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Quantitative determination of BMS186716, a thiol compound, in dog plasma by high-performance liquid chromatography–positive ion electrospray mass spectrometry after formation of the methyl acrylate adduct

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Abstract

As it is extremely unstable in blood, the thiol compound BMS186716 was stabilized by the addition of methyl acrylate (MA) to blood samples. The blood samples were then kept in ice for 10–15 min for completion of the Michael addition reaction to occur between the thiol group of BMS186716 and MA, after which the plasma was separated by centrifugation under refrigeration. For sample analysis, the standard and quality control samples were prepared by spiking blank plasma with the BMS186716–MA adduct. After addition of the internal standard, BMS188035–MA, each sample was acidified with HCl and then extracted with methyl *tert.*-butyl ether. Each reconstituted extract was injected into a high-performance liquid chromatography–positive ion electrospray ionization mass spectrometric system. The electrospray condition was chosen to enhance the $[M+NH_4]^+$ signal at the expense of the $[M+H]^+$ signal. Monitoring the $[M+NH_4]^+$ signal, a lower limit of quantitation of 2.5 ng/ml was achieved, with 0.5 ml plasma. We have thus shown that a sulfhydryl compound (BMS186716) in blood can successfully be stabilized by reacting it with MA and that the adduct produced is adequately stable in blood and plasma to allow the development of a rugged quantitative bioanalytical method.

Keywords: Thiols; BMS186716

1. Introduction

BMS186716 (Fig. 1) is a novel dual metalloprotease inhibitor that is being developed for the treatment of hypertension and congestive heart failure. A high-performance liquid chromatography–positive ion electrospray mass spectrometry (HPLC–MS) method for the quantitative determination of BMS186716 in dog plasma has been developed to support pharmacokinetic, bioavailability and toxicokinetic studies in dog. The objective of this paper is to describe the development of the bioanalytical method and to present the validation data.

Due to the presence of a free sulfhydryl group, BMS186716 is extremely unstable in both blood and plasma; therefore, it was necessary to stabilize the compound in blood and plasma by reacting the sulfhydryl group with methyl acrylate (MA) to form the stable Michael addition product, BMS186716–MA adduct. The use of acrylic acid esters, such as

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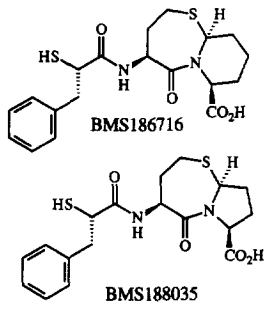

Fig. 1. Structures of BMS186716 and BMS188035, the analytical internal standard.

MA, for the stabilization of thiol compounds has been described previously [1,2]. The advantage of using MA over N-ethylmaleimide (NEM) for the stabilization of thiol compounds has been demonstrated previously [2]. HPLC coupled with electrospray MS, both in the positive and negative ion mode, is a proven technique for the rapid and sensitive quantitative determination of drugs and metabolites in biological matrices [3–8].

2. Experimental

2.1. Reagents and chemicals

BMS186716 and BMS188035 (the internal standard) are characterized products of Bristol-Myers Squibb Pharmaceutical Research Institute. Glacial acetic (Mallinckrodt brand), methyl *tert.*-butyl ether (HPLC grade), and concentrated hydrochloric acid (Mallinckrodt brand) were purchased from Baxter Scientific Products (McGaw Park, IL, USA). Laboratory-deionized water, further purified with a Milli-Q water purifying system (Millipore, Bedford, MA, USA), was used. Ammonium acetate (ACS grade) was from Sigma (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade) and disodium hydrogen phosphate were from Fisher (Fair Lawn, NJ, USA). Methyl acrylate was purchased from Aldrich (Milwaukee, WI, USA).

A 0.01 *M* phosphate buffer was prepared by dissolving 1.42 g of disodium hydrogen phosphate in 100 ml of Milli-Q water and then diluting ten-fold with Milli-Q water. A 0.1 *M* hydrochloric acid solution was prepared by diluting 8.3 ml of concentrated HCl to 1000 ml with Milli-Q water.

Drug-free dog blood was obtained from the Department of Metabolism and Pharmacokinetics, and blank plasma was purchased from Pel Freez Biologicals (Rogers, AK, USA). Mobile phase A was prepared by dissolving 770 mg (10 m*M*) of ammonium acetate in a mixture of 750 ml of Milli-Q water and 250 ml of methanol, and then adjusting the pH to 5.5 by the addition of glacial acetic acid. Mobile phase B was prepared by dissolving 770 mg (10 m*M*) of ammonium acetate in 1000 ml of methanol.

2.2. Equipment

HPLC–MS analysis was performed with a Sciex (Thornhill, Canada) API I mass spectrometer equipped with an articulated ion-spray interface and a Macintosh data system. The API I 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, Hypersil C_{18}, 5 μm, 100×2.0 mm, and the Hypersil C_{18} guard cartridge were from Keystone Scientific (Bellefonte, PA, USA). A Turbovap LV evaporator from Zymark (Hopkinton, MA, USA) was used. The Megafuge centrifuge (bucket rotor with 2×7 ml racks and with the top two spacers removed) was from Baxter Scientific Products. An IEC centrifuge, Model PR-7000M (IEC, Needham Heights, MA, USA) was also used. The shaker used was an Eberbach two-speed shaker, purchased from Baxter Scientific Products. 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. Polypropylene conical microvials (0.25 ml, P/N 200 046, and 0.3 ml, P/N 200 040) were obtained from Sun Brokers (Wilmington, NC, USA).

2.3. Chromatographic and mass spectrometric conditions

The mobile phase used was a combination of mobile phase A and mobile phase B (Section 2.1) at a ratio of 6.5:3.5 (v/v). The flow-rate through the HPLC column was 0.4 ml/min and the effluent was split so that only one-seventh of the effluent was

directed to the mass spectrometer. The injection volume was 15 μl. Laboratory nitrogen was used as the nebulizing gas (60 p.s.i.g) and ultrahigh-purity nitrogen was used as the curtain gas (1.2 l/min). The interface temperature was maintained at 60°C. The sprayer voltage was set at +3500 V and the orifice was set at +45 V. The multiplier voltage was optimized daily.

2.4. Preparation of the MA-adduct

Two separate stock solutions of BMS186716–MA, one to be used for standard curve sets (stock solution A) and the second for the quality control (QC) samples (stock solution B), were prepared during validation of the method. For each stock solution, accurately weighed (12.63 mg for the standard curve set and 12.62 mg for the QCs) BMS186716 was added to a 25-ml volumetric flask containing 12.5 ml of 0.01 *M* phosphate buffer and 5 μl of MA that had been mixed with the buffer. The solution was sonicated for 10 min with intermittent vortex-mixing and inversion, after which, the flask was partially filled with acetonitrile. After the flask content was allowed to come to room temperature, the flask was filled to volume with acetonitrile. The stock solution of the internal standard (BMS188035–MA) was prepared in a 50-ml volumetric flask following the same procedure, but using 1.08 mg of BMS188035. All solutions were kept refrigerated (4°C) when not in use.

2.5. Standard and QC preparations

The calibration set consisted of ten concentrations, each in duplicate, prepared by spiking the specified amount of BMS186716–MA into a specified volume of drug-free dog plasma. The curve range was 2.5 to 500 ng/ml of dog plasma. The highest concentration standard was prepared by diluting 10 μl of stock solution A (described in Section 2.4) to 10 ml with drug-free dog plasma. The rest of the standards were prepared from the highest concentration standard through serial dilutions with drug-free dog plasma. Four levels of QCs, prepared in drug-free plasma and stored at −70°C until analysis, were used for each set of QC samples used for method validation. Three QC levels were in the first quartile, near the mid-point, and in the fourth quartile of the curve. The fourth QC, known as the dilution QC, had a concentration that was several-fold higher than the highest concentration of the calibration set. For the QC preparations, a portion of stock solution B was diluted with acetonitrile to obtain two diluted stock solutions, a two-fold and a ten-fold dilution, respectively. The QCs were prepared by diluting the appropriate volume of the stock solution, or one of the diluted stock solutions, with 10 or 50 ml of drug-free dog plasma.

2.6. Extraction and reconstitution

To a 0.5-ml volume of each dog plasma standard, and QC, 15 μl of the internal standard stock solution was added (to obtain 648 ng/ml of plasma) and then 0.5 ml of 0.1 *M* hydrochloric acid solution was added and mixed. For the dilution QC, the 0.5 ml of plasma was obtained by mixing 0.05 ml of the dilution QC and 0.45 ml of drug-free dog plasma. To each acidified plasma solution, methyl *tert.*-butyl ether (3.0 ml) was added, and the sample was then shaken for 10 min using the high setting of the shaker. The aqueous and the methyl *tert.*-butyl ether layers were separated by centrifugation at the 3000 rpm (2000 *g*) setting of the IEC centrifuge. Each organic layer was transferred to a clean 100×16 mm test tube and the methyl *tert.*-butyl ether was removed by evaporation for 10 min in a Turbovap at 40°C, under nitrogen.

Each dried extract was reconstituted by dissolving it in 60 μl of reconstitution solution (65 parts of mobile phase A and 35 parts of mobile phase B) and vortex-mixing for 1.0 min. Each solution was transferred with a transfer pipet that had an extended fine tip (15.3 cm) to a 0.25-ml polypropylene conical microvial for injection. If the sample solution after reconstitution was brown-colored from a hemolyzed plasma sample or if it contained any particulates, the sample solution was transferred using a transfer pipet with extended fine tip (15.3 cm) to a 0.3-ml polypropylene conical microvial, which is easier to see through than the 0.25-ml conical microvial. The vial was then capped and centrifuged using the Megafuge centrifuge (4000 rpm=3000 *g*, for 5 min) for removal of any particulates. The vial was uncapped, and, leaving the bottom contents in the vial, the sample

solution was then transferred to a 0.25-ml polypropylene conical microvial using a transfer pipet with an extended fine tip (10.4 cm).

2.7. Analysis

Daily, the mass spectrometer was tuned to calibrate for mass axis and to achieve a mass peak width of 0.8 a.m.u. at half height. The samples were analyzed via selected ion monitoring (SIM), employing the $[M+NH_4]^+$ ion of BMS186716–MA (m/z 512) and the $[M+NH_4]^+$ ion of the internal standard, BMS188035–MA (m/z 498).

3. Results and discussion

The reaction of MA with BMS186716 is depicted in Fig. 2. The thiol of BMS186716 undergoes the Michael addition reaction across the conjugated carbon–carbon double bond of MA to give BMS186716–MA. The reaction of BMS186716 with MA in 0.01 *M* disodium phosphate buffer, as described in Section 2.4, was found to be complete after 10 min at room temperature. No BMS186716 was detected after the 10 min reaction, and there was no increase in the amount of BMS186716–MA produced as the reaction period was increased to 20, 30 and 60 min. There was no difference in the amount of BMS186716–MA when the amount of MA used was increased by several fold. The BMS186716–MA adduct stock solution was found to be stable for at least 3.5 months when kept refrigerated.

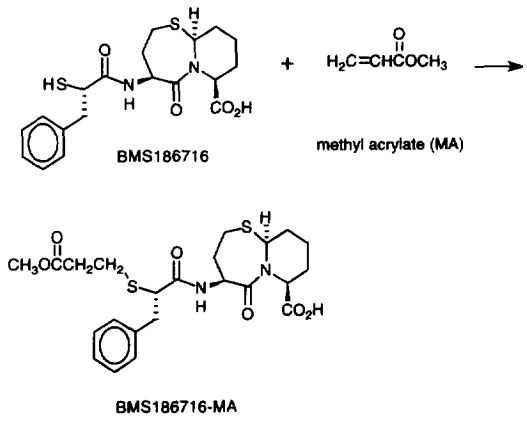

Fig. 2. Reaction of BMS186716 with methyl acrylate (MA).

The positive ion electrospray spectra of BMS186716–MA are shown in Fig. 3. At the orifice voltage of +45 V, the base peak was at m/z 512, due to $[M+NH_4]^+$. At the higher orifice voltage value of +65 V, the base peak was at m/z 495, due to $[M+H]^+$, and additional fragment ions related to the compound were also seen. A similar pattern of change in the mass spectra with the change of the orifice voltage was also seen in the negative ion electrospray mode (Fig. 4). The change of the spectrum with change in the orifice voltage is a well-established property of electrospray, which is due to the collision-induced dissociation (CID) in the region between the electrospray ionization location and the single quadrupole mass analyzer region [9–13]. For the development and validation of the analytical method described in this paper, the electrospray was operated in the positive ion mode with the orifice set at +45 V, and the m/z 512 ion was used for the selected ion monitoring.

The stability of BMS186716 in dog blood and plasma kept in ice and at room temperature for up to 2 h was investigated. Since BMS186716 could not be detected directly with any reliability, the stability of BMS186716 in blood or plasma was assessed indirectly by adding the MA reagent to the blood or

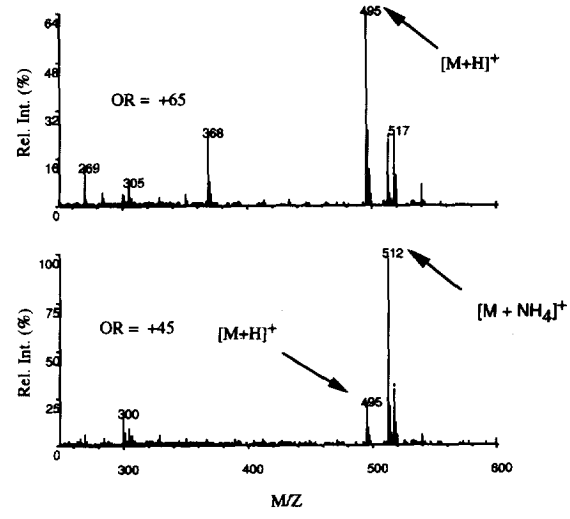

Fig. 3. Positive ion electrospray mass spectra of BMS186716–MA at orifice voltages of +45 and +65 V.

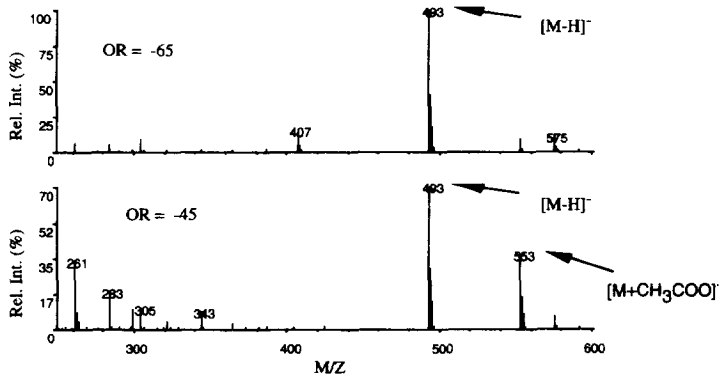

Fig. 4. Negative ion electrospray mass spectra of BMS186716–MA at orifice voltages of −45 and −65 V.

plasma at different times from the time of spiking with BMS186716: after 10 and 30 min and after 1 and 2 h. The reaction of BMS186716 with the MA reagent was then allowed to occur for 10 min either in ice or at room temperature. Blood samples were immediately centrifuged to form plasma. Aliquots (0.5 ml) of the plasma samples were then assayed immediately. The BMS186716–MA adduct values were compared to the adduct value obtained when MA was added to blood and plasma before the addition of the BMS186716, which ensured the reaction of the BMS186716 with MA prior to any degradation of the compound. The results, as depicted in Fig. 5, show that the sulfhydryl compound is not stable in either blood or plasma at room temperature or in ice, due to the probable formation of disulfides. In blood, only 31% of the free sulfhydryl compound is present after 10 min at room temperature (63% in ice). After 2 h, less than 2% is present at room temperature or in ice. In plasma, only 4% is present after 10 min at room temperature (47% in ice). After 2 h, less than 1% is present at room temperature or in ice.

The stability of the BMS186716–MA adduct spiked into dog blood and plasma was investigated for up to 6 h at room temperature and in ice. As shown in Fig. 6, BMS186716–MA was found to be stable for at least 6 h in ice in both blood and in plasma, but at room temperature it was stable only in plasma. At room temperature, BMS186716–MA in blood was not stable. After 1 h, only 54% of the adduct was found and, after 6 h, only 20% was found. It is because of the instability of the adduct in blood at room-temperature that it is specified that the reaction with MA in blood, for the formation of the adduct, should be performed in ice and not at room temperature.

The amount of MA needed for the Michael addition reaction in blood was investigated at both 50 and 500 ng of BMS186716/ml blood. The following amounts of MA were added to 1 ml of blood prior to spiking with BMS186716; 0.5, 2.5, 10 and 20 μl. After a 10-min reaction time in ice, each

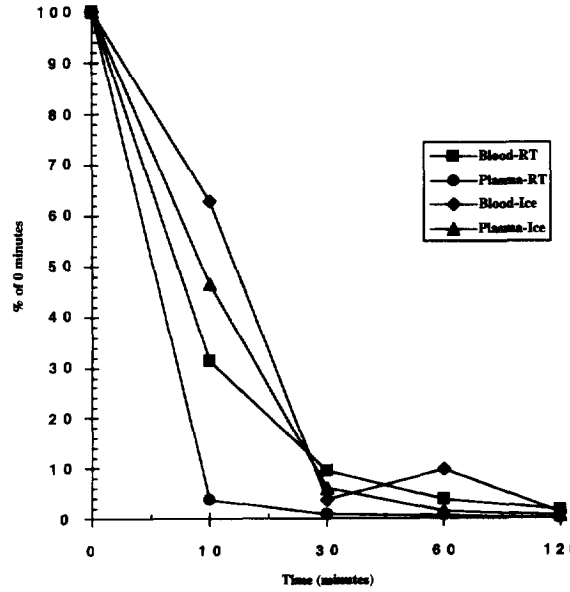

Fig. 5. Stability of BMS186716 in dog blood and plasma (500 ng/ml) at room temperature and in ice. The *Y*-axis shows the BMS186716 concentration at the particular time, expressed as a percentage of the BMS186716 concentration at 0.0 time.

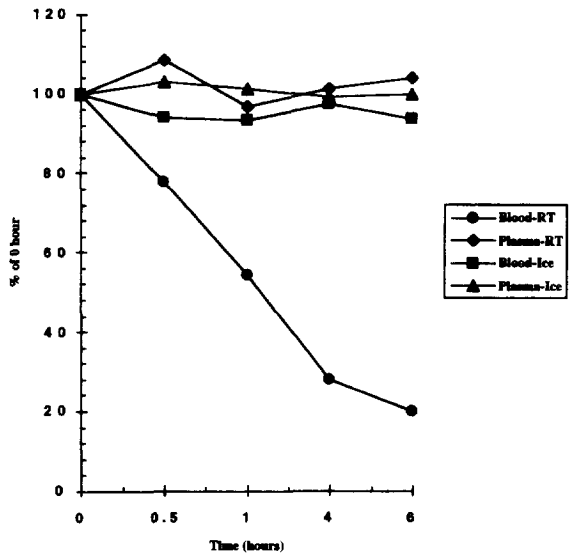

Fig. 6. Stability of the BMS186716–MA in dog blood and plasma (500 ng/ml) at room temperature and in ice. The *Y*-axis shows the BMS186716–MA concentration at the particular time, expressed as a percentage of the BMS186716–MA concentration at 0.0 time.

blood sample was centrifuged to obtain the plasma. The amount of BMS186716–MA in each plasma sample was measured. A 10-μl volume of MA per ml of blood gave the highest concentration of BMS186716–MA at both the 50 and 500 ng/ml levels. Hence, this ratio of μl of MA to ml of blood was used for all subsequent work. The reaction of BMS186716 in blood with MA was taken to be instantaneous, as the amount of BMS186716–MA produced did not increase when the reaction times of 10, 30, 60 and 90 min were used.

The efficiency of the reaction of BMS186716 with MA in blood was evaluated by comparing the amount of BMS186716–MA produced after spiking BMS186716 into blood that already contained MA (10 μl of MA/ml of blood) against the BMS186716–MA spiked into blood. The reaction was quantitative.

The partitioning of BMS186716–MA between the plasma and red blood cell components of blood was studied by spiking BMS186716–MA into blood and then determining the concentration of BMS186716–MA in the plasma obtained by centrifuging the spiked blood. The concentration of BMS186716–MA in the plasma was found to be approximately twice the nominal (added) concentration in blood, indicating that little of the BMS186716–MA was bound to the cellular components of the blood.

Extraction recovery of BMS186716–MA from plasma was determined by dividing the area ratio for the plasma sample spiked with BMS186716–MA before extraction and with internal standard, BMS188035–MA, after extraction (pre/post) by the area ratio of the plasma spiked with both BMS186716–MA and BMS188035–MA after extraction (post/post). The recovery for BMS188035–MA was similarly determined by dividing the area ratio of a post/pre plasma sample by area ratio of a post/post plasma sample. Quantitative recovery (>90%) of both BMS186716–MA and BMS188035–MA was obtained when methyl *tert.*-butyl ether was used during liquid–liquid extraction. About 70–80% recovery was obtained with *n*-butyl chloride, toluene and ethyl acetate for both BMS186716–MA and BMS188035–MA, except for *n*-butyl chloride, for which the recovery was only about 45% for BMS188035–MA.

The method exhibited excellent linearity in dog plasma. The results of a linear regression analysis (weighted to $1/x$, where x is the concentration in ng/ml plasma) of a typical standard curve are shown in Table 1. The accuracy of each standard point and the precision of the duplicate points were very good.

Table 1
Linear regression analysis results of a typical standard curve in dog plasma

Theoretical concentration (ng/ml)	Experimental concentration (ng/ml)		Deviation (%)	
2.51	2.32	2.50	−7.4	−0.4
5.02	4.55	5.17	−9.3	+3.0
10.0	9.88	10.5	−1.5	+4.6
25.1	25.9	25.4	+3.3	+1.4
50.2	49.9	54.2	−0.5	+8.1
100	89.4	110	−10.9	+9.4
151	143	150	−4.7	−0.3
251	257	262	+2.3	+4.4
301	288	321	−4.3	+6.5
502	469	515	−6.5	+2.7

The two values given under the headings "Experimental concentration" and "Deviation" are for the duplicate standards at each level.

The linear regression parameters: Intercept=0.00047; slope=0.00139; R^2=0.999.

Table 2
Summary of the results of dog plasma QC samples analyzed on three different days

Parameter	Nominal concentration (ng/ml)			
	35.09	200.5	350.9	2507
Grand mean (ng/ml)	34.2	201	334	2450
Deviation (%)	−2.5	0.3	−4.8	−2.4
Inter-day precision, C.V. (%)	1.1	0.9	0.0[a]	0.0[a]
Intra-day precision, C.V. (%)	3.6	2.3	3.4	4.0

[a] No significant additional variation was observed as a result of performing the assay on different days.

Table 2 presents the summary of the results of the dog plasma method validation on three different days. The deviation of the grand mean was 5% or less for all QC levels. The inter-day and intra-day precision was 4% or better at all levels.

For the verification of the lower limit of quantitation (LLQ) of the method in dog plasma, six different lots of blank dog plasma were spiked with BMS186716–MA at the lowest level of the standard curve (2.5 ng/ml) and the internal standard. The samples were then analyzed against the standard curve. The six different lots of blank dog plasma were also analyzed after spiking with the internal standard only (QC0), and with no spiking at all, in order to determine if any endogenous plasma constituents co-eluted with the analyte or the internal standard. The values obtained for the LLQ verification are listed in Table 3. The deviation from the nominal value was less than ±15% for all six samples. The precision at this level was excellent with the C.V. of the six values being less than 10%.

Table 3
Lower limit of quantitation (LLQ) for BMS186716–MA in dog plasma

Blank dog plasma lot number	Calculated value for BMS186716–MA (ng/ml)	Deviation from expected (2.50) (ng/ml) (%)
1, 24416A	2.29	−8.3
2, 24416B	2.28	−8.7
3, 24416C	2.79	+11.8
4, 24416D	2.60	+4.3
5, 24416E	2.77	+10.9
6, 24416F	2.37	−5.0
Mean	2.52	
S.D.	0.234	
C.V. (%)	9.3	

Figs. 7 and 8, which compare the chromatogram of a QC0 against that of an LLQ sample (2.5 ng/ml), demonstrate the specificity of the method. In the six lots of blank plasma used, little to no response was detected in the analyte or internal standard channels. A good-sized peak was obtained for BMS186716–MA at the LLQ level.

The stability of QC dog plasma samples was checked at −70°C and at −20°C. BMS186716–MA in dog plasma was found to be stable for at least 4.5 months at −70°C. During this period, the values obtained for the QCs at 35.12, 200.7 and 351.2 ng/ml plasma did not show any pattern for degradation and remained within the acceptance criterion of ±15% deviation. At −20°C, BMS186716–MA was not stable. After storage for one month, the determined concentrations were only 80% of the original value. Extracts dissolved in the reconstitution solutions were found to be stable for at least 48

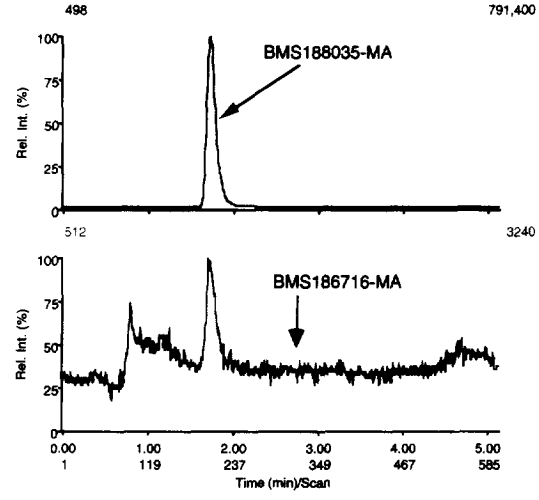

Fig. 7. Chromatogram of BMS186716–MA at 0.0 ng/ml and the internal standard BMS188035–MA at 648 ng/ml in dog plasma.

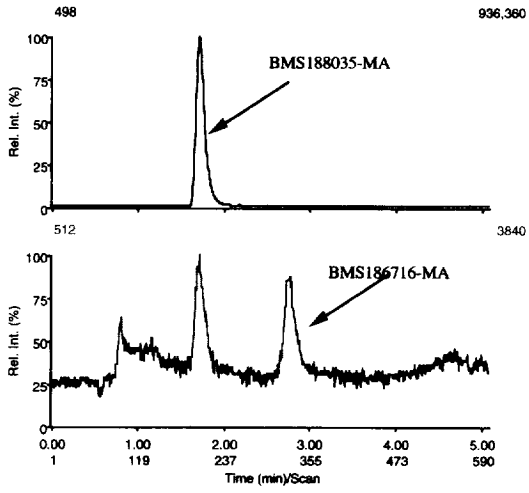

Fig. 8. Chromatogram of BMS186716–MA at 2.5 ng/ml (LLQ) and the internal standard BMS188035–MA at 648 ng/ml in dog plasma.

h at room temperature on the autosampler and for at least 48 h at 4°C.

The method developed has successfully been used to analyze plasma samples obtained from dogs dosed with BMS186716. Blood samples drawn from the animals were immediately put into tubes containing tripotassium EDTA and MA (10 μl of MA per ml of blood). The tubes were inverted gently to allow mixing of the reagents with the sample, placed in an ice bath for 10–15 min, and then centrifuged to harvest plasma. The plasma samples were transferred to a second set of tubes and stored at −70°C until analysis.

4. Conclusion

We have shown that a sulfhydryl compound (BMS186716) in blood can successfully be stabilized by reacting it with MA and that the adduct produced is adequately stable in blood and plasma to allow the development of a rugged quantitative bioanalytical method. The electrospray ionization mass spectra of the adduct, both in the positive and negative ion mode, can be easily changed by simply raising the orifice voltage to bring about CID before entering the single quadrupole mass analyzer. For the development of the bioanalytical method described in this paper, we selected, without further investigation, the positive ion mode and an orifice voltage which enhanced the signal of $[M+NH_4]^+$ at the expense of $[M+H]^+$. Monitoring the $[M+NH_4]^+$ signal under this condition, an LLQ of 2.5 ng/ml plasma was easily achieved. Improvement in sensitivity, when desired, could be explored by simply modifying the HPLC parameters, and/or by investigating the use of higher orifice voltages, both in the positive and negative ion mode.

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