Journal of Chromatography, 570 (1991) 129–138
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5982

High-performance liquid chromatographic method for the determination of nefazodone and its metabolites in human plasma using laboratory robotics

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(First received January 31st, 1991; revised manuscript received April 22nd, 1991)

ABSTRACT

A quantitative analytical method, using high-performance liquid chromatography and ultraviolet detection, has been established for the determination of nefazodone (NEF) and its metabolites, *m*-chlorophenylpiperazine (mCPP), *p*-hydroxynefazodone (PHN), and hydroxynefazodone (HO-NEF), in human plasma. The fully automated, robotic procedure consisted of addition of internal standard (aprindine), extraction with butyl chloride, followed by phase separation, organic phase evaporation, reconstitution of the residue, and injection onto the chromatographic system. The limits of detection for NEF, mCPP, PHN, and HO-NEF were 5, 1, 10, and 5 ng/ml, respectively, at a signal-to-noise ratio of 4. The method had a linear range of 10–1000 ng/ml for NEF and HO-NEF, 20–2000 ng/ml for PHN, and 2.5–250 ng/ml for mCPP. Correlation coefficients of 0.996 or greater were obtained during validation and study sample analysis.

INTRODUCTION

Nefazodone (NEF), 2-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-5-ethyl-2,4-dihydro-4-(2-phenoxyethyl)-3*H*-1,2,4-triazol-3-one hydrochloride (Fig. 1), has a potent effect on serotonergic (5-HT_2) receptors and has exhibited significant antidepressant activity and no cardiotoxicity or anticholinergic activity as seen for tricyclic antidepressants [1]. Studies in animals and humans given NEF identified the major metabolite as hydroxynefazodone (HO-NEF), 2-[3-[4-(3-chlorophenyl)-1-piperazinyl]propyl]-5-(2-hydroxyethyl)-2,4-dihydro-4-(2-phenoxyethyl)-3*H*-1,2,4-triazol-3-one hydrochloride [2]. Another monohydroxy metabolite, 2-[3-[4-(3-chloro-4-hydroxyphenyl)-1-piperazinyl]propyl]-5-ethyl-2,4-dihydro-4-(2-phenoxyethyl)-3*H*-1,2,4-triazol-3-one hydrochloride or *p*-hydroxynefazodone (PHN), and the N-dealkylated product, *m*-chlorophenylpiperazine (mCPP), have also been identified as NEF metabolites in both dogs and humans [3].

The analytical method previously used was modified in order to measure PHN levels in man [4]. This report describes the fully automated liquid–liquid extrac-

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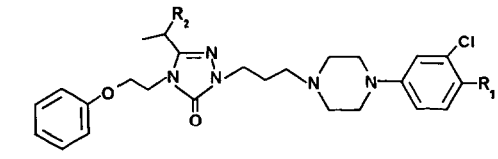
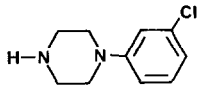


I III IV

Compound	R_1	R_2
Nefazodone	H	H
PHN	OH	H
HO-NEF	H	OH



II

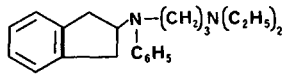


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Fig. 1. Structures of nefazodone (I), mCPP (II), PHN (III), HO-NEF (IV), and aprindine (V).

tion procedure for NEF and its metabolites. This procedure was then used to assay human plasma samples from several clinical studies.

EXPERIMENTAL

Chromatographic apparatus

Chromatography was performed using a Waters Assoc. (Milford, MA, USA) Model 590 programmable pump. Detection was performed using a Waters Assoc. Lambda Max Model 481 variable-wavelength UV detector. Injections were made by the robot using a Zymark (Hopkinton, MA, USA) Model Z311 LC sipping station. The detector output was digitized by a Hewlett-Packard (Palo Alto, CA, USA) 18652A analog-to-digital converter and the data were collected and processed by a Hewlett-Packard HP 3357 laboratory automation system computer.

Chemicals and reagents

NEF, mCPP, PHN, HO-NEF, and aprindine were all obtained as hydrochloride salts from Bristol-Myers Squibb. Acetonitrile and methanol were HPLC grade from J. T. Baker (Phillipsburg, NJ, USA). Phosphoric acid (85%) was a

certified ACS reagent from Fisher Scientific (Fair Lawn, NJ, USA). Butyl chloride was obtained as the reagent grade from Burdick & Jackson (Muskegon, MI, USA). Sodium bicarbonate and dibasic ammonium phosphate were certified ACS chemicals from Fisher Scientific, and tetramethylammonium hydroxide was obtained as reagent grade from Sigma (St. Louis, MO, USA). Distilled water was deionized and filtered through a Millipore (Milford, MA, USA) Milli-Q system. Control human plasma was obtained from Cocalico Biologicals (Reamstown, NJ, USA). Plasma samples and control human plasma were obtained from freshly drawn EDTA-treated blood.

The HPLC mobile phase was prepared by diluting 20 ml of 1 *M* dibasic ammonium phosphate and 20 ml of 1 *M* tetramethylammonium hydroxide (both solutions made to pH 3.0 with phosphoric acid) with 860 ml of deionized water. The solution was thoroughly mixed with 900 ml of acetonitrile and 200 ml of methanol. The resulting mixture was filtered through a 0.22-μm Durapore filter.

Robotic instrumentation

The robotic apparatus used was the Zymate II laboratory automation system (Zymark, Hopkinton, MA, USA). The components included: microprocessor controller with Easy-lab software, laboratory robot, general purpose (GP) hand, two master laboratory stations (for liquid dispensing and operating the extraction and LC sipping stations), a centrifuge, tumble mixer, evaporator, liquid–liquid extraction station, HPLC sipping station, vortex mixer, test tube dispenser, and other miscellaneous assessories (Fig. 2).

Robotic system disposables

Non-uniform 16 mm × 100 mm disposable glass culture tubes (Kimble, Toledo, OH, USA) were tested beforehand to verify tube diameter was within the tolerance limits (15.7 ± 0.02 mm) for the tumble mixer station.

Acquisition of study samples

The study samples that were analyzed were obtained from a single- and multiple-dose pharmacokinetic study in elderly (aged >65) males and females and young (aged 18–40) males and females. A total of 2976 samples was collected during the study.

Standard and quality control preparation

NEF, HO-NEF, mCPP, PHN, and aprindine (internal standard) stock solutions were prepared at 100.0 μg/ml in absolute ethanol. These solutions were used to prepare human plasma standards for each analytical run. The human plasma standards were prepared immediately prior to each analytical run. The internal standard solution was further diluted with absolute ethanol to 4.0 μg/ml for dispensing by the robot.

Human plasma quality controls were prepared to validate the assay and to

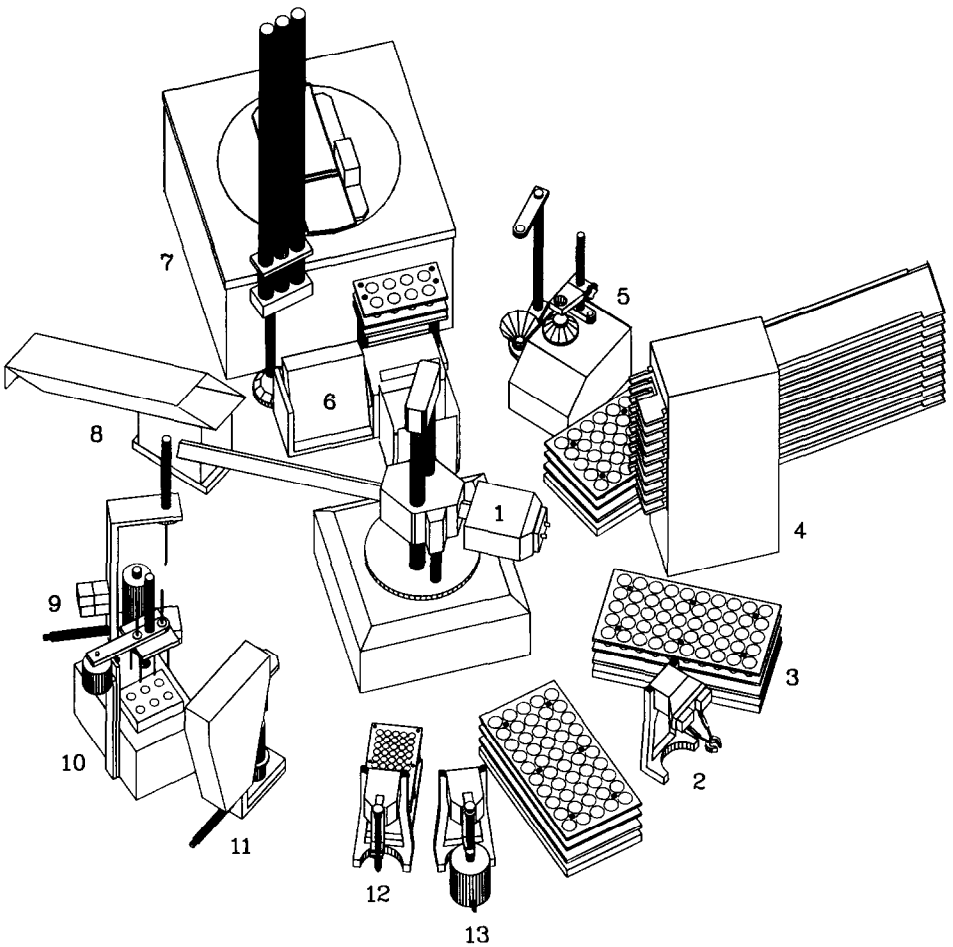

Fig. 2. Schematic drawing of the robotic layout for this method: 1 = Robot arm; 2 = general purpose hand; 3 = sample rack; 4 = test tube dispenser; 5 = vortex mixer; 6 = tumble mixer; 7 = centrifuge; 8 = disposal; 9 = liquid–liquid extraction station; 10 = evaporator station, 11 = LC injector station; 12 = disposable pipet hand; 13 = 5-ml syringe hand.

monitor the performance of the assay during the study sample analysis. For assay validation, three 100-ml pools of control human plasma were spiked with NEF and metabolites at the concentrations listed in Table I. The pools of spiked plasma were divided into 1.0-ml aliquots and stored frozen.

Chromatography

The HPLC mobile phase, acetonitrile–methanol–water, containing 0.01 *M* tetramethylammonium hydroxide and 0.01 *M* dibasic ammonium phosphate (45:10:45, v/v), flowed through a Zorbax Phenyl column (Rockland Technol-

TABLE I

ACCURACY AND PRECISION DATA FOR NEFAZODONE AND METABOLITES IN HUMAN PLASMA

Analyte	Nominal concentration (ng/ml)	Mean observed concentration[a] ($n = 11$) (ng/ml)	Coefficient of variation (%)	
			Intra-assay[b] ($n = 11$)	Inter-assay[c] ($n = 44$)
mCPP	2.50	2.70	25.1 (8.0)[d]	
	11.00	10.39	5.6	3.4
	200.00	189.94	4.1	4.9
	1125.00	1109.10	4.2	5.9
PHN	20.00	24.47	20.3 (22.3)[d]	
	80.00	83.56	1.1	1.1
	1800.00	1858.90	2.4	2.4
	9625.00	9664.84	2.5	2.2
HO-NEF	10.00	11.20	18.7 (12.0)[d]	
	44.00	47.75	2.4	2.9
	800.00	791.73	3.5	2.9
	4875.00	4951.18	3.8	1.7
NEF	10.00	11.97	12.8 (19.7)[d]	
	44.00	45.96	3.1	2.4
	800.00	780.72	4.8	3.0
	4875.00	5167.56	5.3	0.8

[a] Mean value on a single day.
[b] (S.D./mean) × 100 in same assay ($n = 11$).
[c] (S.D./mean) × 100 in four different assays ($n = 11 \times 4$).
[d] Percentage deviation at lower limit of quantitation.

ogies, 250 mm × 4.6 mm I.D., 5 μm) at ambient temperature and a flow-rate of 1.0 ml/min. Detection was performed using UV absorbance at 254 nm.

Liquid–liquid extraction by robot

Plasma samples (1 ml) were buffered with 0.5 ml of saturated sodium bicarbonate prior to robotic processing. The extraction process was performed in three separate steps by the robot. In the first step, the robot removed the sample tube from the sample rack, added 50 μl (200 ng) of internal standard solution, and vortex-mixed the sample for 20 s. Butyl chloride (5 ml) was added to the tube, and the tube was capped and placed in the tumble mixer for 17 min.

In the second step, the robot removed the previously mixed sample from the tumble mixer, uncapped it, and placed it in the centrifuge where it was spun at 600 *g* for 2 min. After centrifugation, the robot transferred the tube to the liquid–liquid extraction station where 4.5 ml of the upper phase were removed. The tube was then returned to its orginal position in the sample rack. The 4.5 ml of extract was collected in a clean tube and placed in the evaporator station, maintained at 42°C, for evaporation of the organic phase over 17 min.

In the third step, the robot removed the previously evaporated sample from the evaporator station, paused 30 s to allow the tube to cool, dispensed 200 μl of 0.01 *M* dibasic ammonium phosphate buffer into the tube, and placed the sample in the vortex mixer. After mixing for 20 s, the robot transferred the tube to the

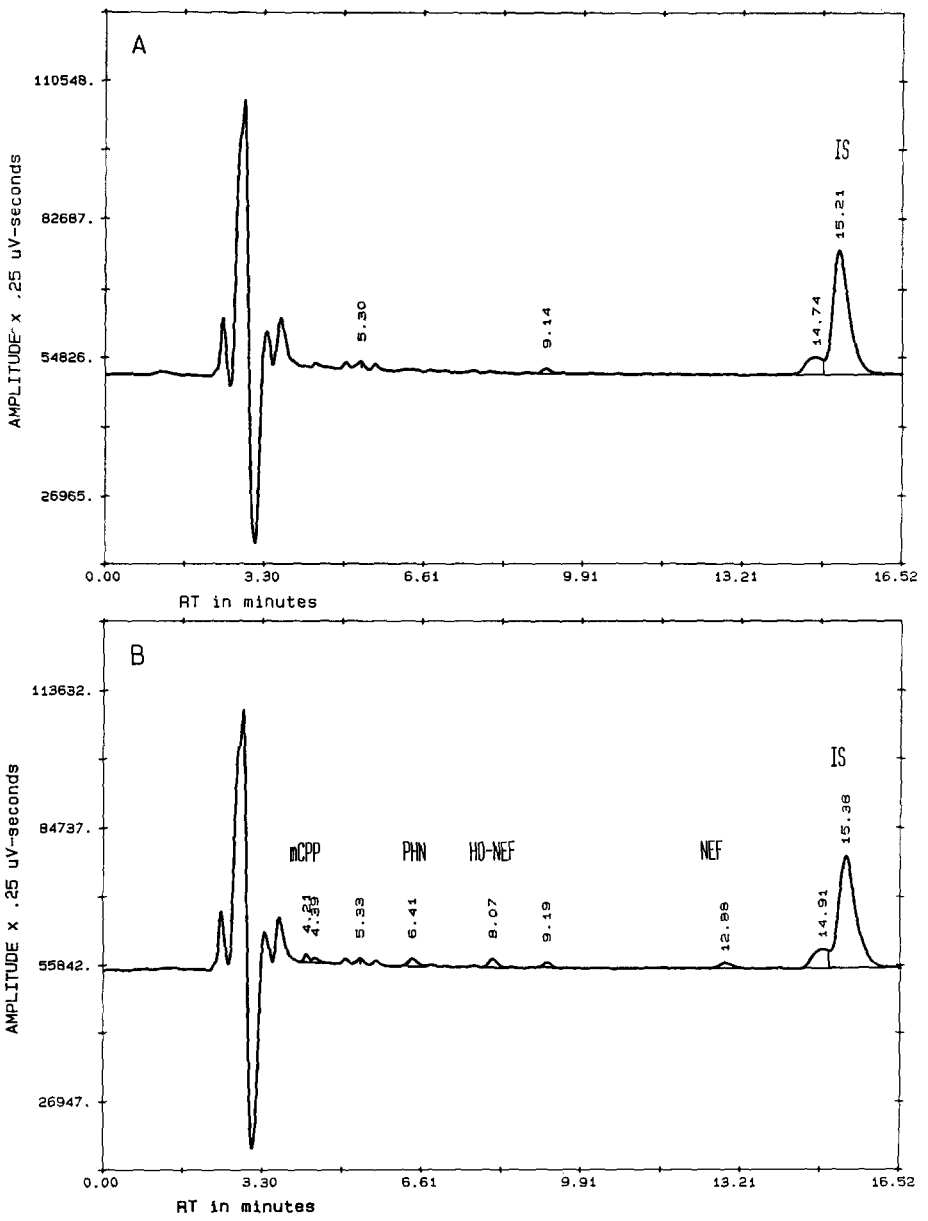

Fig. 3.

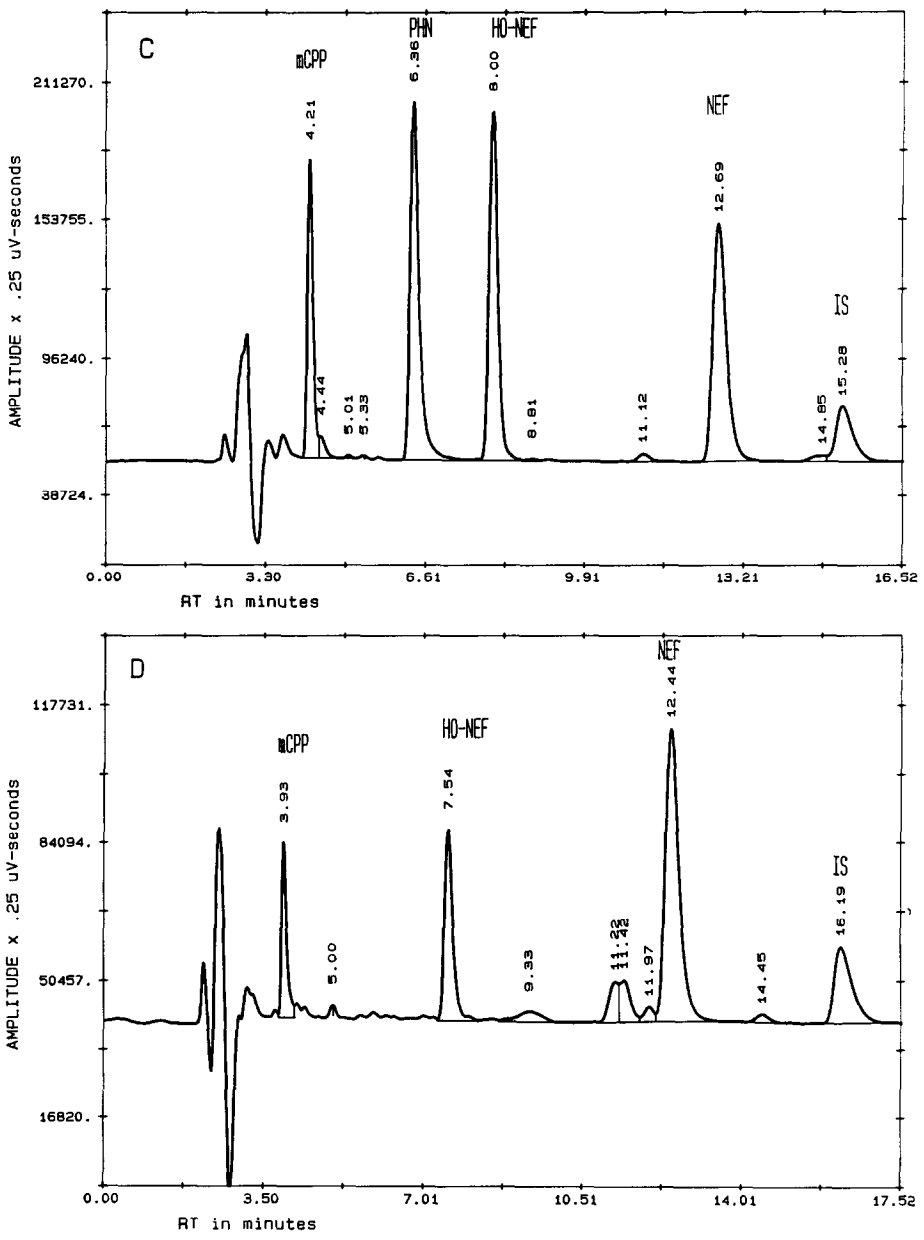

Fig. 3. Chromatograms of extracted human plasma samples. (A) Plasma blank; (B) standard with 2.5 ng/ml mCPP, 20 ng/ml PHN, 10 ng/ml HO-NEF, and 10 ng/ml NEF; (C) standard with 250 ng/ml mCPP, 2000 ng/ml PHN, 1000 ng/ml HO-NEF, and 1000 ng/ml NEF; (D) subject 18, 8 h, 150 mg nefazodone twice daily.

HPLC sipping station for injection onto the HPLC system. The loop was purged with 2.5 ml of unbuffered mobile phase, followed by two bursts of 206.8 kPa air, after each injection, and the liquid–liquid extraction station was washed automatically between samples with 15 ml of butyl chloride.

Data processing

The data management system used to acquire and process data during validation and analysis of clinical samples has been previously described [5].

RESULTS

Validation of assay

Chromatograms obtained from a blank and from processed samples at two different concentrations of NEF and its metabolites are shown in Fig. 3. Acceptable accuracy and precision were obtained for mCPP, PHN, HO-NEF, and NEF at plasma concentrations of 2.5, 20, 10, and 10 ng/ml (Table I). These concentrations were chosen as the lower limits of quantitation for the assay. Standard curves were linear from these low levels to 250, 2000, 1000, and 1000 ng/ml for mCPP, PHN, HO-NEF, and NEF, respectively, and yielded correlation coefficients of 0.999 for each. Recoveries of these compounds were 59.2, 65.8, 68.7, and 68.9% for mCPP, PHN, HO-NEF, and NEF, respectively. The intra-assay precision as measured by the relative standard deviation (R.S.D.) was within 6.0% for all analytes at concentrations in the lower and upper quartiles of the linear range and at concentrations that required dilution to be within the linear range (Table I). The predicted concentrations were within 8.0% of their actual concentrations. Fig. 4 shows that all four analytes were stable in human plasma at room temper-

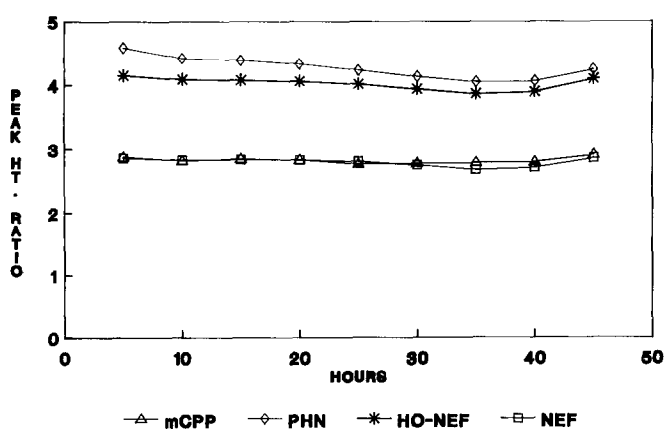

Fig. 4. Stability data for NEF and metabolites in human plasma during robotic processing at ambient temperature.

TABLE II

PHARMACOKINETIC PARAMETERS OF A SINGLE YOUNG FEMALE

C_{max} = maximum obtained plasma concentration; T_{max} = time at which C_{max} is reached; AUC = area under the plasma concentration–time curve.

Analyte	C_{max} (ng/ml)	T_{max} (h)	Terminal half-life (h)	$AUC_{0-\infty}$ (ng/ml h)
NEF	1169.27	0.5	3.45	4400.46
mCPP	24.77	2	3.84	136.60
HO-NEF	398.95	2	2.88	1803.06

ature in the time required to complete robotic processing of 145 samples. The assay was checked for interferences from possible concomitant medications. Interferences were found with two out of ten possible medications (diazepam interfered with HO-NEF and haloperidol with NEF). A total of 27 human predose samples were also checked for interfering substances, and none were observed. During sample analysis, eight pairs of standards were interspersed among samples for each analytical run. Pharmacokinetic data, as determined by this assay, for a young human female after oral administration of NEF are shown in Table II. These data illustrate the applicability of this assay for quantifying samples obtained from biopharmaceutic studies with NEF.

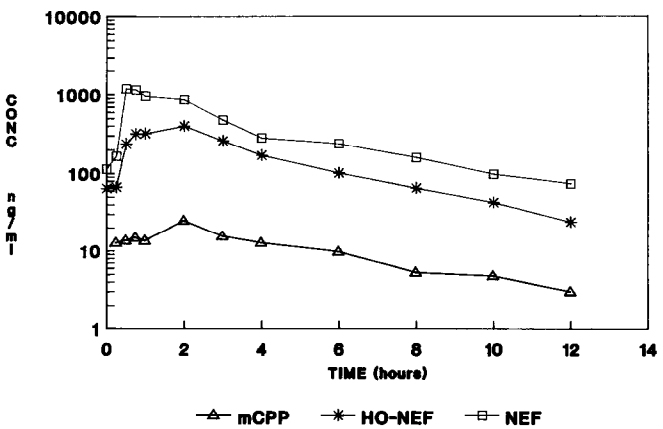

Fig. 5. Logarithmic plot of plasma concentrations of NEF, mCPP, and HO-NEF in a young female volunteer (aged 18–40 years) following twice daily dosage therapy of 150 mg NEF.

DISCUSSION

This assay was developed to analyze for NEF and its metabolites in plasma samples generated by clinical studies of NEF. Baseline separation for NEF, HO-NEF, mCPP, and PHN was achieved in this assay. Results from this study show that PHN concentrations were below the lower limit of quantitation in most subjects even after they received multiple-dose treatment of 300 mg NEF (twice daily dosage). The PHN concentrations that were measurable (20–50 ng/ml) were $< 1\%$ of the NEF concentration; HO-NEF ranged from 25 to 35%, while mCPP ranged from 2 to 10% of the NEF concentration.

To evaluate this procedure against the previous manual method the intra-assay variation was compared between assays. It was found that the robotic procedure yielded less variation than the manual procedure (1.1–5.6% robotic, 2–17% manual). Sample throughput has increased by at least 25% (360 *versus* 288 samples per week), despite a two-fold increase in HPLC analysis time. Reliable, unattended operation of the robotic procedure has led to a reduction of the analyst benchtime required for this assay from 25–35 to 9–15 h per week.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Diem Huynh for providing the drawing of the robotic system and to Frank Dixon for identifying the PHN metabolite of NEF.

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