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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1728–1736
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Liquid–liquid extraction of strongly protein bound BMS-299897 from human plasma and cerebrospinal fluid, followed by high-performance liquid chromatography/tandem mass spectrometry

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Received 27 September 2006; received in revised form 20 November 2006; accepted 22 November 2006
Available online 3 January 2007

Abstract

BMS-299897 is a γ-secretase inhibitor that is being developed for the treatment of Alzheimer's disease. Liquid–liquid extraction (LLE), chromatographic/tandem mass spectrometry (LC/MS/MS) methods have been developed and validated for the quantitation of BMS-299897 in human plasma and cerebrospinal fluid (CSF). Both methods utilized $^{13}C_6$-BMS-299897, the stable label isotope analog, as the internal standard. For the human plasma extraction method, two incubation steps were required after the addition of 5 mM ammonium acetate and the internal standard in acetonitrile to release the analyte bound to proteins prior to LLE with toluene. For the human CSF extraction method, after the addition of 0.5 N HCl and the internal standard, CSF samples were extracted with toluene and no incubation was required. The organic layers obtained from both extraction methods were removed and evaporated to dryness. The residues were reconstituted and injected into the LC/MS/MS system. Chromatographic separation was achieved isocratically on a MetaChem C18 Hypersil BDS column (2.0 mm × 50 mm, 3 μm). The mobile phase contained 10 mM ammonium acetate pH 5 and acetonitrile. Detection was by negative ion electrospray tandem mass spectrometry. The standard curves ranged from 1 to 1000 ng/ml for human plasma and 0.25–100 ng/ml for human CSF. Both standard curves were fitted to a 1/*x* weighted quadratic regression model. For both methods, the intra-assay precision was within 8.2% CV, the inter-assay precision was within 5.4% CV, and assay accuracy was within ±7.4% of the nominal values. The validation and sample analysis results demonstrated that both methods had acceptable precision and accuracy across the calibration ranges.

Keywords: Human plasma; Cerebrospinal fluid; Liquid–liquid extraction; Chromatography; LC/MS/MS; BMS-299897

1. Introduction

BMS-299897 is a putative gamma-secretase inhibitor and has been shown to rapidly and potently inhibit the production of the various iso-forms of the beta-amyloid peptide (Aβ), which is a major component of senile plagues found in the brain of patients with Alzheimer's disease (AD). The production and release of Aβ is a normal physiological event. However, accumulation of the Aβ peptide in brain in the form of plagues is believed to be linked to the pathogenesis of AD. BMS-299897 is being developed as a disease modifying treatment for Alzheimer's disease [1,2].

During the past 15 years, liquid chromatography-tandem mass spectrometry (LC/MS/MS) has become the technique of choice for the quantitation of drug candidates and their reactive metabolites in biological fluids because of its high sample throughput, sensitivity, and selectivity [3]. Even with the tremendous selectivity of the mass spectrometry, development of a good sample cleanup procedure is still essential for minimizing ion suppression and matrix effects and for a successful, reliable bioanalytical method [4–6]. Liquid–liquid extraction (LLE) has always been used as an alternative to solid phase extraction (SPE) because it is cheap and easy to develop, and capable of providing very clean sample extracts [8,9]. The drawback of the technique is that it normally requires a large amount of organic solvent and is less amenable to automation [3,7,8]. In the past 10 years, concerted efforts have been made to study the utility of semi-automated 96-well LLE for the quantitation of drugs and

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doi:10.1016/j.jpba.2006.11.035

their metabolites in biological fluids. Jemal et al. [9] reported 96-well LLE for the determination of a carboxylic acid with methyl *t*-butyl ether. Zhang et al. [10] reported 96-well LLE for the quantitation of diphenhydramine, desipramine, chlorpheniramine, and trimipramine in rat plasma with a selection of organic solvents. Similar 96-well LLE approaches were also used for the determination of methylphenidate in human plasma [11], paclitaxel in human plasma [12], SU11248 in monkey tissues [13], simvastatin and its β-hydroxy acid in human plasma [14], ABT-578 in human blood [15], a novel insulin sensitizer in human plasma [16]. Song reported an LLE, HILIC/MS/MS method for the analysis of omeprazole, and 5-OH omeprazole in human plasma which eliminated both evaporation and reconstitution steps [17].

This paper reports the quantitation of strongly protein bound BMS-299897 in human plasma and human cerebrospinal fluid (CSF). The objectives of this work were: (1) to develop and validate a semi-automated 96-well LLE, LC/MS/MS method for the determination of BMS-299897 in human plasma by incubating samples to release BMS-299897 bound to plasma proteins; (2) to develop and validate a highly sensitive LLE, LC/MS/MS method for the determination of the same drug candidate in human CSF. Validations were performed to assess the accuracy, precision, linearity, and lower limit of quantitation of both methods, and the results presented here demonstrate that both human plasma and CSF methods were reliable and suitable for analyzing BMS-299897 in human plasma and CSF. Both methods were subsequently used to analyze BMS-299897 concentrations in human plasma and CSF samples from a clinical study.

2. Experimental

2.1. Reagents and chemicals

BMS-299897 and $^{13}C_6$-BMS-299897 (internal standard, IS; Fig. 1) were provided by the Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute. The pK_a of the carboxylic acid moiety was experimentally determined to be 5.6. Acetonitrile (HPLC grade), ammonium acetate, and glacial acetate acid were purchased from EM Science (Gibbstown, NJ, USA). Toluene and methyl *t*-butyl ether were also purchased from EM Science. In-house deionized water, further purified with a Milli-Q water purifying system (Millipore Corporation, Bedford, MA, USA), was used. Drug-free human plasma and CSF were purchased from Lampire Biological Laboratories (Pipersville, PA, USA).

2.2. Chromatographic conditions

2.2.1. Human plasma method

BMS-299897 and the IS were separated isocratically, using a solvent system containing 10 mM ammonium acetate (pH 5) in water and acetonitrile (70:30, v:v) with the flow rate of 0.3 ml/min at room temperature. The separation column was a MetaChem C18 Hypersil BDS, 2.0 mm × 50 mm, 3 μm column (MetaChem Technologies, Torrance, CA). The injection volume was 10 μl and the run time was 2 min.

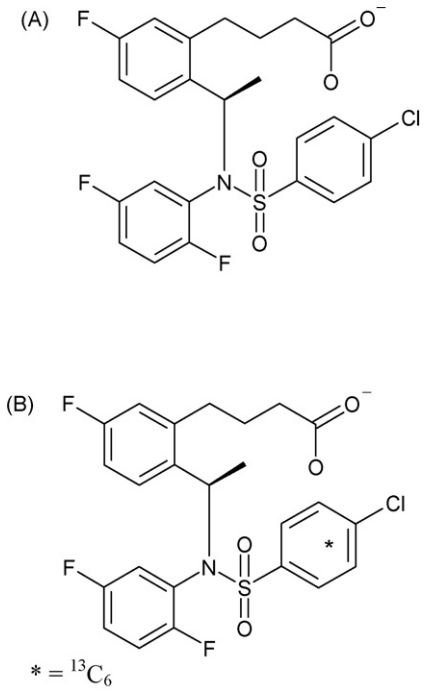

Fig. 1. Chemical structures of BMS-299897 and internal standard ($^{13}C_6$-BMS-299897). (A) BMS-299897: molecular weight = 511.95, elemental formula = $C_{24}H_{21}ClF_3NO_4S$, monoisotopic exact mass = 511, $[M-H]^-$ = 510.05. (B) $^{13}C_6$-BMS-299897 (internal standard): molecular weight = 517.95, elemental formula = $C_{24}H_{21}ClF_3NO_4S$, monoisotopic exact mass = 517, $[M-H]^-$ = 515.99. (*) $^{13}C_6$.

2.2.2. Human CSF method

The same column and mobile phases were used for human CSF method. There were only two minor differences: (1) mobile phase combination (40:60, v:v); (2) the injection volume (20 μl), and the run time (2.5 min).

2.3. Instrumentation

The robotic liquid handling system used was a Tecan Genesis RSP 150 Series with Gemini Software (Tecan US, Research Triangle Park, NC). The collection microtubes, racked in 96-well format, and the microtube caps were purchased from US Scientific (Ocala, FL).

The liquid chromatography separation system used consisted of two Shimadzu LC-10AD pumps (Columbia, MD, USA) and a Perkin-Elmer Series 200 LC autosampler (Norwalk, CT, USA).

2.3.1. Mass spectrometer for human plasma method

A Sciex API 3000 LC MS/MS system (Foster City, CA, USA) operating under Analyst 1.1 software was used. The electrospray ion source was run in a negative ionization mode. The typical ion source parameters were: capillary −3 kV, declustering potential (DP) −31 V, focusing potential (FP) −150 V, entrance potential (EP) 10 V, collision energy (CE) −24 eV, collision cell exit potential (CXP) −19 V, deflector 400 V, channel electron multiplier (CEM) −2400 V, and Source temperature 325 °C. Nebuliser gas (NEB), curtain gas (CUR), and collision gas (CAD) were set to 10, 15, and 4 of the state file parame-

ters, respectively. Nitrogen gas was used for CUR, CAD, NEB, and auxiliary 2. The samples were analyzed via selected reaction monitoring (SRM). The monitoring ions were set as *m/z* 510–302 for BMS-299897 and *m/z* 516–308 for the IS. The scan dwell time was set 0.15 s for both channels.

2.3.2. Mass spectrometer for human CSF method

A Micromass Quattro LC LC/MS/MS system (Beverly, MD, USA) operating under MassLynx 3.4 software was used. The electrospray ion source was run in a negative ionization mode for all human CSF experiments. The typical ion source parameters were: capillary voltage 3 kV, sample cone voltage 25 V, probe temperature 100 °C, desolvation gas 890 l/h, nebulizing gas 78 l/h, and collision energy 20 eV. The multiplier was set at 650 V. The samples were analyzed via SRM. The monitoring ions were set as *m/z* 510–302 for BMS-299897 and *m/z* 516–308 for the IS. The scan dwell times were set 0.3 s for the analyte and 0.1 s for the IS. Fig. 2 shows the electrospray negative ion MS/MS product ion spectra of BMS-299897 and IS on the Micromass Quattro LC (the data from the API 3000 are not shown).

2.4. Preparation of standards, quality control samples and internal standard

2.4.1. Standard preparation

A 1 mg/ml standard stock solution was prepared by dissolving an appropriate amount of BMS-299897 in acetonitrile. This stock solution was further diluted with acetonitrile to obtain a 100 μg/ml working solution. The appropriate dilutions of the 100 μg/ml working solution with drug-free human plasma or human CSF were used to prepare the standard concentrations in human plasma and CSF. The final standard concentrations in human plasma were: 1, 2, 3.9, 7.8, 31.3, 62.5, 250, 500, 750, and 1000 ng/ml. The final standard concentrations in human CSF were: 0.25, 0.5, 2, 4, 25, 50, 75, and 100 ng/ml. Both human plasma and CSF standard curves were prepared fresh daily.

2.4.2. QC preparation

A 1 mg/ml QC stock solution was prepared from a separate weighing of BMS-299897. For human plasma method, the 6,000, 800, 480, and 3 ng/ml QC samples were prepared by appropriate dilutions of the 1 mg/ml stock solution with human plasma. For human CSF method, the 750, 75, 30, and 0.75 ng/ml QC samples were prepared by appropriate dilutions of the 1 mg/ml stock solution with human CSF. Both the human plasma and CSF QC samples were stored in 0.8-ml volumes at −20 °C.

2.4.3. IS preparation

A 1 mg/ml IS stock solution was prepared by dissolving an appropriate amount of the IS in acetonitrile. For human plasma method, the IS working solution, 500 ng/ml, was prepared from the 1 mg/ml stock solution by dilution with acetonitrile. The same procedure was used for the preparation of the 150 ng/ml IS working solution for human CSF method. The IS working solutions were stored at 4 °C.

2.5. Sample processing procedure

2.5.1. Human plasma method

On the Tecan workstation, each calibration standard and QC sample, 0.2 ml, was pipetted from the sample tubes and transferred to collection microtubes on a 96-well plate, followed by the addition of 0.5 ml of ammonium acetate buffer at pH 4.3. The microtubes were vortexed offline for 1 min and allowed to sit for 10 min at room temperature prior to adding 0.5 ml of internal standard. The microtubes were vortexed again for 1 min and allowed to sit for an additional 10 min at room temperature before adding 0.65 ml of toluene. The microtubes were capped (manually) with plug caps and placed on a linear shaker offline for 10 min. The organic layer from each sample was transferred to a clean microtube by the Tecan workstation and then evaporated offline to dryness. The dried extracts were re-dissolved in 0.1 ml of the mobile phase before injection into the Sciex API 3000.

2.5.2. Human CSF method

A very similar extraction procedure was used for human CSF method. Briefly, a 0.05 ml portion of 0.5 N HCl solution was added to 0.2 ml of each sample. The samples were vortexed for 2 min and incubated at room temperature for 10 min prior to adding 0.05 ml of the internal standard. The samples were vortexed again and incubated at room temperature for another 10 min before the addition of 3 ml toluene. After shaking and centrifugation, the organic layer was transferred to a clean tube and evaporated to dryness. The dried extracts were re-dissolved in 0.1 ml of a 40:60 mixture of mobile phases A and B before injection into the Micromass Quattro LC.

3. Results and discussion

3.1. Method optimization

3.1.1. Sample pretreatment and liquid–liquid extraction

3.1.1.1. Human plasma method. Drugs and metabolites are transported in the bloodstream partly in solution as free drug/metabolite and partly bound to blood components. Many plasma proteins can interact with drugs/metabolites. Prior to sample extraction, protein binding must be disrupted; otherwise, the analyte of the interest will not be effectively extracted. In many cases, protein binding is a rapid and reversible process; the analyte can be released instantly during sample extractions. In some cases, the binding between proteins and analytes is strong enough to require special reagents to disrupt it. Ke et al. [18] reported to use a small amount of disodium EDTA, formic acid, trichloroacetic acid, phosphoric acid or acetonitrile to release reserpine from mouse plasma. The similar techniques were used by other authors [19,20].

During the initial human plasma method development for BMS-299897, it was found that the recovery of BMS-299897 from human plasma was lower than that of the stable labeled IS when a routine LLE extraction procedure without incubations was used. The suspected cause for the low analyte recovery was due to incomplete release of BMS-299897 from protein

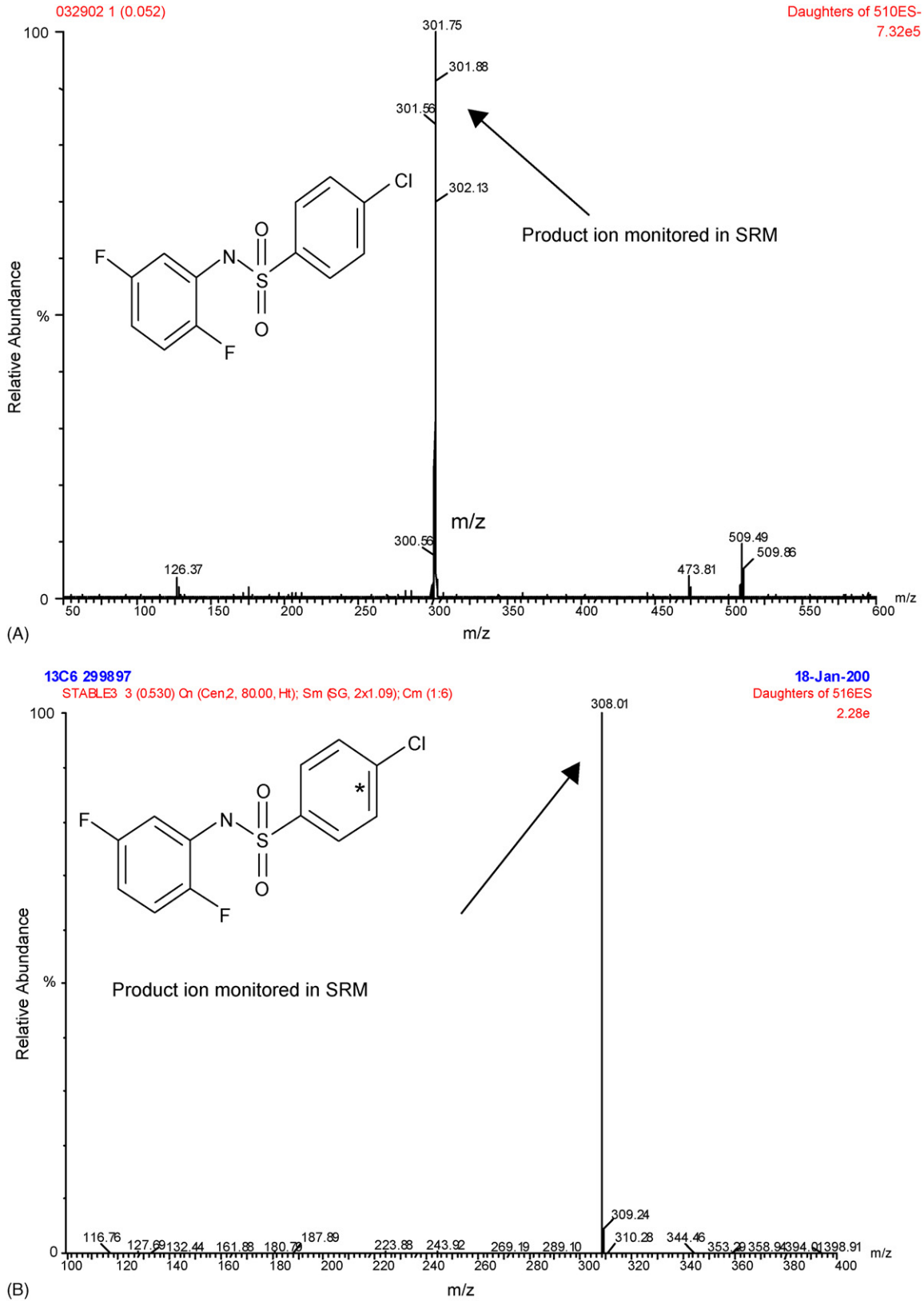

Fig. 2. Electrospray negative ion MS/MS product ion spectra of $[M-H]^-$ for BMS-299897 (A) and its internal standard (B).

binding sites (over 99.3% of BMS-299897 was bound to plasma proteins). To overcome the poor analyte recovery, a two-step sample pretreatment was used. First, 0.2 ml of plasma samples was incubated with 0.5 ml of ammonium acetate buffer at 4.3 for 10 min. The pH change (from pH 7.4 to 4.3) of plasma samples affected the protein conformation, which in turn led to reduced affinity between BMS-299897 and plasma proteins. This was followed by 10 min incubation of the sample mixture with 0.5 ml of acetonitrile (IS solution). Acetonitrile helped partially denature plasma proteins and further release the analyte from the protein binding sites. Partial denaturation without precipitation of plasma proteins reduced the possible loss of the analyte by occlusion in the precipitate. The experimental results showed that the two incubation steps were sufficient to liberate BMS-299897 from protein binding sites, and led to the consistent recoveries for the analyte, being ~40%. In addition, the use of the incubation steps minimized the lot-to-lot variability of human plasma for the analyte recovery. Therefore, the incubation steps were used for subsequent experiments. It should be pointed out that the low analyte recovery was also due to incomplete neutralization of the analyte at the pH 4.3 buffer. However, the 1 ng/ml LLOQ was easily achieved on a sensitive mass spectrometry platform (Sciex API 3000) and deemed sufficient for the measurement of systemic exposure of BMS-299897 in humans. Therefore, no further attempt was used to improve method sensitivity or performance.

3.1.1.2. Human CSF method. BMS-299897 human CSF levels were very low due to the fact that only limited BMS-299897, which was ionized in human plasma, was able to pass the blood/brain barrier and enter CSF circulation. This required setting the LLOQ as low as possible. The availability of the less sensitive Micromass Quattro LC/MS/MS at the time also compounded the difficulty for the development and validation of human CSF method. To overcome these hurdles, the liquid–liquid extraction procedure used for human plasma method was further optimized to maximize extraction recovery. First, three sets of the standard curve were extracted under three different pH buffers (4.3, 3.5, and 2.9), and the peak area responses for BMS-299897 and its IS were used to determine the optimal pH for the extraction buffer. The experimental results showed that the pH 2.9 buffer offered the highest extraction recovery based on the highest peak area counts. This was consistent with the chemical structure of BMS-299897. In samples mixed with this pH buffer, the compound existed in the neutral form, and was readily extracted by toluene. Subsequently, the pH 2.9 buffer was compared with the addition of 0.5 N HCl to the samples. The results indicated that the latter produced even higher peak area responses; therefore, it was used for LLE.

Three different volumes (3, 2, and 0.7 ml) of toluene were evaluated for the extraction. The results revealed that 0.7 ml of toluene were sufficient to extract BMS-299897 from 0.2 ml of human CSF. However, 3 and 2 ml of toluene allowed more complete transfer of the organic layer utilizing the automated liquid handler. An additional experiment compared toluene and methyl *t*-butyl ether extractions using identical pH conditions. The results indicated that both extraction solvents provided equivalent recovery. Therefore, toluene was used for the further experiments. Initially, the targeted LLOQ for human CSF method was 0.1 ng/ml. However, the instrument response was too low for a consistent measurement. Therefore, the LLOQ was raised to 0.25 ng/ml, where sufficient response was obtained for reliable quantitation. The average recoveries for the analyte and its IS from CSF were approximately 90%. The complete neutralization of BMS-299897 at 0.5 N HCl contributed to the significant improvement of the analyte recovery in human CSF method. Human CSF is a much simpler matrix than plasma. With much lower proteins or lipids, the two incubation steps in human plasma method were not required to achieve the consistent recovery for the analyte. However, for the consistency of both methods, both incubation steps were also used for human CSF method.

3.1.2. LC conditions

According to Willoughbu et al. [21], the pH of the mobile phase should have a strong influence on the sensitivity of negative electrospray ionization. Therefore, two different pH (5 and 8) mobile phases were examined. In the first case, the mobile phase pH was slightly below the pK_a of the compound (5.6), and the compound was only partially ionized. In the second case, the mobile phase pH was much greater than pK_a of the compound, and the compound was completely ionized. The test results indicated that increasing pH of the mobile phase did not promote desorption or ionization efficiency in negative electrospray, rather it hindered the process as indicated by the decreased analyte responses at the pH 8 mobile phase. The observation was consistent with what reported by Jemal et al. [22], where the authors concluded the electrospray response was not a simple function of the mobile phase pH. Similar phenomena have been observed for other negative electrospray methodologies (data not shown) within our laboratory. Based on the experimental results, the pH 5 buffer was used for mobile phase preparation. Additionally, it was determined that a higher percentage of acetonitrile or methanol generally gave better sensitivity, but higher flow rates led to reduced responses resulting from insufficient desolvation in the ion source. Methanol did not reduce response due to potential formation of a methyl ester with BMS-299897. Instead, a methanol containing mobile phase resulted in higher peak area responses for the compound. However, acetonitrile containing mobile phases offered better peak shapes. Therefore, acetonitrile was used for further experiments. Furthermore, two other columns (Inertsil and Symmetry) were tested along with Hypersil, and all gave comparable peak shape and minimal tailing. Therefore, the Hypersil column was used for both method validations. For the human plasma method, the retention times of BMS-299897 and the internal standard were 1.0 min and the chromatographic run time was 2.0 min (Fig. 3). For human CSF method, the retention times of BMS-299897 and the internal standard were 1.7 min and the chromatographic run time was 2.5 min (Fig. 3).

3.1.3. Specificity

BMS-299897 has a carboxylic acid functional group, so an acyl glucuronide could be a major circulating metabolite.

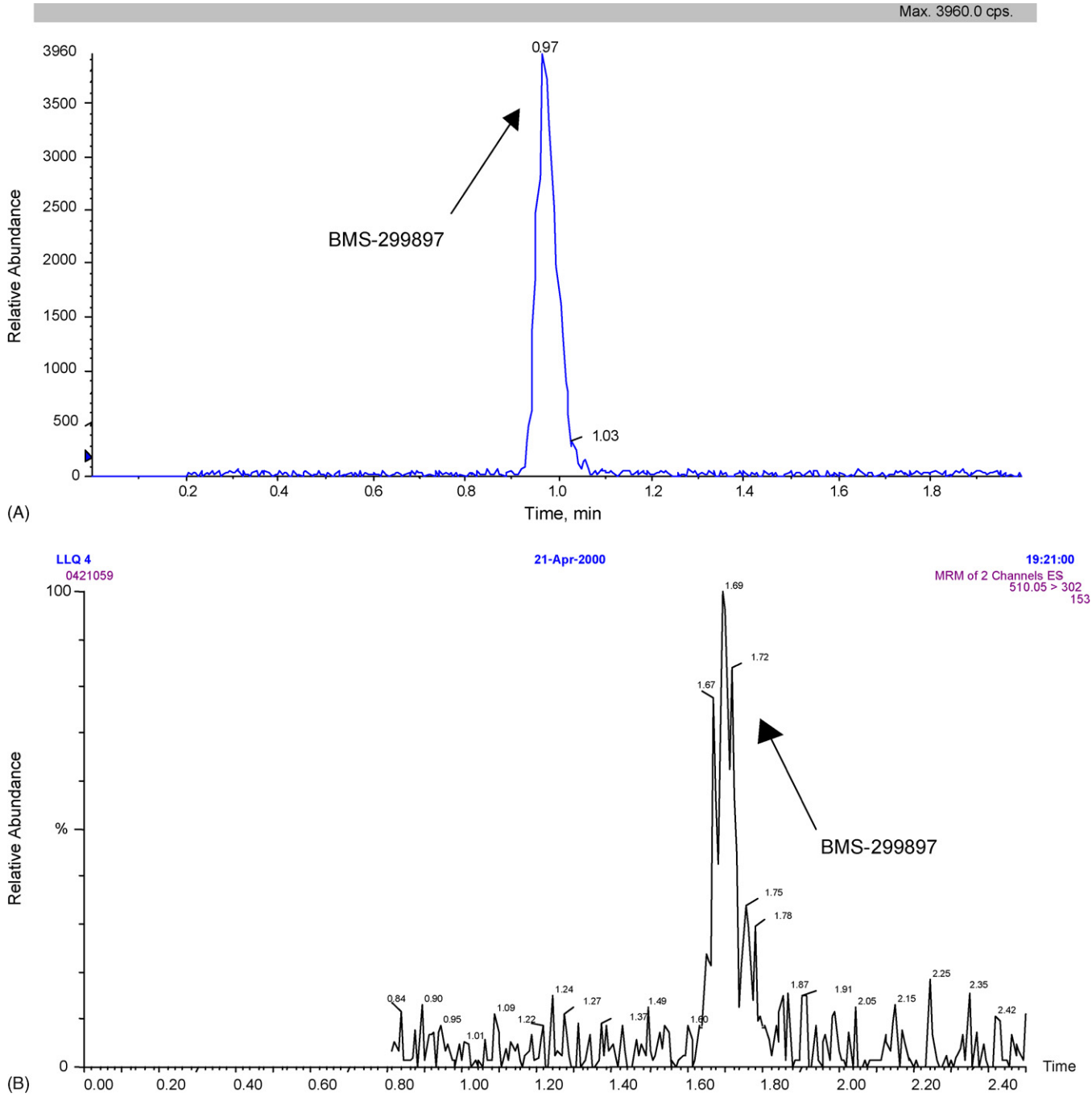

Fig. 3. Selected reaction monitoring chromatograms for BMS-299897 obtained from: (A) LLOQ (1 ng/ml) in human plasma on API 3000; (B) LLOQ (0.25 ng/ml) in human CSF on Quattro LC.

Ideally, an acyl glucuronide and BMS-299897 need to be chromatographically resolved, because the acyl glucuronide could contribute to the BMS-299897 response via in-source conversion back to the parent. However, at the time of method development and validation, acyl glucuronide reference material was not available. Therefore, LLE was used to selectively extract BMS-299897 but not its acyl glucuronide, thereby eliminating the need for chromatographic resolution. A subsequent human ADME study confirmed that there was no significant circuiting acyl glucuronide metabolite in human plasma. This finding also eliminated the need for acidification of human plasma sample at the time of sample collection to stabilize the acyl glucuronide.

Based on in vitro metabolism work, BMS-299897 was observed to form a hydroxyl metabolite (mw = 527) by oxidation of the γ carbon of the carboxylic acid functional group. In addition, the hydroxyl metabolite could lose water and form a lactone (mw = 509). Therefore, both metabolites were screened for potential interference to BMS-299897. Full scan results showed that although the hydroxyl metabolite was extensively ionized in negative electrospray mode, it did not convert back to the parent in the ion source. Therefore, it did not pose any

problem with human plasma and CSF assays for BMS-299897. On the other hand, the lactone metabolite gave a strong positive electrospray signal, but did not produce a negative ion. Therefore, the lactone metabolite did not interfere to the assays either.

In addition, six different lots of control human plasma or CSF were analyzed with and without IS. Their SRM chromatograms were inspected, and no significant interfering peaks from the plasma or CSF were found (Fig. 4).

3.2. Method validation

3.2.1. Standard and QC performance

3.2.1.1. Human plasma method. A 10-point calibration standard curve ranging from 1 to 1000 ng/ml of BMS-299897 in human plasma was used in duplicate with one curve at the beginning and the other at the end to bracket the QC samples from four difference concentrations. Five replicate QC samples at each concentration were analyzed in three core validation runs and triplicate samples at each concentration were analyzed the remaining two validation runs. Peak area ratios of BMS-299897 to IS were used for regression analysis. Both linear and quadratic regression models were evaluated; however, the quadratic model better fitted for calibration standard data. Therefore, a quadratic regression with 1/concentration squared weighting was used to plot the peak area ratio of the analyte to it internal standard versus concentration. Among 100 standards within the five analytical runs, only two had percent deviations of the back-calculated concentrations greater than 15% (20% for LLOQ samples) from the nominal values. The regression coefficients (R^2) were greater than 0.999. Among 84 QC samples used for the validation, only three were rejected due to a preparation error and one additional QC had a percent deviation of the predicted concentration greater than 15% from its nominal value. For the three core validation runs, the intra-day precision was within 8.2% CV, the inter-day precision was within 4.1% CV, and the assay accuracy was within ±6.1% of the nominal values (Table 1).

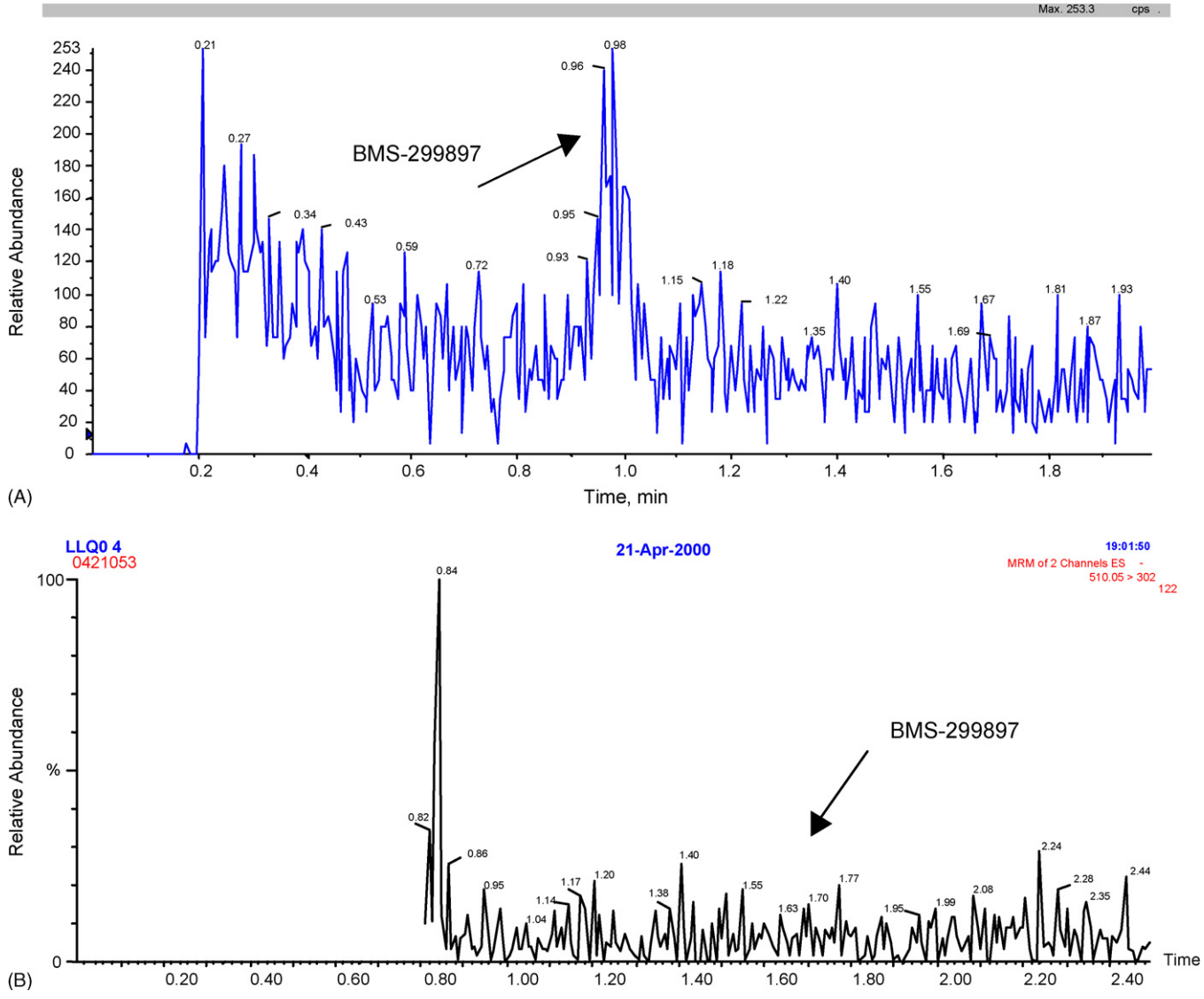

Fig. 4. Selected reaction monitoring chromatograms for BMS-299897 obtained from: (A) matrix blank in human plasma on API 3000; (B) matrix blank in human CSF on Quattro LC.

Table 1
One-way ANOVA accuracy and precision for BMS-299897 during method validations in human plasma (A) and in human CSF (B)

(A) In human plasma during method validation				
Nominal conc. (ng/ml)	3	480	800	6000
Mean observed conc.	2.9	470.2	848.4	6278.6
%Dev	−1.7	−2.1	6.1	4.6
Between run precision (%CV)	4.1	0.0	0.8	0.0
Within run precision (%CV)	8.2	2.6	1.4	4.4
Total variation (%CV)	9.1	2.4	1.6	4.1
N	12	15	15	15
Number of runs	3	3	3	3
(B) In human CSF during method validation				
Nominal conc. (ng/ml)	0.75	30	75	750
Mean observed conc.	0.73	27.8	71.1	710.9
%Dev	−2.2	−7.4	−5.2	−5.2
Between run precision (%CV)	4.6	3.8	3.8	0.0
Within run precision (%CV)	6.8	3.4	2.7	3.6
Total variation (%CV)	8.2	5.1	4.7	3.4
N	20	20	20	20
Number of runs	4	4	4	4

3.2.1.2. Human CSF method. Similarly, an eight-point calibration standard curve ranging from 0.25 to 100 ng/ml of BMS-299897 in human CSF was used in duplicate in each analytical run along with the QC samples at four different concentrations. Five replicate samples at each concentration were analyzed in the first four runs (triplicates for the last run). Peak area ratios of BMS-299897 to IS were used for regression analysis. Among the regression models evaluated, the quadratic model better fitted for calibration standard data. Therefore, a weighted (1/concentration) quadratic regression model was fitted to each standard curve. Out of 80 standards, only 1 of them had a percent deviation of the back-calculated concentration greater than 15% (20% for LLOQ samples) from the nominal value. The regression coefficients (R^2) were greater than 0.998. Among 92 QC samples used in the five analytical runs, only 2 of them had percent deviations of the back-calculated concentrations greater than ±15%. For the remaining QCs, most of them were within 10% of the nominal concentrations. For the four core validation runs used to determine the accuracy and precision of the method (Table 1), the intra-assay precision was within 6.8% CV, inter-assay precision was within 4.6% CV, and the assay accuracy was within ±7.4% of the nominal values.

The standard and QC validation data demonstrated that both plasma and CSF methods were accurate and precise for the determination of BMS-299897 concentrations in human plasma and CSF.

3.2.2. Lower limit of quantitation (LLOQ)

3.2.2.1. Human plasma method. Six LLOQ samples from six different plasma lots were processed and analyzed, and their predicted concentrations determined. The deviations of the predicted concentrations from the nominal value were within ±18.7% for the six LLOQ samples. A typical SRM chromatogram at the LLOQ concentration is shown in Fig. 3 (A). Increasing plasma volume may further improve the method sensitivity. However, because the limited volume (1.2 ml) of microtubes used for the extraction, plasma volume should not exceed 0.6 ml. Otherwise, the extraction may not be efficient due to insufficient mixing.

3.2.2.2. Human CSF method. Similar to the plasma method, six LLOQ samples from six different CSF lots were processed and analyzed. The deviations of the predicted concentrations from the nominal value were within ±15.2% for all of the six LLOQ samples. A typical SRM chromatogram at the LLOQ concentration is shown in Fig. 3 (B). The peak area counts and background noise level of the LLOQ sample indicated that with the current LLE procedure, the LLOQ for human CSF method had reached its LLOQ limit. In comparison with human plasma method, a lower LLOQ (4-fold) was obtained for human CSF method on a less sensitive instrument (Micromass Quattro LC versus Sciex API 3000).

3.2.3. Matrix effect

To evaluate possible matrix effects for human plasma and CSF assays, baseline and the internal standard responses were carefully monitored during method validations and sample analysis. The within-run internal standard responses were very consistent for both methods, and there was no trend in internal standard responses across standards, QCs, and study samples. The use of the stable label internal standard effectively corrected some minor fluctuations during the extraction and sample analysis, including ion suppression and matrix effects in the ion source [23].

3.2.4. Stability

The storage and processing stability of BMS-299897 in human plasma and CSF was evaluated in triplicate using QC samples at two different concentrations. The deviations of the mean predicted concentrations of the QC samples from the nominal concentrations were used as an indicator of the stability of BMS-299897 in human plasma or CSF. The results showed that BMS-299897 was stable in human plasma for at least 48 h at room temperature, at least 9 weeks at −20 °C, and through three freeze–thaw cycles. The processed samples were stable for 48 h on the autosampler at room temperature. Similarly, for the human CSF method, BMS-299897 was stable for at least 48 h at room temperature, at least 2 weeks at −20 °C, and through three freeze–thaw cycles. The processed samples were stable for 48 h on the autosampler at room temperature.

3.3. Applications

Both human plasma and CSF methods were successfully applied for the determination of BMS-299897 in human plasma and CSF samples obtained from a clinical study. More than 350 human plasma samples were analyzed within nine separate runs, and all of them passed the batch acceptance criteria. A one-way ANOVA analysis was performed for the nine runs, and the results are shown in Table 2 (A). The intra-day precision was within 10.4% CV, and the inter-day precision was within

Table 2
One-way ANOVA accuracy and precision for BMS-299897 during study sample analysis in human plasma (A) and in human CSF (B)

(A) Study sample analysis in human plasma				
Nominal conc. (ng/ml)	3	480	800	6000
Mean observed conc.	3.17	505.32	847.41	6344.76
%Dev	8.3	5.3	5.9	5.7
Between run precision (%CV)	5.7	4.9	3.0	5.2
Within run precision (%CV)	10.4	4.4	3.5	3.5
Total variation (%CV)	11.9	6.6	4.6	6.2
N	27	27	27	24
Number of runs	9	9	9	8
(B) Study sample analysis in human CSF				
Nominal conc. (ng/ml)	0.75	30	75	750
Mean observed conc.	0.74	28.34	69.93	780.78
%Dev	−1.7	−5.5	−6.8	4.1
Between run precision (%CV)	0.0	5.6	0.0	N/A
Within run precision (%CV)	9.7	3.4	5.9	N/A
Total variation (%CV)	9.6	6.6	5.6	N/A
N	21	21	21	3
Number of runs	7	7	7	1

N/A: not applicable.

5.7% CV. The assay accuracy was within ±8.3% of the nominal values. Similarly, more than 200 human CSF samples were analyzed within seven separate runs, and all of them passed the batch acceptance criteria. The intra-day precision was within 9.7% CV, and the inter-day precision was within 5.6% CV. The assay accuracy was within ±6.8% of the nominal values (Table 2 (B)). These numbers are comparable to that obtained during the method validations.

4. Conclusions

We have reported human plasma and CSF LC/MS/MS methods for the quantitative determination of BMS-299897, where liquid–liquid extraction was used for sample cleanup. For human plasma method, two incubation steps were required to effectively release BMS-299897 from plasma binding sites. The human plasma extraction method was modified to achieve a greater sensitivity for human CSF method on a less sensitive mass spectrometer. Human plasma method had a calibration range of 1–1000 ng/ml on a Sciex API 3000, and human CSF method had a calibration range of 0.25–100 ng/ml on a Micromass Quattro LC. Three potential circulating metabolites were examined to ensure method specificity. Through rigorous method validations and actual sample analysis, both methods have been proven to be reproducible and robust.

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