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Sensitive determination of nefopam and its metabolite desmethyl-nefopam in human biological fluids by HPLC

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Abstract

Nefopam (NEF) and desmethyl-nefopam (DMN) were assayed simultaneously in plasma, globule and urine samples using imipramine as internal standard. A liquid–liquid extraction procedure was coupled with a reverse phase high-performance liquid chromatography system. This system requires a mobile phase containing buffer (15 mM KH_2PO_4 with 5 mM octane sulfonic acid: pH 3.7) and acetonitrile (77:33, v/v) through (flow rate = 1.5 ml/min) a C_{18} Symmetry column (150 × 4.6 I.D., 5 μm particle size: Waters) and a UV detector set at 210 nm. Internal standard was added to 1 ml of plasma or globule sample or 0.5 ml of urine sample, prior to the extraction under alkaline ambiance with *n*-hexane. The limits of quantification were 1 and 2 ng/ml for both molecules in plasma and globule, respectively; 5 and 10 ng/ml for NEF and DMN in urine, respectively. The method proved to be accurate and precise: the relative error at three concentrations ranged from −13.0 to +12.3% of the nominal concentration for all molecule and biological fluid; the within-day and between-day precision (relative standard deviation %) ranged from 1.0 to 10.1% for all the molecules and biological fluids. The method was linear between 1 and 60 ng/ml for both molecules in the plasma; 2 and 25 ng/ml for both molecules in the globule; 25 and 250 ng/ml for NEF and 50 and 500 ng/ml for DMN in the urine: correlation coefficients of calibration curves (determined by least-squares regression) of each molecule were higher than 0.992 whatever the biological fluid and during the pre-study and in-study validations. This method was successfully applied to a bio-availability study of NEF in healthy subjects.
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Keywords: Nefopam; Desmethyl-nefopam; High-performance liquid chromatography

1. Introduction

Nefopam hydrochloride ((±)-3,4,5,6-tetrahydro-5-methyl-1-phenyl-1H-2,5-benzoxazocine hydrochloride; NEF) is a non-opioid analgesic. Heel et al. [1] emphasized that data concerning its pharmacokinetic characteristics are sparse. Although the desmethyl metabolites constitute

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major metabolic pathways that account for greater than 50% of the product found in human urine, their disposition kinetics have not been documented and it is not known whether these metabolites, mainly desmethyl-nefopam (DMN), are present in human plasma. Moreover, although NEF can be administered orally, no data on its bio-availability has been published.

Few gas chromatography (GC) methods in the literature are reported to allow the quantification of NEF in human plasma [2–4] with flame ionisation [2,3], and nitrogen-selective detection [4]. These methods reached a limit of quantification (LOQ) ranging from 5 to 10 ng/ml using a 1 ml [4], 2 ml [3], or even 5 ml [2] volume of plasma sample. The published methods using reversed-phase liquid chromatography (LC) with ultraviolet [5,6] or electrochemical [7] detection are more sensitive than the GC methods; the LOQ ranged from 1 [7] to 2.5 ng/ml [6] using 1–2 ml volume of plasma [5–7] or milk [5] samples. The GC and LC methods did not allow the quantification of any metabolite, except the LC method developed by Mather et al. [6]. Indeed, Mather et al. [6] validated a LC chiral method for simultaneous determination of NEF and DMN antipodes but, this metabolite did not reach measurable concentration (<1.5 ng/ml) in any of the sample collected from healthy subjects receiving an intravenous infusion of 20 mg NEF. We propose here a method for the simultaneous quantification of NEF and DMN in plasma, urine or globule samples. The samples were obtained in 24 healthy subjects enrolled in an absolute bio-availability study following the recommended crossover design trial.

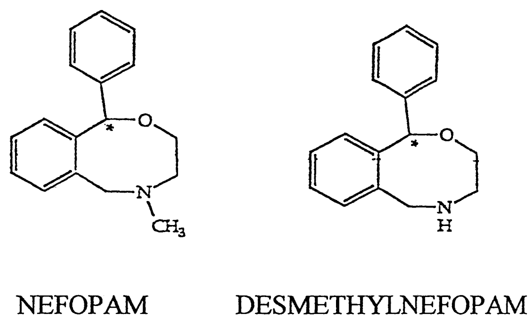

Fig. 1. Chemical structures of NEF and DMN.

2. Materiel and methods

2.1. Experimental

(±)-NEF hydrochloride (pKa 9.36), (±)-DMN and (±)-*N*-oxyde-nefopam (NO-NEF) were supplied by Biocodex pharmaceutical company (Montrouge, France). Imipramine hydrochloride (IS) was purchased from Sigma (St. Louis, USA). The molecular structures are shown in Fig. 1. The molecular weights of NEF hydrochloride, NEF in free base, and DMN in free base are 289.8, 253.3 and 239.3 g/mol, respectively. Stock solutions of each compounds (1 mg/ml) were prepared by dissolving 10 mg of equivalent free and pure base of each substance in 10 ml of methanol. These solutions stored at +4 °C in the dark were stable for months. All working solutions were prepared daily. The working solutions used for spiked plasma preparations were at 5, 0.5 and 0.05 µg/ml for both NEF and DMN and 1.5 µg/ml for IS. The working solutions used for spiked globule preparations were at 0.5, 0.25 and 0.025 µg/ml for both NEF and DMN and 1.5 µg/ml for IS. The working solutions used for spiked urine preparations were at 2.5 and 0.5 µg/ml for NEF, 5 and 1 µg/ml for DMN and 6 µg/ml for IS.

Acetonitrile, methanol and *n*-hexane (Carlo Erba Reagenti, Milan, Italy) were high-performance liquid chromatography (HPLC) graded. Potassium dihydrogenophosphate (KH_2PO_4) (Merck, Darmstadt, Germany), octane sulfonic acid 0.25 M (PIC® Reagent B8-low UV, Waters, Milford, MA), sodium carbonate monohydrate (Na_2CO_3) (Merck), hydrochloric acid 30% (HCl) (Merck) and acetone (Carlo Erba Reagenti) were analytical-reagent graded. Distilled water was purchased from Fresenius France Pharma (Louviers, France). Blank drug-free plasma and globules were obtained from the Pitié-Salpêtrière hospital blood bank. Blank drug-free urine was obtained from healthy subjects before NEF administration.

2.2. Instrumentation

The HPLC system consisted of a 114 M pump (Beckman, Berkeley, CA), solvent delivery module, a wisp 717 Plus Autosampler injector (Waters) set at 19 min/sample for the run time, a 2487 dual λ absorbance (Waters) variable-wavelength ultraviolet detector set at 210 nm (wavelength corresponding to the maximum absorbance of NEF and DMN) and a System Gold 2 integrator (Beckman) for data acquisition and treatment. The separation was achieved at room temperature using a reversed-phase Symmetry 5 μm C_{18} column (150 × 4.6 mm I.D.) (Waters). The analytical mobile phase (500 ml/chromatographic session) consisting of a mixture of buffer and acetonitrile (77:33, v/v) was degassed ultrasonically for 5 min before use. The buffer (pH 3.7) composed of 0.015 M KH_2PO_4 and 0.005 M octane sulfonic acid (2% v/v of PIC® Reagent B8-low UV) was filtered through a 0.22 μm filter Durapore GVWP 047 (purchased from Millipore, Bedford, MA) before adding acetonitrile. The analytical mobile phase recycled during the chromatographic session, was delivered at a flow rate of 1.5 ml/min with an average operating pressure of 1.8 kp.s.i. (1 p.s.i = 6894.76 Pa). At the end of each chromatographic session, the column was washed with 200 ml of acetonitrile–water (50:50, v/v).

2.3. Subjects samples

Twenty four Caucasian males aged 25.5 ± 3.0 (mean $\pm$ S.D.) years, weighing 73.9 ± 8.3 kg were included in the study. Subjects had no clinically significant medical history and had been free of medication for at least 2 weeks before entering the study. All subjects, informed of the aim of the trial gave their written consent. The study was approved by the Ethics Committee, Pitié-Salpêtrière Hospital, Paris. The design was a double blind, double placebo crossover study. Subjects were randomised by treatment order (i.e. IV/oral or oral/IV) with one week interval wash-out. Twenty milligrams of NEF hydrochloride solution or a placebo was administered orally on a sugar cube, simultaneously with a continuous infusion of 20 mg NEF hydrochloride or placebo diluted in 50 ml de 5% dextrose over 30 min, using an electric infuser (Vial médical, France). Blood was drawn from the opposite arm for determination of plasma concentrations of NEF and DMN before (t_0) and at, $t_{0.25}$, $t_{0.5}$, $t_{0.75}$, t_1, $t_{1.25}$, $t_{1.50}$, t_2, $t_{2.5}$, t_3, t_4, t_6, t_8, t_{12}, t_{16}, t_{24} and t_{48} h post-dosing. Samples were collected on lithium heparinate (7 ml) tubes and centrifuged for 10 min at $2000 \times g$ and were immediately frozen (-20 °C) until assay. A 1 ml volume of the blood sediment of t_0, t_2, $t_{2.5}$, t_3 h samples was frozen in order to determine globule concentrations of the drug and its main metabolite. Urine samples were collected before dosing (t_0) and at the following intervals t_{0-12}, t_{12-24} h. Urine collections were refrigerated at 2–4 °C during the 24-h interval. The total volume was recorded, and a 5-ml aliquot was decanted and frozen at $\leqslant -20$ °C until assayed.

2.4. Extraction procedure

Spiked biological samples (plasma, globules and urine) were used for the preparation of the calibration and quality control (QC) samples. The calibration samples were prepared extemporaneously for each chromatographic session. The QC samples used for the pre-study and the in-study validations were prepared before the validation in a sufficient number and stored with the biological samples of healthy subjects (-20 °C). Calibration curve samples, QC samples, and subject's samples from the same biological fluid were extracted simultaneously.

2.4.1. For plasma or globule samples:

Na_2CO_3 (250 μl 2 M) was added to a 15 ml silicone tube (Venoject, Terumo, Belgium) containing 1 ml of plasma or globule (spiked plasma or spiked globule used for calibration and QC samples, and plasma or globule samples from subjects) and the IS (100 μl 1.5 μg/ml) and vortexed for 5 s. N-hexane (8 ml) was added, the tube was then capped, shaken horizontally for 20 min, and centrifuged for 10 min at $2500 \times g$. The tube was placed in a dry ice-acetone bath, the lower aqueous layer was frozen, and the entire upper organic layer was transferred to a clean silicone tube. The tube was evaporated to dryness

Table 1
Accuracy, within-day and between-day (mean (RSD%)) precision for the analysis of NEF and DMN in plasma, globule and urine samples

Theoretical concentrations of NEF and DMN (ng/ml) in QC samples	Mean calculated concentration (RSD%): ng/ml			
	Within-day ($n=7$)		Between-day ($n=7$)	
	NEF	DMN	NEF	DMN
Plasma samples				
NEF and DMN: 2	2.1 (8.7)	2.1 (9.1)	2.0 (8.2)	2.1 (9.5)
NEF and DMN: 10	10.0 (2.9)	10.6 (3.1)	10.5 (5.8)	10.0 (6.5)
NEF and DMN: 50	49.8 (4.2)	55.3 (3.0)	49.6 (5.4)	49.2 (6.9)
Globule samples				
NEF and DMN: 2.5	2.6 (8.5)	2.4 (10.1)	2.5 (9.2)	2.2 (6.0)
NEF and DMN: 10	10.5 (2.4)	10.5 (6.9)	10.1 (6.4)	9.4 (7.4)
NEF and DMN: 25	28.1 (6.8)	28.1 (4.4)	25.4 (8.7)	26.2 (2.8)
Urine samples				
NEF 50; DMN 100	52 (2.8)	101 (6.7)	51 (6.5)	100 (4.2)
NEF 125; DMN 300	122 (3.1)	299 (4.4)	133 (4.2)	299 (5.5)
NEF 250; DMN 500	250 (1.2)	523 (1.0)	239 (5.8)	522 (5.7)

under a gentle stream of nitrogen in a water bath at +40 °C. The residue was then dissolved in 200 µl of mobile phase, and a 100 µl volume was injected in the chromatographic system.

2.4.2. For urine samples:

Na_2CO_3 (250 µl 2 M) was added to a 15 ml silicone tube containing 0.5 ml of urine (spiked urine used for calibration and QC samples, and urine samples from healthy subjects) and the IS (100 µl 6 µg/ml) and vortexed for 5 s. *N*-hexane (8 ml) was added and the tube was capped, shaken, and centrifuged (as described above). The tube was placed in a dry ice-acetone bath, and the entire upper organic layer was transferred to a silicone tube containing 0.5 ml Hcl (0.1 M). Following shaking and centrifugation, the upper organic layer was aspirated to waste. Na_2CO_3 (500 µl 2 M) and *n*-hexane (8 ml) were added for another extraction. After shaking and centrifugation, the tube was then placed in a dry ice-acetone bath, and the entire upper organic layer was transferred to a clean silicone tube. The tube was evaporated (as described before), the residue was then dissolved in 200 µl of mobile phase, 40 µl of which were injected in the chromatographic system.

2.5. Preparation of the calibration curves and QC samples

A calibration curve based on the peak-height ratio was constructed for each assay and biological fluid by adding known amounts of each drug to drug-free human plasma, globule and urine. Eight different spiked plasma samples covering a concentration range of 1–60 ng/ml for both NEF and DMN were assayed. Six different spiked globule samples covering a concentration range of 2–25 ng/ml for both NEF and DMN were assayed. Seven different spiked urine samples covering a concentration range of 5–250 ng/ml for NEF, and 10–500 ng/ml for DMN, were assayed. Linearity of the calibration curves was assessed by unweighted least-squares regression analysis. The concentration ranges of both molecules in spiked plasma, globule and urine used (QC samples) for the pre-study and the in-study validations are listed in Table 1.

2.6. Accuracy, precision, limit of quantification and recovery

Accuracy, between-day and within-day precisions of the method were determined for each

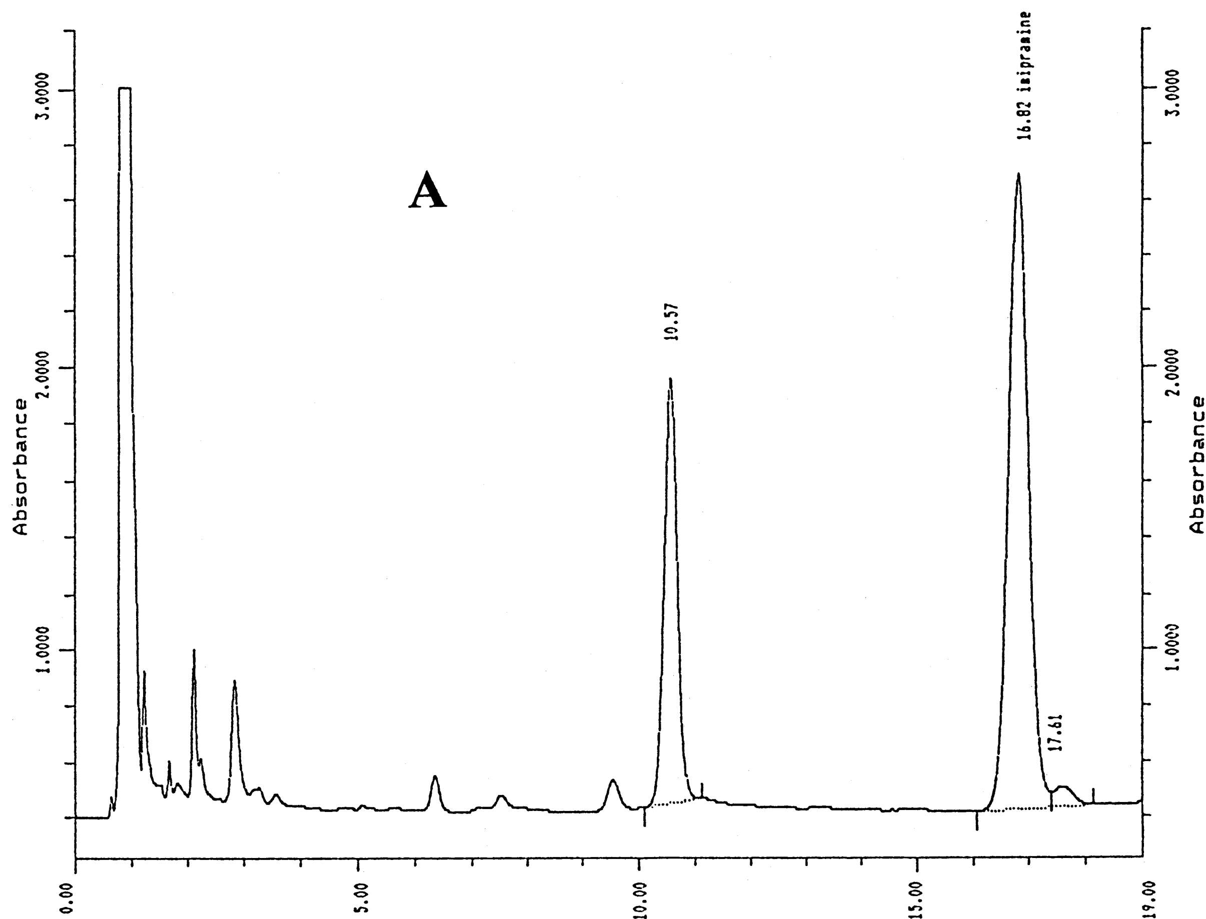

Fig. 2. Chromatograms obtained under described conditions of one subject's plasma sample treated orally with 20 mg of NEF hydrochloride. The retention times of NEF, DMN and internal standard (imipramine) were 4.1 4.4 and 16.8 min, respectively. (A) Blank chromatogram obtained before administration of NEF. (B) Sample obtained 1.25 h after administration of NEF, containing 4.6 and 7.1 ng/ml of NEF and DMN, respectively. (C) Low QC plasma sample spiked with 2 ng/ml of NEF and DMN.

biological fluid and molecule. Seven replicate spiked plasma, globule or urine samples were assayed between-day and within-day at three different concentrations (low, medium, and high QC samples) for NEF and DMN. The concentrations were calculated using calibration curves prepared with the same biological fluid and analyzed in the same run. Accuracy was calculated as percent deviation from the nominal concentration. Within-day and between-day precision were expressed as the relative standard deviation ($RSD\% = 100 \times S.D./mean$) of each calculated concentration. For the concentration to be accepted as the lowest LOQ, the percent deviation from the nominal concentration (mean accuracy) and the relative standard deviation has to be within the range $\pm 20\%$ and less than 20%, respectively. Average recovery of each molecule ($n = 30$) was determined for each biological fluid by comparing the peak-height ratios of the extracts

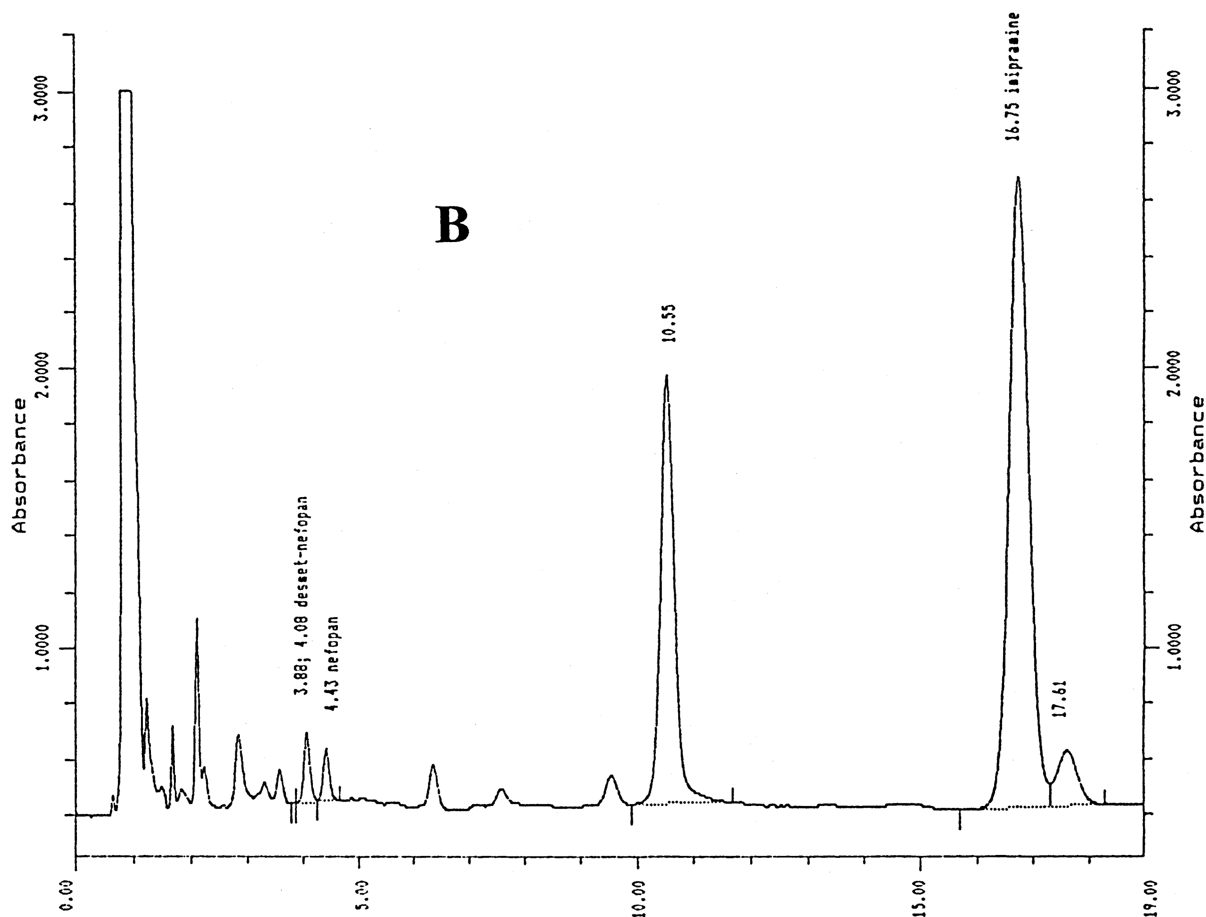

Fig. 2 (*Continued*)

with those obtained by direct injection of the same amount of drug in mobile phase at different concentrations (ten samples for each level).

3. Results and discussion

3.1. Stability

Testing for freeze and thaw analytes stability were determined during three freeze and thaw cycles. Three aliquots of low and high QC of each biological samples, were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the sample was refrozen during 24 h. This cycle was repeated two more times, then the sample was analysed on the third cycle. No degradation (< 10%) occurred whatever the molecule and concentration in each biological fluid.

QC plasma, globule or urine samples at the three levels (for the two molecules) were stable at −20 °C stored in the dark during the whole study period (around 4 months).

Stability after reconstitution was also checked and no degradation was observed with the auto-

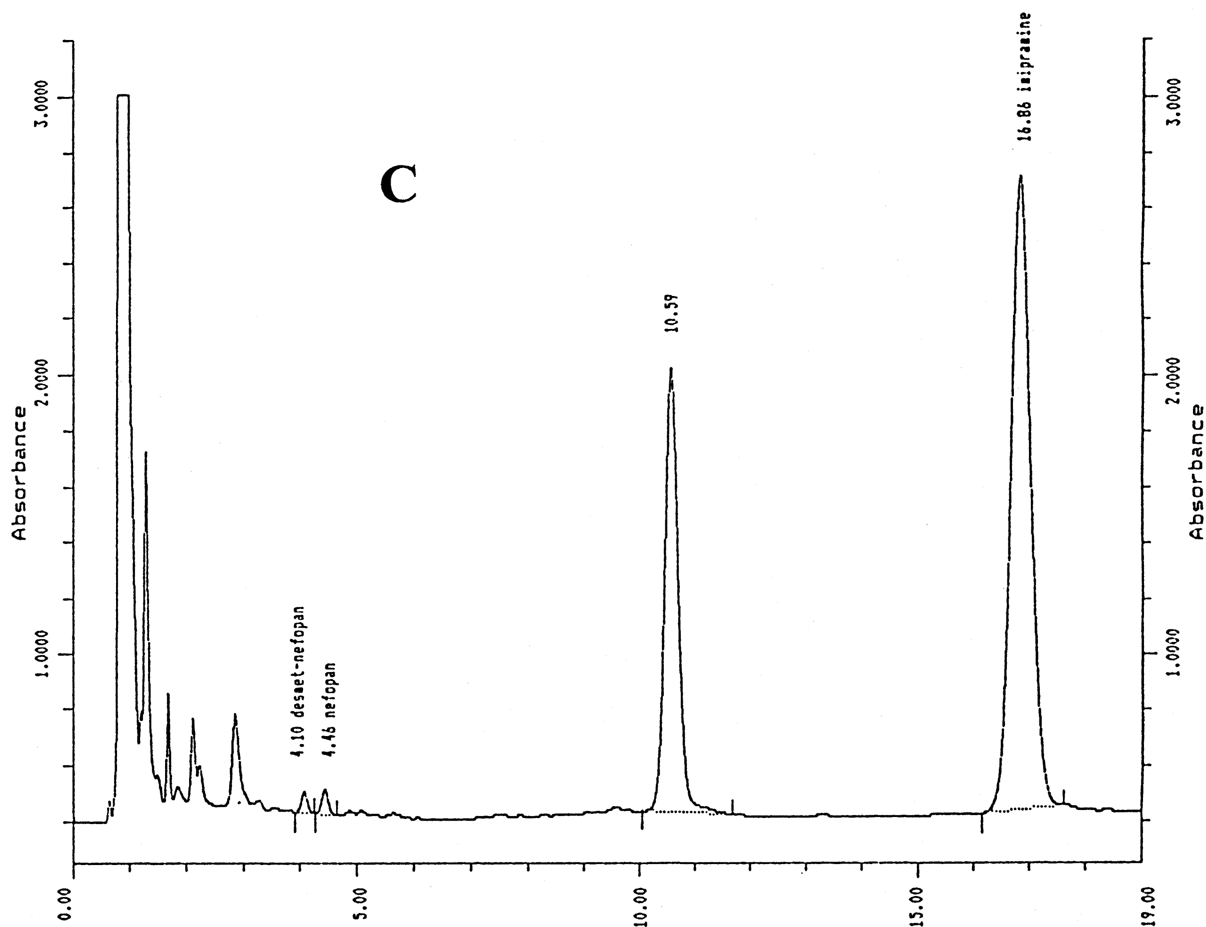

Fig. 2 (*Continued*)

sampler, the two molecules were stable in the mobile phase for 16 h at least at room temperature.

3.2. Selectivity and specificity

Under the described conditions, the retention times of NEF, DMN, and IS were 4.1, 4.4 and 16.8 min, respectively. Fig. 2 shows chromatograms obtained from t_0 and $t_{1.25}$ h (experimental t_{max}) plasma samples of one subject receiving NEF orally; and from a low QC plasma sample. Four hundred samples/column can be analysed without significant loss of resolution. Blank (t_0) plasma, globule and urine showed no interfering peak at the retention times of the molecules studied. Because of the potential interference of late peaks with the two molecules, it was decided to keep a long run time, as the retention time of imipramine permitted.

Two peaks were obtained after direct injection of NO-NEF at 4.2 and 4.4 min, hence very close to DMN and NEF peaks. Nevertheless, NO-NEF does not interfere in this method as it is not extracted under the described conditions. No study was performed to evaluate the interference with

Table 2
Mean recoveries (RSD%) of NEF, DMN and imipramine (IS) in plasma, globule, or urine samples

Biological fluid	Mean recovery (RSD%)		
	NEF ($n=30$)	DMN ($n=30$)	IS ($n=10$)
Plasma	85 (5.2)	81 (5.1)	75 (5.5)
Globule	75 (5.9)	70 (7.1)	69 (5.8)
Urine	66 (2.4)	61 (4.0)	62 (4.9)

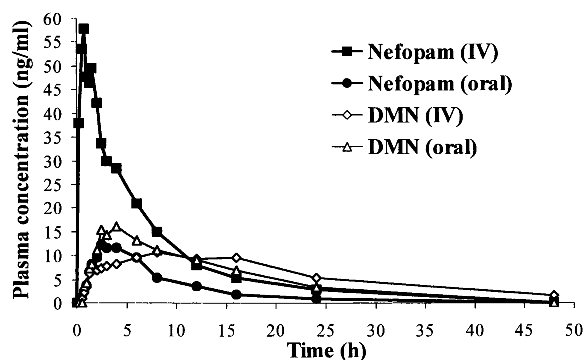

Fig. 3. Pharmacokinetic profiles of NEF and DMN from the same subject after oral and intravenous (infusion duration of 30 min) administration of 20 mg NEF.

the other drugs that are usually co-administered with NEF since this method was developed for a pharmacokinetic study in healthy subjects.

3.3. Limits of quantification

The LOQs as defined previously were 1 ng/ml for both molecules in plasma; 2 ng/ml in globule; 5 and 10 ng/ml for NEF and DMN in urine, respectively. Burton et al. [7] obtained the lowest LOQ for NEF assay (1 ng/ml from 1 ml of plasma sample) using an HPLC method with electrochemical detection. This LOQ was similar to ours but they failed to detect the DMN.

3.4. Accuracy, precision and linearity

The results from the validation of the method in human plasma, globule and urine, are listed in Table 1. The method proved to be accurate and precise: the relative error at three concentrations ranged from −13.0 to +12.3% of the nominal concentration for all molecules and biological fluids; the within-day and between-day precision (RSD%) ranged from 1.0 to 10.1% for all molecules and biological fluids. Correlation coefficients (r) of calibration curves (determined by least-squares regression) of each molecule were higher than 0.992 whatever the biological fluid and during the pre-study and in-study validations.

3.5. Extraction and recoveries

A single liquid–liquid extraction with n-hexane was performed for plasma and globule samples. However, three liquid–liquid extractions were needed for urine samples to remove endogenous interference. In contrast, other LC methods had to use multiple steps for plasma extraction as for example SPE (diol phase) after extraction with cyclohexane [7] and three steps extraction with cyclohexane [5,6]. In our method, we used n-hexane instead of cyclohexane for the two following reasons: (i) n-hexane does not freeze in a dry ice-acetone bath, (ii) the extraction of biological fluid with n-hexane under alkaline conditions permitted to avoid a potential interference with NO-NEF as this molecule is not extracted. Mean recoveries (RSD%) were obtained in 30 samples (10 samples for each level of concentration) for both molecules, and in 10 samples for IS (Table 2). In urine, recoveries are lower as compared to globule and plasma. They however remained remarkably constant.

3.6. Subjects samples

The pharmacokinetic profiles of NEF and DMN from one subject under the two administrations are presented in the Fig. 3. In this subject, $AUC_{0\to\infty}$ (trapezoidal rule) of NEF and DMN were 116 and 253.8 after oral intake and 368.6 and 314.1 ng h/ml after IV infusion, respectively; terminal half life of NEF and DMN were 5.7 and 9.3 after oral intake and 5.9 and 13.5 h after IV infusion, respectively. The F value (0.32) indicated a rather low bio-availability of NEF for this subject.

4. Conclusion

The assay described here is one of the first assays that simultaneously measures NEF and DMN in plasma, urine and globule samples with apparatus commonly available in laboratories. This method is simple, sensitive and may be currently used for pharmacokinetic studies in humans.

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