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Determination of nefazodone and its pharmacologically active metabolites in human blood plasma and breast milk by high-performance liquid chromatography

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Abstract

A method is described for the determination of nefazodone and its active metabolites hydroxynefazodone, the dione BMS-180492 and *m*-chlorophenylpiperazine in blood plasma and expressed human milk based on reversed-phase high-performance liquid chromatography. Measurements were performed on drug-free plasma and expressed human milk spiked with nefazodone and metabolites to prepare and validate standard curves and specimens collected from nursing mothers. Parent drug and metabolites were separated from the biological matrices by solid-phase extraction using CERTIFY columns. Chromatographic separation was achieved with a C_{18} column and compounds were detected by their absorbance at 205 nm. Trazodone was used as an internal standard. The assay was validated for each analyte in the concentration range 200 to 1200 ng/ml.

Keywords: Nefazodone

1. Introduction

Nefazodone, 2-{3-[4-(3-chlorophenyl)-1-piperzinyl]-propyl}-5-ethyl-2,4-dihydro-4-(2-phenoxyethyl)-3H-1,2,4-triazol-3-one hydrochloride, has been demonstrated in double-blind placebo controlled trials to be an effective antidepressant [1]. It is believed to exert its therapeutic effect as a result of its ability to act as an antagonist of post-synaptic 5-HT_2 receptors. Inhibition of pre-synaptic serotonin reuptake may also contribute to its antidepressant effect. The effective dose ranges from 150 to 800 mg/day as a divided oral dose. While the drug has been thoroughly evaluated in major depressive disorders, neither its efficacy in post-partum depression nor the passage of the drug into breast milk have been previously investigated.

Nefazodone is metabolised into several pharmacologically active as well as non-active metabolites [2]. Plasma concentrations of nefazodone and metabolites for single dose and steady state studies have been shown to be within the range of this assay [2,3]. The metabolites hydroxynefazodone and BMS-180492A (dione) are active and BMS-180492A has a significantly longer half life than the parent compound [3]. The metabolite *m*-chlorophenylpiperazine (mCPP) is a known mixed 5HT receptor agonist/antagonist [4]. Structures of nefazodone and its metabolites are given in Fig. 1.

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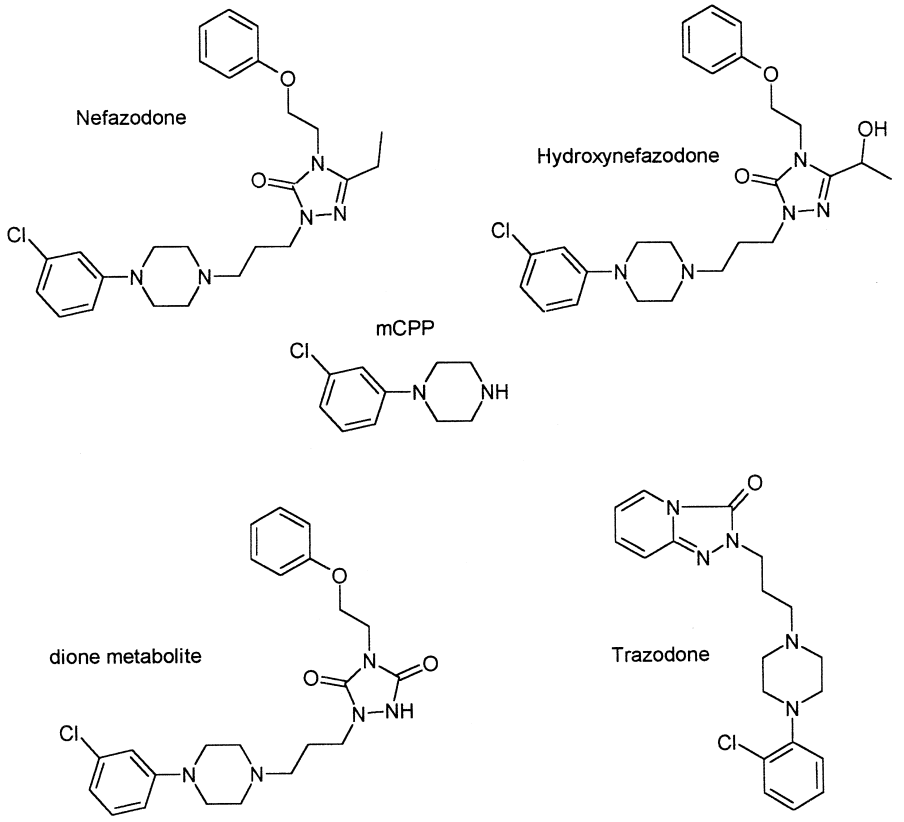

Fig. 1. Structures of nefazodone and its metabolites.

A simple, reliable and validated high-performance liquid chromatography (HPLC) technique to quantitatively determine concentrations of nefazodone and its major metabolites in plasma and human breast milk is described. Previous methods have been reported for the determination of nefazodone and its metabolites (but not the dione [2]) in plasma [3,5–7]. This method is more convenient as it uses solid-phase extraction (SPE) for sample preparation. In addition, determination of nefazodone and its metabolites in human breast milk has not been reported elsewhere.

2. Experimental

2.1. Materials

Dichloromethane, acetonitrile and methanol were purchased from Mallinckrodt (ChromAR HPLC grade, KY, USA). Potassium dihydrogenphosphate was from Rhône-Poulenc (Pronalys analytical-reagent; Melbourne, Australia). Potassium hydroxide and ammonium hydroxide (ammonia solution sp.gr. 0.91) from BDH (AnalaR; Port Fairy, Australia). Glacial acetic acid was from Ajax (Univar analytical-reagent; Sydney, Australia) and isopropanol from Merck (Art. 9634; Darmstadt, Germany). Nefazodone, hydroxynefazodone, BMS-180492A (dione) and mCPP were obtained from Bristol-Myers Squibb (New Brunswick, NJ, USA) and trazodone from Bristol-Myers USPNG (Evansville, IN, USA). Bond Elute Certify SPE columns (3 ml/130 mg) were purchased from Varian (Harbor City, CA, USA).

2.2. Apparatus

SPE from plasma and human breast milk samples were conducted using a 10-port Vac-Elut rack (Analytichem International).

Analysis was conducted using a Shimadzu LC-10AT liquid chromatograph with an SIL-10A$_{XL}$ autoinjector and an SPD-10A UV–Vis detector. Compounds were detected at 205 nm. Data was collected using a CBM-10A communications bus module interfaced to a personal computer. Separation was achieved with an Alphabond C_{18}, 125A, 10 μm, 150×3.9 mm column, with an All-Guard 7.5×4.6 mm guard column obtained from Alltech Associates (Baulkham Hills, Australia).

2.3. Preparation of standard and quality control (QC) samples

Fresh frozen donor plasma used for producing standard curves and quality control samples was obtained from the Haematology Department of the Austin and Repatriation Medical Centre (Melbourne, Australia). Human milk for standard curves and quality control samples was obtained from the Lactation Clinic, Mercy Hospital (Melbourne, Australia). The milk came from a single donor, who was drug-free and three months post-partum. A 150-ml sample was obtained in a single collection and stored at −20°C until required.

Samples were prepared for producing standard curves by spiking human plasma and expressed breast milk with nefazodone, hydroxynefazodone, the dione and mCPP (100 μg/ml in ethanol). Preparations were made of blank plasma and milk samples, and plasma and milk spiked at 50, 250, 500, 1000 and 2000 ng/ml with nefazodone and each metabolite. Measured volumes of nefazodone and metabolite solutions were pipetted separately into 25-ml volumetric flasks. Milk or plasma was then added to bring the flasks to volume. The solutions were mixed, divided into 1.5-ml disposable tubes and stored at −20°C. Quality control samples were prepared by the same technique.

2.4. Collection of plasma and milk clinical samples

Human blood and expressed breast milk samples were obtained from three depressed women treated with nefazodone as their only medication. All had been treated for at least three weeks prior to the samples being collected. Each specimen was collected prior to the morning dose of drug, 10 to 15 h after the previous evening dose of nefazodone. Blood was collected into 10-ml lithium–heparin tubes and centrifuged for 15 min at 3360 *g*. The plasma was decanted into plain tubes. Breast milk, approximately 25 ml, was expressed directly into plain collection jars and stored whole. Milk and plasma were stored at −20°C until required.

2.5. Extraction procedure

The following solutions were used in the extraction procedure: (1) phosphate buffer: 0.1 *M* KH_2PO_4 adjusted to pH 6.0 with 1.0 *M* KOH. Stored at 4°C and used within 30 days. (2) 1.0 *M* Acetic acid prepared from glacial acetic acid and distilled water. (3) SPE eluent: dichloromethane–isopropanol–ammonium hydroxide (78:20:2) prepared fresh daily as required.

2.5.1. Plasma

Thawed plasma specimens (patient samples, standards and QCs) (1.0 ml) were added to 0.1 *M* phosphate buffer (2 ml) in polyethylene test tubes. Trazodone (100 μl of 10 μg/ml ethanol solution) was added as an internal standard and the tubes gently vortexed to ensure thorough mixing. SPE columns were conditioned by vacuum drawing in order, 3 ml of methanol, 3 ml of distilled water and 1 ml of phosphate buffer through the column. The column was not permitted to dry during this process. The prepared samples were applied to conditioned columns and allowed to slowly pass through the columns over a period of at least 2 min with gentle vacuum applied. Columns were then washed with 3 ml of water, 1 ml of 1.0 *M* acetic acid and 3 ml of methanol. Each column was allowed to dry by drawing air through it for 5 min using the vacuum pump. Drugs retained by the column were collected into a glass vial by allowing 3 ml of eluent to pass slowly through the column with low suction. The eluate was evaporated to dryness at 55°C under a stream of air. The dried extract was reconstituted in 120 μl of HPLC mobile phase and vortex mixed. An aliquot (80 μl) of the extract solution was injected onto the HPLC system. Standards, QCs and patient samples were injected by batch schedule using an autoinjector.

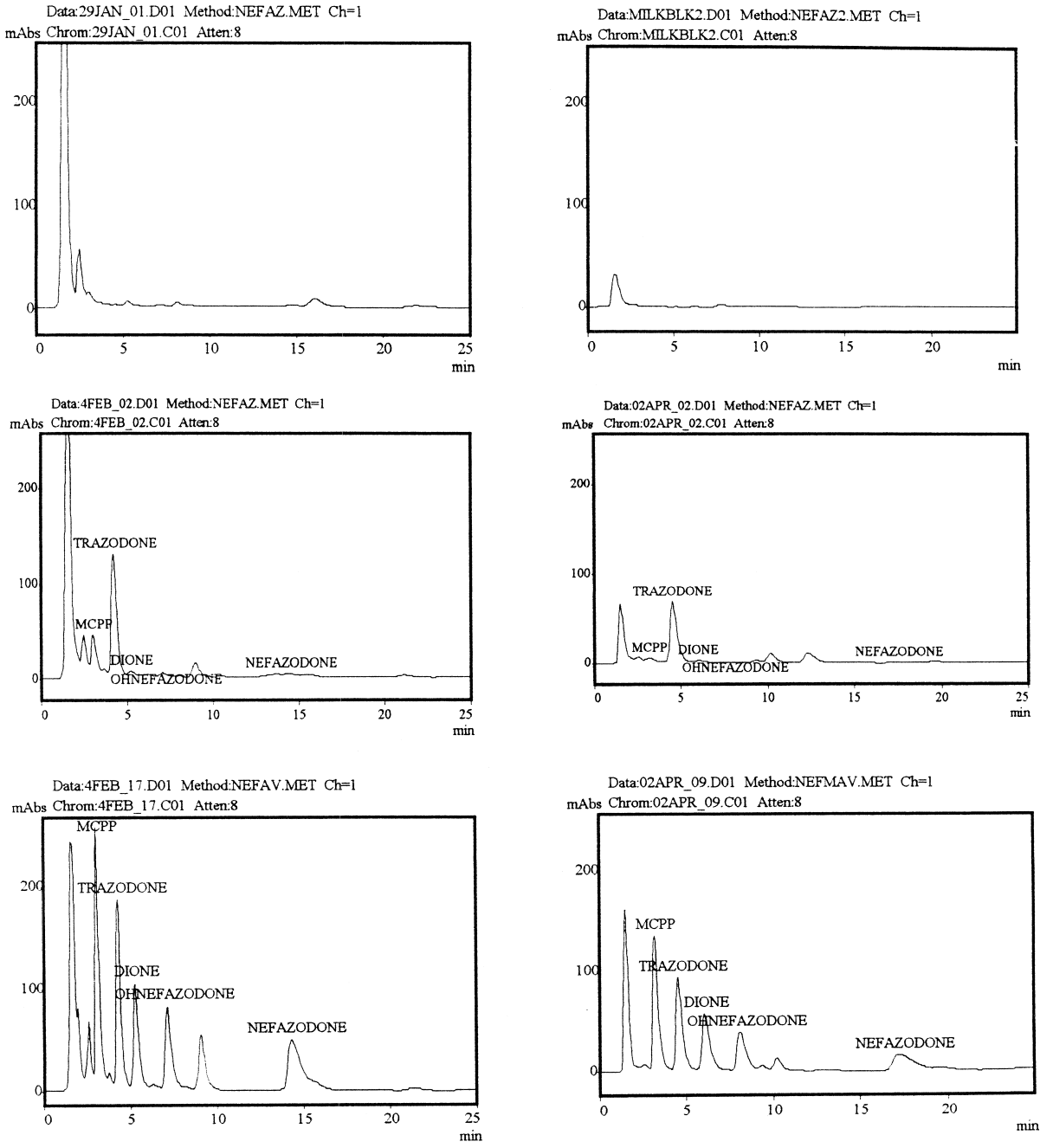

Fig. 2. Chromatograms of blank plasma (top left), blank milk (top right), 50 ng/ml (middle left) and 1200 ng/ml (bottom left) spiked plasma and 50 ng/ml (middle right) and 1200 ng/ml (bottom right) spiked milk.

2.5.2. Milk

Nefazodone and its metabolites were extracted from milk by the same method as used for plasma. The milk samples, even though diluted, passed through the SPE columns with difficulty and generally higher pressures were required.

2.6. Chromatographic conditions

The mobile phase was acetonitrile–50 m*M* KH_2PO_4 (aqueous) (40:60) degassed by filtration. Mobile phase and column were kept at room temperature. The flow-rate was 1 ml/min. Retention times were as follows; mCPP, 3.1; trazodone, 4.4; dione, 5.8; hydroxynefazodone, 7.5; and nefazodone, 16.0 min. Chromatograms for blank and spiked plasma and breast milk extracts are given in Fig. 2. Chromatograms for patient plasma and breast milk extracts are given as Fig. 3.

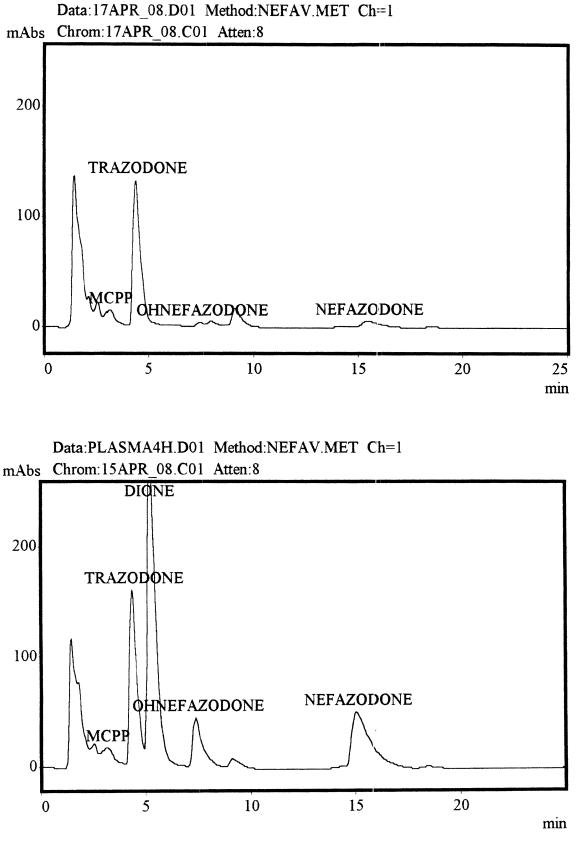

Fig. 3. Chromatograms of patient breast milk (top) containing <50 ng/ml MCPP, undetectable dione, <50 ng/ml OH-nefazodone and 132 ng/ml nefazodone; and patient plasma (bottom) containing <50 ng/ml MCPP, 3984 ng/ml dione, 611 ng/ml OH nefazodone and 1212 ng/ml nefazodone. Both samples obtained 4 h after 200 mg nefazodone.

3. Results

3.1. Validation of the method

Standard curves were constructed using six calibration levels: 0, 50, 250, 500, 1000 and 2000 ng/ml for nefazodone and each metabolite. Standard curves were produced by linear regression of concentration on the peak area ratio, where peak area ratio was calculated for each concentration as the analyte peak area relative to that of trazodone. For each set of standards in both plasma and breast milk regression coefficients exceeded 0.975.

Quality control specimens for plasma and milk were spiked at 200, 800 and 1200 ng/ml with nefazodone and each metabolite. The inter-assay variability of the method was determined from the results of the assay of these control specimens which are shown in Table 1. Between-day variability of the slopes of the regression curves was <10%. Only one set of controls produced a relative standard deviation (RSD) >15%. Precision of the plasma assay is greater than that of the breast milk assay. This can be attributed to the cleaner extraction and higher recovery obtained from the plasma samples than the milk samples.

3.2. Recovery with solid-phase extraction.

Recovery from plasma of the extracted nefazodone and metabolites was calculated from the ratio of peak areas between extracted spiked plasma and non-extracted samples. Recovery from plasma ranged from 60 to 80% and was generally better than recovery from milk which ranged from 40 to 60%. The higher fat content in milk was probably the cause of the low recovery.

3.3. Analysis of patient specimens

Trough plasma and breast milk concentrations of nefazodone and metabolites for three paired sets of patient specimens are presented in Table 2.

4. Discussion

A rapid, reliable method for the quantitative

Table 1
Between-day precision and accuracy of nefazodone and metabolites assay for plasma and human breast milk (data are mean±SD with RSD, %)

Nominal concentration	Nefazodone	Hydroxynefazodone	Dione	mCPP
Plasma				
200 ng/ml				
Mean±SD (ng/ml)	195±19	174±22	211±19	190±25
RSD (%)	9.5	12.6	9.1	13.0
% Accuracy	97.5	87	94.8	95
n	*n*=6	*n*=6	*n*=5[a]	*n*=6
800 ng/ml				
Mean±SD (ng/ml)	851±88	803±35	863±30	710±69
RSD (%)	10.3	4.4	3.5	9.8
% Accuracy	94	99.6	92.7	88.8
n	*n*=6	*n*=6	*n*=6	*n*=6
1200 ng/ml				
Mean±SD (ng/ml)	1233±48	1113±51	1173±53	1201±70
RSD (%)	3.9	4.6	4.6	3.3
% Accuracy	97.3	92.8	97.8	99.9
n	*n*=6	*n*=6	*n*=6	*n*=6
Breast milk				
200 ng/ml				
Mean±SD (ng/ml)	214±30	193±10	196±48	158±23
RSD (%)	14.3	5.2	24.5	14.6
% Accuracy	93.5	96.5	98	79
n	*n*=7	*n*=6[a]	*n*=6[a]	*n*=6[a]
800 ng/ml				
Mean±SD (ng/ml)	800±55	854±30	964±134	832±81
RSD (%)	6.9	3.6	13.9	9.8
% Accuracy	100	93.7	83	96.2
n	*n*=7	*n*=7	*n*=6[a]	*n*=7
1200 ng/ml				
Mean±SD (ng/ml)	1261±112	1280±137	1335±178	1172±172
RSD (%)	8.9	10.7	13.3	14.7
% Accuracy	95.2	93.8	89.9	97.7
n	*n*=7	*n*=7	*n*=7	*n*=7

[a] Interfering peak in chromatogram meant that one sample of a series could not be quantitated.

measurement by HPLC of nefazodone and its major metabolites in plasma and in expressed human breast milk has been presented. The assay demonstrates a high degree of precision and adequate sensitivity for patients at steady-state plasma concentration levels. Sensitivity is probably not sufficient for measuring mCPP levels or drug levels several half lives after the administration of a single nefazodone dose. The possibility of other drugs, such as benzodiazapines coprescribed with nefazodone, interfering with the assay has not been investigated. This is not a problem for the present study as patients received no medications other than nefazodone. There does not appear to be any published data on the use of nefazodone in post-partum depression or on nefazodone pharmacokinetics in breast milk. The

Table 2
Trough plasma and breast milk concentrations of nefazodone and metabolites in three patients[a]

Subject	Nefazodone (ng/ml)	Hydroxynefazodone (ng/ml)	BMS-180492A (ng/ml)	mCPP (ng/ml)
Plasma				
1	617	288	3603	ND[b]
2	<50	ND	63	ND
3	<50	ND	442	ND
Breast milk				
1	57	ND	ND	ND
2	687	<50	ND	ND
3	213	104	ND	ND

[a] Data represent mean of duplicate determinations.
[b] ND=Not detected: below the limits of detection of the assay for this analyte. Note subjects 2 and 3 are the same patient at two different dose levels of nefazodone studied three weeks apart; all samples collected 10 to 15 h after the evening dose of nefazodone and immediately prior to the morning dose. Doses are 200 mg b.i.d. for subject 1, 50 mg b.i.d. for subject 2 and 50 mg morning and 100 mg evening for subject 3.

present data suggests that the diffusion of nefazodone and its metabolites into breast milk may be low, though further data is needed.

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