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Determination of nefazodone and its metabolites in plasma by high-performance liquid chromatography with coulometric detection*

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Abstract: Nefazodone and its metabolites together with the internal standard are extracted from plasma using CN Bond-Elut sorbent columns. Chromatography and detection are performed using isocratic reversed-phase high-performance liquid chromatography (HPLC) with a CN column and coulometric end-point detection. The lower limits of quantitation for nefazodone, *m*CPP, and OH-nefazodone was 0.9, 0.2 and 0.6 ng ml^{-1} when 1 ml of plasma was extracted. The inter-assay relative standard deviations were less than 6% for nefazodone and its metabolites. Recovery averaged over 80%. Plasma profiles of nefazodone and its metabolites following oral dosing are presented.

Keywords: *Nefazodone; metabolites; internal standard; HPLC; coulometric detection.*

Introduction

Nefazodone (NEF), 2-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-5-ethyl-2,4-dihydro-4-(2-phenoxyethyl)-3H-1,2,4-triazol-3-one hydrochloride (Fig. 1) is a novel drug similar in structure to the well known antidepressant trazadone. It has potent effects on serotonergic ($5\text{-}HT_2$) receptors and has exhibited significant antidepressant activity, no cardiac toxicity or anticholinergic activity as shown by tricyclic antidepressants [1].

Nefazodone has two major metabolites; hydroxynefazodone (OH-NEF) and *m*-chlorophenyl piperazine, *m*CPP (Fig. 1). Both OH-NEF and *m*CPP are pharmacologically active compounds and so it is most important to monitor them as well as the parent compound. OH-NEF has a similar pharmacological profile to nefazodone itself, whilst *m*CPP has been shown to cause changes in serotonin (5-HT) synthesis and turnover consistent with post-synaptic receptor activity [2].

The assessment of nefazodone and its metabolites in plasma requires a highly sensitive and selective assay procedure to measure the very low levels present. To date only one other method has been reported. Franc *et al.* [3] used an automated high-performance liquid chromatography (HPLC) with ultraviolet detection. Analytes were extracted from alkalinized plasma with butyl chloride followed by phase separation and evaporation of the organic phase. Aprindine was used as internal standard. This procedure had limits of detection of 5.0, 1.0 and 5 ng ml^{-1} of plasma for NEF, *m*CPP and OH-NEF, respectively.

The simple method described here is based on solid-phase sorbent extraction of nefazodone and its metabolites from plasma followed by isocratic reversed-phase HPLC with coulometric detection. The method uses gepirone (Fig. 1) as an internal standard.

Experimental

Materials

Nefazodone and hydroxynefazodone, and gepirone (the internal standard), were kindly donated by Bristol Myers (Uxbridge, UK). *m*CPP was purchased from Aldrich (Gillingham, Dorset, UK). All other agents used were of the highest grade. Plasma for the preparation of standards was obtained from voluntary blood donors. All water was deionized and glass-distilled prior to use.

Cyanopropyl (CN) sorbent columns (Bond Elut) for extraction were purchased from

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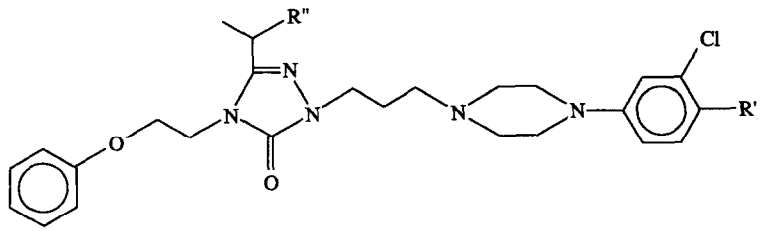

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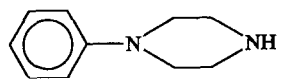

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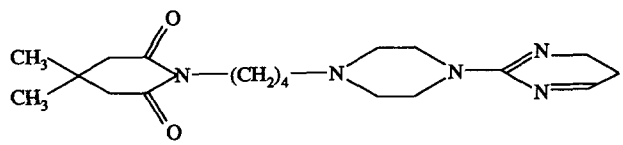

Figure 1
Structures of nefazodone (1), hydroxynefazodone (2), *m*CPP (3) and gepirone, the internal standard (4).

Varian (UK). Stock standard solutions of all analytes and the internal standard were prepared at concentrations of 100 μg ml^{-1} in methanol. These were stored at 4°C and were stable for up to 6 months. Stock solutions for each analyte were serially diluted in water for each assay run and finally made up to the required concentration in drug-free plasma.

Chromatographic apparatus and conditions

The HPLC system comprised of a Milton Roy Constametric 3000 pump (LDC, Stone, UK), a manual Rheodyne 7125 injection valve equipped with a 50 μl loop, a 5 μm particle size cyanopropyl analytical column (150 × 4.6 mm i.d.) protected by a 5 μm particle size C2 guard column (Capital HPLC, Edinburgh, UK). The detection system consisted of a Model 5100 A Coulometric detector and a model 5020 guard cell (RSA, Bedford, MA, USA). The detector was linked to an LDC Cl-4000 integrator (LDC, Stone, UK).

The potentials for detectors 1 and 2 were selected after injection of fixed amounts of the analytes and the internal standard over the range +0.2–0.85 V for analytical electrode (Fig. 2). The selected potentials for the guard

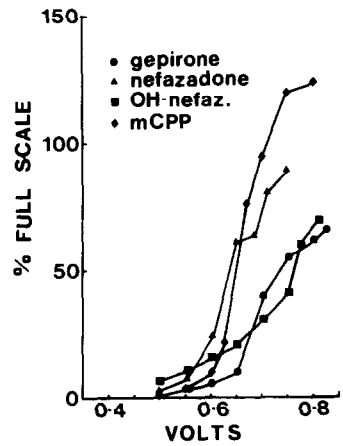

Figure 2
Voltammograms of nefazodone and its metabolites together with the internal standard, gepirone for detector 2 at different oxidation potentials. The voltammograms were determined when the guard cell and detector 1 were at zero potential.

cell and detectors 1 and 2 in the oxidation mode were 0.80, 0.75 and 0.5 V, respectively. The response time was set at 2 s.

The mobile phase consisted of 0.04 M potassium phosphate buffer adjusted to pH 6.6 with 2 M KOH, HPLC grade acetonitrile and methanol (600:225:175, v/v/v). The mobile

phase was filtered and de-gassed prior to use. The flow rate was 1.4 ml min^{-1}.

Peak heights rather than areas in the chromatograms were normally measured. Concentrations of nefazodone and its metabolites were assessed by using the slope of the standard curve for peak-height ratios for the analyte and the internal standard.

Procedures

Blood samples were collected into tubes containing lithium heparin as anticoagulant, centrifuged and the plasma separated and stored at −25°C until required for assay.

Standard curves for each analyte were freshly prepared daily. These consisted of 0, 5, 10, 20, 50 and 100 ng ml^{-1} of each analyte in drug-free plasma. To each 1 ml volume of standard or sample was added 30 ng of the internal standard, gepirone, prior to column addition. Cyanopropyl Bond Elut sorbent columns (100 mg) were initially conditioned with full column volumes of methanol and water, respectively. The vacuum was diverted to keep the columns from drying out and the standards and samples were transferred to the columns. The vacuum was again applied allowing the materials to pass completely through. Each column was washed in turn with two column volumes of water. Columns were taken to dryness under vacuum. The vacuum was again diverted, the manifold needles were wiped dry and a collection tray containing 10 × 75 mm glass tubes was inserted into the Vac Elut Manifold system. Analytes were eluted with one column volume of 10% NH_3–methanol. The evaluates were evaporated to dryness under vacuum at 40°C. Samples were reconstituted in 150 µl mobile phase, vortex mixed and prepared for injection into the HPLC.

Results

Resolution and sensitivity was determined by injection of an extracted plasma standard (Fig. 3). The retention times of nefazodone, hydroxynefazodone, *m*CPP and the internal standard were 3.2, 4.0, 5.0 and 5.6 min, respectively. The linearity of both the extraction procedure and the detector response (determined from the peak height) was verified over the assay range (0–100 ng ml^{-1}). The linearity was determined by assaying pooled drug-free plasma (which had been previously screened for extraneous peaks) spiked with known amounts of each of the analytes. Calibration curves were calculated for each analyte using its concentration and the peak height ratio of analyte to internal standard over the studied range.

The equations for each analyte calibration curve were $y = 84.1x + 4.88$ ($r = 0.987$) for nefazodone, $y = 65.7x + 3.42$ ($r = 0.996$) for

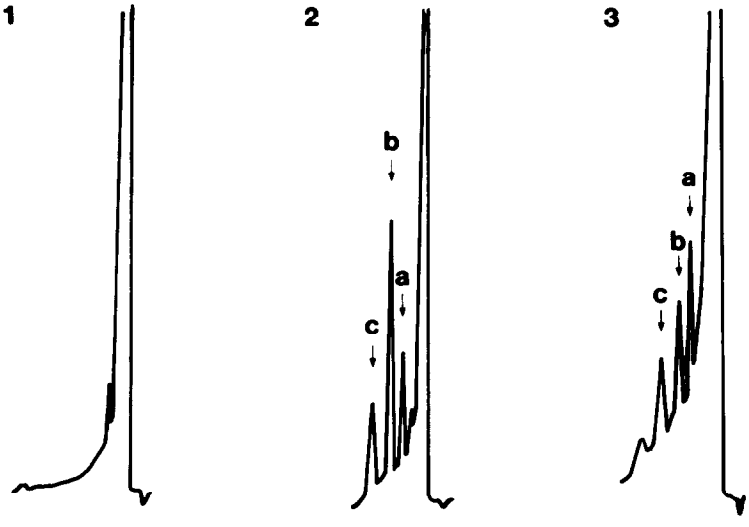

Figure 3
Chromatograms of (1) blank drug-free plasma, (2) drug-free plasma spiked with 50, 20 and 30 ng ml, respectively, of nefazodone (a), *m*CPP (b) and the internal standard (c). (3) Sample from volunteer following oral administration of nefazodone HCl, nefazodone peak equivalent to 22.9 ng ml^{-1} and the *m*CPP peak equivalent to 5.1 ng ml^{-1}, respectively. These analyses were made 1 week after extraction.

Table 1
Intra-assay precision (RSDs) and accuracy of the determination of nefazodone and metabolites in plasma (n = 6)

Actual value (ng ml^{-1})	Observed value (ng ml^{-1})			RSD (%)*		
	Nef	OH-Nef	*m*CPP	Nef	OH-Nef	*m*CPP
5	5.03 ± 0.5	4.73 ± 0.58	4.86 ± 0.3	10.1	12.3	6.3
10	10.58 ± 0.81	11.02 ± 0.41	10.02 ± 0.3	7.7	3.7	3.0
20	20.3 ± 0.62	19.74 ± 0.86	20.2 ± 0.86	3.1	4.4	6.9
100	103.7 ± 4.6	105.2 ± 4.7	100.98 ± 4.69	4.4	4.5	4.6

*The RSD of the method was calculated from results for pooled drug-free plasma spiked with known amounts of nefazodone, hydroxynefazodone and *m*CPP.

hydroxynefazodone and $y = 0.74x + 12.3$ (r = 0.997) for *m*CPP. The intra-assay relative standard deviations (RSDs) are presented in Table 1. The mean inter-assay RSDs for nefazodone and *m*CPP were 5.2 and 5.9%, respectively, for a spiked plasma quality control pool containing 10 ng ml^{-1} of each analyte (n = 8). The absolute extraction recovery for all analytes ranged from 70 to 90%. Sample extracts were stable for up to 1 week when stored out of light at 4°C.

Plasma profiles of nefazodone and its metabolites following oral administration of 200 mg of nefazodone in one male volunteer subject are shown in Fig. 4.

Discussion

Described here is a simple and highly selective HPLC assay procedure which utilizes coulometric detection, solid-phase sorbent extraction and an internal standard, gepirone, for monitoring extraction recovery and detector variation. Gepirone was chosen as the internal standard because it was known to have similar extraction and electrochemical properties to those of NEF and its metabolites [4]. The detection limit (i.e. peak height equal to three times baseline noise) was 0.3 ng for nefazodone, 0.2 ng for hydroxynefazodone and 0.07 ng for *m*CPP which allows for routine measurements of 0.9 ng ml^{-1} of nefazodone, 0.6 ng ml^{-1} of hydroxynefazodone and 0.2 ng ml^{-1} of *m*CPP from a 1 ml plasma sample. Inter-assay RSD data for OH-nefazodone is not presented as we have only recently received reference material and has allowed little time for measurement. It has been established that the ratio between the analytical recovery of the analytes and that of the internal standard submitted to the same operations were constant over a wide concentration range. Also, the detector response for both compounds was linear over the ranges used. The requirements for an internal standard assay procedure were thus satisfied.

Extracted samples were stable for 1 week following the described assay procedures when stored out of light at 4°C as can be seen from the chromatographic traces shown in Fig. 3. It was noted that hydroxynefazodone was found in only one subject of eight recently run, whilst *m*CPP was found in all.

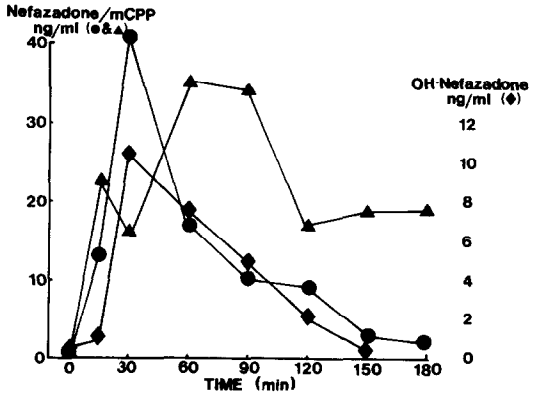

Figure 4
Plasma concentrations of nefazodone and its metabolites following oral dosing (200 mg) of nefazodone HCl in one male volunteer.

Conclusions

A novel technique using HPLC with coulometric detection has been described for the measurement of nefazodone and its metabolites. This has been used successfully to analyse plasma concentrations of nefazodone and its metabolites following oral dosing of nefazodone. It is fast, simple, reliable and relatively cheap to run.

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