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Short communication

High-throughput quantitation of nefazodone and its metabolites in human plasma by high flow direct-injection LC–MS/MS

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Abstract

A rapid, selective and sensitive high-performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method coupled with high flow direct-injection on-line extraction has been developed and validated for the simultaneous quantitation of nefazodone and its three active metabolites, hydroxynefazodone, triazole-dione (BMS-180492) and *m*-chlorophyenylpiperazine (*m*CPP) in human plasma. The method utilized d_7-nefazodone, d_7-hydroxynefazodone, d_4-BMS-180492 and d_4-*m*CPP as internal standards (IS). The plasma samples were injected into the LC–MS/MS system after simply adding the internal standard solution and centrifuging. The required extraction and chromatographic separation of the analytes were achieved on an Oasis® HLB column (on-line extraction column, 1 mm × 50 mm, 30 μm) and a conventional Luna C8 column (analytical column, 4.6 mm × 50 mm, 5 μm). Detection was by positive ion electrospray tandem mass spectrometry. The total analysis run time for each sample was 2 min, which included the time needed for on-line extraction, chromatographic separation and LC–MS/MS analysis. The assay was validated for each analyte and the concentrations ranged from 2.0 to 500 ng/ml for nefazodone, hydroxynefazodone and *m*CPP and from 4.0 to 1000 ng/ml for BMS-180492, respectively. The assay was used for the high-throughput sample analysis of thousands of pharmacokinetic study samples and was proven to be rapid, accurate, precise, sensitive, specific and rugged.

Keywords: Nafazodone quantitation; High-throughput; Direct-injection; On-line extraction; LC–MS/MS

1. Introduction

Nefazodone (Fig. 1), an inhibitor of neuronal uptake of serotonin, achieves its clinical efficacy by inhibiting serotonin and norepinephrine reuptake and by antagonizing the postsynaptic 5-hydroxytryptamine 2-receptor and α_1-adrenergic receptors [1,2]. Nefazodone was completely and rapidly absorbed after oral administration and metabolized into several pharmacologically active as well as non-active metabolites [3–5]. Three active nefazodone metabolites have been identified as hydroxynefazodone, triazole-dione (BMS-180492) and *m*-chlorophenylpipeazine (*m*CPP) [6]. To support nefazodone clinical pharmacokinetic studies, bioanalytical assays were investigated for the quantitation of nefazodone and its active metabolites in human plasma.

Several methods have been reported for the quantitative determination of nefazodone and its active metabolites in biological fluids using high-performance liquid chromatography (HPLC) with ultraviolet (UV) [7,8] or coulometric detection [9] methods, and liquid chromatography–mass spectrometry (LC–MS) assay [10,11]. All the published HPLC methods suffer from long and arduous sample preparation, long chromatographic run times, low sensitivity and low selectivity. Although the reported LC–MS assay simplified the sample preparation by protein precipitation, reduced chromatographic run time to 6 min and improved sensitivity [10,11], single MS is inherently inferior to tandem MS (MS/MS) and the 6 min chromatographic run time is still long by today's industrial high-throughput standard.

Our laboratory developed a simple high flow direct-injection on-line extraction LC–MS/MS system for high-throughput quantitative bioanalysis, in which there is no sample preparation except for sample aliquotting, internal standard (IS) addition and centrifugation [12–16]. As a continued effort to increase sample throughput in the quantitation of drugs and their biotransformation products in biological matrices by LC–MS/MS, in this

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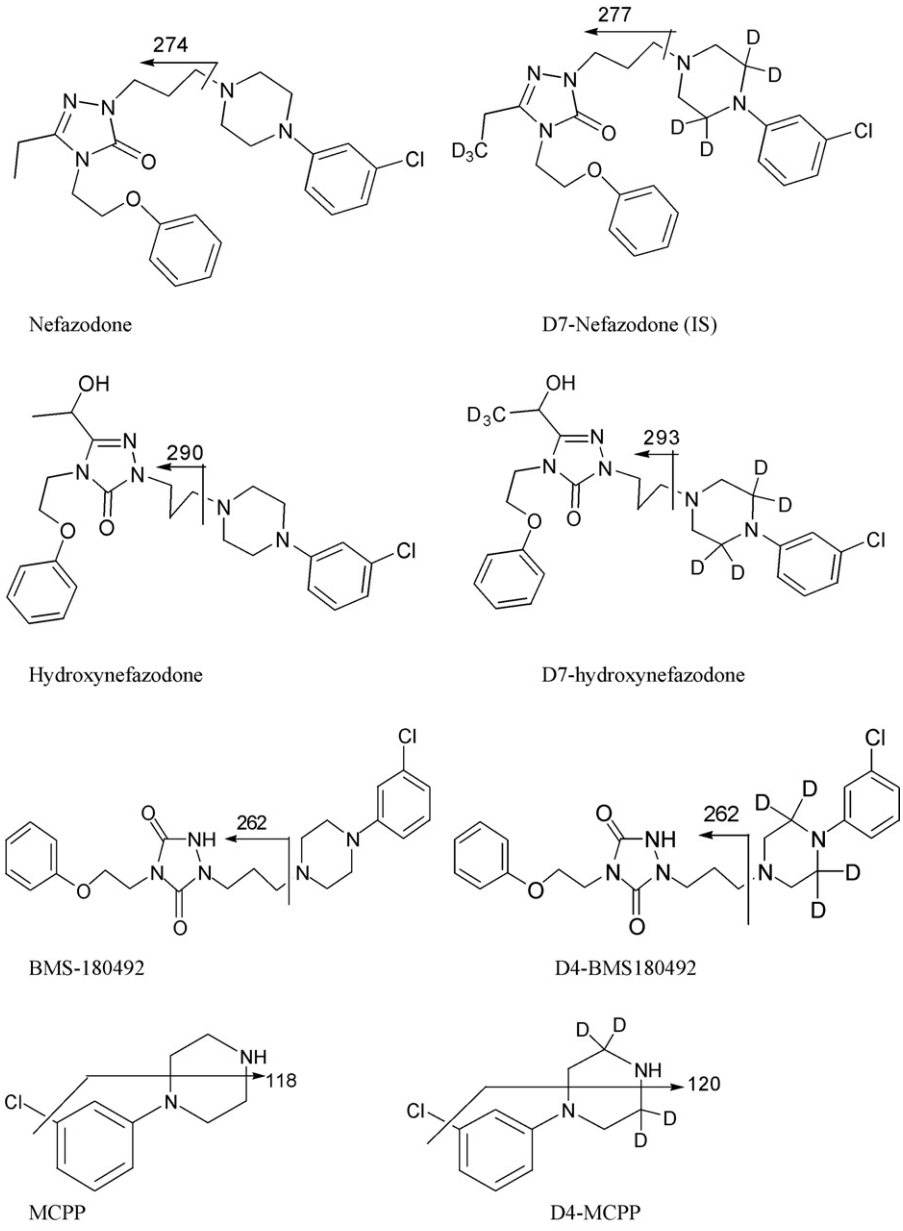


Fig. 1. Chemical structures of nefazodone, its metabolites and internal standards.

paper, we report the simultaneous quantitation of nefazodone and its three active metabolites using stable isotope labeled internal standards by high flow direct-injection LC–MS/MS technique. The total run time for each sample was only 2.0 min, which included the time needed for on-line extraction, chromatographic separation and LC–MS/MS analysis.

2. Experimental

2.1. Chemicals and reagents

Nefazodone, hydroxynefazodone, BMS-180492, mCPP and stable isotope-labeled internal standards, d_7-nefazodone for nefazodone, d_7-hydroxynefazodone for hydroxynefazodone, d_4-BMS-180492 for BMS-180492 and d_4-mCPP for mCPP, used in this study were all characterized products of BMS Pharmaceutical Research Institute. The full chemical structures of the compounds are shown in Fig. 1. Acetonitrile (ACN, HPLC grade), methanol (MeOH, HPLC grade), trifluoroacetic acid (TFA) and ammonium formate were purchased from VWR Scientific (Piscataway, NJ, USA). House deionized water that was further purified with an in-house Milli-Q water purifying system from Millipore (Bedford, MA, USA) was used. Drug-free control human plasma was purchased from Lampire Biological Laboratories (Pipersville, PA, USA). Mobile phase A was prepared by dissolving 0.32 g of ammonium formate in 1000 ml water containing 0.05% of TFA and mobile phase B was a mixture of 50% ACN and 50% MeOH. House high-purity nitrogen (99.999%) was used, and argon (99.999%) was purchased from Airgas (Radnor, PA, USA).

2.2. Materials and equipment

Polyethylene microvials (0.25 ml) were purchased from Scientific Resources Inc. (Eatontown, NJ, USA). An Oasis® HLB column, 1 mm × 50 mm, 30 μm (Waters, Milford, MA, USA) was used as on-line extraction column and a Luna C8 column, 4.6 mm × 50 mm, 5 μm (Phenomenex, Torrance, CA, USA) was used as analytical column. A Finnigan TSQ-7000 triple-stage quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an atmospheric pressure ionization (API-I) electrospray interface, six-port switching valve and Interactive Chemical Information System, was used for the LC–MS/MS analysis. Waters 2690 Separations Module Alliance HPLC System (Waters, Milford, MA, USA) and two Shimadzu LC-10AD VP pumps (Shimadzu, Columbia, MD, USA) were utilized for sample injections and mobile phase delivery.

2.3. Preparation of standard and quality control samples

Separate nefazodone and its three metabolites stock solutions, 1000 μg/ml, were prepared in ethanol. A portion of each of the stock solutions was diluted to 10 ml with water to yield a combined stock solution of 10 μg/ml for nefazodone, hydroxynefazodone and mCPP, and 20 μg/ml for BMS-180492. A combined plasma stock solution of 500 ng/ml for nefazodone, hydroxynefazodone and mCPP, and 1000 ng/ml for BMS-180492 was prepared by appropriate dilutions of the combined stock solution with the control plasma. Separate and combined quality control (QC) stock solutions and combined QC plasma stock solution were prepared the same as above, but from separate weighing. Separate internal standard stock solutions, 1000 μg/ml, were prepared by dissolving d_7-nefazodone, d_7-hydroxynefazodone, d_4-BMS-180492 and d_4-mCPP in ethanol. A combined IS working solution containing 500 ng/ml of each IS compound was prepared from the separate IS stock solutions by dilution with acetonitrile.

The calibration curve consisted of eight plasma standards, 2.00, 4.00, 10.0, 50.0, 100, 250, 350 and 500 ng/ml for nefazodone, hydroxynefazodone and mCPP, and 4.00, 8.00, 20.0, 100.0, 200, 500, 700 and 1000 ng/ml for BMS-180492. The calibration standards were freshly prepared with each extraction set by serial dilutions of the combined plasma stock solution with the control plasma. The QC samples, 5, 200, 400 and 2500 ng/ml for nefazodone, hydroxynefazodone and mCPP, and 10.0, 400, 800 and 5000 ng/ml for BMS-180492, were prepared by serial dilutions of the combined QC plasma stock solution with control

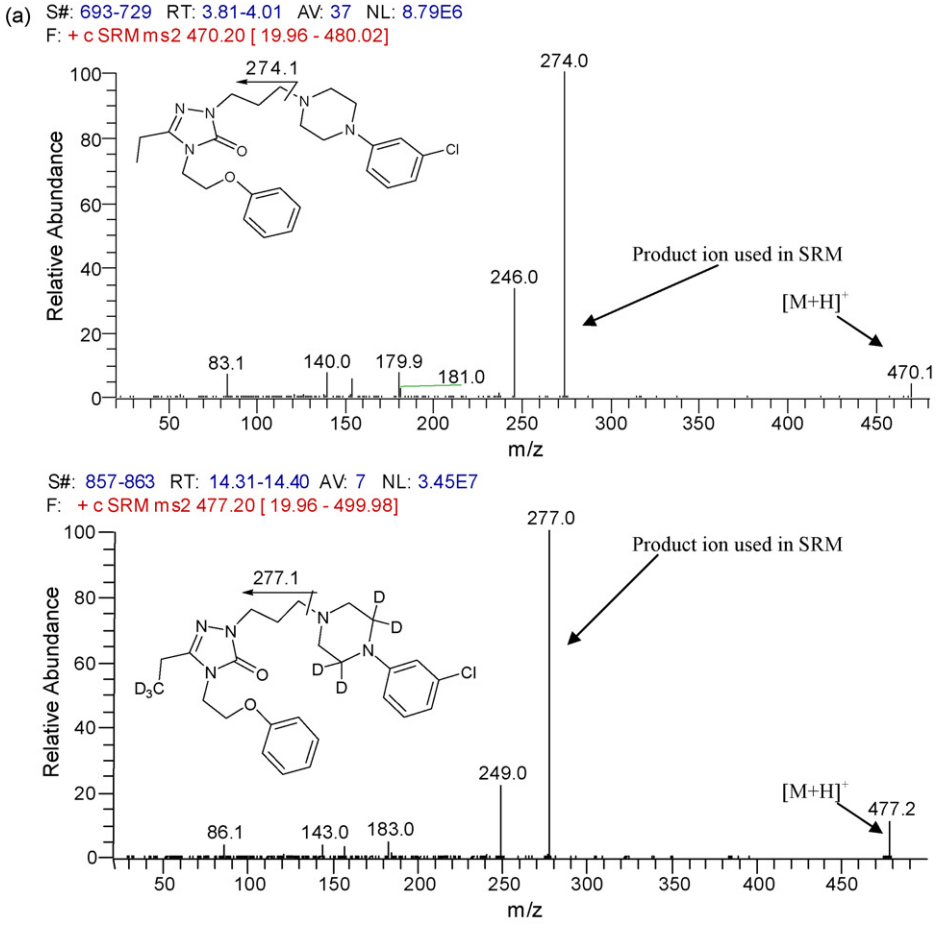


Fig. 2. (a) Electrospray positive ion MS/MS product ion spectra of $[M+H]^+$ for nefazodone (top) and its internal standard (bottom). (b) Electrospray positive ion MS/MS product ion spectra of $[M+H]^+$ for hydroxynefazodone (top) and its internal standard (bottom). (c) Electrospray positive ion MS/MS product ion spectra of $[M+H]^+$ for BMS-180492 (top) and its internal standard (bottom). (d) Electrospray positive ion MS/MS product ion spectra of $[M+H]^+$ for mCPP (top) and its internal standard (bottom).

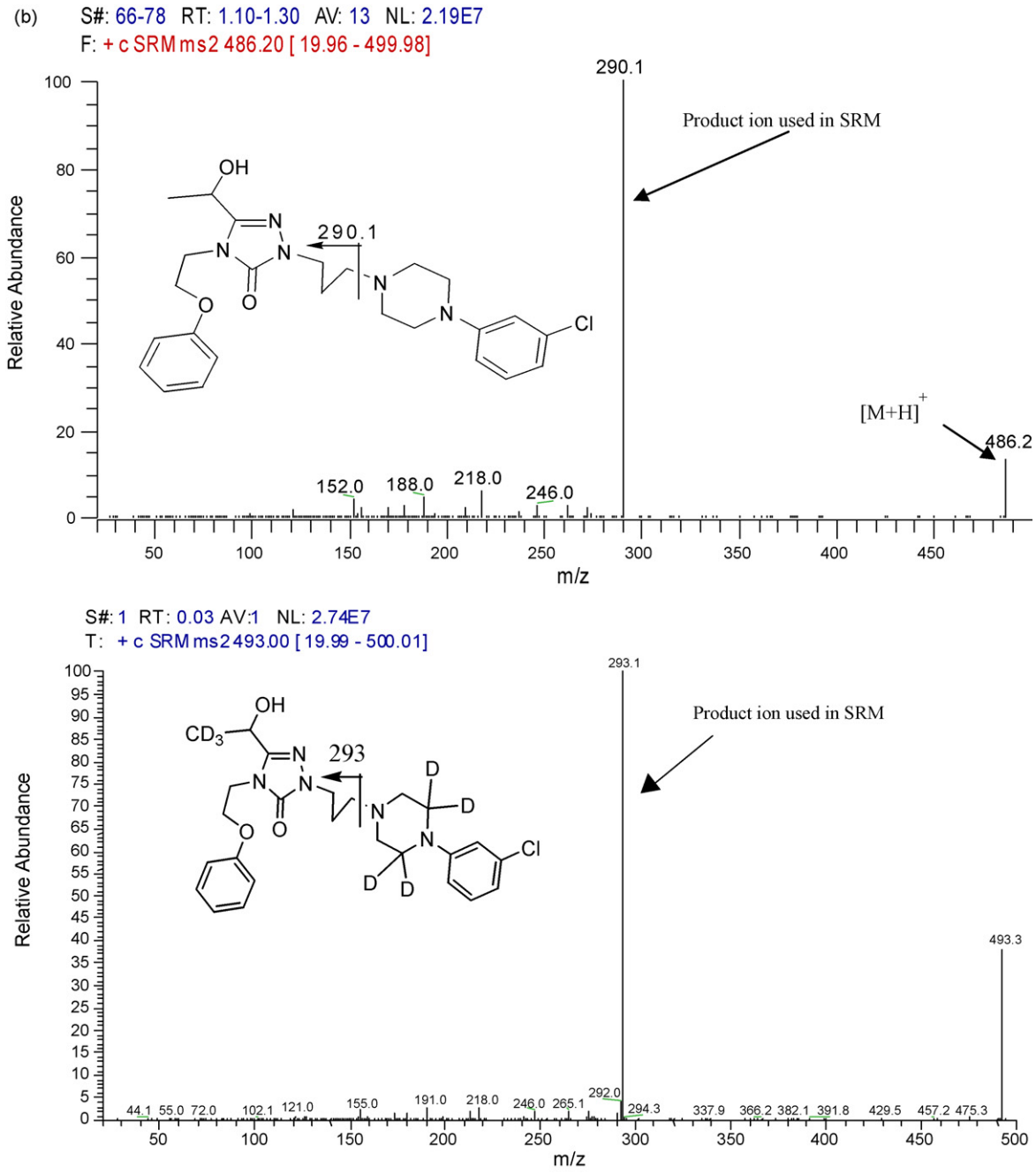


Fig. 2. (*Continued*)

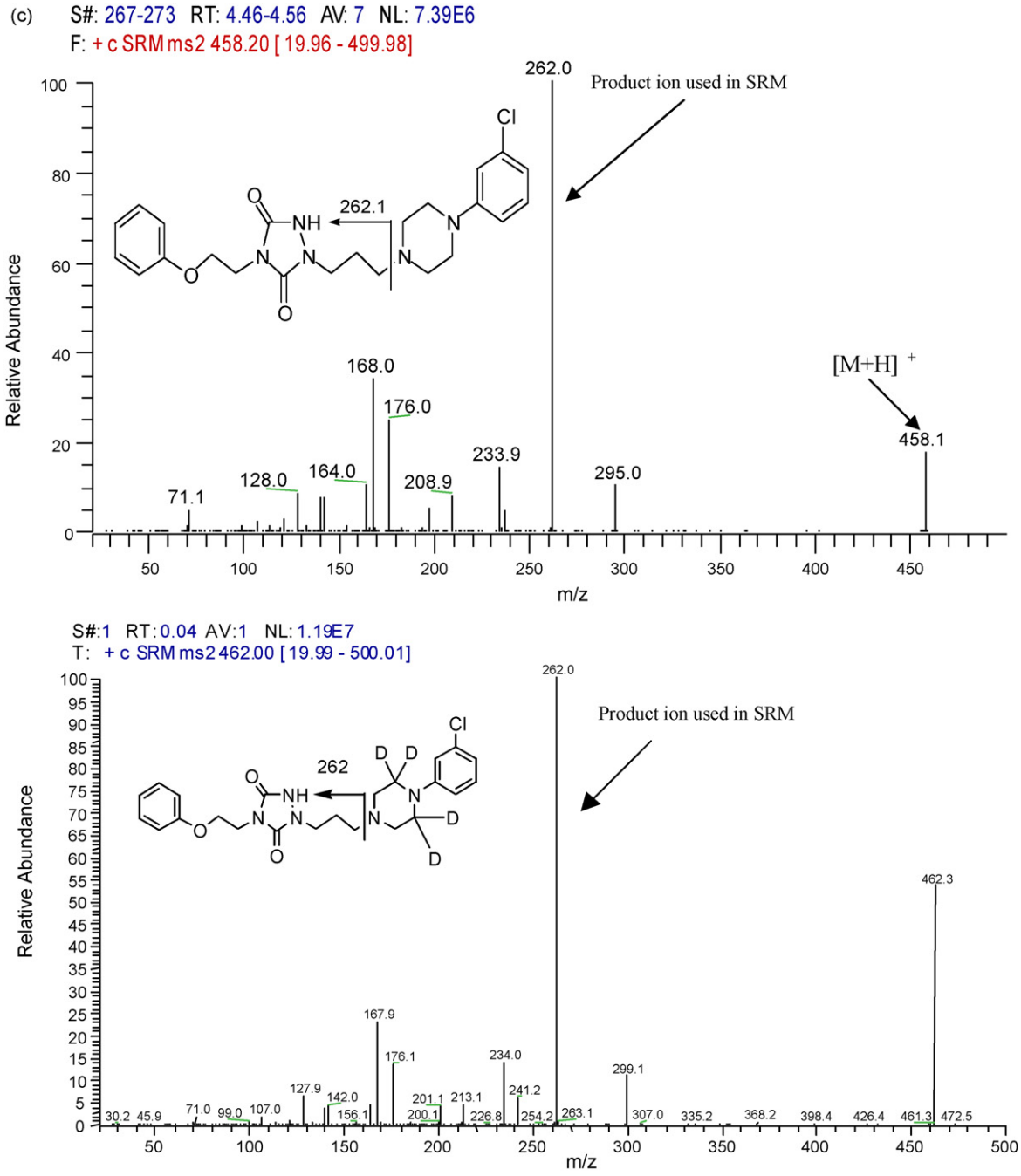
plasma and stored at −20°C in 600 μl aliquots. The 2500 and 5000 ng/ml QC samples (dilution QCs) were diluted 1:9 with drug-free human plasma before analysis.

2.4. Sample preparation

After the addition of 0.1 ml of the IS working solution to 0.1 ml of each calibration standard and QC sample in a polyethylene microvial, the samples were vortexed for 30 s and centrifuged for 5 min. The concentration of the IS in the samples was 250 ng/ml of plasma. A 20-μl portion of the processed samples was then injected into the LC–MS/MS system.

2.5. On-line extraction and chromatography

The on-line extraction set up and procedures have been described in detail previously [12]. Briefly, chromatography was carried out using a Shimadzu LC-10AD VP pump and a Waters 2690 HPLC system. A 20-μl portion of the processed plasma sample in vials placed in a chilled (4 °C) autosampler tray was injected. The loading mobile phase was 5 mM ammonium formate with 0.05% TFA at a flow rate of 4 ml/min. The eluting mobile phase consisted of 47% 5 mM ammonium formate with 0.05% TFA and 53% ACN/MeOH (50/50) at a flow rate of 1.35 ml/min.



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2.6. Electrospray ionization tandem mass spectrometry

The Finnigan TSQ-7000 triple quadrupole mass spectrometer was operated in positive electrospray ionization (ESI) mode. The spray voltage was set to 5.0 kV. The heated capillary tube temperature was set to 350 °C. Nitrogen was used as the sheath and auxiliary gas and set to 80 psi and 40 (arbitrary) units, respectively. The argon collision gas pressure was set to 2.5 mTorr. The collision energy was set to −30 eV for all of the analytes and internal standards. For selected reaction monitoring (SRM), the transitions monitored were *m/z* 470–274 for nefazodone, *m/z* 477–277 for d_7-nefazodone, *m/z* 486–290 for hydroxynefazodone, *m/z* 493–293 for d_7-hydroxynefazodone, *m/z* 458–262 for BMS-180492, *m/z* 462–262 for d_4-BMS-180492, *m/z* 197–118 for mCPP and *m/z* 201–121 for d_4-mCPP. The half-height mass-peak width was set to one unit (0.7 Da) for both Q1 and Q3 and the dwell time was 0.5 s for each SRM channel.

The SRM chromatographic peak area ratios of analytes to internal standards were used for the quantitation and each calibration curve was fitted to a $1/x$ weighted linear regression model.

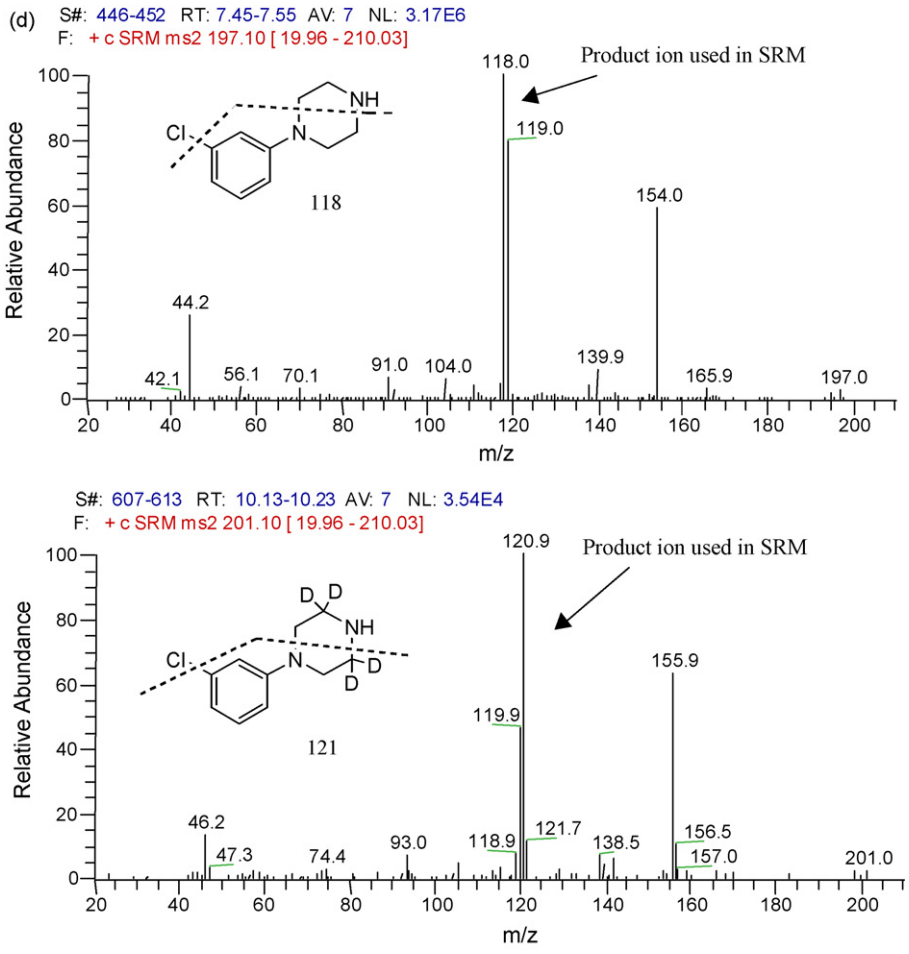


Fig. 2. (*Continued*).

2.7. Method validation

An LC–MS/MS method was first fully validated for the quantitation of nefazodone and its three motabolites in human EDTA plasma using d_7-nefazodone as the internal standard for nefazodone, hydroxynrfazodone and BMS-180492, and d_4-mCPP for mCPP. After the stable labeled analogs of hydroxnefazodone and BMS-180492 became available, this fully validated assay was then modified and revalidated for the quantitation of nefazodone, hydroxynefazodone, BMS-180492 and mCPP in 0.1 ml of human EDTA plasma by incorporating all stable isotope analogs as internal standards to reduce potential matrix effects in sample analysis. For each analyte, an eight-point calibration standard curve in human plasma, ranging from 2.00 to 500 ng/ml for nefazodone, hydroxynefazodone and mCPP and from 4.00 to 1000 ng/ml for BMS-180492, was used in duplicate. The accuracy at the lowest level of quantitation (LLOQ) for each analyte was assessed by spiking six different lots of human plasma with nefazodone, hydroxynefazodone and mCPP at 2.00 ng/ml and BMS-180492 at 4.00 ng/ml and internal standards at 250 ng/ml. The LLOQ samples were then analyzed as unknown against the standard curves. For the specificity of the assay, six different lots of blank human plasma were analyzed after they had been spiked with the internal standards only (QC0), or with no spiking at all. For the determination of assay precision and accuracy, QC samples at four concentrations were assayed in replicates of five on three different days for the full assay validation and 1 day for the cross validation. Since the sample analysis was performed using the assay with all four stable isotope analogs as internal standards, only the 1 day assay validation data will be discussed later in Section 3.3.

3. Results and discussion

3.1. Mass spectrometry

In positive ESI mode under the experimental conditions, the most abundant ions observed were protonated molecular ions of nefazodone, its metabolites and the internal standards (data not shown). Collision-induced dissociation (CID) of these protonated molecular ions produced their corresponding product ions (Fig. 2(a–d)). Selected reaction monitoring was used for the quantitation. The proposed CID fragmentation of all the analytes are shown in Fig. 2(a–d).

During the MS scanning, in addition to the proton adduct $[M+H]^+$ ions, signals from the ammonium adduct $[M+NH_4]^+$ ions were observed as well, and the relative intensities of these two ions varied depending on the experimental conditions used

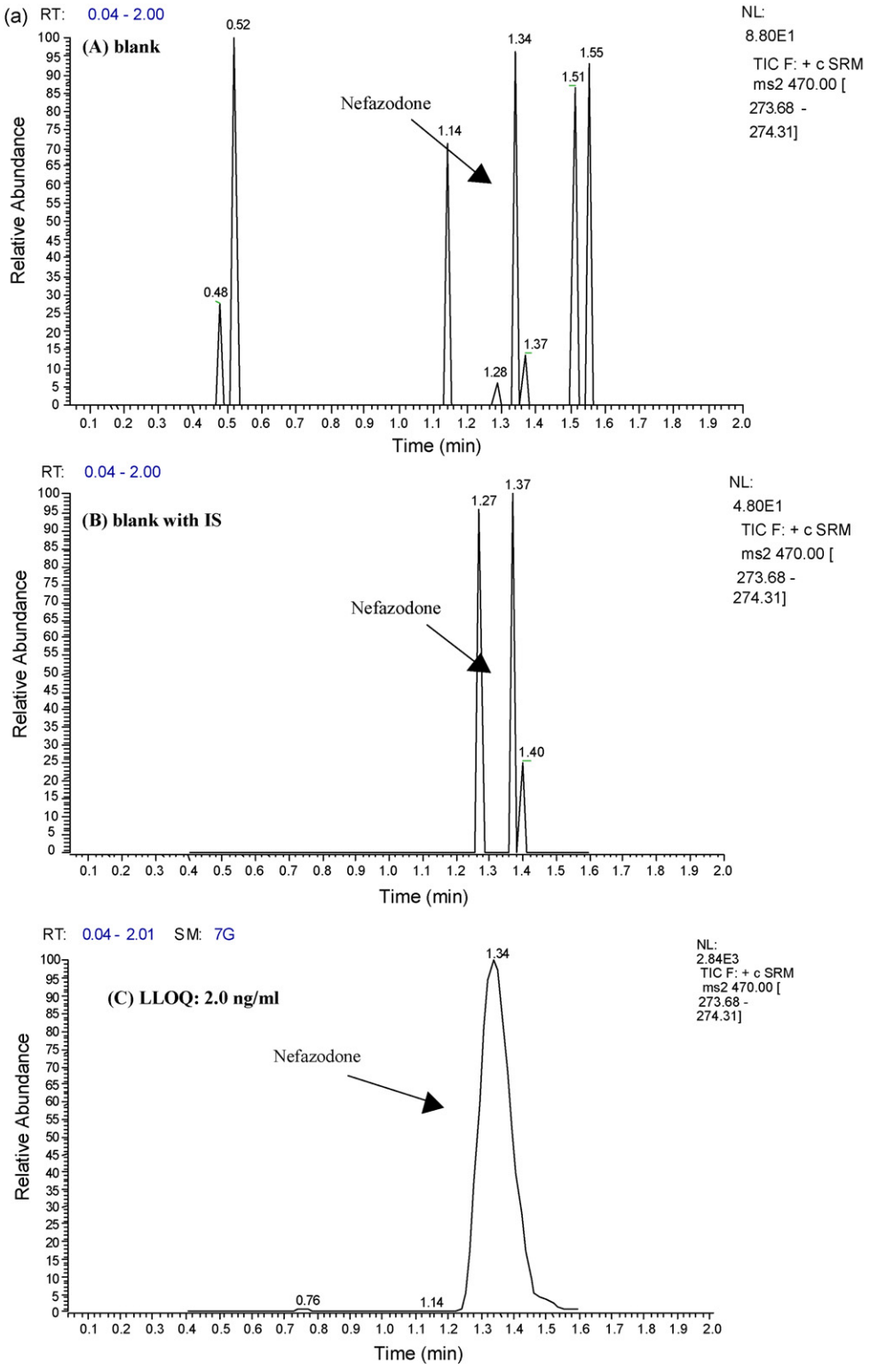
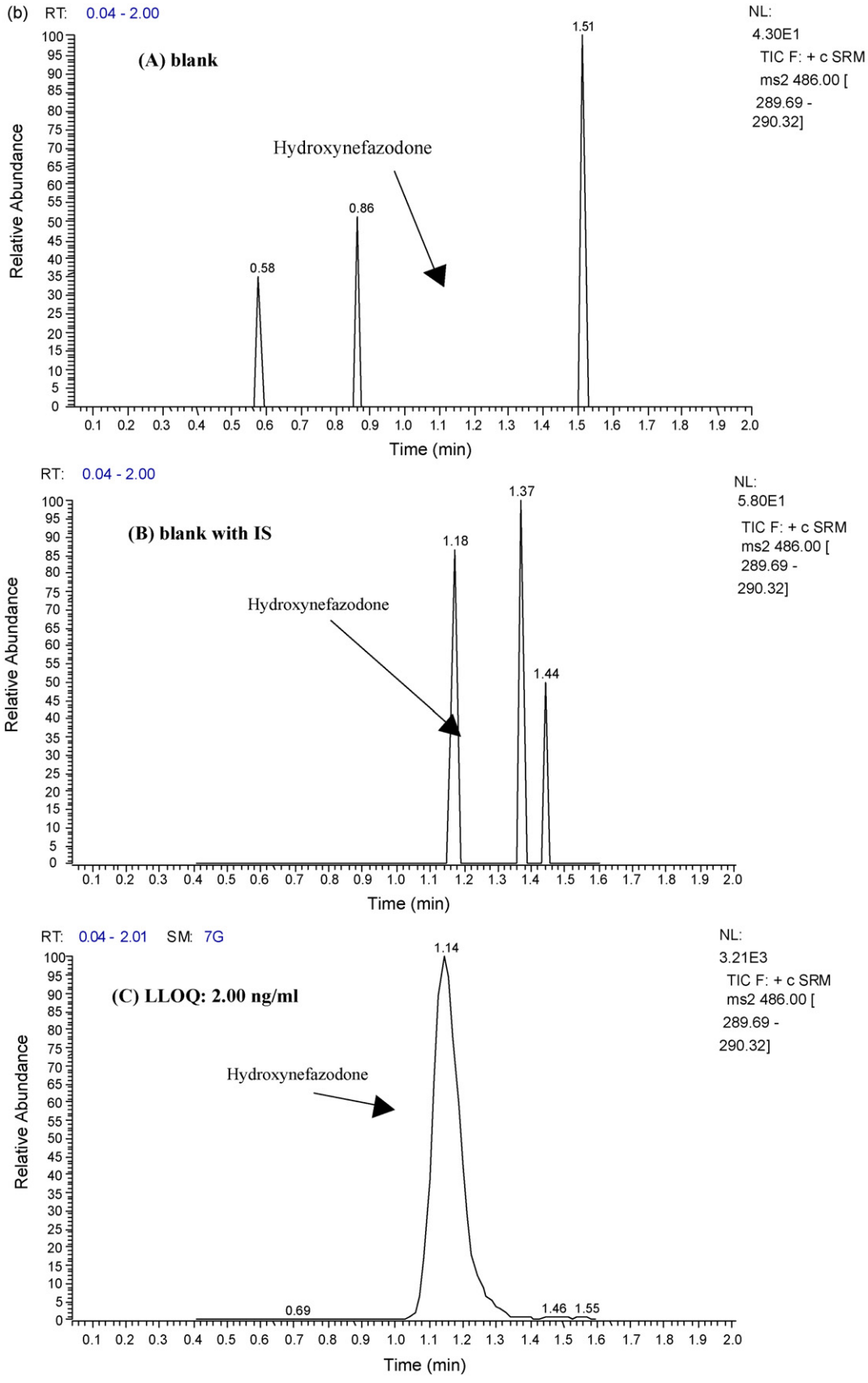


Fig. 3. (a) Selected reaction monitoring chromatograms for nefazodone (*m/z* 470 → 274) obtained from: (A) blank human plasma; (B) human plasma containing only internal standard at 250 ng/ml; (C) human plasma containing nefazodone at lower limit of quantitation (2.00 ng/ml) and its internal standard at 250 ng/ml. (b) Selected reaction monitoring chromatograms for hydroxynefazodone (*m/z* 486 → 290) obtained from: (A) blank human plasma; (B) human plasma containing only internal standard at 250 ng/ml; (C) human plasma containing nefazodone at lower limit of quantitation (2.00 ng/ml) and its internal standard at 250 ng/ml. (c) Selected reaction monitoring chromatograms for BMS-180492 (*m/z* 458 → 262) obtained from: (A) blank human plasma; (B) human plasma containing only internal standard at 250 ng/ml; (C) human plasma containing nefazodone at lower limit of quantitation (4.00 ng/ml) and its internal standard at 250 ng/ml. (d) Selected reaction monitoring chromatograms for mCPP (*m/z* 197 → 118) obtained from: (A) blank human plasma; (B) human plasma containing only internal standard at 250 ng/ml; (C) human plasma containing nefazodone at lower limit of quantitation (2.00 ng/ml) and its internal standard at 250 ng/ml.



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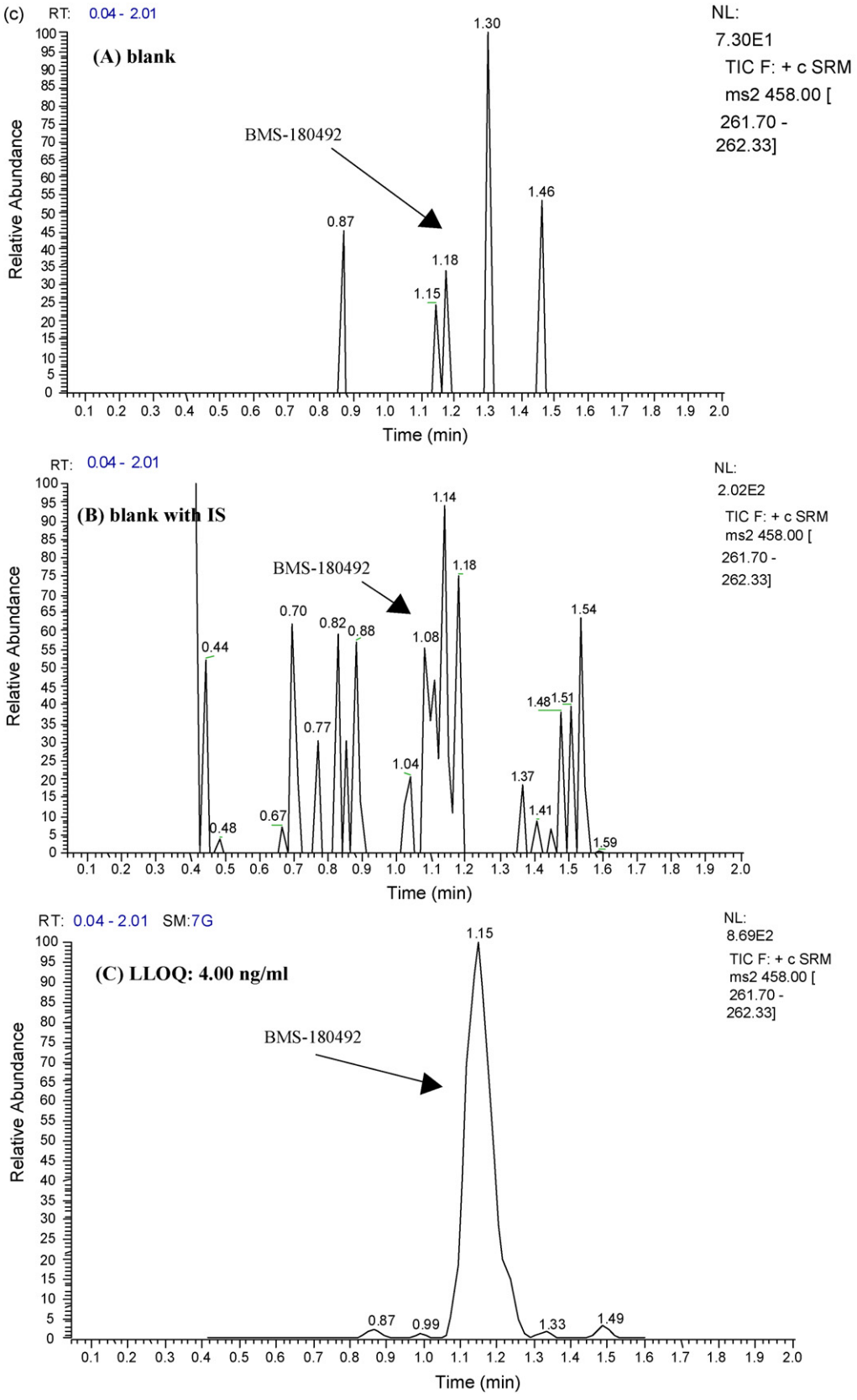

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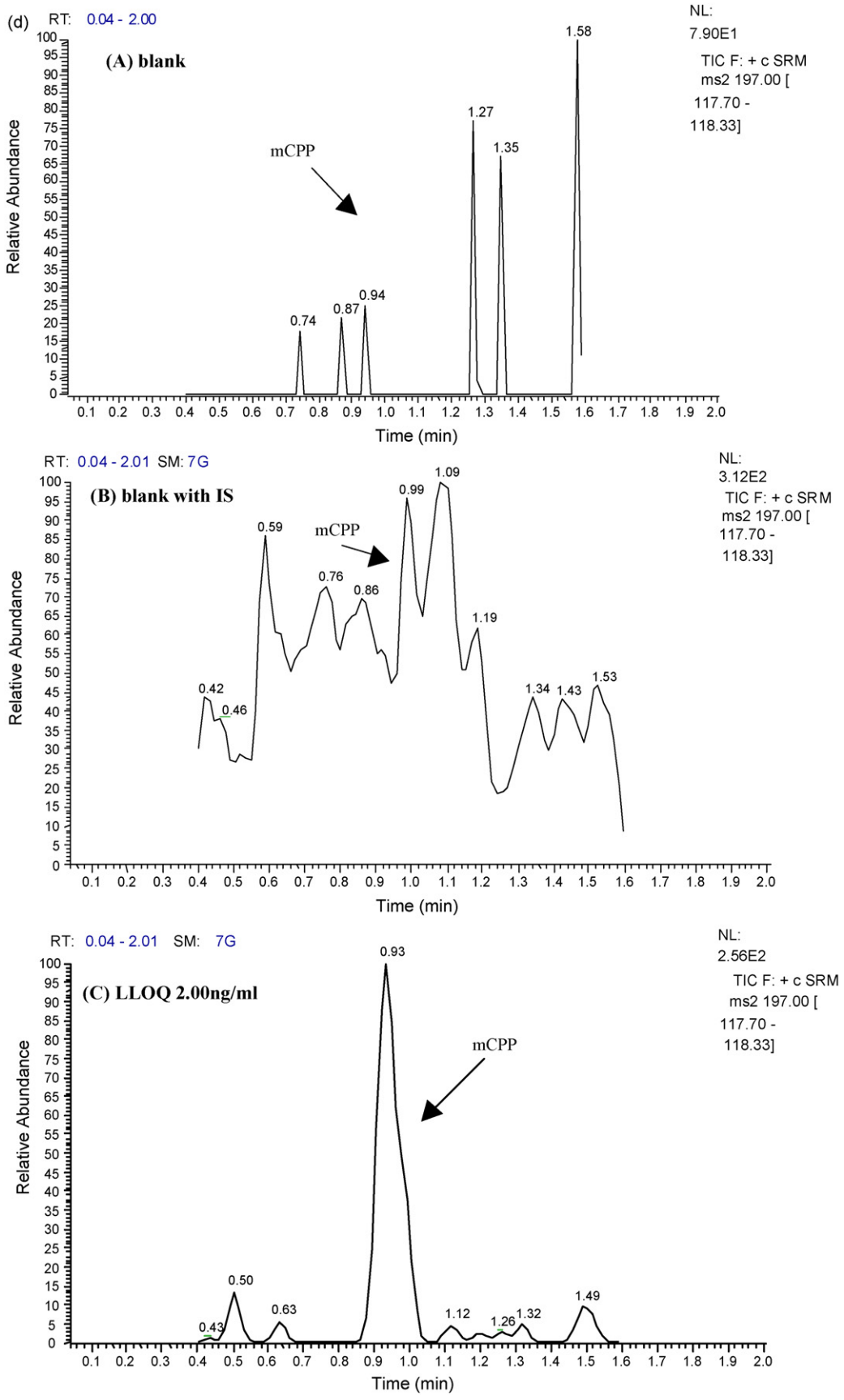

Fig. 3. (*Continued*).

(data not shown). If the $[M+NH_4]^+$ (*m*/*z* 487) of nafazodone is used for the quantitation, a potential interference will exist resulting from the A + 1 of the $[M+H]^+$ of hydroxynefazodone (*m*/*z* 486). This is true even if Q1 (MS) operated under a unit-mass resolution [14]. Therefore, the favorable conditions for the formation of the protonated ions were selected and the $[M+H]^+$ (*m*/*z* 470) of nafazodone was used for the quantitation.

3.2. High-flow chromatographic separation for high-throughput LC–MS/MS

The unique feature of the on-line extraction technique employed in this study is the use of a narrow-bore LC extraction column (1 mm × 50 mm) packed with large particles of a stationary phase material (30 μm), with a very high flow of the mobile phase (4.0 ml/min). The combination of the fast flow and large particle sizes provides the desired chromatographic behavior that allows the achievement of rapid passage of the large biomolecules in the biological sample and simultaneous retention of the small molecules of interest [16]. Because of the inherent specificity and sensitivity of the tandem mass spectrometry, the high flow LC–MS/MS has made it possible to drastically reduce the chromatographic run time.

3.3. Method validation

Chromatographic data acquisition and integration were performed using the system quantitation software. Peak area ratios (analyte area/IS peak area) versus nominal concentrations of the standards were fitted to a linear regression equation, weighting each standard by the reciprocal of its concentration. Linear standard curves were constructed ranging from 2.00 to 500 ng/ml for nefazodone, hydroxynefazodone and mCPP and from 4.00 to 1000 ng/ml for BMS-180492, respectively. The deviations of the back-calculated concentrations from their nominal values were within ±11.8% for calibration standards of all analytes.

To assess the specificity of the assay, six different lots of control human plasma were analyzed with and without internal standards followed by inspection of the chromatograms to determine whether any endogenous plasma constituents interfered with the analytes or the internal standards. No significant interfering peaks from the plasma were found at the retention times and in the ion channels of analytes (Fig. 3(a–d)).

For the verification of the lower limit of quantitation (LLOQ) of the assay, six different lots of control human plasma were spiked with the analytes at the lowest levels of their standard curves, 2.00 ng/ml for nefazodone, hydroxynefazodone and mCPP and 4.00 ng/ml for BMS-180492, respectively, and the internal standards at 250 ng/ml. The LLOQ samples were then analyzed with standard and QC samples and their predicted concentrations were determined. Typical chromatograms from each analyte at the LLOQ concentration are shown in Fig. 3(a–d). The mean deviation of the predicted concentrations from the spiked value for each analyte was within 8.0% for all LLOQ samples.

To assess the accuracy and precision of the method, QC samples at three concentrations, 5.00, 200 and 400 ng/ml for nefazodone, hydroxynefazodone and mCPP and 10.00, 400 and 800 ng/ml for BMS-180492, respectively, were prepared and analyzed at five replicates in one run. A fourth QC sample with concentrations higher than the upper limits of the standard curve ranges, 2500 ng/ml for nefazodone, hydroxynefazodone and mCPP and 5000 ng/ml for BMS-180492, respectively, was also analyzed. This QC sample, known as dilution QC, was diluted 1:9 with control human plasma. A portion (0.1 ml) of the dilute QC sample was processed. Concentration of each analyte in QC samples was determined by inverse prediction from the calibration curve. The assay precision and accuracy were obtained during the validation of the method. The intra-assay precision was within 7.7% CV and assay accuracy was within ±8.8% of the nominal values for all four analytes (data not shown).

The accuracy, precision and ruggedness of the assay were further demonstrated by its successful application to several clinical studies. More than 5000 human plasma samples were assayed for the determination of the concentrations of nefazodone, hydroxynefazodone, mCPP and BMS-180492 using the above validated assay. A one-way ANOVA analysis was performed for the 12 runs of one clinical study (data not shown). All of the 12 analytical runs met the following acceptance criteria established a priori: (1) the predicted concentrations of at least three-fourths of all calibration standards shall be within ±15% of their nominal concentrations (±20% for the LLOQ); (2) at least one replicate of the lowest concentration in the standard curve shall be within ±20% of the nominal concentration for that level to qualify as the LLOQ and (3) the predicted concentrations of at least two-thirds of all QC samples shall be within ±15% of their individual nominal concentrations, with at least one (1) acceptable QC sample at each level meeting the acceptance criteria. For all analytes, the intra-day precision was within 7.3% CV and inter-day precision was within 5.7% CV; the assay accuracy was within ±2.7% of the nominal concentrations.

4. Conclusions

A high flow direct-injection LC–MS/MS method was developed and validated in human plasma over the concentration ranges of 2.0–500 ng/ml for nefazodone, hydroxynefazodone and *m*-chlorophyenylpiperazine and 4.0–1000 ng/ml for triazole-dione (BMS-180492), respectively. The required extraction and chromatographic separation of the analytes were achieved with a total run time of 2 min per sample. The assay was successfully applied to nefazodone pharmacokinetic studies for high-throughput sample analysis and was proven to be rapid, accurate, precise and rugged.

References

[1] O. Spigset, S. Hagg, A. Bate, Int. Clin. Psychopharmacol. 18 (2003) 157–161.
[2] A.S. Eison, M.S. Eison, J.R. Torrente, R.N. Wright, F.D. Yocca, Psychopharmacol. Bull. 26 (1990) 311–315.
[3] R.F. Mayol, C.A. Cole, G.M. Luke, K.L. Colson, E.H. Kerns, Drug Metab. Dis. 22 (1994) 304–311.
[4] R.H. Barbhaiya, U.A. Shukla, P. Chaikin, D.S. Greene, P.H. Marathe, Eur. J. Clin. Pharmacol. 50 (1996) 101–107.

[5] L.L. Von Moltke, D.J. Greenblatt, B.W. Granda, J.M. Grassi, J. Schmider, J.S. Harmatz, R.I. Shader, Psychopharmacology 145 (1999) 113–122.
[6] S. Kaul, U.A. Shukla, R.H. Barbhaiya, J. Clin. Pharmacol. 35 (1995) 830–839.
[7] J.E. France, G.F. Duncan, R.H. Farmen, K.A. Pittman, J. Chromatogr. B 570 (1991) 129–135.
[8] S. Dodd, A. Buist, G.D. Burrows, K.P. Maguire, T.R. Norman, J. Chromatogr. B 730 (1999) 249–255.
[9] M. Franklin, J. Pharm. Biol. Anal. 11 (1993) 1109–1113.
[10] M. Yao, V.R. Shah, W.C. Shyu, N.R. Srinivas, J. Chromatogr. B 718 (1998) 77–85.
[11] M. Yao, N.R. Srinivas, Biomed. Chromatogr. 14 (2000) 106–112.
[12] M. Jemal, Y.-Q. Xia, D.B. Whigan, Rapid Commun. Mass Spectrom. 12 (1998) 1389–1399.
[13] M. Jemal, Z. Ouyang, Y.-Q. Xia, M.L. Powell, Rapid Commun. Mass Spectrom. 13 (1999) 1462–1471.
[14] M. Jemal, Z. Ouyang, Rapid Commun. Mass Spectrom. 14 (2000) 1757–1765.
[15] M. Jemal, M. Huang, Y. Mao, D. Whigan, A. Schuster, Rapid Commun. Mass Spectrom. 14 (2000) 1023–1028.
[16] M. Jemal, Biomed. Chromatogr. 14 (2000) 422–429.