar{P}_1$ = molar polarization of pure Solvent 1
$\bar{P}_2$ = molar polarization of pure Solvent 2
P_m = total molar polarization of solvent mixture
$P_m = X_1\bar{P}_1 + X_2\bar{P}_2$
M_m = weight of 1 mole of mixture
$M_m = (n_1M_1 + n_2M_2)/(n_1 + n_2) = [(n_1M_1)/(n_1 + n_2)] + [(n_2M_2)/(n_1 + n_2)]$
$M_m = X_1M_1 + X_2M_2$
V_m = volume of 1 mole of mixture
$V_m = (n_1\bar{V}_1 + n_2\bar{V}_2)/(n_1 + n_2) = [(n_1\bar{V}_1)/(n_1 + n_2)] + [(n_2\bar{V}_2)/(n_1 + n_2)]$
$V_m = X_1\bar{V}_1 + X_2\bar{V}_2$
$\rho_m = (X_1M_1 + X_2M_2)/(X_1\bar{V}_1 + X_2\bar{V}_2)$, with ρ_m = (weight of 1 mole of mixture)/(volume of 1 mole of mixture)

Debye (10) reasoned that $P_m = X_1\bar{P}_1 + X_2\bar{P}_2$ and $\bar{P}_1$ is constant. $\bar{P}_2$ is considered constant here based on the assumptions of ideality of the mixture of the two solvents; however, it is not constant in real mixtures.

For nonpolar liquids and dilute solutions of polar solutes in nonpolar solvents, Eq. 1 is available (11):

$$P_m = [(D - 1)/(D + 2)](M/\rho) \qquad \text{(Eq. 1)}$$

where P_m is the molar polarization. This quantity has the dimensions of volume and is really a molar polarizability. D is the dielectric constant, M is the gram molecular weight of the dielectric, and ρ is the density of the dielectric. The total molar polarization quantity, P_m, includes contributions from both the induced dipoles (induction effect) and the permanent dipoles (orientation