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Negative ion electrospray high-performance liquid chromatography–mass spectrometry method development for determination of a highly polar phosphonic acid/sulfonic acid compound in plasma Optimization of ammonium acetate concentration and in-source collision-induced dissociation

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Abstract

A method, based on negative ion electrospray ionization (ESI) single-stage mass spectrometry coupled with HPLC, was developed for the determination of a squalene synthase inhibitor, BMS-187745, in human plasma. BMS-187745, a highly polar compound with both phosphonic acid and sulfonic acid groups, presented difficulties in developing plasma extraction and HPLC procedures. Precipitation of the plasma protein with methanol was finally chosen as the basis for sample preparation since extraction with water-immiscible solvents or with solid-phase extraction columns failed. It was essential to add ammonium acetate to the HPLC mobile phase, not only to enhance the retention of BMS-187745 but also to ensure a well-shaped chromatographic peak. While the use of ammonium acetate had the desired chromatographic effects, it had the undesirable consequence of suppressing the negative ion ESI signal. With the plasma extracts, the $[M–H_2O–H]^-$ ion (m/z 367) showed significantly lower chemical noise than the $[M–H]^-$ ion (m/z 385), and was thus chosen as the analytical ion for the selected ion monitoring. The signal of the m/z 367 ion was significantly enhanced by the optimization of the in-source collision-induced dissociation (CID) of m/z 385 to m/z 367. © 1997 Elsevier Science B.V.

Keywords: BMS-187745; Enzyme inhibitors

1. Introduction

The blockade of cholesterol biosynthesis has been established as a method for the treatment of hypercholesterolemia in humans [1,2]. BMS-187745 (Fig. 1) blocks cholesterol biosynthesis via the inhibition of the enzyme squalene synthase. This enzyme catalyzes the reductive dimerization of farnesyl diphosphate (FPP) to form squalene, which is subsequently converted to cholesterol [3]. We were charged with the task of developing a bioanalytical method for the quantitative determination of BMS-187745 in human plasma samples. In this paper, we describe the method development approaches and the optimization of the conditions for the HPLC, nega-

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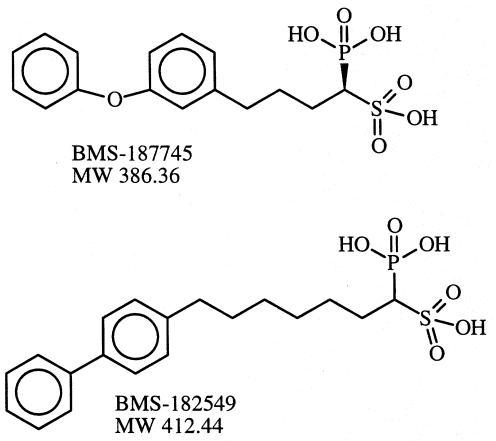

Fig. 1. Structures of BMS-187745 and BMS-182549, the analytical internal standard.

tive ion electrospray mass spectrometry and the plasma extraction.

2. Experimental

2.1. Reagents and chemicals

BMS-187745 (in the adamantanamine salt form, lot 34942-118-32) and the internal standard BMS-182549 (in the free acid form, batch 2) were characterized products of Bristol-Myers Squibb Pharmaceutical Research Institute. Methanol, HPLC grade, acetonitrile, HPLC grade, ammonium acetate, ACS grade, and triethylamine, ACS grade, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). House deionized water, further purified with a Milli-Q water purifying system (Millipore Corporation, Bedford, MA, USA), was used.

Drug-free human plasma was obtained from Biological Specialty (Colmar, PA, USA). Mobile phase A was Milli-Q water; mobile phase B was methanol; mobile phase C was 1 *M* ammonium acetate in water. Reconstitution solution was prepared by combining 25 ml of methanol with 75 ml of water.

2.2. Equipment

The HPLC–MS analysis was performed with a Hewlett-Packard (Avondale, PA, USA) 5989B mass spectrometer with electrospray interface equipped with a hexapole ion guide and a Windows-based (DOS) software, G1047A, version C.02.00. The mass spectrometer was coupled to a Hewlett-Packard (Palo Alto, CA, USA) 1090L HPLC system equipped with a ternary pumping unit and a variable volume automatic sampler. The HPLC column was YMC Basic, 2.0×100 mm from YMC (Willmington, NC, USA). The IEC centrifuge, model PR-7000M (IEC, Needham Hts, MA, USA), was used. Disposable polyethylene transfer pipets with extended fine tips (1-ml draw, 10.4 cm, and 3-ml draw, 15.3 cm) were purchased from Fisher Scientific (Fair Lawn). Polypropylene conical microvials (0.25 ml) were obtained from Sun Brokers (Wilmington, NC, USA). Savant concentrator, model AES2000-220, was purchased from Savant Instruments (Farmingdale, NY, USA). Cryule vials with color-coded caps (Wheaton, 2 ml) were purchased from Baxter Scientific Products (McGaw Park, IL, USA).

2.3. Chromatographic and mass spectrometric conditions

A gradient mobile-phase scheme (Table 1) consisting of mobile phases A, B, and C (Section 2.1) was used. The HPLC column was maintained at 40°C. The flow-rate through the HPLC column was 0.4 ml/min and the entire effluent was directed to

Table 1
Mobile-phase gradient system

Time (min)	% of mobile phase B	% of mobile phase C	Flow-rate (ml/min)
0	5	2.5	0.4
5	90	2.5	0.4
6	90	2.5	0.4
7	5	2.5	0.4
8	5	2.5	0.4

Mobile phase A, water; mobile phase B, methanol; mobile phase C, 1 *M* ammonium acetate in water.

the mass spectrometer. The injection volume was 25 μl. The mass spectrometer was operated in the negative ion mode using a high purity house nitrogen as the nebulizing gas (set at 80 psig) and as the drying gas (set at 50 psig). The drying gas temperature was set to 290°C. The quadrupole temperature was maintained at 150°C. The lens voltages were optimized daily. Typical voltage values used were: V_{cap} +3000, V_{end} +2000, V_{cyl} +4000, Cap Ex −170. The dwell time was set to 50 ms.

2.4. Preparation of stock solutions

Two separate stock solutions of BMS-187745, one to be used for standard curve (stock solution A) and the second for the QC samples (stock solution B), were prepared during validation of the method. For each stock solution, accurately weighed (13.16 mg for the standard curve and 12.98 mg for the QC samples) BMS-187745 was dissolved in 25 ml of a 50:50 mixture of acetonitrile and water in a 10-ml volumetric flask. The stock solution of the internal standard (BMS-182549) was prepared in a 25-ml volumetric flask in 100% water, using 10.77 mg of BMS-182549. Appropriate dilutions of the stock solution were prepared by diluting with water. All solutions were kept refrigerated (4°C) when not in use.

2.5. Standard and QC preparations

The standard curve consisted of nine concentrations, each in duplicate, each prepared by spiking the specified amount of BMS-187745 into a specified volume of drug-free human plasma. The curve range was 21.5–2690 ng of BMS-187745/ml of human plasma. The highest concentration standard was prepared by diluting the appropriate volume of stock solution A (described in Section 2.4) to 10 ml with drug-free human plasma. The rest of the standards were prepared from the highest concentration standard through serial dilutions with drug-free human plasma. Three levels of QCs, prepared in drug-free plasma and stored at −20°C until analysis, were used for method validation. The three QC levels were in the first quartile, near the mid-point, and in the fourth quartile of the curve. The QCs were prepared by diluting the appropriate volume of the stock solution to 50 ml with drug-free human plasma. The QC samples were frozen in 1.5-ml aliquots using cryule vials at −20°C.

2.6. Extraction and reconstitution

To a 0.5-ml portion of each human plasma standard and QC in a 16×100 mm screw cap test tube, 25 μl of the internal standard working solution (to obtain 4040 ng/ml of plasma) was added. To each plasma sample, methanol (3 ml) was added, and the sample tubes were then vortexed. The samples were centrifuged using the 3500 rpm setting of the IEC centrifuge. The supernate from each tube was transferred to a 13×100 mm tube. To each transferred supernate, 0.5 ml of water and 2 ml toluene were added and the tube vortexed. The tubes were then centrifuged at 3500 rpm for 10 min. The toluene (upper) layer from each sample was removed and discarded. The tubes were then dried in a Savant evaporator on the high setting for approximately 3 h.

Each dried extract was dissolved in 100 μl of reconstitution solution. The test tubes were vortexed on a multitube vortexer for 1 min and each sample was transferred to a microcentrifuge tube and centrifuged at 5000 rpm for 10 min. The solution from each tube was then transferred to a polypropylene conical microvial using a transfer pipet with an extended fine tip (1 ml draw). The vials were crimp-capped, ready for injection.

2.7. Analysis

The samples were analyzed via selected ion monitoring (SIM), employing the $[M-H-H_2O]^-$ ion of BMS-187745 (m/z 367) and the $[M-H]^-$ ion of the internal standard BMS-182549 (m/z 411).

3. Results and discussion

Because BMS-187745 was a highly polar compound containing sulfonic and phosphonic acid groups, it was anticipated that established extraction approaches such as liquid–liquid extraction with water-immiscible solvents and reversed-phase solid-phase extraction (SPE) would not be feasible. This was confirmed by experiments with phenyl, C_{18} and

cyclohexyl (CH) SPE extraction columns, which showed little retention of the analyte. We then assessed the feasibility of applying an approach reported in the literature [4–6], where retention of sulfate compounds on reversed-phase SPE columns was achieved by adding a high concentration of ammonium acetate to the sample before loading onto the SPE columns. Thus, to the plasma samples containing BMS-187745, we added ammonium acetate, at a concentration ranging from 5 to 20% w/v, before loading onto the SPE columns (C_{18}, CH and phenyl). Although decent recovery of the analyte was achieved in some cases, the results were inconsistent and the approach was thus deemed unreliable and abandoned. Anion-exchange SPE also gave low and inconsistent recoveries. With the failure of the extraction approaches that were expected to provide cleaner extracts, we resorted to the plasma protein precipitation approach, which is the least desirable approach in terms of expected extract cleanliness. Among the three plasma protein precipitating solvents investigated (methanol, acetone and acetonitrile), methanol gave the highest recovery, with acetone a close second. Thus, methanol was chosen. Washing the methanol extract with a less polar organic solvent prior to evaporation of the methanol made the extract physically cleaner as evidenced by the ease of reconstituting the dried extract to obtain a clearer solution. After investigating toluene, *n*-hexane, ethyl acetate, methyl *tert*.-butyl ether and dichloromethane, toluene was chosen as the wash solvent based upon the ESI response, clarity of the reconstituted solution and ease of use (e.g. distinctness of the methanol layer from the added organic solvent layer). A reconstitution solution of 25% methanol and 75% water was chosen based on the ESI response and clarity of the extract after reconstitution.

The negative ion electrospray mass spectra for BMS-187745 at different capillary exit voltage values are shown in Fig. 2a–c. At a capillary exit value of −100 V, only m/z 385, due to $[M-H]^-$, was seen. At and beyond the capillary exit voltage of −150 V, m/z 367, due to $[M-H-H_2O]^-$, was seen, in addition to m/z 385. At about −200 V, the m/z 367 response was approaching that of the m/z 385. At and beyond the capillary exit voltage of −200 V, m/z 79 was seen, in addition to m/z 367 and m/z 385. At −400 V, an additional fragment at m/z 63 was seen. The signals at m/z 79 and m/z 63 are due to $[PO_3]^-$ and $[PO_2]^-$, respectively [7,8]. Thus, by increasing the capillary exit voltage gradually from −100 to −400 V, the formation of the product ions due to the in-source CID [9–13] was clearly demonstrated. Increasing the capillary exit voltage affected not only the relative response at the different m/z values but also the absolute total response (i.e., the sum of responses at the different m/z values). For instance, the total response at −250 V was only about 10% of the total response at −150 V.

With the plasma extracts, we found out that the m/z 385 channel had extraneous peaks interfering with the analyte signal, whereas the m/z 367 channel was relatively clean. The m/z 79 channel was also investigated and found to have extraneous signals interfering with the analyte signal. Thus, the m/z 367 channel was finally adopted as the analytical ion for the selected ion monitoring, although the m/z 385 signal of the analyte was larger than the m/z 367 signal of the analyte at all capillary exit voltage values. The capillary exit voltage that gave the highest absolute response for m/z 367 was utilized. This voltage value, which was optimized daily, ranged from −160 to −180 V. For the internal standard, the m/z 411 channel, due to $[M-H]^-$, was utilized. No apparent advantage was seen in using the m/z 393 channel, due to $[M-H-H_2O]^-$.

Because we had a preliminary indication that the presence of any additive (e.g. ammonium acetate) in the mobile phase would diminish the electrospray response of the analyte, our initial objective was to develop and validate a method that did not utilize any additive in the mobile phase. For this, several HPLC columns were investigated by chromatographing a BMS-187745 standard (with no plasma) using a water/acetonitrile mobile phase. Only a YMC Basic column, provided the required retention of the analyte (>4 times the void volume) with a well-shaped chromatographic peak. Other columns failed to meet this criterion. For instance, a YMC Polymer C_{18} gave a broad tailing peak near the void volume, whereas a BDS Hypersil phenyl column gave a well-shaped peak eluting at the void volume.

Following repeated exposure to plasma extracts, even the YMC Basic column required the incorporation of a mobile phase additive in order to maintain a

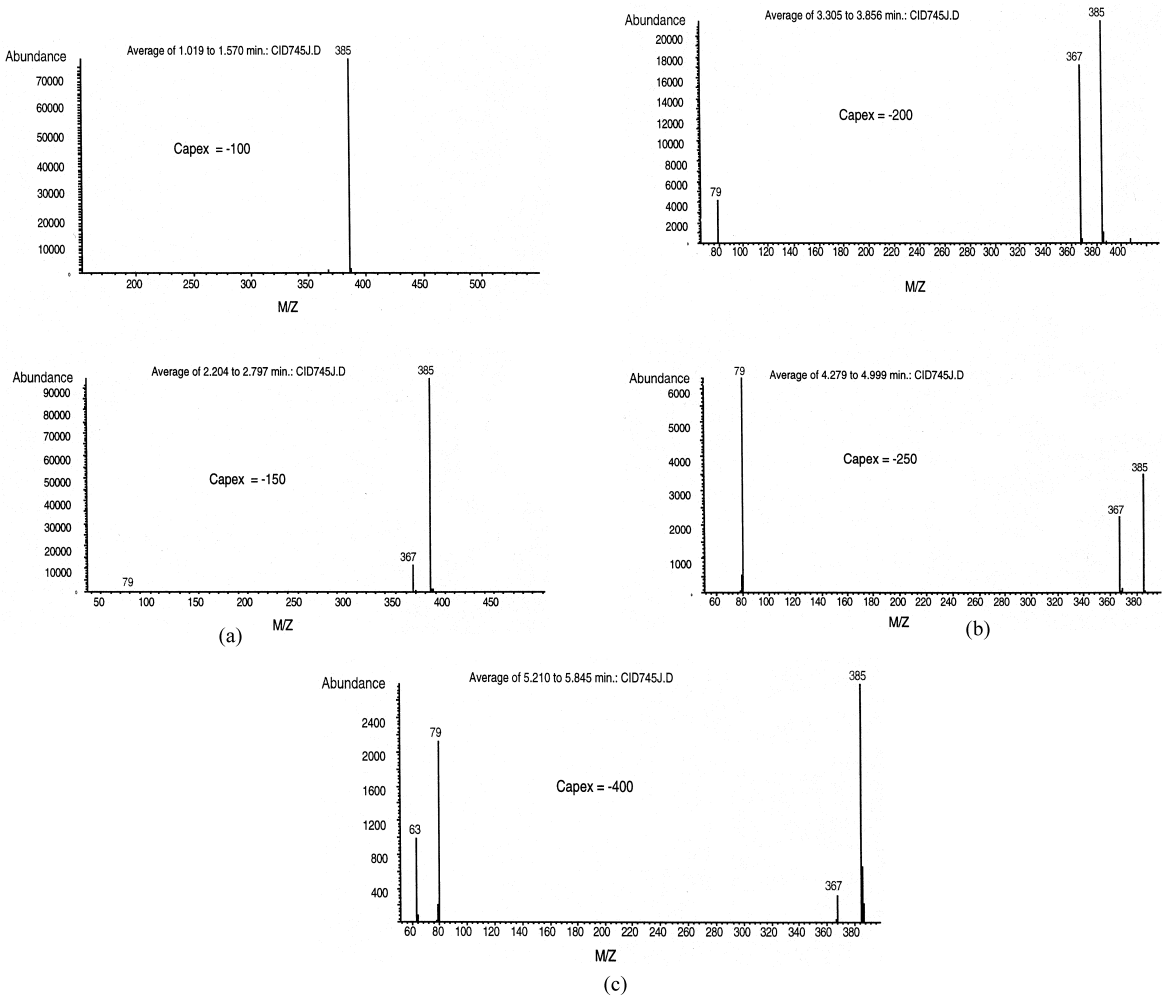

Fig. 2. (a) Negative ion electrospray mass spectra of BMS-187745 at capillary exit voltage values of −100 and −150 V; m/z 385 due to $[M–H]^-$ and m/z 367 due to $[M–H–H_2O]^-$. (b) Negative ion electrospray mass spectra of BMS-187745 at capillary exit voltage values of −200 and −250 V; m/z 385 due to $[M–H]^-$, m/z 367 due to $[M–H–H_2O]^-$ and m/z 79 due to $[PO_3]^-$. (c) Negative ion electrospray mass spectrum of BMS-187745 at capillary exit voltage value of −400 V; m/z 385 due to $[M–H]^-$, m/z 367 due to $[M–H–H_2O]^-$, m/z 79 due to $[PO_3]^-$ and m/z 63 due to $[PO_2]^-$.

good analyte peak shape that was well removed from the void volume. The mobile phase additives investigated early on included: ammonium acetate with unadjusted pH, ammonium acetate with pH adjusted to 5.0 with acetic acid, triethylamine with pH adjusted to 9.0 with acetic acid, triethylamine with pH adjusted to 5.0 with acetic acid. The addition of ammonium acetate or triethylamine to the mobile phase not only increased the analyte retention on the column but also improved the analyte peak shape. We subsequently focussed on the use of ammonium acetate with unadjusted pH because of its simplicity and the ruggedness of the HPLC column under this condition.

Fig. 3a–c depict the effect of different concentrations of ammonium acetate in the mobile phase on the peak shape, peak retention time and peak size (response) using a YMC Basic HPLC column that had previously been exposed to plasma extracts. The analyte retention increased with an increase in the

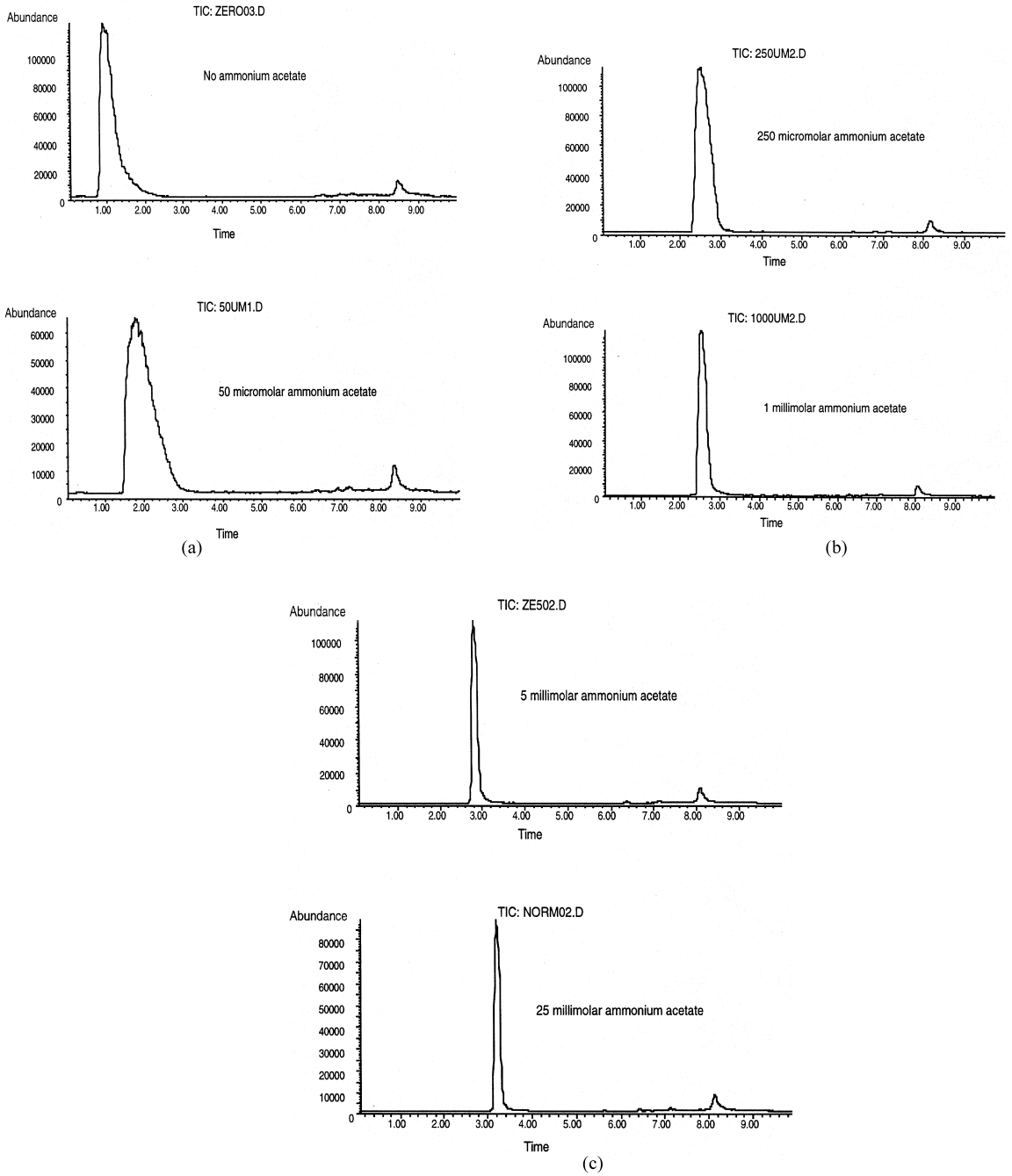

Fig. 3. (a) Chromatograms of a plasma extract containing BMS-187745, with ammonium acetate in the mobile phase at 0 and 50 μ*M*. (b) Chromatograms of a plasma extract containing BMS-187745, with ammonium acetate in the mobile phase at 250 and 1000 μ*M*. (c) Chromatograms of a plasma extract containing BMS-187745, with ammonium acetate in the mobile phase at 5 and 25 m*M*.

ammonium acetate concentration, from about 1.0 min at zero concentration to 3.2 min at a concentration of 25 m*M*. The chromatographic peak shape gradually improved (sharper and more symmetrical) with increasing ammonium acetate concentration. On the other hand, the analyte electrospray response (as measured by the area of the chromatographic peak) decreased with increase in ammonium acetate concentration. The response with 25 m*M* ammonium acetate was about 20% of the response with zero or 50 μ*M* ammonium acetate. The decrease in mass spectrometric response due to the presence of ammonium acetate was more dramatic when the sample was introduced via infusion; for instance, the response with 5 m*M* ammonium acetate was only about 10% of the response with no ammonium acetate. Considering the desirable effects on the HPLC and undesirable effects on the ESI response, an ammonium acetate concentration of 2 m*M* was selected and utilized for some time. However, after a prolonged exposure of the HPLC column to plasma extracts, the 2 m*M* concentration was not adequate to maintain peak shape and/or peak retention. The finally adopted concentration was 25 m*M*.

The method parameters described in Section 2 were finally adopted to validate the method. The validated method was then successfully used to analyze human plasma samples from clinical studies. The results obtained during validation are briefly presented below.

Extraction recovery of BMS-187745 from plasma was determined by comparing the response of the pre-spiked sample against the response of the post-spiked sample. The pre-spiked sample was prepared by spiking the plasma with both BMS-187745 and the internal standard before initiating the plasma extraction described in Section 2.6. BMS-187745 was spiked at three concentrations while keeping the internal standard concentration the same. The post-spiked sample was prepared by spiking the plasma with only the internal standard, extracting the plasma sample and then spiking the methanol extract with BMS-187745, at the same levels utilized for the pre-spiked samples. Extraction recovery of BMS-187745 from plasma at each concentration was determined by dividing the area ratio (area response of analyte divided by the area response of internal standard) of the pre-spiked sample by the area ratio of the post-spiked sample. As shown in Table 2, the extraction recovery of BMS-187745 from human plasma at the three concentrations evaluated ranged from 68 to 78%.

Table 2
Extraction recovery of BMS-187745 from human plasma

BMS-187745 concentration (ng/ml)	% recovery
269	78
1080	68
2690	78

During the validation of the method, it was determined that a linear regression (weighted to $1/x$, where x is the concentration in ng/ml plasma) was well suited to get the best fit for the curve range used. The results of a typical standard curve are shown in Table 3. The accuracy of each standard point and the precision of the duplicate points were good. Table 4 presents the summary of the plasma QC results obtained during the method validation on three different days. The inter- and intra-day C.V. was $\leq$12 and $\leq$9%, respectively. The deviation of the grand mean from the nominal value was $\pm$3% at all levels.

Fig. 4 shows the chromatogram of a plasma sample that contains BMS-187745 at the lowest concentration of the standard curve range (21.5 ng/ml), and Fig. 5 shows the chromatogram of a plasma sample containing BMS-187745 at a higher concentration (1080 ng/ml).

BMS-187745 QC samples in human plasma were found to be stable for at least 6 months at -20°C at all concentrations evaluated. The QC samples were stable after they were subjected to a three-cycle freeze–thaw process.

4. Conclusion

We have shown that in the HPLC-negative ion electrospray of a highly polar compound containing sulfonic and phosphonic acid groups, the use of ammonium acetate in the mobile phase has the desirable effect of prolonging the retention of the analyte on the reversed-phase HPLC column and

Table 3
Regression analysis results of a typical standard curve in human plasma

Nominal concentration (ng/ml)	Deviation from nominal concentration (%)	C.V. (%)
21.5	−18.5, +1.3	15
43.0	−17.3, +2.2	15
86.1	+9.2, 16.2	4.4
269	−6.2, +8.0	9.9
538	+1.8, +4.5	1.8
1080	−2.0, −1.0	0.7
1610	+4.4, +3.7	0.5
2150	−1.4, −2.6	0.8
2690	−3.4, +1.1	3.2

The two values given under the heading Deviation from nominal concentration are for the duplicate standards at each level. The C.V. (%) values give the precision of the duplicate standard points. The regression parameters are: intercept=0.0215; slope=0.000282; R^2=0.999.

Table 4
Summary of the results of human plasma QC samples analyzed on three different days

Nominal concentration (ng/ml)	Mean concentration (ng/ml)	Deviation from nominal concentration (%)	Inter-day precision C.V. (%)	Intra-day precision C.V. (%)
265	260	−1.9	11.6	9.1
1060	1030	−2.8	6.4	6.1
2120	2140	+0.9	2.0	4.3

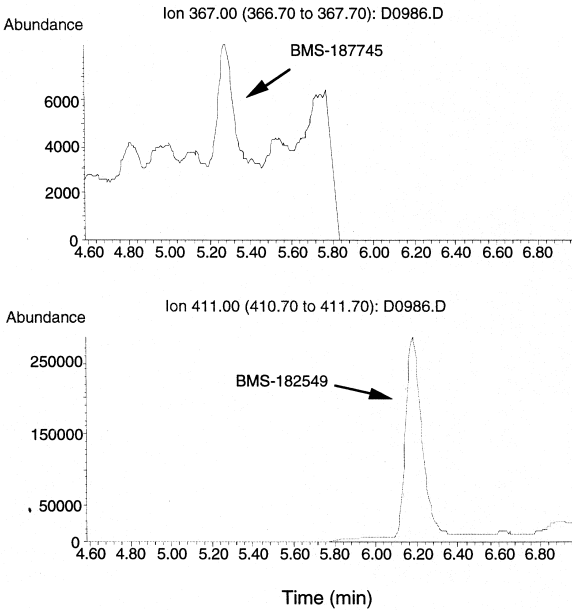

Fig. 4. Chromatogram of the extract of a plasma sample containing BMS-187745 at 21.5 ng/ml and the internal standard BMS-182549 at 4040 ng/ml of plasma.

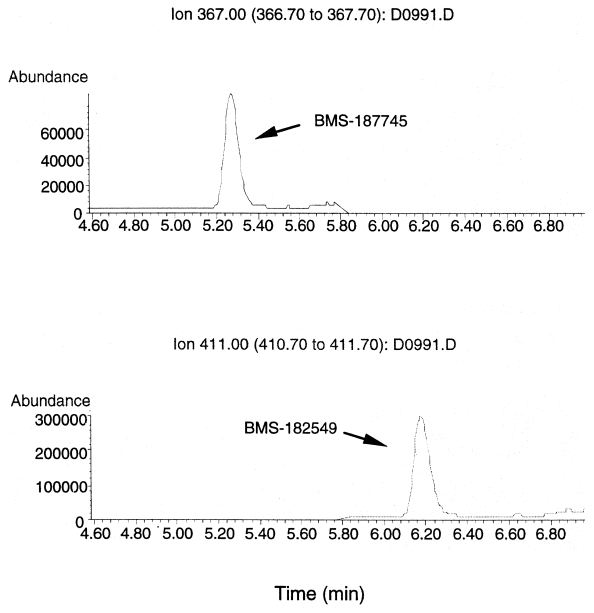

Fig. 5. Chromatogram of the extract of a plasma sample containing BMS-187745 at 1080 ng/ml and the internal standard BMS-182549 at 4040 ng/ml of plasma.

improving the peak shape, but has the undesirable effect of diminishing the electrospray response. To maintain the desirable chromatographic effects following repeated exposure of the HPLC column to plasma extracts, an adequate concentration (about 25 m*M*) of ammonium acetate had to be used. We have also shown that a product-ion obtained via in-source CID was a better analytical ion than the precursor ion, as the plasma extract was cleaner in the product-ion channel than in the precursor-ion channel. For highly polar compounds, such as BMS-187745, protein precipitation is the most practical approach for plasma sample extraction, since other traditional approaches do not provide adequate and reliable recovery of the analyte.

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