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Analysis of brefeldin A and the prodrug breflate in plasma by gas chromatography with mass selective detection

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Abstract

Breflate is a water soluble prodrug developed to facilitate parenteral administration of the investigational antineoplastic agent brefeldin A (BFA). Previously, using analytical methods based upon reversed-phase high performance liquid chromatography (HPLC) with low wavelength UV detection or gas chromatography (GC) with electron capture detection following derivatization with heptafluorobutyrylimidazole, it was demonstrated that breflate undergoes rapid and efficient conversion to BFA following bolus i.v. injection in mice and dogs. However, plasma concentrations of the drug and prodrug achieved during the administration of nontoxic doses of breflate to beagle dogs as a 72 h continuous i.v. infusion were undetectable (< 0.1 μg ml^{-1}) by these methods. The sensitivity and specificity required for therapeutic drug level monitoring were achieved by GC with selected-ion mass spectrometry (MS) detection. Breflate, BFA and 1-eicosanol, the latter added to the sample as an internal standard (IS), were extracted from plasma into *tert*-butyl methyl ether (TBME) and esterified with trifluoroacetic anhydride. Methanol was added to the reaction mixture to effect the convenient removal of excess reagent as the volatile methyl ester during evaporation of the solvent. The residual material was analyzed directly upon reconstitution by capillary GC with automated splitless injection. Electron-ionization (70 eV) MS detection was performed by sequentially scanning ions at m/z 58, 202 and 325. The lowest concentration of either analyte quantified with acceptable reproducibility, as defined by an inter-day R.S.D. of about 20%, was near 10 ng ml^{-1} in plasma using a sample volume of 100 μl. The assay has proven to be sufficiently sensitive, specific and reproducible for the routine analysis of pharmacokinetic specimens acquired during IND (investigational new drug)-directed toxicology studies in dogs.

Keywords: Brefeldin A; Bio-analysis; Gas chromatography/mass spectrometry; Pharmacokinetics; Anticancer agents; Plasma

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1. Introduction

Brefeldin A (BFA) (see Fig. 1), a naturally occurring macrolide [1], exhibits a diversity of

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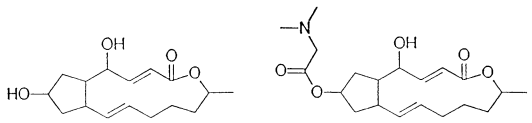
biological activities which include antibiotic, antiviral, cytostatic, antimitotic and antitumor effects [2]. Its importance as a molecular tool for studying signal transduction pathways in mammalian cells resulted from the discovery that it inhibits the transport of proteins from the endoplasmic reticulum to the Golgi complex, notably resulting in the reversible disassembly of the Golgi complex [3]. Recent interest in the potential utilization of BFA as a chemotherapeutic agent followed from its evaluation in the National Cancer Institute's in vitro anticancer screen [4]. Against the panel of ≈ 60 human tumor cell lines, BFA showed particular sensitivity toward the melanoma subpanel and many of the colon and renal cancer cell lines. Exposure to 0.1 μM BFA (0.028 μg ml^{-1}) for at least 24 h was required to irreversibly inhibit the growth of cultured human melanoma and prostate cancer cell lines [5]. However, pharmacokinetic studies in mice revealed that BFA was rapidly eliminated from systemic circulation, as plasma levels remained above 0.1 μg ml^{-1} for only 1 h after giving a single bolus i.v. injection of the drug near its maximum tolerated dose [6]. Thus, in the absence of significant oral bioavailability [6], it would be necessary to administer BFA by continuous i.v. infusion in order to sustain potentially therapeutic concentrations of the drug in plasma [5].

Whereas the limited solubility of BFA precluded its direct administration in a clinically acceptable parental formulation, the compound possesses two 2° hydroxyl groups that could function as the site of a bioreversible structural modification for enhanced water solubility. Among the compounds that were prepared and evaluated as candidate prodrugs, the 7-*N*,*N*-dimethylglycinate ester of BFA (Fig. 1), hereafter referred to as breflate, exhibits solubility and stability, characteristics appropriate for delivery by continuous i.v. infusion [7,8]. Moreover, the compound behaves as a true prodrug, undergoing rapid and essentially quantitative conversion to the parent drug following bolus i.v. injection in both mice and dogs. Breflate exhibited impressive activity against xenografts of several human melanoma cell lines implanted subcutaneously in nude mice when given as a 3-day continuous infusion [9].

During studies to further assess the suitability of breflate as a clinical candidate, it became apparent that a highly sensitive analytical method would be required to determine plasma concentrations of the drug and prodrug achieved during the infusion of nontoxic doses of breflate to beagle dogs [10]. The analysis of BFA as the di-*O*-heptafluorobutyryl derivative by capillary gas chromatography (GC) with electron capture detection, for which the lower limit of quantitation (LLQ) was 0.10 μg ml^{-1} in plasma, did not provide adequate sensitivity [6]. Furthermore, being substantially less volatile than the derivative of the parent drug, the prodrug was not amenable to quantitation by this method. Subsequent studies revealed that the two compounds were readily separable from each other and extractable endogenous constituents by high performance liquid chromatography (HPLC) [8]. However, applicable methods of direct detection, including low wavelength UV absorption and thermospray ionization mass spectrometry (MS), did not provide the sensitivity required within the constraints of acceptable precision. Precolumn derivatization for enhanced detectability during HPLC also proved unsatisfactory for either compound. As documented in this report, GC with mass selective detection provided the basis of an alternative analytical method for the concurrent determination of BFA and breflate in plasma with a LLQ near 0.01 μg ml^{-1} for both compounds. This assay was found to be adequately sensitive for monitoring steady state plasma levels of the parent drug during the infusion of potentially therapeutic doses of breflate [10].



Brefeldin A Breflate

Fig. 1. Chemical structures of brefeldin A and its prodrug, breflate.

2. Experimental

2.1. Reagents and chemicals

Brefeldin A (NSC 89671) and breflate (NSC 656202) were obtained from the National Cancer Institute. Dimethyl sulfoxide, 1-eicosanol and trifluoroacetic anhydride (TFAA) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). The acetonitrile, *tert*-butyl methyl ether (TBME) and methanol were 'Baker Analyzed' HPLC-grade solvents (J.T. Baker, Phillipsburg, NJ). All chemicals were used as received, with the exception of the TFAA and TBME, which were distilled and used immediately thereafter. Human plasma was acquired from the American Red Cross (Baltimore, MD).

2.2. Analytical solutions

Stock solutions of BFA and breflate were made by dissolving the compounds, accurately weighed on a Cahn C-31 microbalance (Cahn Instruments, Cerritos, CA), in dimethyl sulfoxide within class A borosilicate glass volumetric flasks (Kontes, Vineland, NJ) to provide a concentration of 0.2 mg ml^{-1}. The stock solutions were stored at 5°C. They were thawed at ambient temperature and thoroughly mixed before use. A stock solution of 1-eicosanol, the internal standard (IS), with a concentration of 0.2 mg ml^{-1} in TBME was similarly prepared. Standard solutions were made daily by serially diluting the BFA and breflate stock solutions with human plasma to provide concentrations of 1.0, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025 and 0.010 μg ml^{-1}.

2.3. Sample preparation

Samples were prepared for analysis in 10 ml (16×100 mm) borosilicate glass culture tubes (Sun Brokers, Wilmington, NC) and centrifuge tubes (Fisher Scientific, Pittsburgh, PA) which were sealed with Teflon-lined phenolic screw caps. The tubes were washed with Alconox detergent (Alconox, New York, NY), thoroughly rinsed with distilled water and oven dried. Glassware used in the assay was not surface deactivated. The sample extraction solution was made daily by diluting the IS stock solution to a concentration of 33.3 ng ml^{-1} with TBME in a 100 ml volumetric flask. The TBME-IS solution was dispensed directly from the volumetric flask using a class A borosilicate glass volumetric pipette.

A 100 μl aliquot of plasma and 3 ml of TBME-IS solution were pipetted into a 10 ml centrifuge tube and tightly capped. After vigorous mixing on a vortex action stirrer for about 30 s, the sample was extracted for an additional 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI), then centrifuged for 4 min at $2500 \times g$. The upper organic phase was carefully transferred to a round-bottomed culture tube and concentrated to dryness under a stream of nitrogen using a Meyer N-EVAP (Organomation, Berlin, MA) with a bath temperature of 45–50°C. TBME (1 ml) and chilled neat TFAA (50 μl) were added directly to the tube containing the plasma extract, which was capped and mixed by vortexing. Derivatization was effected by placing the tube in a Dri-Bath (Barnstead/Thermolyne, Dubuque, IA) set to provide a solution temperature of 40°C. After heating for 4 h, the reaction mixture was cooled over ice, before adding methanol (50 μl) to the tube and vortexing (30 s) to consume the remaining TFAA. The sample was allowed to stand at ambient temperature for about 10 min then evaporated as described above. The residue was reconstituted with 100 μl of TBME, then transferred into a borosilicate glass conical insert which was placed in a 12×32 mm autosampler vial and sealed with a silicone/PTFE Snap-Cap (Scientific Resources, Somerset, NJ).

2.4. Chromatography

Analyses were performed using a 5890 Series II gas chromatograph (Hewlett-Packard) equipped with a capillary inlet system and a 5791A Mass Selective Detector (Hewlett-Packard), controlled through a DOS-series MS ChemStation (Hewlett-Packard, Palo Alto, CA). The inlet was fitted with a deactivated quartz direct injection liner (2.0 mm, I.D., 140 μl vol.) and operated in the splitless mode. Samples (5 μl) were introduced into the gas chromatograph using a model 7673 automatic

injector (Hewlett-Packard) with a 10 μl syringe (Hamilton, Reno, NV). Acetonitrile was used as the injector syringe wash solvent. Separations were performed on a 15 m × 0.25 mm fused silica capillary column wall-coated with 0.25 μm DB-5 cross-linked poly(5%-diphenyl-95%dimethyl)siloxane (J&W Scientific, Folsom, CA). Helium was employed as the carrier gas at a linear velocity of 37.5 cm s^{-1} (pentane, 60°C) using flow rates of 3 and 35 ml min^{-1} at the septum purge and split vents, respectively. Temperatures were 250°C at the injection port and 275°C at the transfer line to the detector. Injections were made at an initial oven temperature of 60°C. The inlet purge was activated at 1 min postinjection. The oven temperature was held isothermally at 60°C for 2 min, then increased linearly to 180°C at 10°C min^{-1} and finally ramped from 180°C to 275°C at 25°C min^{-1}. The final temperature was maintained for 2.2 min to desorb less volatile components of the sample. Mass spectral detection (EI, 70 eV) was performed by selected ion monitoring, sequentially measuring ions at m/z 58, 202 and 325, with a 75 ms dwell time. Data was collected between 5 and 15 min postinjection. The ion chromatograms were integrated to provide peak areas.

2.5. Quantitation

A series of plasma standards were prepared for analysis and run together with pharmacokinetic plasma specimens on a daily basis. Ratios of the chromatographic peak area for BFA or breflate to that of the IS were calculated. Standard curves were constructed by plotting the peak area ratios against the respective analyte concentration in the plasma standards. Linear least squares regression was performed using a weighting factor of $1/y_{obs}$, without inclusion of the origin, to determine the slope, y-intercept and correlation coefficient of the best fit line. Analyte concentrations in unknown samples were calculated using results of the regression analysis. Each unknown sample was initially assayed in duplicate on separate days, with additional analyses performed if the replicate determinations deviated from their average by $> 10\%$. Specimens with concentrations exceeding the upper limit of the standard curve were reassayed upon appropriate dilution with blank plasma.

2.6. Assay validation

Accuracy and precision of the assay were determined by analyzing the backcalculated sample concentrations and regression parameters from a series of standard curves of BFA and breflate in plasma that were prepared and analyzed on separate days. The R.S.D. of the mean predicted concentrations for the independently assayed standards provided the measure of precision. The LLQ was defined as the minimum concentration amenable to analysis with an inter-day R.S.D. not $> 20\%$. Accuracy of the assay was assessed by expressing the mean predicted analyte concentration as a percentage of its known concentration in the standard solutions.

2.7. Pharmacokinetics

Dosing and sample collection procedures were performed by Corning Hazleton (Vienna, VA) in full compliance with current NIH guidelines on the humane care and use of laboratory animals in research. Breflate was formulated as a 7.5 mg ml^{-1} solution in Sterile Water for Injection, USP, adjusted to pH 3.5–4.0 with 1 N hydrochloric acid. The dosing solution was passed through a 0.2 μm filter shortly before administration. A dose of 21.6 mg kg^{-1} breflate was given to a single male beagle dog over a period of 24 h by continuous i.v. infusion. Serial blood specimens (1.0 ml) were collected in disposable plastic syringes before dosing, at 11 time points during the infusion. Immediately upon collection, the blood was expelled into an Eppendorf heparin-coated polypropylene microcentrifuge tube (Curtin Matheson Scientific, Houston, TX), mixed by inversion, and centrifuged at 12 000 × g for 2 min. The plasma was separated, frozen over dry ice and maintained at −70°C until assayed. The actual dose was calculated from the body weight of the dog, gravimetric determination of the volume of dosing solution delivered and the assayed prodrug concentration in the formulation.

3. Results and discussion

3.1. Evaluation of HPLC methods

Initially, efforts were focused to develop a reversed-phase HPLC-based analytical method to quantitate both BFA and potential water-soluble prodrugs with greater sensitivity than achieved with the GC procedure for BFA alone [6]. Isocratic conditions to separate BFA and breflate were readily established. Briefly, chromatography was performed on a 3.9 mm × 15 cm Nova-Pak Phenyl column (Waters, Milford, MA) using a mobile phase composed of acetonitrile-potassium phosphate buffer (pH 7.2; 0.05 M) (26:74, v/v) containing 10 mM tetrabutylammonium hydrogen sulfate at a flow rate of 1.0 ml min^{-1}. Both compounds were efficiently isolated from plasma by liquid/liquid extraction with TBME, without alkalinization of the sample, even though the *N*,*N*-dimethylglycinyl promoiety is fully protonated at physiological pH. Unfortunately, sensitivity was limited by the absence of a strongly absorbing chromophore, fluorophore or an electrochemically active functional group. As a consequence, even though the TBME extracts of plasma specimens were sufficiently clean to enable elution of the analytes to be monitored by uv absorbance at 215 nm when the sample volume was restricted to 100 μl, as demonstrated in Fig. 2a, the LLQs for BFA and breflate were only about 0.1 and 0.25 μg ml^{-1}, respectively. The relative magnitude of endogenous peaks eluting in the presence of the analytes precluded the use of significantly larger sample volumes for greater sensitivity.

Attempts to enhance detectability of the two compounds by derivatization with fluorescent reagents prior to HPLC, although intensively evaluated, proved unsatisfactory. It was then observed that BFA and breflate responded directly to thermospray ionization MS following HPLC. The mobile phase was modified by replacing the potassium phosphate buffer, which is incompatible with thermospray LC/MS detection, with buffers containing an ammonium salt. An eluent composed of acetonitrile-ammonium acetate buffer (pH 4.7; 0.15 M) (30:70, v/v), at a flow rate of 0.7 ml min^{-1}, afforded optimum detector response for the two analytes and a very satisfactory separation on the Nova-Pak Phenyl column (Fig. 2b). Despite encouraging preliminary results, instrumental parameters that provided maximum sensitivity could not be controlled with acceptable precision. The inherent fluctuations in operating conditions during and between runs, while seemingly minor, resulted in unacceptably large variations in detector response. Using instrumental

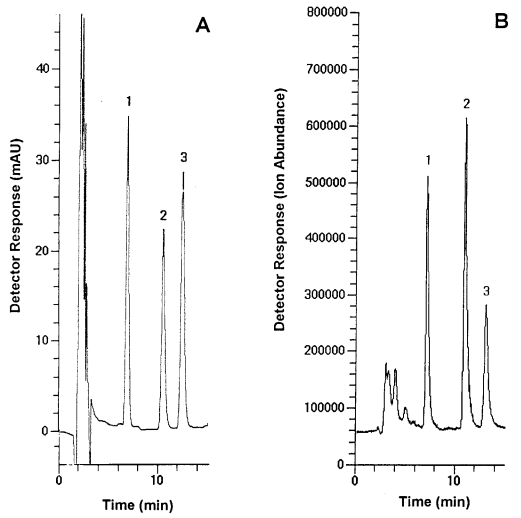


Fig. 2. Liquid chromatograms of plasma, to which brefeldin A ((A), 5 μg/ml; (B), 6 μg/ml) and breflate ((A), 0.5 μg/ml; (B), 0.6 μg/ml) were added at a concentration of about 0.5 and 0.6 μg/ml, respectively, monitored by UV absorbance at 215 nm (A) and thermospray ionization MS with selected ion detection (B). The samples (100 μl) were prepared for analysis by liquid/liquid extraction with TBME as described in Section 2. Separation conditions and detection parameters were as follows: (A) column, Nova-Pak Phenyl (4 μm, 3.0 mm × 15 cm); mobile phase, acetonitrile-potassium phosphate buffer (pH 7.2; 0.05 M) (26:74, v/v) with 10 mm tetrabutylammonium hydrogen sulfate; flow rate, 1.0 ml min^{-1}; flow cell, 10 mm path length; slit, 8 nm; sample wavelength, 215 nm (10 nm bandwidth); reference wavelength, 325 nm (50 nm bandwidth); (B) column, Nova-Pak Phenyl (4 μm, 3.0 mm × 15 cm); mobile phase, acetonitrile-ammonium acetate buffer (pH 4.7; 0.15 M) (30:70, v/v); flow rate, 0.7 ml min^{-1}; ion source temperature, 290°C; discharge ionization, on; fragmentor electrode, 0 V; stem temperature, 112°C; ions monitored: IS, *m*/*z* 204.20, BFA, *m*/*z* 245.25 and 263.25, breflate, *m*/*z* 366.35; dwell time, 400 ms. Chromatographic peaks: 1, brefeldin A; 2, breflate; 3, IS (7-dimethylamino-4-methylcoumarin).

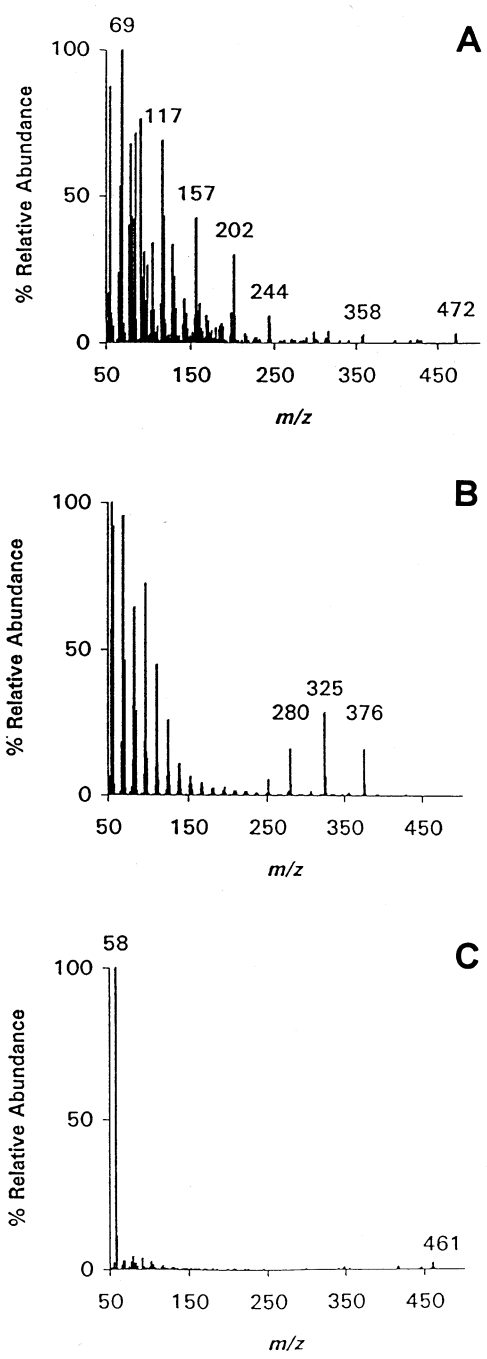


Fig. 3. Electron ionization (70 eV) mass spectra (50–500 u) acquired during GC separation of the *O*-trifluoroacetate derivatives of brefeldin A (A), 1-eicosanol (the IS; (B)), and breflate (C).

parameters to achieve the minimal reproducibility required for quantitative analysis, the sensitivity of thermospray LC/MS with selected ion monitoring for BFA and breflate was comparable only to that achieved by HPLC with UV detection.

3.2. Gas chromatography/mass selective detection

As previously described [6], BFA is not amenable to direct GC analysis, presumably due to insufficient volatility combined with thermal instability. However, esterification of its two 2° hydroxyl groups with heptafluorobutyrylimidazole afforded a derivative that was readily separable by GC. This reagent was chosen primarily to enable detection by electron capture. Aside from sensitivity issues, the method was not applicable to the analysis of potential amino acid ester prodrugs of BFA, such as breflate, since their heptafluorobutyryl derivatives eluted among strongly retained derivatives of endogenous constituents extracted from plasma. Accordingly, an alternate esterification reagent was required to evaluate the potential of GC with mass selective detection as the basis of a specific assay for BFA and breflate in plasma with improved sensitivity. TFAA proved to be ideal in this application since it is readily available from commercial sources in high purity and is relatively easy to handle. Reaction with a large molar excess of TFAA in TBME at 40°C reproducibly converted both BFA and breflate into their corresponding *O*-trifluoroacetate esters within 4 h. Rigorously anhydrous conditions were not required. In fact, the reaction proceeded without even taking precautions to exclude atmospheric water vapor from contacting the reagent or reaction mixture. Thus, the procedure was amenable to the batchwise preparation of a large number of samples for analysis. The trifluoroacetate derivatives of BFA and breflate were readily separable by capillary GC, had molecular weights within the scanning capability of the mass selective detector (i.e. < 600 d), and exhibited adequate stability in TBME to permit overnight analyses using an autosampler.

Under the chromatographic conditions employed, the di-*O*-trifluoroacetate derivative of BFA eluted at 8.6 min and yielded a mass spec-

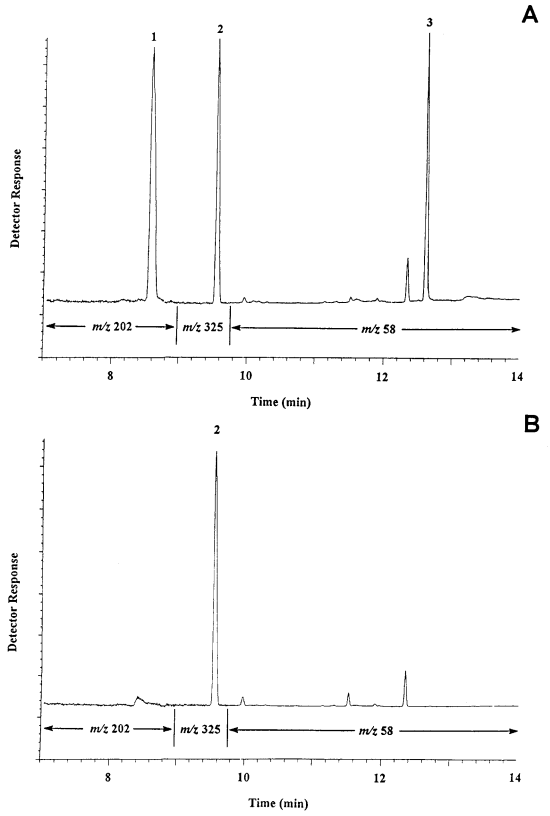


Fig. 4. Time-selected ion profile reconstructed from data acquired during GC separation with selected ion detection of (A) an extract of plasma spiked with BFA and breflate, each at a concentration of 0.75 μg ml^{-1} and (B) an extract of plasma to which only the IS had been added. Both samples were treated with TFAA prior to GC/MS analysis. Peak assignments: 1, brefeldin A; 2, IS (1-eicosanol); 3, breflate.

trum (Fig. 3a) showing a molecular radical cation (m/z 472) of moderate relative abundance (3%), and a substantial number of fragments. The presence of fragment ions at m/z 358 (3%) and m/z 244 (10%) can be attributed to the loss of one and two molecules of trifluoroacetic acid, respectively. The fragment ion at m/z 202 (31%) represents further loss of the elements of ketene from m/z 244. These assignments were verified by exact mass measurements. The uniqueness and relatively high abundance of m/z 202 (31%) was key to choosing this particular ion for specifically monitoring BFA in extracts of plasma specimens. GC/MS analysis of a blank plasma extract treated with TFAA (Fig. 4b) and plasma spiked with BFA that was similarly prepared (Fig. 4a) clearly demonstrates the uniqueness of the m/z 202 ion for specific detection of the BFA derivative.

The *O*-trifluoroacetate derivative of 1-eicosanol (used as the IS) eluted at 9.6 min and yielded a mass spectrum (Fig. 3b) showing a barely detectable molecular radical cation at m/z 394. The ion chosen for the assay is an appreciably abundant, high mass fragment ion (m/z 325, 28%), which results from the molecular radical cation losing a trifluoromethyl radical (CF_3).

In contrast to BFA, GC/MS analysis of the *O*-trifluoroacetate derivative of breflate, which exhibited a retention time of 12.6 min, afforded a mass spectrum (Fig. 3c) showing a molecular radical cation (m/z 461) of moderate-to-low relative abundance (2%) and a single major fragment at m/z 58 (100%). The base peak corresponds to ionization at the nitrogen center of the dimethylamino group followed by α-cleavage. While low mass ions are not commonly used for selected ion detection, comparison of the chromatograms obtained by analyzing extracts of blank plasma (Fig. 4b) and plasma spiked with breflate (Fig. 4a) by this procedure demonstrates that m/z 58 is adequately unique for specifically monitoring the derivative of breflate.

3.3. Accuracy and precision

1-eicosanol was used as an IS for the assay. It was added during the initial extraction step and thereby served to monitor recovery of the analytes from plasma and consistency of the derivatization procedure. In addition, since quantitation was based upon the chromatographic peak area of the analyte relative to that of the IS, the IS also served to minimize variability associated with manual sample manipulations and the autosampler.

The BFA-to-IS and breflate-to-IS peak area ratios were directly proportional to the added concentration of the respective analyte in plasma over the range 0.010–1.0 μg ml^{-1}. Linear regression performed with weighting according to the reciprocal of the observed peak area ratio yielded the best fit of the standard curves. Accuracy and

Table 1
Accuracy and precision for assaying brefeldin A and breflate in plasma[a]

Concentration added (μg ml^{-1})	Brefeldin A		Breflate	
	Recovery (%)	Precision (%)	Recovery (%)	Precision (%)
1.0	100.1	2.53	110.4	4.43
0.75	99.0	2.20	101.1	7.02
0.50	101.4	2.54	93.7	7.94
0.25	99.5	3.57	95.8	10.58
0.10	100.9	5.93	90.0	15.43
0.075	96.9	6.51	83.0	15.58
0.050	100.5	12.72	93.4	12.45
0.025	100.9	8.67	111.8	21.00
0.010	103.8	21.54	135.7	16.58

[a] Accuracy and precision of the assay were assessed from 14 standard curves of brefeldin A and breflate in plasma analyzed over an 8-week period.

precision of the method were assessed from 14 standard curves of BFA and breflate in plasma assayed during an 8-week period. The BFA standard curves had a mean slope of 3.91 ± 0.43 (S.D.), a value of 0.0196 ± 0.0223 for the y-intercept, with a correlation coefficient of 0.999 ± 0.001. Mean values ($\pm$S.D.) of the regression parameters for the breflate standard curves were: slope, 110.1 ± 12.82; y-intercept, -0.787 ± 0.771; and correlation coefficient, 0.991 ± 0.009. As shown in Table 1, the R.S.D. for inter-day quantitation of BFA in plasma was generally $< 10\%$ at concentrations > 0.025 μg ml^{-1}, increasing to 21.5% at 0.010 μg ml^{-1}, the lowest concentration included in the standard curve. The mean recovery of BFA in the plasma standards ranged from 96.9 to 101.4% of the added concentration. Quantitation accuracy of BFA as measured by recovery did not appear to be dependent upon its concentration. There was a noteably greater degree of variability and diminished accuracy for the quantitation of breflate in comparison to the parent drug. Nevertheless, the reproducibility was still generally better than 20% with recoveries ranging from 83.0 to 135.7% at concentrations encompassing the standard curve. On the basis of this data, the LLQ for both BFA and breflate was established as 10 ng ml^{-1} in plasma, corresponding to about 25 pg of the (underivatized) compounds injected onto the column.

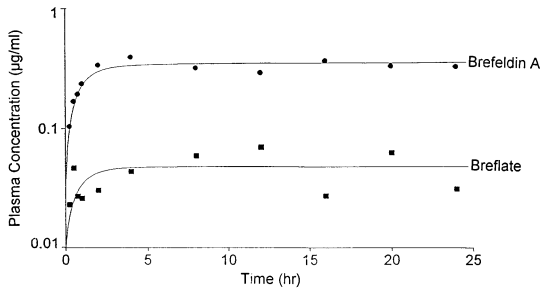


Fig. 5. Plasma concentration-time profiles of brefeldin A (●) and breflate (■) determined during the continuous i.v. infusion of breflate at a rate of 0.9 mg kg^{-1} hr^{-1} to a male beagle dog. The GC/MS method was used for the analysis of serial plasma specimens acquired from the dog. The apparent steady state concentrations of breflate and brefeldin A in plasma were 0.129 μM (0.047 μg ml^{-1}) and 1.207 μM (0.338 μg ml^{-1}), respectively.

It should be noted that when only breflate was added to blank plasma and the sample then processed and analyzed as described above, BFA was not detectable, thereby indicating that the prodrug is not subject to significant conversion to BFA during the preparation of samples for analysis. Furthermore, there were no significant differences between the concentrations of BFA and breflate in plasma specimens that were reassayed after storage at -70°C for a period of 14 months (data not shown).

3.4. Plasma pharmacokinetics of breflate in a dog

The GC/MS assay was found to be suitable for routine use during pharmacokinetic studies to determine the steady state plasma concentrations of BFA and breflate provided by continuous i.v. infusion of the prodrug. Fig. 5 shows the plasma concentration-time profiles of the drug and the prodrug in a male beagle dog treated with 0.9 mg kg^{-1} hr^{-1} of breflate for 24 h. Onset of steady state for both compounds was achieved at ≈ 4 h after starting the infusion. The apparent steady state plasma concentration of BFA, 1.207 μM (0.338 μg ml^{-1}), was about 10 fold higher than the administered prodrug, 0.129 μM (0.047 μg ml^{-1}), indicative of its facile conversion to the parent drug upon presentation to the blood stream.

4. Conclusions

A specific assay for concurrently determining BFA and breflate, a prodrug for parenteral administration, has been developed. The method involves preliminary isolation of the two compounds from plasma by extraction into TBME followed by derivatization with TFAA. The resulting derivatives were separated by GC and detected by EI-MS with selected ion monitoring. The lowest concentration of either analyte that could be quantified with acceptable reproducibility (R.S.D. $< 20\%$) in 100 μl of plasma was near 0.010 μg ml^{-1}. The assay has been shown to be specific, accurate and reproducible, thereby rendering the procedure appropriate for monitoring plasma levels of the drug and prodrug during pharmacokinetic studies.

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