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Sensitive liquid chromatographic–mass spectrometric assay for the simultaneous quantitation of nefazodone and its metabolites hydroxynefazodone *m*-chlorophenylpiperazine and triazole-dione in human plasma using single-ion monitoring

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Abstract

A sensitive, selective, accurate, precise and reproducible high-performance liquid chromatographic–mass spectrometric (LC–MS) assay was developed and validated for the simultaneous determination of nefazodone (NEF), hydroxynefazodone (OH-NEF), *m*-chlorophenylpiperazine (mCPP), and triazole-dione (Dione) in human plasma using trazodone (TRZ) as the internal standard (I.S.). The method involved simultaneous protein precipitation with acetonitrile and liquid–liquid extraction with methylene chloride, after which the organic layer was evaporated to dryness. The residue was reconstituted in 25% acetonitrile in 10 m*M* ammonium formate (pH 4.0), and an aliquot was injected onto a BDS Hypersil C_{18} column at a flow-rate of 0.3 ml/min. The mobile phase comprising of 10 m*M* ammonium formate (pH 4) and acetonitrile in 55:45 (v/v) was used in an isocratic condition. The mass spectrometer was programmed to admit the protonated molecules at *m*/*z* 197.0 (mCPP), 372.0 (I.S.) 470.4 (NEF), 458.1 (Dione), and 486.2 (OH-NEF). Standard curves were linear ($r^2 \geq 0.995$) over the concentration range of 4–1000 ng/ml for Dione and 2–500 ng/ml for other analytes. The lowest standard concentrations were the lower limit of quantitation for each analyte. The mean predicted quality control (QC) concentrations for all analytes deviated less than −12.1% from the corresponding nominal values; the intra-assay and inter-assay precision of the assay for all analytes were within 7.0% relative standard deviation. All analytes including I.S. were stable in the injection solvent at room temperature for at least 24 h. The extraction recovery of the various analytes ranged from 67.3 to 86.5%. The validated assay was applied to a pharmacokinetic study of nefazodone.

Keywords: Nefazodone; Hydroxynefazodone; *m*-Chlorophenylpiperazine; Triazole-dione

1. Introduction

Nefazodone (Serzone™ NEF), chemically 2-[3-4-(3-chlorophenyl)-1-piperazinyl]-propyl]-5-ethyl)-2,4-dihydro- 4 -(2 - phenoxy-ethyl)- 3 *H* - 1,2,4,-triazol-3-one-hydrochloride (Fig. 1), is a new antidepressant [1]. NEF is structurally unrelated to the tricylic and tetracyclic antidepressants. The clinical antidepressant activity of NEF is attributed to the potentiation of the central serotonergic system. Preclinical studies

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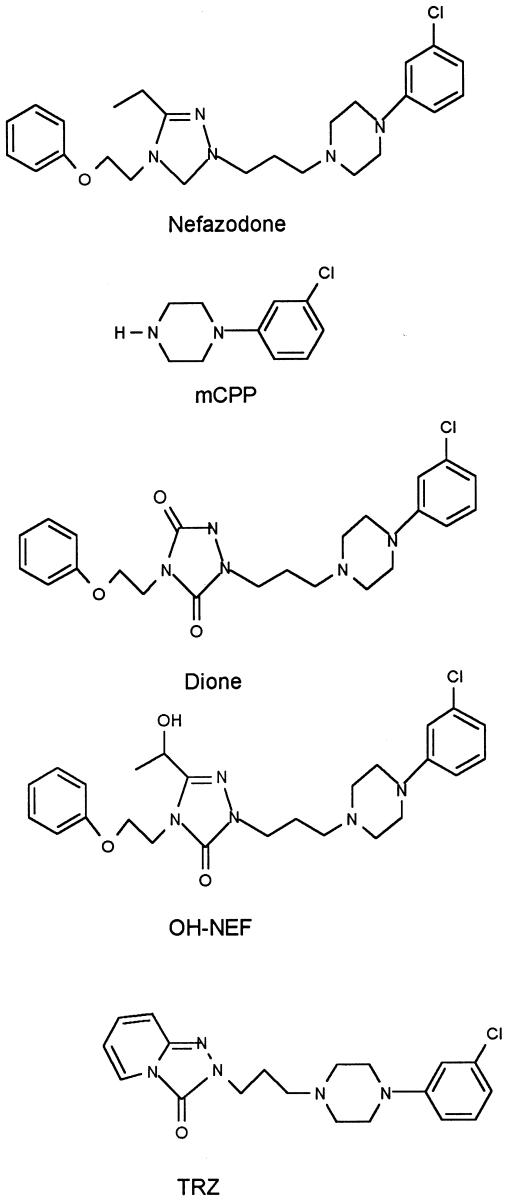

Fig. 1. Structural representation of nefazodone, mCPP, dione, OH-NEF, and trazodone (I.S.).

have confirmed that NEF is a strong inhibitor of the neuronal uptake of serotonin [2,3]. NEF undergoes extensive first pass metabolism in humans with an absolute bioavailability of approximately 20% [4]. The main metabolites of NEF are hydroxy-nefazodone (OH-NEF), triazole-dione (Dione), and *m*-chlorophenylpiperazine (mCPP) (Fig. 1) [4]. In vitro serotonin uptake studies have shown that both OH-NEF and Dione have stronger activity compared to mCPP.

High-performance liquid chromatographic methods employing either ultraviolet [5] or coulometric detection [6] are available for the determination of NEF and its metabolites. However, since the published methods were time consuming with regard to both sample preparation and chromatographic run time, an improved method which would adequately address both of these shortcomings is developed and validated.

2. Experimental

2.1. Chemicals and reagents

NEF (purity 99.7%), OH-NEF (purity 98.0%), Dione (purity 94.1%) and trazodone (I.S.; purity 100%) were obtained from Bristol-Myers Squibb Company (Wallingford, CT, USA). mCPP (purity 99.4%) was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskeson, MI, USA). Methylene chloride was purchased from EM Science (Gisstown, NJ, USA). Water was purified by Mill-Q-System from Millipore Corp. (Milford, MA, USA). Ammonium formate (Avocado Research Chemicals, Ltd., Wordhill, MA, USA), formic acid (J.T. Baker, Phillipsburg, NJ, USA) were of analytical grade. Control human plasma was purchased from Biological Specialty Corp. (Calmar, PA, USA). All other reagents were of analytical grade and were used without further purification.

2.2. HPLC and API-MS condition

HPLC was performed on a BDS Hypersil 3-μm ODS column (100×2 mm) (Keystone Inc., Wilmington, NC, USA) using an alliance 2690 HPLC system containing autosampler (Waters Corp., Milford, MA, USA). The mobile phase consisted of acetonitrile and 10 m*M* ammonium formate (pH 4, adjusted by formic acid) (45:55, v/v). The flow-rate was 0.3 ml/min.

Mass spectrometric detection was carried out using a PE Sciex API 100 single-stage quadruple

instrument (Perkin-Elmer, Foster City, CA, USA) operating in the positive API mode. The mass spectrometer was programmed to admit the protonated molecules at the mass to charge ratios (m/z) of 197.0 (mCPP), 372.0 (I.S.), 458.1 (Dione), 470.4 (NEF), and 486.2 (OH-NEF). The positive turbo-ion spray voltage (V) was 4500 mV. The orifice and ring voltages were set at 48 and 335 mV, respectively. The dwell time was 200 ms. Curtain gas and nebulizing gas (nitrogen) pressure were set at 8.26 and 4.82 kPa, respectively. The flow-rate of heated gas (Gas 2) was operated at 5 l/min. TurboIonSpray temperature was set at 350°C. Analytical data were acquired by the PE Sciex Software (LC Tune1.3) and the peak areas measured using MacQuan software (MacQuan 1.5).

2.3. Standard solutions

Stock solutions of NEF, OH-NEF, Dione, mCPP, and I.S. were prepared in ethanol (200–300 μg/ml) and stored at −20°C. A working I.S. solution was prepared daily at a concentration of 50 ng/ml in acetonitrile and was used to spike the samples prior to extraction.

2.4. Standard curve and quality control samples

A serial dilution technique was employed to obtain the final concentrations with 2–500 ng/ml of NEF, OH-NEF and mCPP, and 4–1000 ng/ml of Dione in human plasma. These spiked samples, containing eight different concentrations, were used to construct the standard curve. Quality control (QC) samples were prepared from independent stock solutions in human blank plasma to contain concentrations of NEF and its metabolites within the standard curve range. In addition, a QC sample containing concentrations of analytes above the upper limit of the standard curve was prepared in blank plasma to serve as a diluting QC sample.

2.5. Sample preparation

To a 0.1-ml aliquot of the human plasma standard, blank, QC or study sample in a clean screw-capped tube, was added 0.2 ml of acetonitrile containing 50 ng/ml of I.S. to precipitate the protein. Subsequently, 0.1 ml methylene chloride was added to the sample and it was vortexed for 2 min. After centrifugation at 20 200 *g* for 5 min, upper layer organic phase was transferred to a 4-ml glass tube and evaporated under a gentle stream of nitrogen at 30°C. The residue was reconstituted with 0.1 ml of 25% acetonitrile in 10 m*M* ammonium formate (pH 4.0, adjusted with formic acid), vortexed and centrifuged at 2000 *g* for 3 min. Then a 20-μl volume of the reconstituted sample was injected onto the LC–MS system.

2.6. Validation of the assay

2.6.1. Calibration

Calibration curves were constructed by plotting peak area ratios of the analyte to the I.S. against the analyte's concentration. The weighted ($1/x$) linear regression was fitted over the concentration range (i.e., 2–500 ng/ml for NEF, OH-NEF, and mCPP; and 4–1000 ng/ml for Dione).

2.6.2. Accuracy and precision

The accuracy and precision of the assay were determined by assaying QC samples (10, 200, and 400 ng/ml for NEF, OH-NEF, and mCPP; and 20, 400 and 800 ng/ml for Dione) in six replicates on at least three different days. In addition, a fourth QC sample (dilution QC) was spiked above the highest standard (i.e., 2000 ng/ml for Dione and 1000 ng/ml for the other analytes) and diluted with blank plasma prior to sample preparation. The dilution QC samples were analyzed in a single run. The inter-assay precision was evaluated by one-way ANOVA to obtain the treatment mean square (TMS), error mean square (EMS), and grand mean (GM). Inter-assay precision, expressed as %R.S.D., was defined for each of the concentration as:

$$\%\text{R.S.D.} = 100[(\text{TMS} - \text{EMS})/N]^{0.5}/\text{GM}$$

Alternatively, if EMS is greater than TMS, the inter-assay precision can be calculated as the %R.S.D. of the individual sequence means about the grand mean.

2.6.3. Lower limit of quantitation (LLQ)

Control plasma samples were obtained from 10 individuals. For each individual, a blank plasma and

a spiked plasma containing NEF (2 ng/ml), OH-NEF (2 ng/ml), mCPP (2 ng/ml), and Dione (4 ng/ml) were prepared and analyzed. The performance of the assay on accuracy at the LLQ was calculated as the percentage deviation (%DEV) of the mean observed concentration from the nominal concentration for each analyte in the 10 spiked individual samples. The precision of the assay at the LLQ was expressed as the %R.S.D. of the observed concentration for each analyte in the 10 spiked individual samples.

2.6.4. Extraction recovery

Two sets of standards, in the concentration range of 2–500 ng/ml for mCPP, OH-NEF and NEF, and in the range 4–1000 ng/ml for Dione, were prepared. One set was prepared in human plasma and the other set was prepared in the mobile phase. Plasma standards were processed and chromatographed as previously described; while standards prepared in mobile phase were injected onto the column directly. The extraction recovery was calculated by the following equation.

$$\%\text{Recovery} = \frac{\text{Peak area slope of standard curve prepared in plasma}}{\text{Peak area slope of standard curve prepared in mobile phase}}$$

2.6.5. Autosampler stability

The stability of Dione, mCPP, NEF, OH-NEF and I.S. in the injection solvent while waiting for injection in the autosampler was determined by periodically injecting replicate preparations of processed samples at 0, 4, 8 and 24 h. The peak area obtained at 0 h for each analyte was used as the reference in calculating the relative ratios for each analyte at the various time intervals.

3. Results and discussions

3.1. Method development

LC–MS technology has progressed tremendously in recent years; it offers single–quadruple mass spectrometers for online HPLC–MS, which is now routinely used for quantitative determination of drugs and their metabolites. The drugs or metabolites with different masses can be unambiguously identified and determined accurately with single–quadruple mass spectrometers even when they cannot be separated adequately by the HPLC column. However, if there are some endogenous compounds that have the same molecular weight as the analyte, this will interfere in the quantification of the analyte. In this situation, separation of the analyte of interest from endogenous interference by HPLC will be critical. In this regard, when we employed a protein precipitation extraction method (with acetonitrile), we observed an endogenous interference from plasma extract at the retention time of the mCPP peak (a very polar metabolite eluting close to the void volume on the HPLC). Instead of changing the HPLC conditions to resolve this problem, we modified the extraction procedure. Through a series of experiments, it was observed that acetonitrile could be easily separated from the aqueous phase by addition of methylene chloride at a ratio of 2:1:1 (acetonitrile:plasma:methylene chloride, see Section 2.5). Since most of the polar endogenous interference remained in the aqueous phase, no interfering components were observed on the HPLC at the retention time of mCPP peak and a clean single ion monitoring of mCPP was achieved. Prior to the protein precipitation method, a liquid–liquid extraction method using toluene was attempted, but due to a poor recovery of Dione this method was not considered further.

Initially, two types of ODS column (100×2 mm) with or without treatment of basic deactivated silica (BDS) were employed. The BDS-treated ODS column provided better resolution of all analytes, which are basic compounds, than the ODS column without BDS treatment. Since specially treated silica and special bonding chemistry are used to produce a very deactivated packing material, the BDS column appears to be well suited for the separation of multiple basic compounds.

The mobile phase or a component of the mobile phase is usually used as a reconstitution solvent. However, when the mobile phase was used as a reconstitution solvent, we were not able to get a satisfactory baseline separation for mCPP and I.S. peaks, and also all analytes showed broader peaks (see panel I in Fig. 2). Therefore, we were interested to see whether or not the alteration of the com-

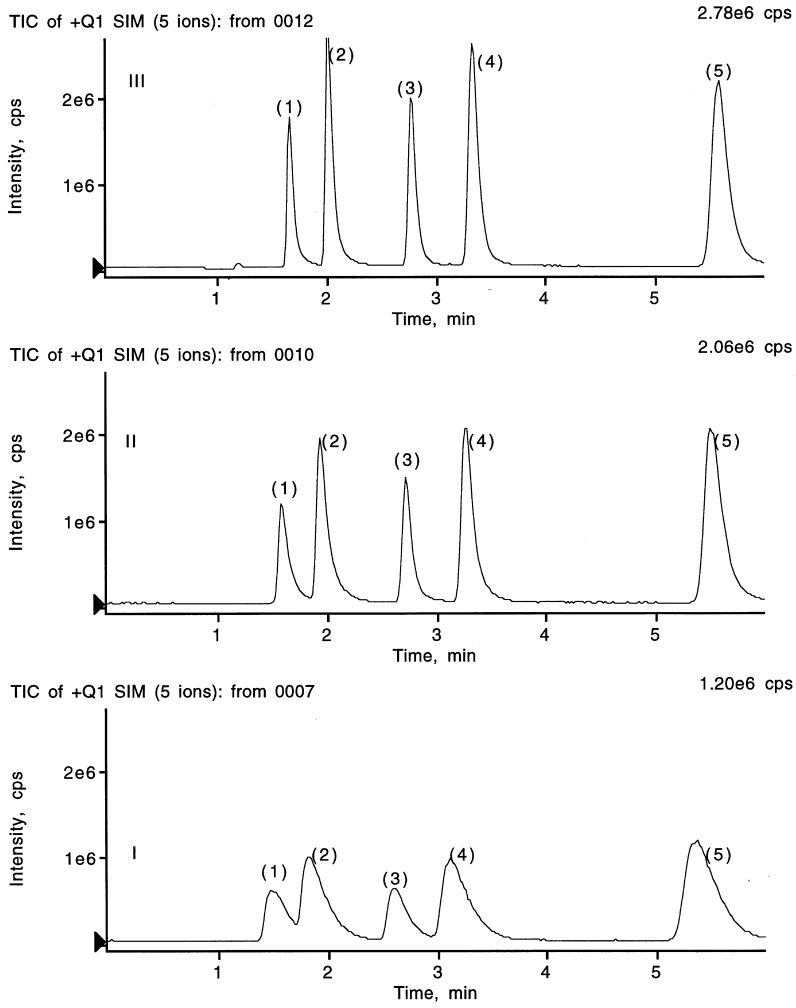

Fig. 2. Optimization of acetonitrile in the reconstitution solvent; (I) 45% of acetonitrile; (II) 25% of acetonitrile; (III) 0% of acetonitrile. (1) mCPP; (2) I.S.; (3) Dione; (4) OH-NEF; (5) NEF.

position of the reconstitution solvent produced better peak shapes and separation characteristics. Use of a lower percentage of acetonitrile in the reconstituted solvent resulted in both a better separation as well as sharper peak shapes for the various peaks of interest (Fig. 2). Although the reconstitution solvent containing 0% acetonitrile gave the best peak characteristics for all analytes, the reconstitution solvent containing 25% acetonitrile was considered ideal because of factors, such as increased solubility of the analytes and compatibility with the mobile phase.

Numerous experiments were carried out to test the effect of pH of the mobile phase on the separation characteristics of the various analytes. It revealed that changes in pH between 3 and 4 range produced no improvement in either peak shapes or resolution.

3.2. Validation of assay

3.2.1. Specificity

Chromatograms were obtained and compared between the blank and spiked human plasma matrices (Fig. 3). No significant interfering peaks were detected at the retention times of the peaks of interest. The nominal retention times for mCPP, I.S., Dione, OH-NEF and NEF were 1.55, 1.83, 2.41, 3.02 and

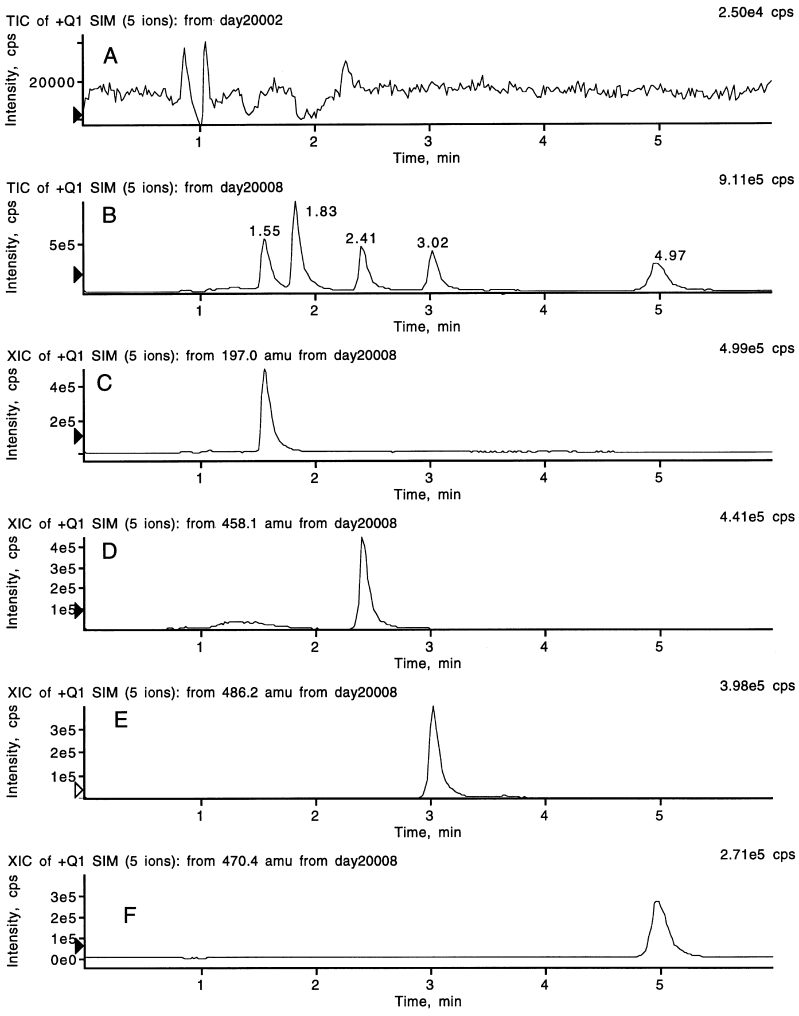

Fig. 3. Representation of the total ion chromatogram (TIC) and single ion monitoring (SIM) chromatograms; (A) Blank plasma TIC; (B) spiked plasma standard showing mCCP (1.55 min), I.S. (1.83 min), Dione (2.41 min), OH-NEF (3.02 min), and NEF (4.97) peaks; (C) SIM for mCCP (m/z 197.0); (D) SIM for Dione (m/z 458.1); (E) SIM for OH-NEF (m/z 486.2); and (F) SIM for NEF (m/z 470.4).

4.97 min, respectively. All analyte peaks showed baseline separation.

3.2.2. Linearity

A weighted linear regression of the peak area ratios versus standard concentrations was performed for four analytes using a weight of 1/concentration. The observed peak area ratios were linear over the concentration range of 2–500 ng/ml for mCPP, OH-NEF and NEF, and 4–1000 ng/ml for Dione in human plasma. The values of r^2 ($\geq$0.995) and the consistency in slope values (Table 1) demonstrated that the standard curve had a reliable response over the studied concentration range.

3.2.3. Lower limit of quantitation (LLQ)

The predicted mean concentrations in LLQ samples for Dione, mCPP, OH-NEF, and NEF were 3.88, 1.84, 1.99 and 1.84 ng/ml, which deviated less than $\pm$8.0% from the respective nominal values. The precision estimates for the LLQ samples for various analytes were within 7.5% R.S.D. Therefore, the

Table 1
Mean (±S.D.) standard curve ($n=3$) summary of nefazodone and its metabolites in human plasma

Analyte	Slope ($\times 10^3$)	Intercept ($\times 10^3$)	r^2
NEF	4.27±0.16	4.93±3.67	0.997±0.001
OH-NEF	3.65±0.124	4.01±2.3	0.996±0.002
Dione	1.91±0.03	−0.5±2.6	0.999±0.001
MCPP	4.14±0.37	0.82±1.98	0.995±0.003

Model: area ratio = (slope)·(concentration + intercept).

LLQ for Dione in human plasma was established at 4.0 ng/ml; and for mCPP, OH-NEF, and NEF it was established at 2.0 ng/ml.

3.2.4. Intra- and inter-assay accuracy and precision

The intra- and inter-assay accuracy and precision values for QC samples of all four analytes are provided in Table 2. The intra- and inter-assay precision values (%R.S.D.) at the various concentrations for the four analytes were less than 7.0%. The accuracy (%DEV) values for all four concentrations deviated less than −12.1% from the corresponding nominal concentrations.

Table 3
Extraction recovery of nefazodone and its metabolites from human plasma

Analytes	Slope value		% Recovery
	Extracted	Non-extracted	
mCPP[a]	11 471	17 053	67.3
Dione[b]	7017	10 215	68.7
OH-NEF[a]	12 871	14 875	86.5
NEF[a]	15 727	18 976	82.9

[a]Range, 2–500 ng/ml.
[b]Range, 4–1000 ng/ml.

3.2.5. Extraction recovery

The results of the comparison of neat standards versus plasma-extracted standards in the concentration range 2–500 ng/ml for mCPP, OH-NEF, and NEF, and in the range 4–1000 ng/ml for Dione, indicated that the extraction of the analytes from human plasma was 67.3–86.5%, Table 3).

3.2.6. Autosampler stability

The absolute peak areas at three concentration levels for Dione, mCPP, OH-NEF, NEF and I.S. were generally found to be within 2.5–8.6% of the

Table 2
Intra- and inter-assay accuracy and precision results for nefazodone and its metabolites

Analytes	Nominal conc. (ng/ml)	Mean observed conc. (ng/ml)	Accuracy (%DEV)	Precision (%R.S.D.)	
				Within run	Between run[a]
Dione	20	20.18	0.88	6.22	6.95
	400	397.5	−0.62	6.53	0.00*
	800	808.9	1.11	4.27	0.00*
	2000[b]	1918.8	−4.06	2.21	N/A
OH-NEF	10	9.246	−7.54	5.74	4.81
	200	184.6	−7.70	4.72	1.22
	400	354.1	−11.5	3.85	1.19
	1000[b]	887.3	−11.3	1.99	N/A
MCPP	10	9.727	−2.73	5.75	5.13
	200	190.1	−4.97	6.65	5.56
	400	384.0	−3.99	4.58	7.76
	1000[b]	880.5	−12.0	2.60	N/A
NEF	10	9.052	−9.48	4.05	5.51
	200	180.7	−9.66	5.81	0.00*
	400	351.9	−12.0	3.88	0.00*
	1000[b]	894.4	−10.56	2.97	N/A

[a]%R.S.D. = 100[(TMS − EMS)/N]$^{0.5}$/GM (see Section 2.6.2).
[b]Dilution QC samples (analyzed in a single run).
*No significant additional variation was observed as a result of performing the assay in different runs.

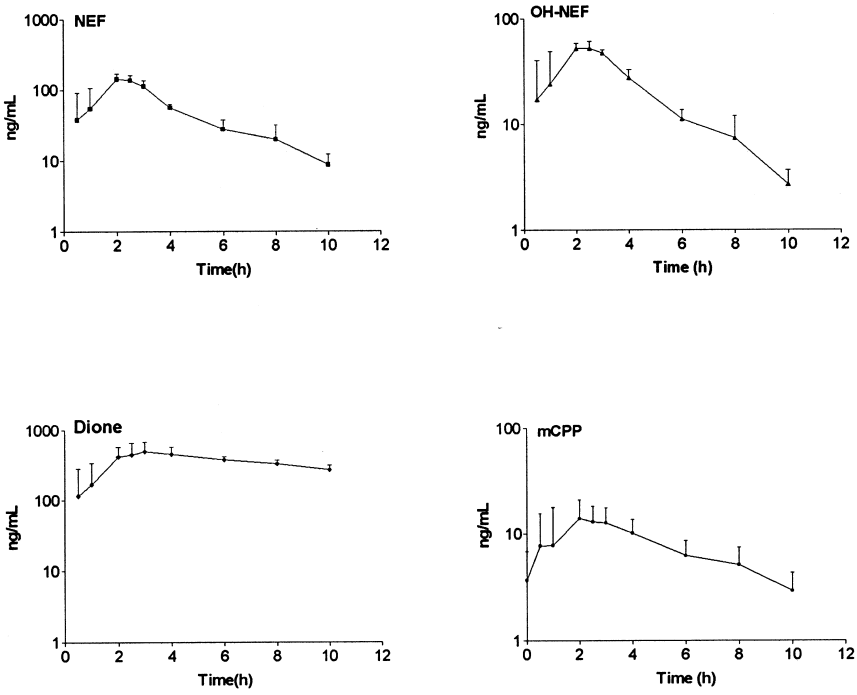

Fig. 4. Mean (±S.D.) plasma concentration versus time profiles of nefazodone and its metabolites in healthy subjects ($n=3$) following a single 100-mg oral dose administration.

corresponding peak areas at time zero during the course of this stability experiment (i.e., 24 h).

3.3. Application

The method was applied to determine the levels of Dione, mCPP, OH-NEF, and NEF in human subjects ($n=3$) receiving a 100-mg oral dose of NEF. The mean plasma concentration versus time profiles for Dione, mCPP, OH-NEF, and NEF are depicted in Fig. 4, and the mean (S.D.) pharmacokinetic parameters are tabulated in Table 4.

4. Conclusions

A sensitive, selective, accurate, and precise HPLC procedure with single ion monitoring by single quadruple mass spectrometer was developed and validated for determination of nefazodone and its

Table 4
Mean (±S.D.) pharmacokinetic parameters of nefazodone and its metabolites in healthy subjects ($n=3$) following a single 100-mg oral dose administration

Analyte	C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{inf} (h·ng/ml)
NEF	151.1 (21.83)	2.17 (0.29)	2.0 (0.53)	538.3 (91.4)
Dione	532.0 (129.2)	4 (1.73)	—n.d	5628.42[a] (3127.7)
MCPP	15.0 (6.13)	2.5 (0.50)	3.3 (0.30)	92.5 (45.42)
OH-NEF	55.8 (5.89)	2.5 (0.50)	2.0 (0.27)	217.7 (41.07)

n.d., not determined due to lack of adequate time points in the terminal phase. C_{max}, peak plasma concentration; T_{max}, time for attainment of C_{max}; $t_{1/2}$, terminal elimination half-life; $AUC_{(0-T)}$, area under the plasma concentration–time curve up to time T; AUC_{inf}, area under the plasma concentration–time curve up to time infinity.
[a] $AUC_{(0-T)}$.

three metabolites in human plasma. The reported method offers several advantages, such as a rapid and clean extraction scheme and a short chromatographic run time.

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