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Determination of ABT-089 in human plasma by high performance liquid chromatography using in situ precolumn derivatization with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole

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Abstract

ABT-089 is a potent, selective neuronal cholinergic channel modulator with cognition enhancing activity in several animal paradigms. A simple and sensitive chromatographic method for the specific determination of ABT-089 in human plasma has been developed and validated. The method utilizes in situ precolumn fluorescence derivatization of the sample with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) prior to liquid-liquid extraction followed by reverse phase HPLC and fluorescence detection (λ_{ex} 495 nm, λ_{em} 533 nm). The described method significantly simplifies sample preparation. The derivatized product was separated from interference using a YMC ODS-AQ, 5 μm, 250 × 4.6 mm i.d. column using a mobile phase consisting of 30:5:65 (v:v:v) acetonitrile/methanol/aqueous buffer at a flow rate of 0.75 ml min^{-1}. The aqueous buffer consisted of 0.01 M tetramethylammonium perchlorate, 0.1% (v:v) trifluoroacetic acid, pH 3.0. An Alltech Absorbosphere CN, 5 μm, cartridge guard column was also used before the analytical column. Plasma samples were alkalinized with 0.1 M $NaHCO_3$, 300 μl of a 1 mg ml^{-1} ethanolic solution of NBD-F was added and the samples were heated in a water bath for 10 min at 50°C. The samples were then extracted with tert-butylmethylether, evaporated to dryness and then reconstituted in mobile phase. For 1 ml of plasma, a limit of quantitation (LOQ) of 1.6 ng ml^{-1} was obtained. The method was linear from 1.6 to 836 ng ml^{-1}. Inter and intra-day assay RSD ($n = 6$) were less than 9%. Accuracy determinations showed the quality control samples to range between 88–114% of the theoretical concentration.

Keywords: Reversed-phase HPLC; Fluorescence detection; Pre-column derivatization; NBD-F; Bioanalysis; Cholinergic channel modulator; ABT-089

1. Introduction

ABT-089, shown in Fig. 1, is a potent cholinergic channel activator under evaluation for the treatment of Alzheimer's disease and is in a class

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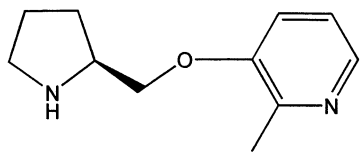
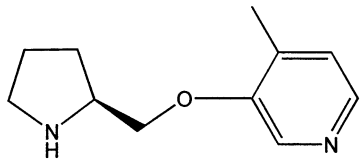
of drugs which selectively bind to the central nervous system nicotinic receptors and releases acetylcholine. These compounds may have enhanced cognitive effect and have been shown to have an improved side-effect profiles relative to nicotine, including reduced cardiac and gastrointestinal effects. ABT-089 also has good oral bioavailability and lacks the induced tolerance effects of nicotine.

For pharmacokinetic studies, a very sensitive and selective analytical method for the determination of ABT-089 in human plasma is required. Because of the lack of significant native fluorescence or significant electrochemical activity, pre-column fluorescence derivatization was chosen as a detection technique for ABT-089. 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was chosen as a derivatization reagent because it forms highly fluorescent derivatives with secondary amines using relatively mild conditions.

Pre-column fluorescence derivatization with benzofurazans has mostly been applied to the determination of amino acids, peptides and their metabolites [1–5] but has had relatively few applications for the analysis of drugs or other endogenous compounds from biological matrices [6–11]. The reaction of NBD-F with secondary amines can be performed in aqueous or organic solvents and has an optimum pH of 8–9. The hydrolysis product 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) is only weakly fluorescent and can easily be separated from the desired fluorescent products. NBD-F has also been shown to react ten times faster than its 4-chloro analog [1,12].



ABT-089



ABT-840 (internal standard)

Fig. 1. Structure of ABT-089 and ABT-840 (internal standard).

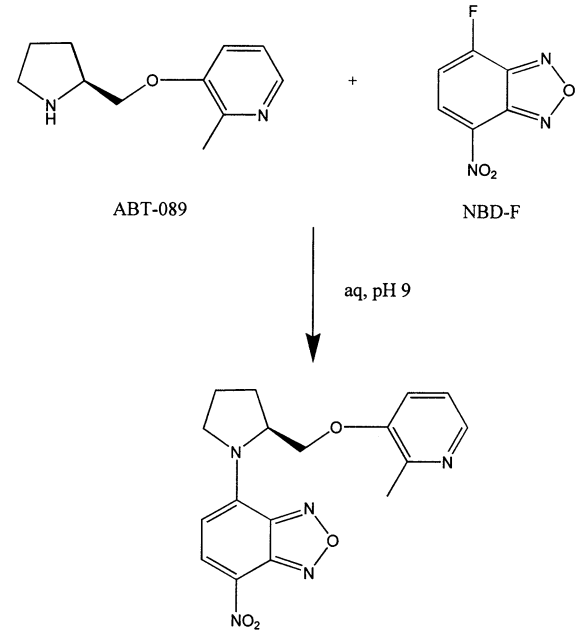


Fig. 2. Derivatization reaction of ABT-089 with NBD-F (see text for reaction conditions).

ABT-089 is a relatively polar and volatile compound in its free base form. Previous methods for the analysis of compounds similar to ABT-089 have yielded sample preparation methods consisting of multiple steps including: organic solvent extraction, back-extraction into an aqueous solvent, derivatization with NBD-F, solvent extraction, evaporation and sample reconstitution steps [10,11]. In situ pre-column derivatization of ABT-089 with NBD-F directly in plasma samples eliminates the need for one of the organic solvent extraction and the back-extraction steps, significantly simplifying the method.

Reaction for the alkylation of ABT-089 with NBD-F is shown in Fig. 2. Because of its analogous structure, ABT-840 (Fig. 1) was chosen as the internal standard. This paper describes the method and validation for a sensitive and selective HPLC method for the determination of ABT-089 in normal human plasma with in situ pre-column fluorescent derivatization by NBD-F.

2. Experimental

2.1. Chemicals and reagents

All aqueous solutions including the HPLC mobile phase were prepared with purified deionized water (Milli-Q Plus, Millipore Corp., Milford, MA). HPLC grade acetonitrile and methanol were purchased from EM Science, Gibbstown, NJ. Tetramethylammonium perchlorate and *tert*-butylmethylether were from Sigma Chemical, St. Louis, MO. Sodium bicarbonate (analytical reagent grade) was from Mallinckrodt, Pans, KY. Trifluoroacetic acid (HPLC/spectro grade) was from Pierce, Rockford, IL. 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was purchased from Regis Chemical, Morton Grove, IL. Drug free normal human plasma, was purchased from Pel-Freez, Inc., Brown Deer, WI. ABT-089 dihydrochloride salt, and ABT-840 dihydrochloride salt were obtained from the Abbott Laboratories chemical stockroom.

2.2. HPLC apparatus and conditions

The HPLC system consisted of either a Model LC-600 or a Model LC-10AD pump, a Model SIL-10A or a Model SIL-9A autosampler, a Model RF-551 or a Model RF-10A fluorescence detector (λ_{ex} 495 nm and λ_{em} 533 nm) (Shimadzu Corp., Kyoto, Japan). The analyte and internal standard were separated with an Adsorbosphere CN guard cartridge, 5 mm (Alltech Assoc., Deerfield, IL) and an ODS AQ, 5 μm, 120 Å, 250 × 4.6 mm i.d. analytical column (YMC, Corp., Wilmington, NC) using a mobile phase consisting of acetonitrile/methanol/aqueous buffer, 30:5:65 (v:v:v). The aqueous buffer consisted of 0.01 M tetramethylammonium perchlorate and 0.1% (v:v) trifluoracetic acid, pH 3.0. The mobile phase flow rate was 0.75 ml min^{-1}.

2.3. Other apparatus

Samples were mixed using a Model 58816-115, Multi-tube vortexer, (VWR, West Chester, PA). Centrifugation was performed by a Model CR 4.11 (Jouan, Winchester, VA).

Sample evaporation was performed on a Model 114 Multivap Evaporator, (Organomation, South Berlin, MA). The sonicator was a Model B-5200R-4 Sonic cleaner, (Branson Ultrasonic Co., Shelton, CT).

2.4. Data processing

Chromatographic data was acquired and processed by TurboChrom-4 (PE Nelson, Inc., Cupertino, CA). For sample quantitation, a set of standard samples in plasma including at least two blank normal (drug-free) plasma human and a set of quality control (QC) samples in replicate (three or more) were assayed in each analytical batch. The ratios of the observed peak heights of the analyte to those of the internal standard from the standard curve samples were subjected to weighted (1/concentration) linear regression against the theoretical plasma concentrations to derive a standard curve. The analyte concentration of the QC samples or unknown samples were determined by application of the standard curve linear regression expression to the sample peak height ratio. The QC samples were used to provide in-process monitoring of both the accuracy and precision of the assay.

2.5. Preparation of standard and quality control samples

ABT-089 was obtained as a dihydrochloride salt. Salt weights were converted to the free base weights by multiplying the dihydrochloride salt weight by a factor of 0.731 (i.e. 1000 μg ml^{-1} ABT-089 dihydrochloride is equivalent to 731 μg ml^{-1} ABT-089 free base). All subsequent concentration will be expressed as free base equivalents unless otherwise specified.

Standard and QC stock solutions of ABT-089 and ABT-840 (internal standard) were prepared from their dihydrochloride salts and were dissolved in 0.05 M KH_2PO_4. Stock solutions were stored at ≈ 5°C. Standard curve spiking solutions were volumetrically diluted in 0.05 M KH_2PO_4 to give solutions ranging in concentration from 16.33 to 8363 ng ml^{-1}. The standard curve was prepared daily by spiking 100 μl of the standard curve spiking solutions into 1 ml of pooled blank human plasma. The resulting standard curve concentrations ranged from 1.63 to 836.3 ng ml^{-1}. QC stock solutions were prepared in 0.05 M KH_2PO_4 from a separate weighing than the standard stock solutions. QC samples were volumetrically diluted in pooled blank human plasma to produce ABT-089 concentrations of, 8.911, 44.55 and 178.2 ng ml^{-1}. For storage, 1.0 ml aliquots of the QC samples were transferred into 16×100 mm test tubes. The test tubes were capped, placed in a rack, covered with aluminum foil and stored at ≈ -20°C until used. The internal standard solution (20 μg ml^{-1}, ABT-840 dihydrochloride) was prepared in 0.05 M KH_2PO_4 .

2.6. Sample preparation

NOTE It is important to protect the NBD-F and the derivatized samples from light whenever possible. When sample manipulation was performed in the light, the steps were performed as quickly as possible. Sample racks with derivatized drug were covered with aluminum foil to protect them from light.

Blank plasma and QC samples were allowed to come to room temperature and 1.0 ml of the blank plasma was pipetted into individual 16×100 mm test tubes. The QC samples remained in their original test tubes. The standard curve was prepared by adding 100 μl of the standard curve working solutions into tubes containing 1.0 ml of plasma into individual test tubes (16×100 mm). To each of the tubes containing the standards and QC samples, 50 μl of internal standard solution was added. No internal standard was added to the blank samples. A 500 μl aliquot of 0.1 M $NaHCO_3$ was added to each tube and the sample were thoroughly mixed by vortexing.

After addition of $NaHCO_3$ to the samples, the derivatizing agent was prepared (It is important to prepare the NBD-F reagent immediately before use). A 1.0 mg ml^{-1} ethanolic solution of NBD-F was made in an actinic volumetric flask. To each sample, 300 μl of the NBD-F solution was added. The test tubes were capped, thoroughly mixed and placed in a heated sonicator bath for 10 min at 50°C. After reaction, the tubes were placed in an ice bath. When cool, 500 μl of 0.1 M $NaHCO_3$ was added and the tubes were thoroughly mixed by vortexing.

The samples were extracted by adding 6 ml of *tert*-butylmethylether. After recapping the test tubes, the samples were mixed by vortexing at high speed for 2 to 3 min. The samples were centrifuged at 3000 rpm for 10 min. The organic layer was transferred to a 13×100 mm test tube and the solvent was evaporated under a gentle stream of dry nitrogen at ambient temperature.

The samples were each reconstituted with 150 μl of mobile phase and thoroughly mixed for 1 min. Before transferring to a 250 μl vial insert in an amber vial, the samples were centrifuged for 10 min at 3000 rpm. A 100 μl aliquot of the sample was injected into the HPLC for analysis.

2.7. Determination of relative recovery

A set of neat standard curves in triplicate was processed and consisted of the following levels: 6.53, 13.07, 52.27, 104.5, 209.1, and 418.1 ng ml^{-1}. The neat standard curve was prepared in 0.1 M $NaHCO_3$ instead of plasma. The average value of the three replicates at each level was used in the calibration curve. Three sets of each level of the QC samples were used to determine the relative recovery. The standard and QC samples were extracted as described above, except that a 4.0 ml aliquot of the organic layer was transferred to the 13×100 mm test tubes for evaporation. The internal standard spiking solution was prepared from 1.0 ml of the internal standard stock solution and was derivatized, extracted and dried along with the standards and QC samples. The internal standard sample was then quantitatively transferred and brought to volume in a 25 ml volumetric flask with mobile phase. The internal

standard sample, after dilution to 25 ml was used to reconstitute the standards and QC samples. The standards and QC samples were reconstituted in 150 μl of this solution. A 125 μl aliquot was transferred and 100 μl was injected on the HPLC. The relative recovery of ABT-089 was determined using the averaged neat standard curve and the average results from the three replicates of the 8.91, 44.55, and 178.2 ng ml^{-1} QC samples. The concentrations were not corrected for the fraction of the organic layer used since the standards and QC samples were treated identically.

2.8. Precision and accuracy

Evaluation of the accuracy and precision of the method was performed by analyzing a set of samples consisting of two drug-free plasma samples, six samples of each level of QC samples, and one set of the ten point standard curve in the concentration range of 1.63 to 836.3 ng ml^{-1} ABT-089 on five separate occasions. On two separate occasions only three samples of each QC level were used. Four analysts performed the validation over a period of 4 weeks on seven separate days.

2.9. Determination of limit of quantitation (LOQ)

The lower limit of quantitation was determined using one set of the standard curve and six replicates each of the two lowest points on the standard curve (1.63 and 3.27 ng ml^{-1}). The samples were extracted as described in the sample preparation section. These twelve replicates were not used as part of the standard curve.

2.10. Freeze–thaw stability in plasma

The stability of ABT-089 in human plasma following repeated freeze–thaw cycles was assessed using QC samples spiked with ABT-089. The samples were stored at -20°C between thaw cycles. The samples were thawed by allowing them to stand at room temperature protected from light for ≈ 2 h. The samples were then returned to the freezer. The stability of ABT-089 was assessed after four freeze–thaw cycles. The samples were processed using the same procedure as described in the sample preparation section.

2.11. Long term stability in plasma

An evaluation of the long term stability of ABT-089 in human plasma was established from the results of QC samples stored at -20°C during the entire validation study (4 weeks).

2.12. Stability of ABT-089 in an autosampler

The stability of derivatized ABT-089 after sample preparation was determined using the samples for freeze–thaw stability in plasma. The samples were housed in the autosampler at room temperature for at least 25 h after preparation.

3. Results and discussion

Because of a lack of significant native fluorescence or electrochemical activity, pre-column fluorescence derivatization was chosen as a technique to increase the detectability of ABT-089. 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) was chosen as a derivatization reagent because it forms highly fluorescent derivatives with secondary amines using relatively mild reaction conditions [13]. The NBD-F reaction with secondary amines can also be performed in aqueous solvents. Derivatizing ABT-089 in plasma in situ prior to extraction significantly simplified the method and increased extraction efficiency into organic solvents by increasing lipophilicity with the benzofurazan moiety.

Initial attempts at sample preparation for analogs of ABT-089 focused on extensive sample clean-up prior to derivatization [10]. Attempts were made to extract the drugs from plasma using liquid–liquid extraction under alkaline conditions, the drugs were then back-extracted into dilute aqueous hydrochloric acid. The pH was then adjusted back to alkaline where NBD-F was added for derivatization. The derivatized drugs were then extracted into an organic solvent prior to evaporation and reconstitution. In addition to being labor intensive, the method suffered from low recoveries due to the drug's lack of lipophilicity prior to derivatization.

For the sample preparation method for ABT-089, even though performing the derivatization directly in plasma significantly improves the simplicity of the method, other problems became apparent. In situ derivatization was initially attempted with 0.1 mg ml^{-1} NBD-F, with 30 min reaction time at 50°C (see Fig. 3). Using 0.1 mg ml^{-1} NBD-F for in situ derivatization, the observation was made that the chromatogram showed many more peaks than when the drug was derivatized after an organic solvent extraction. This result would be expected because by performing the derivatization directly in plasma there would be many more endogenous compounds (including proteins) which could be derivatized by NBD-F. These endogenous compounds would also consume the derivatizing agent and caused the recovery to decrease. In an attempt to improve recovery, various protein clean-up steps were evaluated, including protein precipitation and ultrafiltration.

As shown in Fig. 4, little improvement was shown by solvent protein precipitation with acetonitrile, methanol, ethanol or isopropanol. A slight improvement was seen with 0.1 M perchloric acid protein precipitation prior to solvent extraction. The greatest recovery improvements

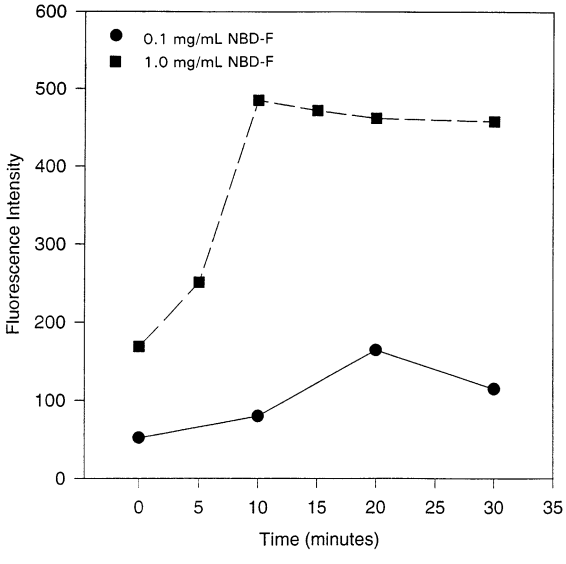


Fig. 3. Time course of the derivatization reaction of ABT-089 with 0.1 and 1.0 mg ml^{-1} NBD-F at 50°C.

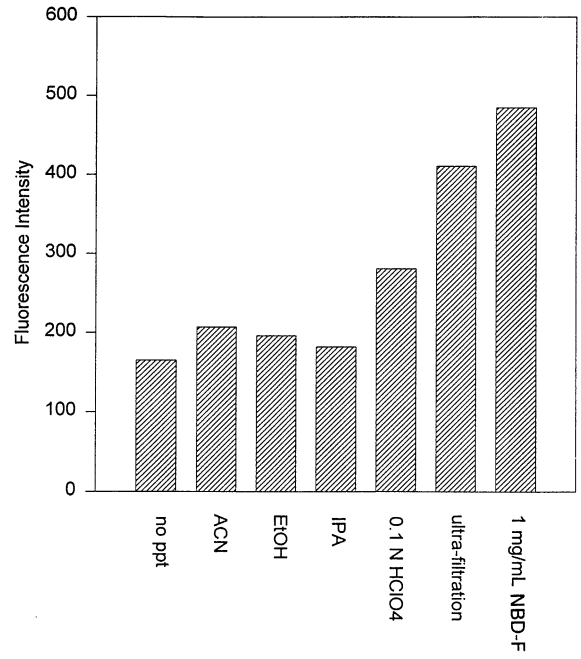


Fig. 4. Effects of protein removal methods compared to in situ derivatization of ABT-089 with 1.0 mg ml^{-1} NBD-F in human plasma.

were seen with ultrafiltration (Centrifree micropartition system, Amicon, Beverly, MA) prior to extraction and also with the use increased derivatization reagent (1.0 mg ml^{-1} NBD-F). These results gave evidence that the plasma proteins were the major source of competition for the derivatization reagent. It would follow that by removing the protein or by adding far excess reagent to saturate the derivatizable sites on the proteins, the recovery could be improved. Also, the chromatograms obtained from the ultrafiltered samples were similar to those seen when ABT-089 was extracted prior to derivatization. The ultafiltration method for protein removal was not considered a viable option because it took over 3 h at 3000 rpm to filter just 0.5 ml of plasma. Therefore, the option of increasing the concentration of the derivatizing reagent was chosen. It should be noted that approximately linear increases in recovery were achieved when the NBD-F concentration was increased between 0.1, 0.2, 0.3, and 0.5 mg ml^{-1}. The slope of the NBD-F concentration versus fluorescence inten-

sity line significantly decreased between 0.5 and 1.0 mg ml^{-1} NBD-F suggesting the approach to an optimal NBD-F concentration. Further increase in NBD-F concentration was not attempted due to concerns of reagent solubility and cost associated with using increased amounts of derivatization reagent. The effects of NBD-F concentration and time on the formation of derivatized ABT-089 at 50°C are shown in Fig. 3. These results show that using 1.0 mg ml^{-1} NBD-F, reaction times longer than 10 min did not increase the amount of derivatized ABT-089. For temperatures less than 50°C, longer times to maximum fluorescence intensity were observed.

In order to select the an appropriate extraction solvent, various organic solvents various combinations of hexane, ethyl acetate, isopropanol and *tert*-butylmethylether were used. *tert*-Butylmethylether was chosen as the extraction solvent because it gave the highest recovery and the cleanest chromatograms.

During method development, the chromatographic conditions consisted of a YMC ODS AQ guard column, YMC ODS AQ 5 μm, 250 × 4.6 mm i.d. analytical column and a mobile phase consisting of 35:65 (v:v) acetonitrile–aqueous buffer at a flow rate of 1.0 ml min^{-1}. The aqueous buffer consisted of 0.01 M tetramethylammonium perchlorate and 0.1% (v:v) trifluoroacetic acid at pH 3.0. To obtain the stated LOQ, improvements in resolution between the derivatized analyte and other interference were required and were achieved by the addition of 5% (by volume) methanol to the mobile phase. Also, the mobile phase flow rate was decreased to 0.75 ml min^{-1}. Each change gave a slight improvement in resolution and cumulatively were sufficient to achieve the required sensitivity. After final mobile phase selection, the optimum excitation (495 nm) and emission (533 nm) wavelength maxima were determined for the derivatized analyte.

3.1. Sensitivity and limit of quantitation

The lowest limit of quantitation was determined as the lowest concentration where the RSD is less than 20% for six replicate samples. Results in Table 1 show that the method could detect and quantitate ABT-089 with a LOQ of 1.63 ng ml^{-1}. The LOQ was established using a 1 ml plasma sample. The RSD for ABT-089 at this concentration was 6.9% ($n = 6$).

3.2. Specificity and selectivity

Specificity was indicated by the absence of interference as evaluated by chromatograms of blank (drug-free) human plasma, and plasma spiked with ABT-089 and the internal standard ABT-840. Figs. 5–7 show typical chromatograms for an extract from a normal human blank (drug-free), human plasma spiked with ABT-089 at the LOQ (1.63 ng ml^{-1}) and human plasma spiked at 209.1 ng ml^{-1}, respectively.

3.3. Standard curve linearity

Linearity was indicated by the closeness of the calibration curve coefficient of determination to unity. The mean coefficient of determination (r^2) obtained during the entire validation ($n = 10$) was 0.9992 and the results ranged from 0.9974 to 0.9999. The regression line (with a 1/concentration weighting) agreed very well with the observed response for each standard point. The mean back-calculated concentration for the standards from which the curves were derived ranged from 94 to 106% of the theoretical. A summary of the calibration curve data are shown in Table 2.

3.4. Accuracy and precision

Accuracy for the method was indicated by good agreement of the measured concentrations for the QC samples with their theoretical values (Table 3). The inter-assay accuracy results for the QC samples through the entire validation period ranged from 88 to 114% of the target concentrations. The mean results for the QC levels of 8.91, 44.55, and 178.2 ng ml^{-1} were 102, 102, and 101% of the theoretical, respectively. Intra-assay precision was indicated by the reproducibility of the assayed results from the QC samples ($n = 6$) performed on five different days by different analyst. The RSD were less than 8.9%. Inter-assay precision was indicated by the RSD for all valida-

Table 1
Lower limit of quantification of for the determination of ABT-089 from human plasma

	Replicate						Theoretical concentration (ng ml^{-1})	Mean	Mean % theory	SD	RSD
	1	2	3	4	5	6					
Back-calculated concentration (ng ml^{-1})	1.67	1.57	1.42	1.64	1.43	1.61	1.633	1.56	95	0.11	6.9
% Theory	102	96	87	101	88	99					
Back-calculated concentration (ng ml^{-1})	[a]	3.68	3.24	3.73	3.65	3.27	3.267	3.51	108	0.24	6.8
% Theory		113	99	114	112	100					

[a] Apparent outlier.

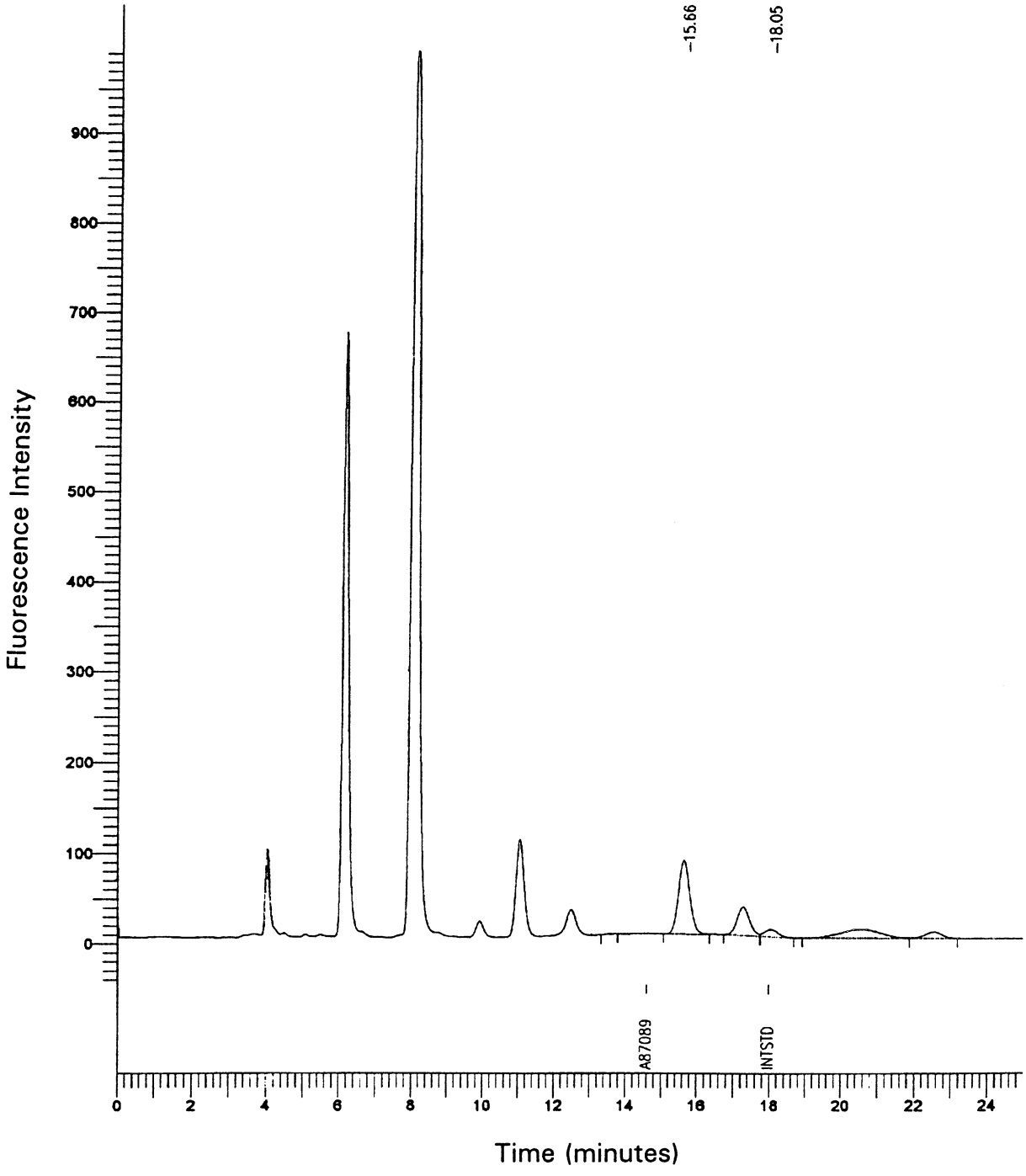


Fig. 5. Representative chromatogram of an extract from blank normal human plasma.

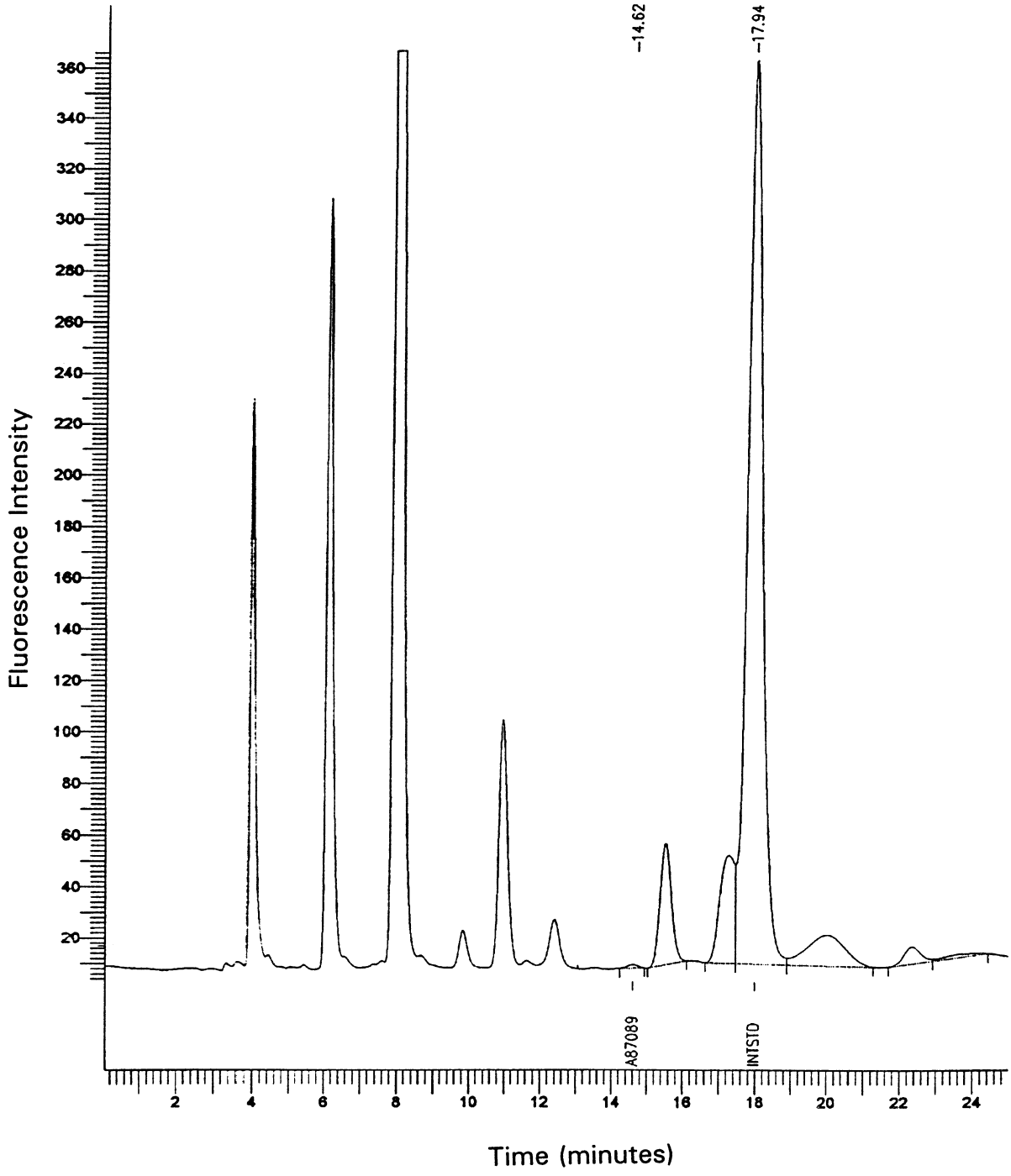


Fig. 6. Representative chromatogram of an extract from the limit of quantitation (LOQ) of ABT-089 in normal human plasma.

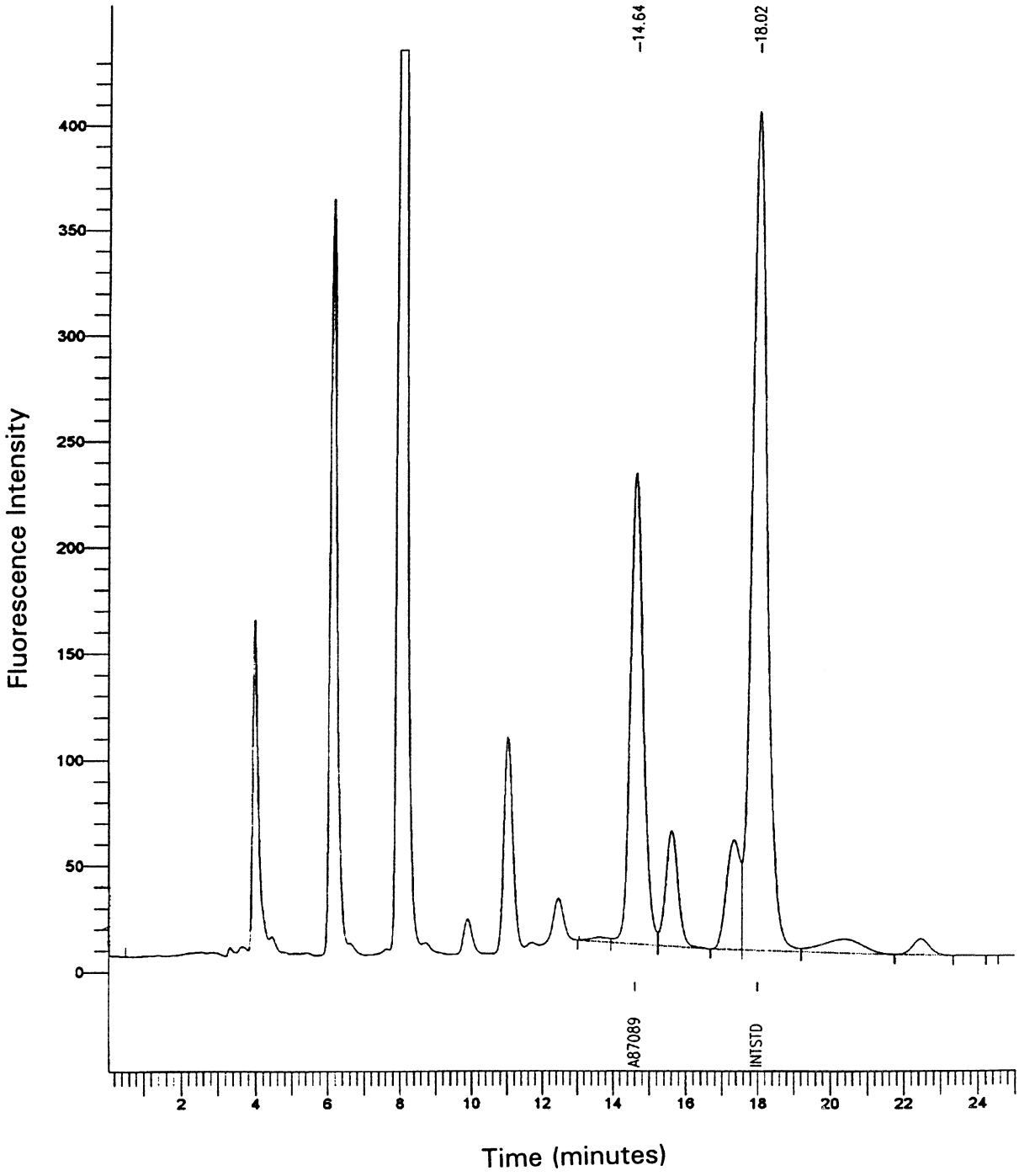


Fig. 7. Representative chromatogram of an extract from a calibration standard (209 ng ml^{-1}) of ABT-089 in normal human plasma.

Table 2
Summary of the calibration curve data for the determination of ABT-089 from human plasma

	Theoretical concentration (ng ml^{-1})										r^2	Slope	Intercept
	1.633	3.267	6.534	13.07	26.13	52.27	104.5	209.1	418.1	836.3			
Mean	1.68	3.47	6.88	12.61	26.15	48.96	101.68	206.79	423.87	838.97	0.9992	0.0028	0.0017
Mean% theory	103	106	105	97	100	94	97	99	101	100			
SD	0.12	0.15	0.46	0.57	1.15	1.16	3.04	8.05	7.56	11.9			
RSD	7.3	4.2	6.7	4.5	4.4	2.4	3.0	3.9	1.8	1.4			
Max	1.84	3.70	7.64	13.40	27.61	50.69	104.83	226.18	431.47	856.29			
Min	1.53	3.19	6.03	11.51	24.09	47.40	94.32	198.64	410.27	822.78			
Max% theory	112	113	117	103	106	97	100	108	103	102	0.9999	0.0035	0.0074
Min% theory	94	98	92	88	92	91	90	95	98	98	0.9974	0.0022	−0.0026
Count	8	8	9	10	10	10	10	10	10	10			

Table 3
Summary of the intra and inter-assay accuracy and precision data for the determination of ABT-089 from human plasma

	Theoretical concentration (ng ml^{-1})	Analyst	Intra-assay[a]					Inter-assay[b]				
			Daily mean (ng ml^{-1})	Mean % theory	SD	RSD	Count	Grand mean (ng ml^{-1})	Mean % theory	SD	RSD	Count
QC low	8.91	1	9.79	110	0.28	2.8	6					
		2	8.89	100	0.46	5.2	6					
		3	8.97	101	0.65	7.2	5					
		4	9.69	109	0.33	3.5	5					
		5	8.55	95	0.23	2.7	6	9.13	102	0.63	6.9	34
QC mid	44.55	1	46.85	105	1.17	2.5	6					
		2	44.36	100	3.96	8.9	6					
		3	45.20	101	0.86	1.9	6					
		4	47.48	107	1.42	3.0	6					
		5	45.73	103	0.51	1.1	6	45.58	102	2.41	5.3	36
QC high	178.2	1	180.8	101	2.2	1.2	6					
		2	169.3	95	4.8	2.8	6					
		3	179.3	101	4.3	2.4	6					
		4	191.3	107	6.2	3.2	6					
		5	180.7	101	2.2	1.2	5	179.4	101	8.8	4.9	35

[a] Calculated for daily validation runs using six replicates of each QC level. Results considered as apparent outlier were omitted.
[b] Calculated for all validation runs. Each run included at least three replicates of each QC level. Results considered as apparent outlier were omitted.

Table 4
Summary of stability data for the determination of ABT-089 from human plasma

		Freeze–thaw cycle				−20°C (4 weeks)
		1	2	3	4	
QC low	Mean (ng ml^{-1})	9.41	9.31	9.46	9.59	8.89
8.91 ng ml^{-1}	% Theory	106	104	106	108	100
QC mid	Mean (ng ml^{-1})	44.46	45.50	43.68	44.95	44.16
44.55 ng ml^{-1}	% Theory	100	102	98	101	99
QC high	Mean (ng ml^{-1})	166.7	166.4	166.2	170.5	169.3
178.2 ng ml^{-1}	% Theory	94	93	93	96	95

tion runs having a value less than 6.9%. The mean RSD results for the QC levels of 8.91, 44.55, and 178.2 ng ml^{-1} were 6.9, 5.3, and 4.9%, respectively.

3.5. Recovery

Recovery of ABT-089 from plasma was determined relative to the same concentration derivatized in water. Absolute recovery was not determined because of the need to isolate and purify the NBD-F derivatized ABT-089. The derivatization and extraction recoveries of ABT-089 from plasma at concentrations of 8.91, 19.58, and 48.96 ng ml^{-1} were 21, 21, and 22%, respectively.

3.6. Stability

Table 4 shows the results of the analyses of the QC samples following repeated freeze–thaw cycles and storage at −20°C. ABT-089 was shown to be stable in frozen plasma at −20°C for at least four freeze–thaw cycles because there was no significant change in concentration of the QC samples during the experiment. An evaluation of the stability of ABT-089 frozen at −20°C in human plasma was obtained from the quality control samples values acquired during the entire validation process. There was no significant change in concentration of the QC samples during the 4 week period.

Data from the validation run for freeze–thaw stability in plasma were used to determine the effect of time at room temperature before analysis on the results obtained from QC samples. There was no significant change in the concentration of the ABT-089 between the first and the last set of QC samples injected over a 25 h period. These results show that ABT-089 and its NBD derivative are stable under normal laboratory conditions and the described extraction conditions.

4. Conclusions

The analytical procedure and validation results shown in this paper for the determination ABT-089 from normal human plasma proved the method to be accurate, precise and linear within the range from 1.63 to 836.3 ng ml^{-1} for a 1 ml sample volume. The in situ derivatization of ABT-089 directly in the plasma samples with an ethanolic solution of NBD-F imparts simplicity to the method. In addition to the expected increase in sensitivity with the alkylation of ABT-089 with a fluorophore, the sample preparation time is significantly improved.

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