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Sensitive detection of selected cholinergic channel activators derivatized with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole ☆

Yu-hua Hui *, Kennan C. Marsh

Abbott Laboratories, D-46W, AP9, 100 Abbott Park Rd., Abbott Park, IL 60064, USA

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Abstract

A high-performance liquid chromatographic method with fluorescence detection was developed for a series of cholinergic channel activators using 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as a fluorescent labeling reagent. This method includes three separate steps: extraction of parent compound and internal standard with organic solvent from plasma, reaction of parent and internal standard in NBD-F precolumn to yield a fluorescent product and extraction of the resultant fluorophores with organic solvents. The extraction and reaction procedures were optimized for this series of structurally new compounds. The method showed high sensitivity, selectivity and reproducibility and proved useful for the determination of ng ml^{-1} plasma levels of selected cholinergic channel activators.

Keywords: Cholinergic channel activators; Reversed-phase high-performance liquid chromatography; Fluorescence detection; NBD-F derivatization; Plasma concentrations

1. Introduction

A series of new compounds, known as cholinergic channel activators (CChA), which may prove useful in the treatment of Alzheimer's disease are under evaluation at Abbott Laboratories [1,2]. From an analytical perspective, these compounds may be divided into two distinct groups. The first contains an *N*-methyltetrahydropyrrole ring (tertiary amine) that is amenable to electrochemical detection [3]; these compounds are not considered in this paper. The second contains an *N*-demethylated tetrahydropyrrole ring (secondary amine). A-82695 and its enantiomer, A-79814, are examples in this series. The high potency, in combination with low dosages, requires that analytical methods for the quantitation of the parent drug in plasma samples be developed with low ng ml^{-1} sensitivity. The compounds do not contain the requisite UV chromophore for sensitive detection,

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* Corresponding author.

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nor do they have endogenous electrochemical activity. However, this series of compounds contains a secondary amine functional group which was accessible for derivatization, resulting in products with high molar absorptivity and/or fluorescence with the requisite sensitivity.

7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was introduced as a fluorescent labeling reagent for amines by Imai and Watanabe in 1981 [4]. NBD-F reacts readily in aqueous solution with primary and secondary amines to yield highly fluorescent products; NBD-F is superior to NBD-Cl and NBD-Br in reactivity [4]. This reagent has been used to determine amino acids [5–12], proteins and peptides [13], forensic fingerprints [14] and biologically important amines [15]. Fluorogenic reagents having a benzofurazan structure in liquid chromatography have been reviewed [16].

The purpose of this investigation was to develop a rapid and sensitive method for the quantitation of selected *N*-demethylated cholinergic channel activators in plasma. This paper discussed the optimization of derivatization conditions for A-82695, using NBD-F as a derivatizing reagent for sensitive HPLC detection, with the application of this method to the quantitation of the parent drug in plasma samples derived from pharmacokinetic studies in dog and monkey.

2. Experimental

2.1. Materials

NBD-F was purchased from Regis Chemical (Morton Grove, IL, USA). Trifluoroacetic acid (TFA), methanol and acetonitrile were obtained from EM Sciences (Gibbstown, NJ, USA). All other chemicals were of analytical-reagent grade and all solvents were of HPLC grade. Solutions of NBD-F in ethanol were prepared just prior to use [17].

2.2. HPLC apparatus and conditions

The HPLC system 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 Instruments, Chicago, IL, USA) and a Model RE-551 fluorescence detector (Shimadzu Scientific, Columbia, MD, USA) with a PeakPro data collection system (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^{-1}.

The reaction products of the title compounds were separated from contaminants on a 5 cm × 4.6 mm i.d. 3 μm Spherisorb ODS-2 column (Regis Chemical) with acetonitrile (30:70, v/v) water as the mobile phase at a flow rate of 1.0 ml min^{-1} with fluroescence detection of the analytes (λ_{ex} = 480–487 nm, λ_{em} = 529–530 nm).

2.3. LC–MS–MS apparatus conditions

An API III$^+$ LC–MS–MS system from Perkin-Elmer Sciex Instruments (Ontario, Canada) was used to identify the reaction product of NBD-F with A-82695. The A-82695-NBD derivative was separated from the plasma contaminants on a 5 cm × 4.6 mm i.d. 3 μm Spherisorb ODS-2 column with an acetonitrile–water (30:70, v/v), as the mobile phase at a flow rate of 1.0 ml min^{-1} 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 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 measured 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

Effects of reaction temperature and time

To evaluate the effects of reaction temeprature and reaction time on the formation of the NBD derivative, a series of samples containing A-82695

and A-83030 (internal standard) were subjected to identical extraction procedures. The compounds were partitioned from an aliquot of plasma with 5.0 ml of ethyl acetate–hexane (1:1, v/v) under alkaline conditions; the compounds were removed from the organic phase through liquid–liquid extraction with 300 µl of 0.01 N HCl. The aqueous phase was alkalinized with the addition of 10 µl of 0.5 M sodium carbonate followed by reaction with 200 µl of 0.5 mg ml^{-1} NBD-F in ethanol. The reaction conditions with NBD-F were investigated at temperatures of 40, 50 and 70 °C at 2, 5, 10, 20, 30, 40 and 60 min intervals.

Effect of pH and/or different alkaline conditions

The derivatization of the cholinergic channel activators with NBD-F was also evaluated as a function of pH and buffer strength. In a series of experiments, the aqueous solution containing the compounds of interest (in 0.01 N HCl) were adjusted by the addition of different concentrations (0.1–0.5 M) and amounts (10–500 µl) of sodium carbonate; a second study evaluated the effect of addition of variable volumes of 0.5 M phosphate buffer (pH 9.86).

Effect of reagent concentration

The effect of reagent concentrations on the fluorescent response following derivatization was evaluated in a series of experiments in which the concentration of the NBD-F was varied from 0.01 to 1 mg ml^{-1}. In each case, a constant 300 µl volume of freshly prepared NBD-F in ethanol was added to plasma extracts prepared using the standard sample procedure described above.

Effect of extraction solvents on extraction recovery

The effect of organic solvent on the extraction recovery of the cholinergic channel activators from plasma and on the recovery of the CChA–NBD derivative from the 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 greatest detail. The standard sample procedure for extraction/derivatization employing 5 ml aliquots of the organic solvent under evaluation was utilized as described above.

2.5. Recommended extraction and reaction procedure

The parent compounds were separated from the plasma matrix utilizing a two-step liquid–liquid partitioning followed by reaction with NBD-F precolumn to yield a fluorescence product. The conditions for the optimized procedure for quantitation in plasma were as follows. Dog and monkey plasma samples or standards (1.0 ml) containing 100 µl of internal standard were combined with 500 µl of (0.5 M) sodium carbonate and extracted with 5.0 ml of ethyl acetate. After vortex mixing for 30 s followed by centrifugation for 10 min at 1819*g*, the organic layer was transferred in to a 5 ml screw-capped disposable centrifuge tube; the extracted aqueous plasma layer was discarded. Hydrochloric acid (0.01 N; 300 µl) was vortex mixed with the ethyl acetate fraction for 30 s followed by centrifugation at 1918*g* for 10 min. The organic layer was aspirated to waste; the pH of the aqueous layer was adjusted through the addition of a 50 µl aliquot of phosphate buffer (pH 9.86; 0.5 M). The NBD-F derivatization reagent (300 µl; 0.5 mg ml^{-1} in ethanol) was added and briefly mixed with each sample. The reaction mixture was heated at 50 °C for 5 min followed by immersion in an ice-bath to quench the derivatization reaction. The reaction products were extracted from the derivatization mixture with 5 ml of ethyl acetate. After vortex mixing 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 methanol–water (1:1, v/v) for injection (100 µl) into the HPLC system.

2.6. Preparation of standards

Stock solutions of A-82695, A-79814 and A-83030 (as hydrochloride salts, synthesized by D47W, Abbott Neuroscience Discovery) were prepared by dissolving the compounds in HPLC-grade water at concentrations of ~ 100 µg ml^{-1}; stock solutions were refrigerated when not in use. Calibration solutions were prepared by spiking 1.0 ml of drug-free dog or monkey plasma with

100 µl of intermediate stock solutions to achieve final concentrations of about 1, 10, 100, 300 and 500 ng ml^{-1}. An internal standard working solution of 2.22 µg ml^{-1} A-83030 was prepared by appropriate dilution of the stock standard solution with HPLC-grade water.

2.7. Validation study

The intra-day precision and accuracy of the method were evaluated by triplicate analyses of spiked dog plasma standards of each of three separate concentrations. The assay precision was based on the calculation of the relative standard deviation (RSD). 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.

2.8. Data processing

The peak area of the compounds of interest were derived 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 standards versus concentration.

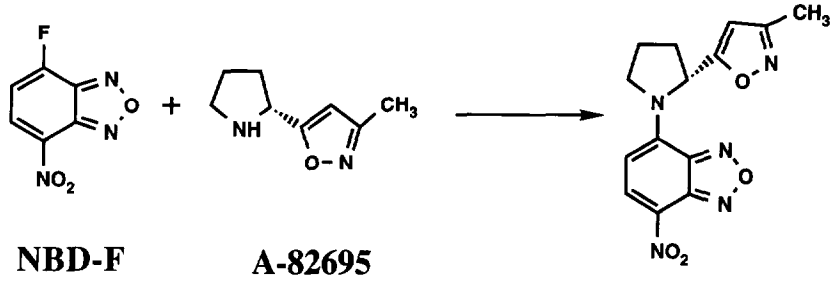
2.9. Protocol for pharmacokinetic investigation

Compounds were 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, each containing three animals. One group of dogs or monkeys received a 100 nmol kg^{-1} intravenous dose while a second group of animals in each species received a 200 nmol kg^{-1} oral dose administered by gavage. Heparinized blood samples were withdrawn from each animal prior to dosing and at selected time points throughout the 6 h following drug administration. Plasma was separated from the red cells by centrifugation (1819g at 4 °C) and frozen (−20 °C) until analysis.

3. Results

The sample preparation for the quantitation of selected cholinergic channel activators in plasma samples requires three separate, individually optimized steps: (1) the selective extraction of the compounds from plasma, (2) the reaction of compounds with NBD-F to yield fluorescent analytes and (3) optimization of the HPLC signal-to-noise response. The general aim of the optimization experiments was to achieve the best possible compromise between a rapid reaction time and high fluorescence intensity versus degradation of the fluorophore during sample processing. The proposed scheme for the derivatization is detailed in Fig. 1.

The effect of temperature (40, 50 or 70 °C) and heating time (2, 5, 10, 20, 30, 40 or 60 min) on the reaction of the cholinergic channel activators with NBD-F was evaluated in a series of experiments which utilized fixed extraction conditions for sample processing. Both the temperature of the reac-



NBD-F A-82695

Fig. 1. Proposed reaction between the cholinergic channel activator A-82695 and NBD-F.

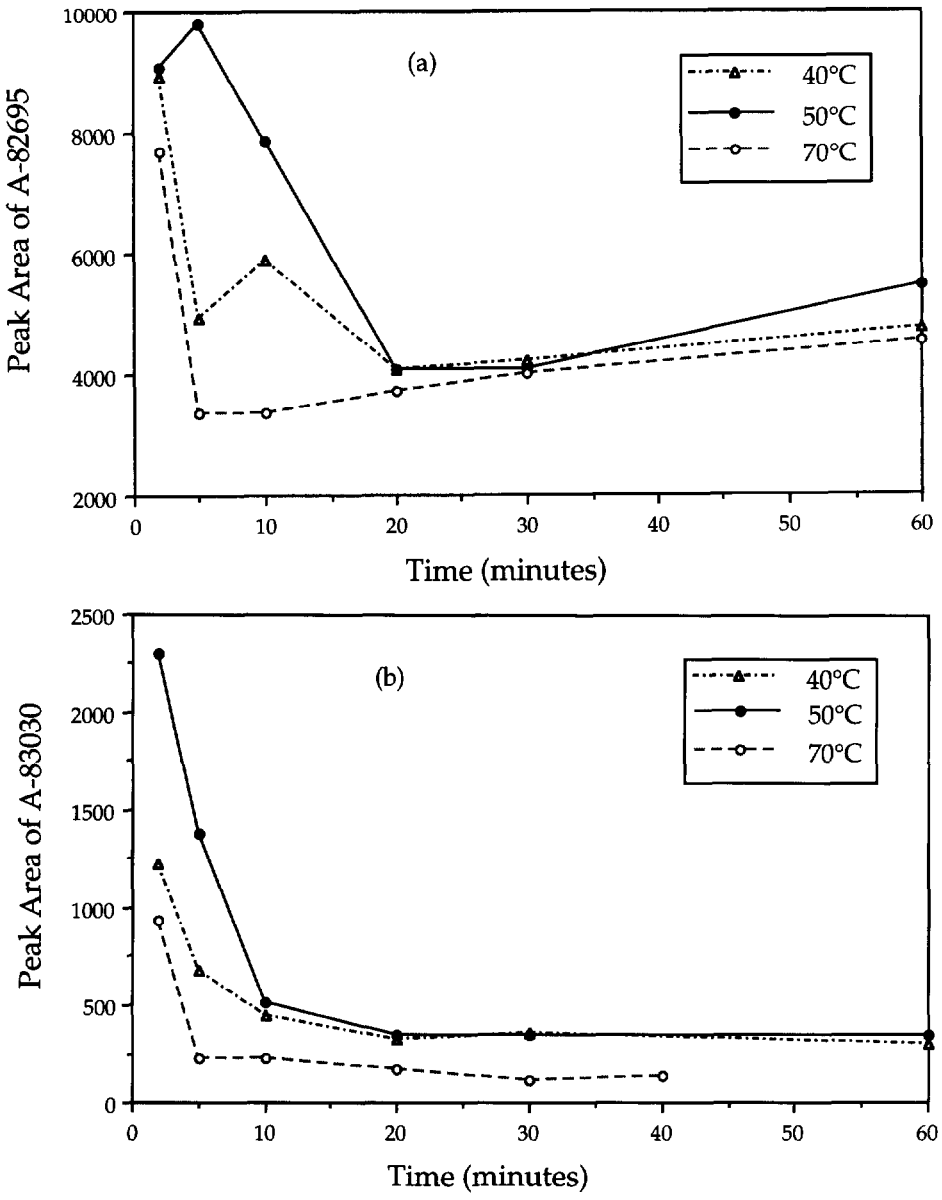


Fig. 2. Effect of reaction temperature and reaction time on the formation of (a) A-82695–NBD derivative or (b) A-83030–NBD fluorescent derivative.

tion and the time the samples were held at elevated temperature were found to contribute to the yield of the fluorescent products (see Fig. 2). A higher yield of the reaction product was obtained at elevated temperature, but the peak area decreased with increases in the length of time the samples were held at the high temperature. The decrease in fluroescence response was attributed to degradation of reaction products at elevated temperature. The peak areas of the derivatives obtained at 50 °C were larger than those obtained at either 40 or 70 °C. The reaction was completed in 5 min. Longer reaction times will only cause more degradation of the reaction product. A

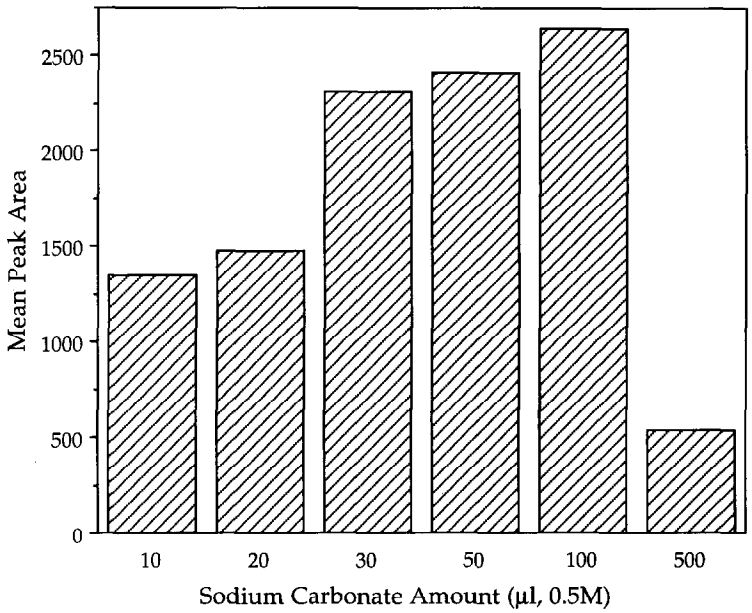


Fig. 3. Effect of pH (adjusted with different amounts of 0.5 M sodium carbonate) on the reaction of A-82695 with NBD-F. Extraction and reaction procedures as described in the text.

5 min reaction time at 50 °C was selected for the optimum yield for the derivatization of both A-82695 and the internal standard, A-83030.

According to the literature, the reaction of NBD-F with secondary amines is influenced by the pH of the solution [4]. A series of experiments designed to evaluate the effect of pH on the reaction of the cholinergic channel activators with NBD-F demonstrated an increase in fluorescence with an increase of sodium carbonate concentration (0.1–0.5 M) when added to the reaction mixture in 50–100 µl increments. Larger amounts (500 µl) of sodium carbonate, however, caused a sharp decrease in the peak area of the fluorescent product (see Fig. 3). Aliquots of phosphate buffer (pH 9.86; 0.5 M) provided the required pH adjustment with the addition of buffering capacity to the sample (data not shown). An aliquot (50 µl) of phosphate buffer (pH 9.86; 0.5 M) was therefore utilized for the adjustment of the hydrochloric acid to the required alkaline conditions for optimum derivatization.

A third series of experiments designed to evaluate the effect of reagent concentration on the fluorescent yield demonstrated on increased response with increasing concentrations of reagent over the 0.01–0.5 mg ml^{-1} concentration range (see Fig. 4). No additional increases in response were noted at higher concentrations (1 mg ml^{-1}) of NBD-F. Owing to the relatively high cost of the derivatizing reagent, a 0.5 mg ml^{-1} concentrations was selected for use in validation studies.

The recoveries of both the parent compound and the derivatized product during the two separate extraction steps were each individually influenced by the nature of the organic extraction solvent (see Table 1). The recovery, as measured by the fluorescent response at the end of the derivatization, increased with increasing polarity of the extraction solvent. The highest response was achieved through the use of ethyl acetate as the extraction solvent in each of the two partitioning steps. A-79814, a structural analog of A-82695, was extracted and derivatized under conditions indentical with those used for A-82695 and A-83030.

4. Discussion

NBD-F has been developed as a fluorogenic reagent for amino acids, peptides, proteins and

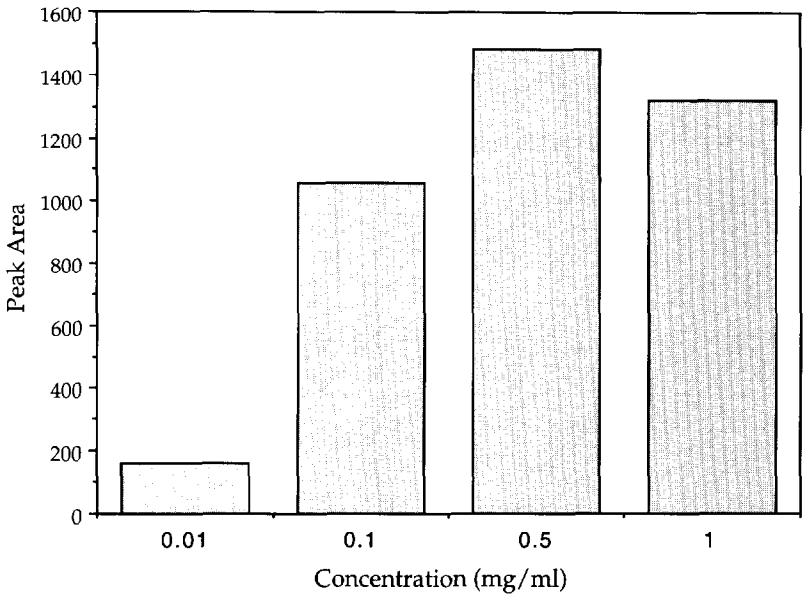


Fig. 4. Effect of NBD-F concentrations on the formation of the A-82695–NBD reaction product. Extraction and reaction procedures as described in the text.

biological amines. To date, no reports have been found on its application in the pharmaceutical industry. In the present study, optimization and application of this derivatization method for the analysis of a series of new pharmaceutically important compounds in biological fluid were demonstrated. Advantages of this method in the determination of plasma concentrations of selected cholinergic channel activators are the high sensitivity and selectivity and excellent reproducibility.

Preliminary studies with selected cholinergic channel activators compared the fluorescence response obtained from derivatization with dansyl chloride with that obtained with NBD-F (see Fig. 5). The advantages of the NBD-F derivatization reaction are readily apparent from the comparison of these two chromatograms. The NBD-F derivatization reaction provided a superior signal-to-noise ratio for the compounds of interest. Additional advantages were noted in the selectivity of the reaction, as evidenced by the cleanliness of the chromatograms. Additionally, no endogeneous contaminants interfered with the detection of the targeted NBD derivatives in plasma sam-

Table 1
Effect of extraction solvents 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/product extraction)	A-82695-NBD		A-83030-NBD	
	Mean peak area	SD (%)	Mean peak area	SD (%)
EtOAc–hexane (1:1)/EtOAc–hexane (1:1)	3578.17	7.4	215.09	8.1
EtOAc–hexane (1:1)/EtOAc–hexane (9:1)	9066.04	0.6	2299.14	20.9
EtOAc–hexane (9:1)/EtOAc–hexane (9:1)	14024.31	4.4	2235.34	16.3
EtOAc–hexane (9:1)/EtOAc	69492.75	6.0	16512.70	18.3
EtOAc/EtOAc	72007.69	5.3	17653.22	2.4

[a] Mixture of ethyl acetate and hexane are expressed by volume.

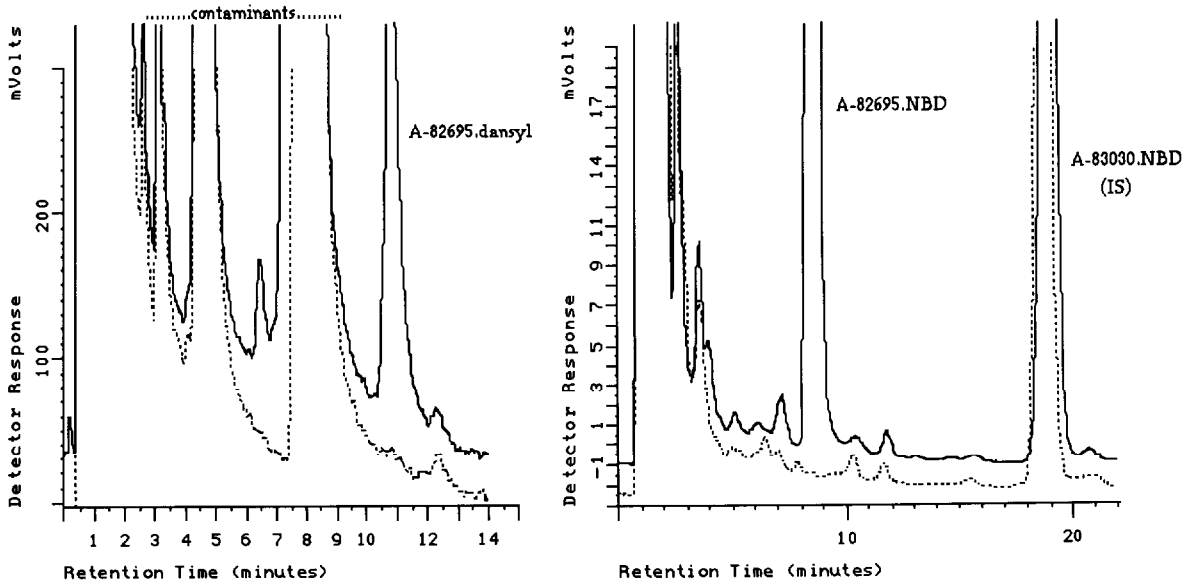


Fig. 5. Comparison of the reaction products derived from A-82695 and (a) dansyl chloride and (b) NBD-F in dog plasma.

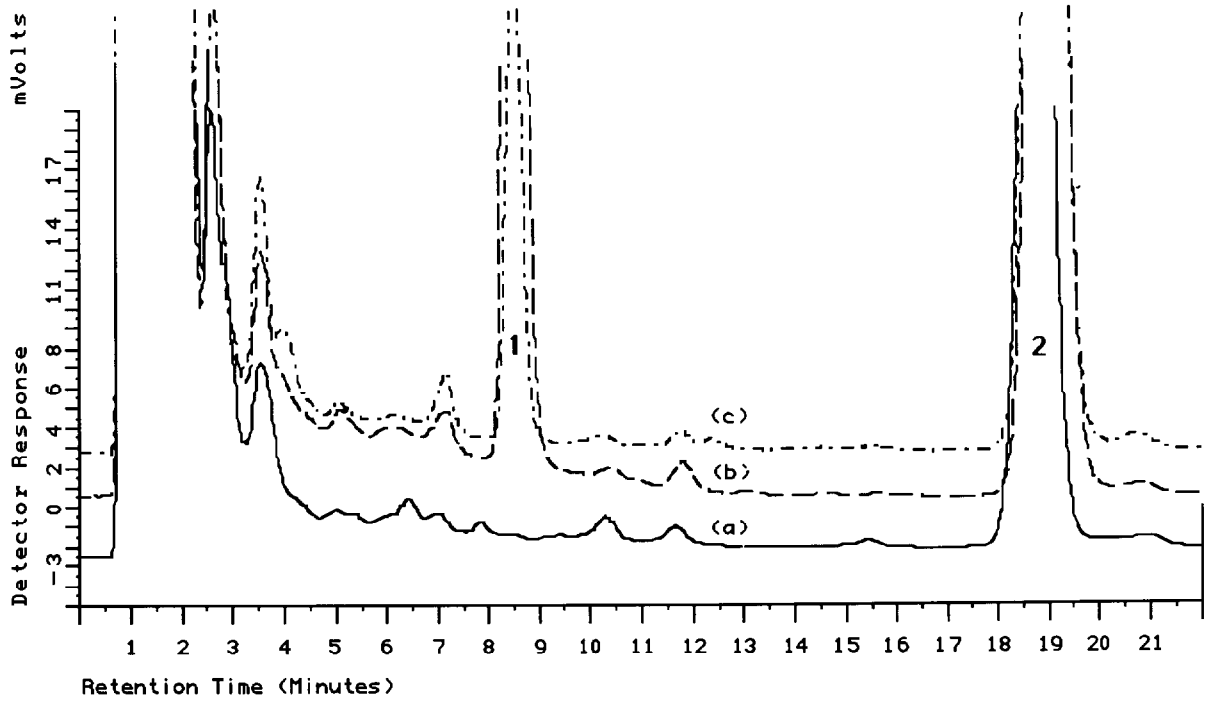


Fig. 6. Representative chromatograms obtained from precolumn derivatization with NBD-F. Peak 1 is the A-82695–NBD derivative and peak 2 the A-83030–NBD derivative (internal standard). Chromatographic conditions as described in the text. (a) Blank plasma containing internal standard; (b) spiked plasma standard, (c) dog plasma sample.

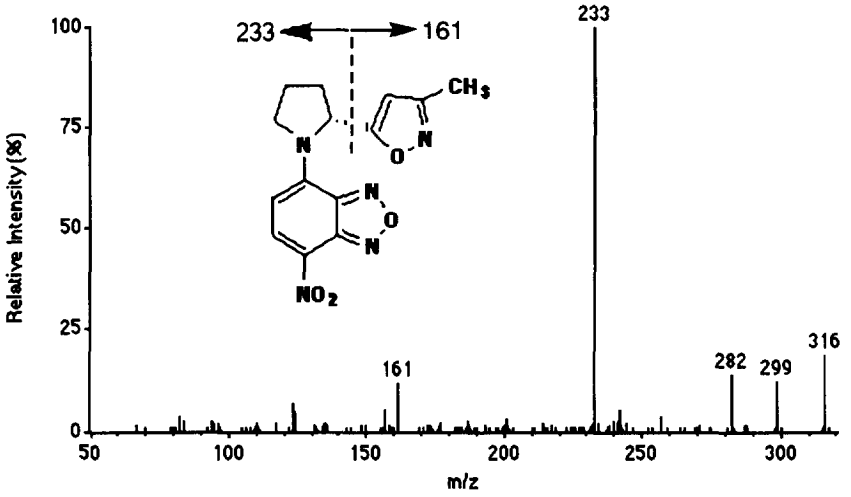


Fig. 7. Mass spectrum of A-82695–NBD derivative: daughter ion scan of m/z 316 (MH^+).

ples (see Fig. 6). To verify the derivative identity, LC–MS–MS was applied to the A-82695 reaction product. The A-82695-NBD derivative provided a MH^+ ion at m/z 316 in APCI, which corresponded to the expected molecular weight of 315. Its daughter spectrum (see Fig. 7) gave major fragment ions at m/z 233 and 161, from cleavage between the *N*-demethylated tetrahydropyrrole ring and the α-heteroaromatic ring. This provided confirmatory evidence for the chemical identity of the proposed A-82695–NBD derivative.

All NBD-F derivatives for this series of compounds exhibited similar excitation and emission maxima at wavelengths in the ranges 480–487 and 529–530 nm, respectively. No substantial differences were noted in the rates of derivatization or decay amonst the compounds evaluated in this structurally similar class. An optimal re-

Table 2
Reproducibility and accuracy of the method for the quantitation of A-82695 in dog plasma

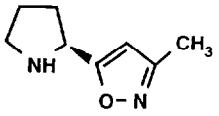
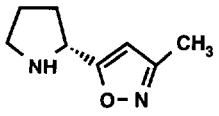
Intra-day variability					Inter-day variability			
Concentration spiked (ng ml^{-1})	Found (ng ml^{-1})	SD (ng ml^{-1})	Precision: SD (%)	Accuracy (% Diff)[a]	Day	Mean concentration[b] (ng ml^{-1})	Grand mean (ng ml^{-1})	RSD (%)
532.00	549.92			3.37	1	532.18		
	522.91	15.37	2.89	−1.70	2	526.95	531.61	0.8
	523.71			−1.56	3	535.71		
106.40	107.04			0.60	1	106.06		
	103.04	2.67	2.52	0.60	2	105.83	101.23	8.1
	108.11			1.60	3	91.87		
10.64	11.34			6.55	1	11.19		
	10.54	0.59	5.23	−0.93	2	10.73	10.05	15.8
	11.68			9.78	3	8.24		

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

Table 3
Summary of A-82695 calibration graphs for dog plasma

Day	Intra-assay RSD (%)	Slope	r^2	Inter-assay	
				Mean slope	SD (%)
1	3.89	6.9503×10^{-3}	1.000		
2	4.94	7.8685×10^{-3}	0.999	7.5×10^{-3}	6.51
3	6.16	7.7023×10^{-3}	0.999		

Table 4
Pharmacokinetic evaluation of A-82695 and A-79814 in dog and monkey after a single 100 nmol kg^{-1} intravenous or 200 nmol kg^{-1} oral dose[a]

Compound	Structure	Dog				Monkey			
		$t_{1/2}$ (h)	C_{max} (ng ml^{-1})	T_{max} (h)	F (%)	$t_{1/2}$ (h)	C_{max} (ng ml^{-1})	T_{max} (h)	F (%)
A-79814		0.71	8.05	0.39	29.9	1.51	4.83	1.67	18.4
A-82695		0.77	12.23	0.39	55.1	2.2	12.47	2.17	68.8

[a] $t_{1/2}$ = plasma elimination half-life; C_{max} = peak plasma concentration following oral dosing; T_{max} = time of peak plasma concentration; F = apparent oral bioavailability.

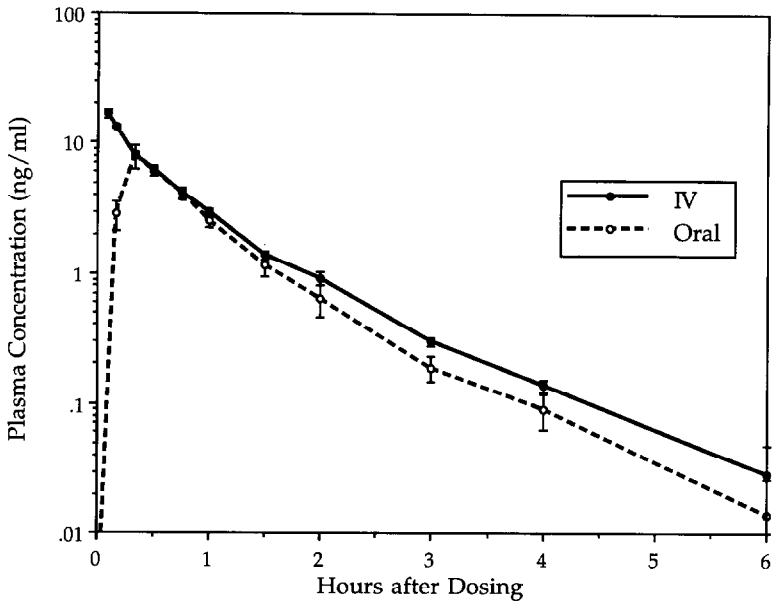


Fig. 8. Mean (± SEM, $n = 3$) plasma concentrations of A-79814 after a 100 nmol kg^{-1} intravenous or 200 nmol kg^{-1} oral dose in dogs.

sponse to the derivatization was obtained when the samples were heated for 5 min at 50 °C. An optimal response was obtained with higher concentrations of reagent (0.5 mg ml^{-1}) added to the reaction mixture in 300 µl aliquots. The use of ethyl acetate under alkaline conditions provided maximal recovery of the parent drug and internal standard from the plasma sample; an additional extraction with ethyl acetate following derivatization provided an additional improvement in the signal-to-noise ratio.

The method for the quantitation of A-82695 in plasma samples employing liquid–liquid extraction followed by derivatization with NBD-F was linear (correlation coefficient > 0.999) over the concentration range 0–532 ng ml^{-1}. The RSDs for the analyses of triplicate samples at 532, 106.4 and 10.6 ng ml^{-1} averaged 2.9, 2.5 and 5.2%, respectively, with relative errors (accuracy) ranging from −1.7 to 9.8% of theory. The mean inter-day precision, as evaluated from triplicate analyses of spiked standards on three consecutive days, averaged 0.8, 8.1 and 15.8% (RSD) at concentrations of 532, 106.4 and 10.6 ng ml^{-1}, respectively (see Table 2). The standard deviation derived from the slopes of the calibration graphs over three different days was 6.51% (see Table 3). The lower limit of quantitation was defined as 0.01 ng ml^{-1} following the extraction of a 1.0 ml plasma sample by actual derivatization. Analysis of control blank plasma indicated the absence of interfering compounds, confirming adequate assay specificity. The high selectivity and desired sensitivity for cholinergic channel activators in biological samples makes this assay useful for the pharmacokinetic evaluation of these compounds.

4.1. Application to pharmacokinetic studies

The method was applied to the pharmacokinetic evaluation of A-82659 and A-79814 in both beagle dog and cynomolgus monkey (see Table 4). The plasma concentrations of A-79814 in dog as a function of time after a 100 mmol kg^{-1} intravenous does or a 200 mmol kg^{-1} oral dose are depicted in Fig. 8. Peak plasma concentrations averaged less than 20 ng ml^{-1}, declining the levels below the limit of quantitation 6 h after dosing. The proposed method proved useful in delineating the pharmacokinetic behavior of a new series of cholinergic channel activators with significant oral bioavailability.

In conclusion, the method described here, which employs two sequential liquid–liquid partitioning steps followed by derivatization with NBD-F, is suitable for the pharmacokinetic evaluation of selected cholinergic channel activators in animals by virtue of its high sensitivity and reproducibility.

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