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# Development of a sensitive method for quantitation of ABT-089 in plasma using fluorescence labeling with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole

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**Abstract**

A high-performance liquid chromatography method for the quantitation of ABT-089 [2-methyl-3-(2-(*S*)-pyrrolidinylmethoxy)pyridine] (I), a new structural type of cholinergic channel modulators (ChCM), is described in this paper using 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as a fluorescent-labeling reagent. The method combined an optimized liquid–liquid extraction from plasma followed by pre-column derivatization to yield a fluorescence product. The selectivity, sensitivity, and reproducibility of this method were found to be excellent. This method was applied to the determination of ng/ml plasma and tissue levels of ABT-089 and similar compounds in biological samples. © 1997 Elsevier Science B.V.

*Keywords:* 2-Methyl-3-(2-(*S*)-pyrridinylmethoxy)pyridine

## 1. Introduction

Fluorometric analysis, in combination with high-performance liquid chromatography, is an important technique for the sensitive detection of primary and secondary amines in biological samples. Fluorogenic-labeling reagents such as NBD-F (7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) have been successfully used for this purpose [1–8].

ABT-089 (I) is a novel compound, recently synthesized as a cholinergic channel modulator [9]. This compound has shown excellent safety, with oral efficacy in a variety of preclinical models of cognitive enhancement, which has lead to its approval for clinical development [10]. Previously, compounds having similar activity but containing a *N*-methyl tetrahydropyrrole ring (tertiary amine) were quantified using electrochemical detection [11]. Compounds containing a *N*-demethylated tetrahydropyrrole ring (secondary amine) with an attached α-hetero aromatic ring (Type A compounds, N-C-Het) were determined by derivatization with NBD-F followed by HPLC with fluorescence detection [12]. Compound I has a similar *N*-demethylated tetrahydropyrrole ring but it differs in that it contains a β-aromatic oxygen (Type B, N–C–C–X). These two types of N–H compounds, as shown in Fig. 1, were characterized by different chemical characteristics and reaction properties during fluorescence derivatization with NBD-F.

This paper describes the development and optimization of a rapid and sensitive analytical method for

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A-82695 [II] ABT-089 [I]

Fig. 1. Comparison of Type A ChCM, II (N-C-Het), and Type B ChCM, I (N-C-C-X).

the determination of compound I in plasma and tissue samples obtained from pharmacokinetic studies. The differences of Type A and Type B compounds in reactivity and optimization conditions are also discussed.

## 2. Experimental

### 2.1. Materials

Compound I and related compounds were synthesized by D47W, Abbott Laboratories Neuroscience Discovery [9]. NBD-F (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) was purchased from Regis (Morton Grove, IL, USA). TMAP (tetramethylammonium perchlorate) was purchased from Sigma (St. Louis, MO, USA). TFA (trifluoroacetic acid), methanol, and acetonitrile were obtained from EM Sciences (Gibbstown, NJ, USA). All chemicals were of analytical-reagent grade unless otherwise noted. Solvents were of HPLC grade.

### 2.2. HPLC apparatus and conditions

The HPLC instrument consisted of two Model 400 isocratic pumps (Applied Biosystems, Foster City, CA, USA), a Model 491 high-pressure dynamic mixer (ABI), a Model 655A-40 autosampler (Hitachi, Tokyo, Japan) and a Model RF-551 fluorescence detector (Shimadzu Scientific, Columbia, MD, USA) using a VAX based data acquisition system (PeakPro, Beckman Instruments, Allendale, NJ, USA). All HPLC mobile phases were filtered and degassed before use. All chromatographic runs were made at ambient temperature using a flow-rate of 1 ml/min.

The NBD reaction products of compound I and the internal standard were separated from contaminants on a 10 cm×4.6 mm I.D., 5 μm YMC-basic column (YMC, Wilmington, NC, USA) with an acetonitrile–methanol–0.01 *M* TMAP–0.1% TFA (18:10:72, v/v) mobile phase at a flow-rate of 1.0 ml/min with fluorescence detection ($\lambda_{ex}$=495 nm, $\lambda_{em}$=533 nm). The chromatographic conditions and fluorescence detection wavelengths of the reaction products of other Type B ChCMs can be found in Table 1.

### 2.3. LC–MS–MS apparatus and conditions

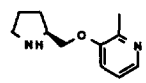
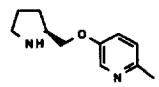
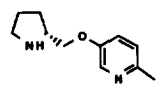
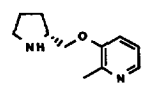
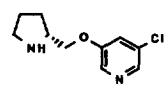
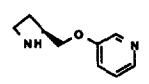
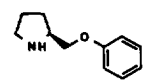
An API III$^+$ LC–MS–MS system (Perkin Elmer Sciex Instruments, Thornhill, Canada) was used to identify the reaction product of compound I with NBD-F. The I-NBD derivative was separated on a 5 cm×4.6 mm, 3 μm Spherisorb ODS-2 column (Regis, Morton Grove, IL, USA) with a 50% acetonitrile in 0.1% TFA mobile phase at a flow-rate of 1.0 ml/min using a Model 500D syringe pump (ISCO, Lincoln, NE, USA). A heated nebulizer (450°C, 70 psi) with an APCI (atmospheric pressure chemical ionization) source was used as the interface between the LC and MS–MS systems. This interface uses nebulizer and make-up gas (air) to atomize the HPLC effluent. The full scan APCI spectrum (Q1 scan) was performed to obtain the molecular ion. Under MS–MS conditions with 70-eV collision energy and argon as the collision gas, structurally informative CAD (collisionally activated dissociation) spectra (daughter scan) were generated.

### 2.4. Optimization studies

#### 2.4.1. Reaction temperature and time

A series of samples containing I, were subjected to identical extraction and derivatization conditions. Compound I was partitioned from an aliquot of plasma with 5.0 ml of ethyl acetate under alkaline conditions; the compound was removed from the organic phase through liquid–liquid extraction with 300 μl of 0.01 *M* HCl. The aqueous phase was alkalized with the addition of 50 μl of 0.5 *M* sodium phosphate buffer (pH 9.86) followed by reaction with 300 μl of 0.5 mg/ml NBD–F in ethanol. The reaction product was extracted again with 5.0 ml of ethyl acetate and dried at room temperature with a gentle stream of dry air. The reaction between compound I and NBD-F was investigated at room

Table 1
Chromatographic conditions and fluorescence maximum for the analysis of NBD derivatives of selected Type B cholinergic channel modulators

| Compound | Structure | Spectroscopic $EX_{max}$ | Spectroscopic $EM_{max}$ | Chromatographic Column | Chromatographic Mobile Phase |
|---|---|---|---|---|---|
| [I] |  | 490 | 533 | 100x4.6mm YMC-basic | 18:10:72* |
| A-87048 |  | 490 | 533 | 100x4.6mm YMC-basic | 18:10:72* |
| A-94219 |  | 488 | 540 | 100x4.6mm YMC-basic | 18:10:72* |
| A-94224 |  | 489 | 540 | 100x4.6mm YMC-basic | 18:10:72* |
| A-94220 |  | 486 | 534 | 100x4.6mm YMC-basic | 30:10:60* |
| A-85380 |  | 496 | 550 | 100x4.6mm YMC-basic | 18:10:72* |
| A-93761 |  | 488 | 531 | 50x4.6mm Spherisorb ODS2 | 50:50 ACN:$H_2O$ |

* Acetonitrile–MeOH–(0.01 *M* TMAP/0.1% TFA).

temperature, 40, 50 and 70°C at 2, 5, 10, 20, 30 and 60 min intervals.

### 2.4.2. pH

The derivatization of compound I with NBD-F was evaluated following the addition of variable volumes of 0.5 *M* sodium phosphate buffer (pH 9.86) using the same procedure described above.

### 2.4.3. Reagent concentrations

The effect of reagent concentration on the fluorescent response following derivatization was evaluated in a series of experiments in which the concentration of NBD-F was varied from 0.01 to 2 mg/ml. In each case, a constant 300-μl aliquot of freshly prepared NBD-F in ethanol was added to plasma extracts using the same procedure.

### 2.4.4. Extraction recovery

The effect of organic solvent on the extraction recovery of compound I from plasma and on the recovery of the I-NBD derivative from reaction mixture was evaluated in a separate series of experiments. The recoveries with ethyl acetate containing various fractions of hexane (0, 10 or 50%, v/v) were evaluated in detail. In each experiment, 5.0 ml of organic solvent was used as described above.

## 2.5. Recommended extraction and reaction procedure for plasma samples

The parent compound (and internal standard) were separated from the plasma matrix utilizing a two step liquid–liquid partitioning followed by reaction with NBD-F to yield a fluorescence product. Rat, dog and

monkey plasma samples or standards (1.0 ml for monkey and dog, 0.2 ml for rat) containing 100 μl of internal standard were combined with 500 μl of 0.5 *M* sodium carbonate and extracted with 5 ml of ethyl acetate–hexane (9:1, v/v). After vortexing for 30 s, followed by centrifugation for 10 min at 1819 *g*, the organic layer was transferred to a 5-ml screw-capped disposable centrifuge tube; the extracted aqueous plasma layer was discarded. Hydrochloric acid (0.01 *M*, 300 μl) was vortex-mixed with the organic fraction for 30 s, followed by centrifugation at 1819 *g* for 10 min. The organic layer was aspirated to waste; the pH of the aqueous layer was adjusted through the addition of a 100-μl aliquot of phosphate buffer (pH 9.86, 0.5 *M*). The freshly prepared NBD-F derivatization reagent (300 μl; 0.1 mg/ml in EtOH) was added and briefly mixed with each sample. The reaction mixture was heated at 50°C for 30 min followed by immersion in an ice-bath to quench the derivatization reaction. The reaction products were extracted from the derivatization mixture with 5.0 ml of ethyl acetate–hexane (1:1, v/v). After vortexing and centrifuging, the organic layer was transferred into a conical centrifuge tube and evaporated to dryness with a gentle stream of dry air at room temperature. The samples were reconstituted in 200 μl of MeOH–$H_2O$ (1:1, v/v) for HPLC analysis.

### 2.6. Preparation of brain tissue samples

Rat brain tissue was combined with two volumes of normal saline (0.9% NaCl in water) and homogenized thoroughly. An aliquot of the brain homogenate (200 μl) was extracted and derivatized using the method described for plasma samples above. Total hemoglobin present in the homogenate was determined using Drabkin's reagent [13].

### 2.7. Preparation of standards

The stock solution of compound I was prepared in HPLC-grade water at a concentration of 148 μg/ml; stock solution was refrigerated when not in use. This solution was diluted in water to yield a series of intermediate spiking solutions. An aliquot (100 μl) of these solutions was spiked into 1.0 ml of rat, dog or monkey plasma to obtain standards ranging from 5 to 100 ng/ml. The internal standard (A-93840) stock solution (137.62 μg/ml) in HPLC-grade water was diluted with water to obtain a 2 μg/ml working solution.

### 2.8. Validation study

The intra-day precision and accuracy of the method were evaluated by triplicate analysis of spiked dog plasma standards at each of three separate concentrations. The assay precision was based on the calculation of the relative standard deviation (R.S.D.). An indication of accuracy was based on the relative error of the samples, i.e. $[(F-T)/T]\times 100$, in which the deviation between the found concentration ($F$) and the theoretical concentration ($T$) was calculated. The inter-day precision for the plasma analysis was assessed from the results of intra-day assays on three separate days.

### 2.9. Data processing

The peak areas of the compounds of interest were obtained from the PeakPro data system. The concentration of parent drug in the plasma samples was calculated by least squares linear regression analysis (unweighted) of the peak area ratio (parent/internal standard) of the spiked plasma or brain tissue standards versus concentration.

### 2.10. Protocol for pharmacokinetic investigation

The test compound was prepared in normal saline at a slightly acidic pH (4.5–5) shortly before dosing. The experiments on monkeys and dogs were parallel studies with two groups of animals. One group of female cynomolgus monkeys or beagle dogs received a 500 nmol/kg intravenous dose while a second group received a 500 nmol/kg oral dose administered by gavage. Heparinized blood samples were withdrawn from each animal prior to dosing and at selected time points throughout the 8 h following drug administration. Plasma was separated from red cells by centrifugation (1819 *g* at 4°C) and frozen (−20°C) until analysis. A 62 μmol/kg oral dose of compound I was administered by gavage to male Sprague–Dawley rats. At selected time points after dosing, groups of three rats were euthanized with

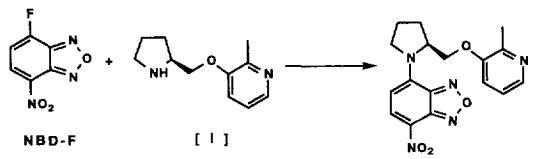



Fig. 2. Proposed reaction scheme for the derivatization of compound I with NBD-F.

$CO_2$ and exsanguinated by cardiac puncture. The brain tissue was removed from each animal for homogenization.

## 3. Results

The proposed derivatization reaction is shown in Fig. 2. The reaction conditions were shown to have a great effect on fluorescence response of the product [12]. A series of experiments was performed to optimize reaction conditions to obtain the highest detection response with the least degradation of reaction product during sample processing.

The effect of reaction temperature and time on the I+NBD-F derivatization was evaluated at room temperature, 40°C, 50°C and 70°C for 2, 5, 10, 20, 30 and 60 min utilizing fixed extraction conditions for sample processing. Both the reaction temperature and the time samples held at elevated temperature were found to contribute to the yield of the fluorescent product (see Fig. 3). The peak areas increased with the increase in reaction time, but response was limited by decomposition at extended time intervals. At elevated temperatures such as 40°C and 50°C, optimized peak area was obtained after a 30-min reaction time. The response of the derivatives obtained at 50°C was larger than those obtained at either 40°C and 70°C. A 30-min reaction time at 50°C was selected for the optimum yield for the derivatization of compound I with NBD-F.

The pH of the reaction mixture was shown to be crucial for the product formation [12]. The effect of

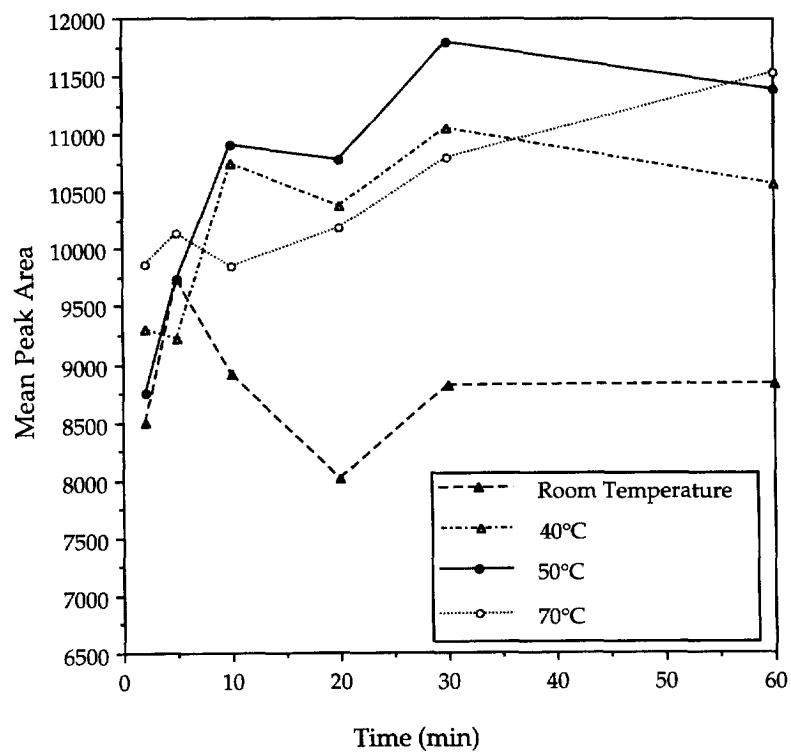



Fig. 3. Effect of reaction temperature and time on the formation of compound I-NBD derivative.

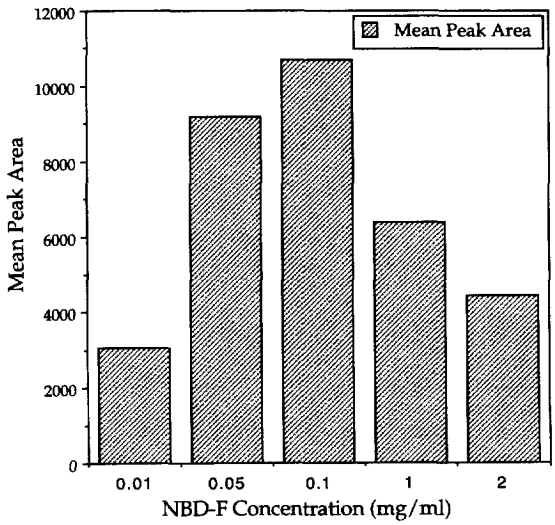



Fig. 4. Effects of reagent concentration on the formation of compound I-NBD derivative. Extraction and reaction procedures as described in Section 2.5.

different amounts of phosphate buffer was investigated by adding 100 μl and 500 μl of 0.5 *M* phosphate buffer (pH 9.86) to the 0.1 *M* HCl solution derived from the extracted plasma sample. An aliquot of 100 μl phosphate buffer (pH 9.86; 0.5 *M*) was sufficient for the adjustment of the hydrochloric acid to the required alkaline conditions for optimum derivatization.

The effect of reagent concentration (0.01, 0.05, 0.1, 1 and 2 mg/ml of NBD–F in ethanol) on the fluorescence yield was evaluated using a fixed-sample preparation procedure. The fluorescent response increased with increasing concentrations of reagent over the range of 0.01–0.1 mg/ml (see Fig. 4). Further increases in reagent concentrations (1 and 2 mg/ml of NBD–F in ethanol) resulted in a decrease in the fluorescence response. A reagent concentration of 0.1 mg/ml was selected for use in the analysis of plasma samples.

The recovery of the compounds of interest from plasma and recovery of the derivatized reaction product from the reaction mixture during the two separate extraction steps were influenced by the nature of the organic solvent (see Table 2). Solvent mixtures containing different fractions of hexane in ethyl acetate were used to extract compound I and internal standard from plasma and the NBD derivatives from the reaction mixture. The fluorescence response of the final derivative increased with decreasing fractions of hexane in the ethyl acetate to a maximum response using 10% hexane in ethyl acetate. The NBD derivatives are more lipophilic which translated into better recovery from a less polar solvent such as 50% ethyl acetate in hexane (see Table 2).

## 4. Discussion

We have previously reported on the derivatization of A-82695 (compound II) (Type A compound, N-C-Het) with NBD-F [12]. In the present study, an application of the NBD-F fluorescence-labeling reagent in the analysis of compound I (Type B, N-C-C-X) in biological fluids is demonstrated and optimized. This method has been used for the quantitation of Type B ChCM compounds in plasma and brain homogenate, providing high sensitivity, selectivity and reproducibility.

Table 2
Effect of extraction solvent on the recovery of the parent compound from plasma and on the recovery of the NBD derivative from the reaction mixture

| Solvent conditions[a] (plasma extraction/reaction product extraction) | Mean peak area | |
|---|---|---|
| | ABT-089 | S.D. (%) |
| 1:1EtOAc–hexane/1:1EtOAc–hexane | 7448.79 | 7.21 |
| 1:1EtOAc–hexane/9:1EtOAc–hexane | 8220.73 | 8.65 |
| 9:1EtOAc–hexane/9:1EtOAc–hexane | 9786.07 | 18.80 |
| 9:1EtOAc–hexane/1:1EtOAc–hexane | 11994.64 | 10.68 |
| EtOAc/EtOAc | 9730.19 | 18.45 |

[a] Mixture of ethyl acetate (EtOAc) and hexane are expressed by volume mean and standard deviation derived from triplicate analysis.

Representative chromatograms obtained from compound I and internal standard NBD derivatives in both plasma and brain homogenate are provided in Figs. 5 and 6, respectively. No endogenous contaminants interfered with the detection of the target derivative in either plasma samples or brain homogenate, indicating adequate assay specificity.

LC–MS–MS was performed for the I-NBD reaction product to elucidate the chemical structure of the derivative. The I-NBD derivative provided a $MH^+$ of 356 in APCI which corresponded to the expected molecular mass of 355. Its daughter spectrum (see Fig. 7) gave several characteristic fragments for the structural identification. This provided confirmatory evidence for the chemical identity of the proposed I-NBD derivative.

All NBD-F derivatives of Type B compounds exhibited similar excitation and emission maxima at wavelength in the ranges of 486–496 nm and 531–550 nm, respectively (Table 1). No significant differences were observed in the rates of derivatization or decay among the compounds evaluated in this structurally similar class. The chromatographic conditions were similar for all but one compound as shown in Table 1. The optimal response for the derivatization reaction was obtained when the samples were heated for 30 min at 50°C, at the relatively low reagent concentration of NBD-F (0.1 mg/ml) added to the reaction mixture in 300-μl aliquots. The utilization of 90% ethyl acetate in hexane provided maximal recovery for the parent from plasma and additional extraction of the NBD derivative with 50% ethyl acetate in hexane provided an improvement in the signal-to-noise ratio.

The assay for the quantitation of Type B compounds in plasma samples employing liquid–liquid extraction followed by derivatization with NBD-F provided excellent linearity and reproducibility. Type B compounds provided correlation coefficient >0.999 for all assays over the concentration range of 0–532 ng/ml. The R.S.D.s for the analysis of triplicate samples at 100.51, 40.21 and 9.65 ng/ml averaged 2.07, 2.07 and 1.40%, respectively, with relative error (accuracy) ranging from −1.74 to 12.79% of the theory. The mean inter-day precision, as evaluated from triplicate analysis of spiked standards on three separate days, averaged 6.8, 2.5 and 4.5% (R.S.D.) at concentrations of 100.51, 40.21 and

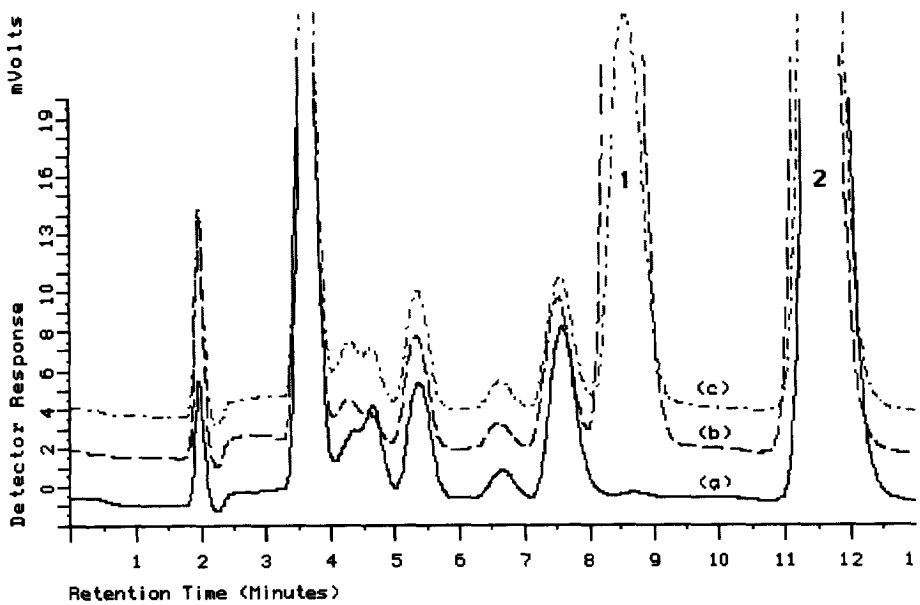



Fig. 5. Representative chromatograms obtained from precolumn derivatization with NBD-F. Peak 1 is the compound I-NBD derivative and peak 2 is A-93840-NBD derivative (internal standard). Chromatographic conditions described in Section 2.2. (a) Blank plasma containing internal standard, (b) spiked plasma standard, and (c) dog plasma sample.

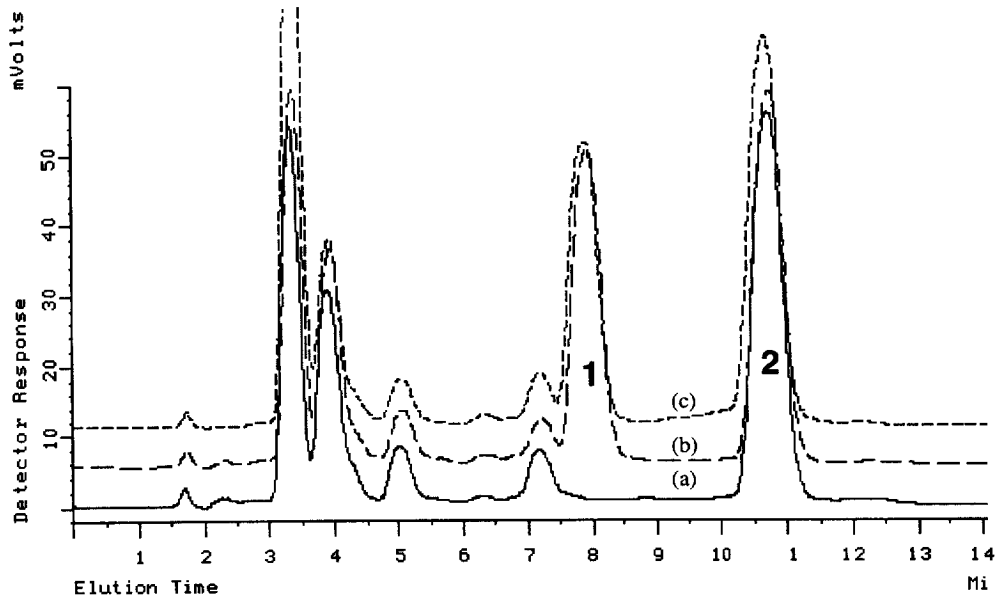



Fig. 6. Representative chromatograms obtained from precolumn derivatization of compound I in brain homogenate with NBD-F. Peak 1 is the compound I-NBD derivative and peak 2 is A-93840-NBD derivative (internal standard). (a) Blank brain homogenate containing internal standard, (b) spiked brain homogenate standard, and (c) unknown rat brain homogenate sample.

9.65 ng/ml, respectively (see Table 3). The standard deviation derived from slopes of the calibration graphs over three different days was 1.86% (see Table 4). The lower limit of quantitation was defined as 0.1 ng/ml following the extraction of a 1.0 ml plasma sample by actual derivatization at the minimum sensitivity settings on the fluorescence detector (gain=1); additional sensitivity may be obtained by increasing the gain. The high selectivity and excellent sensitivity for ChCMs in biological samples

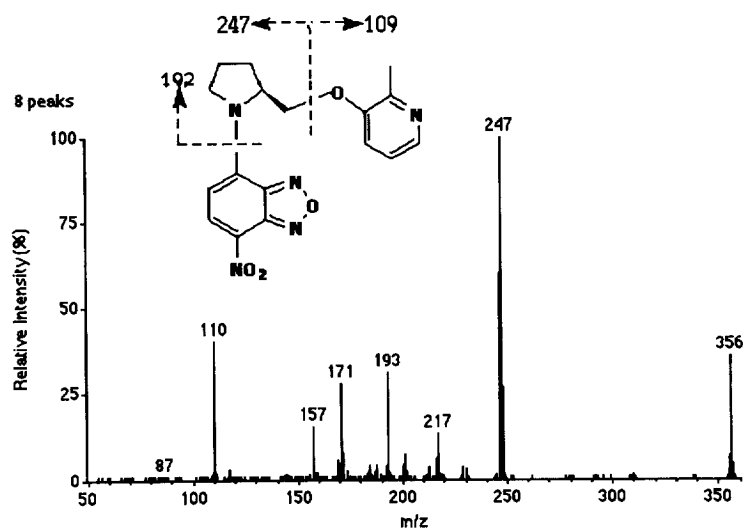



Fig. 7. Mass spectrum of the compound I-NBD derivative: daughter ion scan of 356 ($MH^+$).

Table 3
Reproducibility and accuracy of the NBD-F derivatization method for the quantitation of compound I in dog plasma

| Intra-day variability | | | | | Inter-day variability | | | |
|---|---|---|---|---|---|---|---|---|
| Concentration | | S.D. (ng/ml) | Precision (% S.D.) | Accuracy (% Diff)[a] | Day | Mean concentration[b] (ng/ml) | Grand mean (ng/ml) | R.S.D. (%) |
| Spiked | Found | | | | | | | |
| 100.51 | 87.66 | | | 12.79 | 1 | 89.12 | | |
| | 91.20 | 1.85 | 2.07 | 9.27 | 2 | 101.01 | 99.65 | 6.8 |
| | 88.50 | | | 11.96 | 3 | 99.81 | | |
| 40.21 | 39.48 | | | 1.81 | 1 | 39.96 | | |
| | 39.48 | 0.83 | 2.07 | 1.82 | 2 | 41.23 | 40.14 | 2.5 |
| | 40.91 | | | −1.74 | 3 | 39.80 | | |
| 9.65 | 8.70 | | | 9.82 | 1 | 8.80 | | |
| | 8.94 | 0.12 | 1.40 | 7.42 | 2 | 9.58 | 9.12 | 4.5 |
| | 8.75 | | | 9.35 | 3 | 8.98 | | |

[a] % difference=[(found conc.−spiked conc.)/spiked conc.]×100%.
[b] Mean of triplicate determinations on each day.

makes this assay useful for the pharmacokinetic evaluation of these compounds.

Some interesting and significant differences in both the reactivity during derivatization and in the subsequent stability of the reaction product were found for the derivatization of compound I (Type B ChCM) and II (Type A ChCM). The yield of fluorescent product derived from the Type A compounds+NBD-F was greatest with only a 5-min reaction at 50°C. Type B compounds required a longer reaction time, with maximum yield following 30 min at the same temperature. This phenomena indicated that Type A compounds not only reacted faster but also decayed faster than the Type B compounds. Higher concentrations (0.5 mg/ml) of reagent were also needed for Type A compounds versus the Type B compounds (0.1 mg/ml). Another difference of these two series of compounds was their polarity. Type A compounds were more polar than the Type B compounds. Therefore, Type A compounds and their reaction products were extracted with more polar solvents such as ethyl acetate. The highest recovery of Type B compounds was obtained with 90% ethyl acetate in hexane for the plasma extraction and with 50% ethyl acetate in hexane to remove the NBD derivatives from the reaction mixture. The two types of compounds also provided different limits of quantitation due to differences in reactivity and product fluorescence intensity. Type A compounds have better sensitivity (about 0.01 ng/ml) than Type B compounds (about 0.1 ng/ml) using the optimized derivatization and detection parameters for each series.

### 4.1. Application to pharmacokinetic studies

The derivatization method was successfully applied to the pharmacokinetic evaluation of selected Type B cholinergic channel modulators following a single intravenous or oral dose in Sprague–Dawley derived rats, cynomolgus monkeys and beagle dogs. The plasma concentration profile of compound I following a 500 nmol/kg intravenous dose in dog, as measured using the fluorescence response of the

Table 4
Summary of calibration curves derived from the analysis of I-NBD derivatives in dog plasma

| Day | Intra-assay R.S.D. (%) | Slope | $r^2$ | Inter-assay | |
|---|---|---|---|---|---|
| | | | | Mean slope | S.D. (%) |
| 1 | 1.37 | $1.2241\times10^{-2}$ | 1.000 | | |
| 2 | 1.40 | $1.2657\times10^{-2}$ | 1.000 | $1.24\times10^{-2}$ | 1.86 |
| 3 | 2.60 | $1.2275\times10^{-2}$ | 1.000 | | |

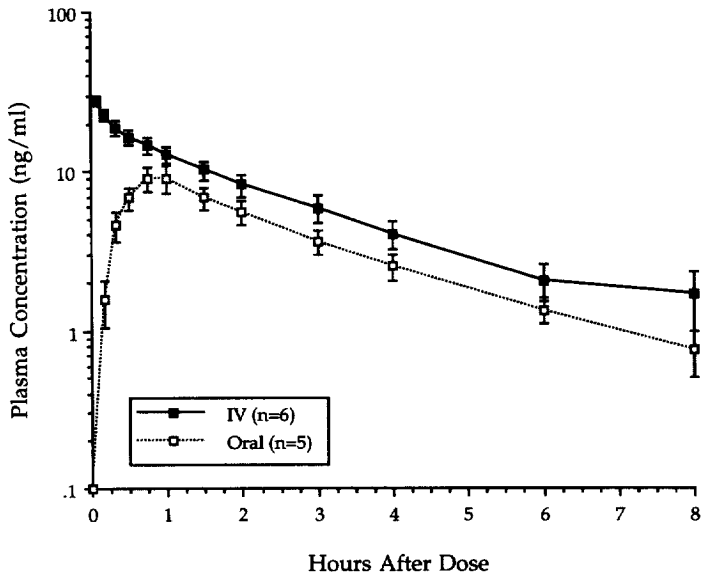



Fig. 8. Mean (±S.E.M.) plasma concentrations of compound I after a 500 nmol/kg intravenous or oral dose in dogs.

NBD derivative, is provided in Fig. 8. Plasma concentrations, 6 min after the bolus intravenous dose, averaged ~30 ng/ml, declining with a half-life of 2 h. Parent compound was rapidly absorbed after oral dosing, with peak concentrations ($C_{max}$=13.0 ng/ml) recorded in the first hour. Brain concentrations of compound I increased more slowly than those in the plasma following a single oral dose in rat (see Fig. 9), declining with an elimination half-life of 1.5 h. The high degree of sensitivity attained by this assay permitted pharmacokinetic profiling at therapeutically relevant doses.

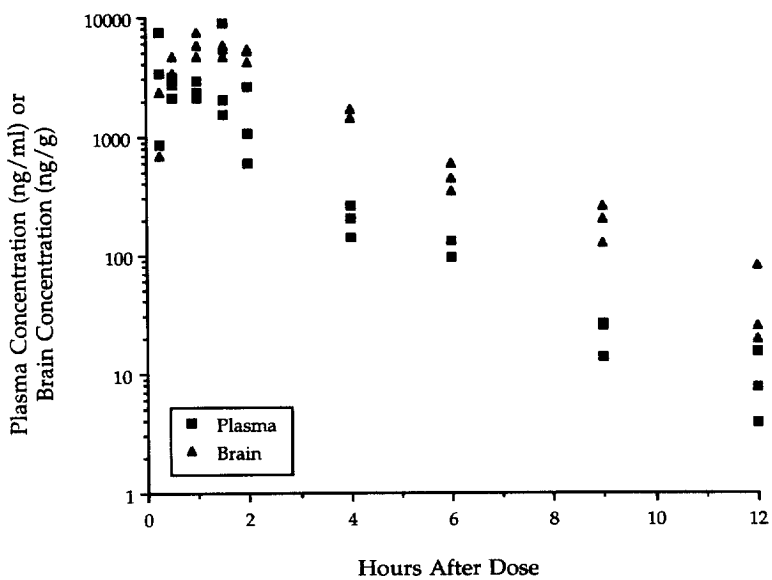



Fig. 9. Plasma and brain concentrations of compound I following a 62 μmol/kg oral dose in rat.

## 5. Conclusions

A highly sensitive, specific and rapid fluorometric HPLC assay has been developed to quantitate Type B cholinergic channel modulators (see Table 1) in rat, dog and monkey plasma and in rat brain tissue. The method utilizes a two step liquid–liquid extraction and derivatization procedure to remove essentially all plasma and tissue interferences. The sensitivity of the assay is about 0.1 ng/ml, which is sufficient for the pharmacokinetic characterization of Type B cholinergic channel modulators in Sprague–Dawley rats, cynomolgus monkeys and beagle dogs.

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