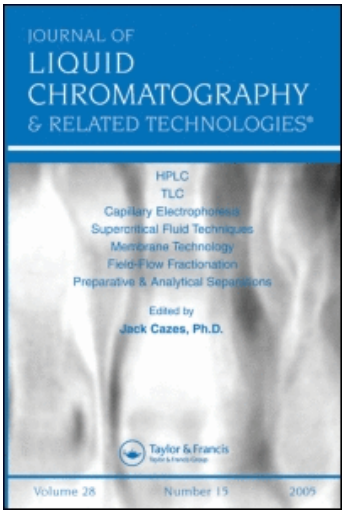

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Rapid Fluorimetric Assay for Plasma Nefopam Using High-Performance Liquid Chromatography

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To cite this Article Chang, L. -C. and Wang, D. -P.(1994) 'Rapid Fluorimetric Assay for Plasma Nefopam Using High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 17: 9, 1971 — 1980
To link to this Article: DOI: 10.1080/10826079408013472
URL: http://dx.doi.org/10.1080/10826079408013472

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 1971-1980 (1994)

RAPID FLUORIMETRIC ASSAY FOR PLASMA NEFOPAM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and rapid liquid chromatographic method for the determination of nefopam in plasma is presented. Plasma samples after de-protein management were directly analyzed by HPLC system with fluorimetric detector. The separation was achieved on a NOVA-PAK C_{18} column with a mixture of acetonitrile and 0.05M phosphate buffer at pH 3.0 using sodium propanesulfonate as the ion-pair agent. Low detection limit (0.5 ng) and linearity of calibration curve validate the suitability. Peak purity of nefopam in the chromatograms was checked by an additional photodiode-array detector (PAD). Recoveries of nefopam in plasma by de-protein and liquid-liquid extraction were 94.79 and 94.31%, respectively. In the in vivo iontophoresis study, remarkable differences of penetration effect were obtained.

It is suggested that this HPLC method could be used for pharmacokinetic study of nefopam.

INTRODUCTION

Nefopam hydrochloride (Fig 1) , structurally related to antiparkinson (orphenadrine) and antihistamine (diphenhydramine) drugs, is a non-narcotic

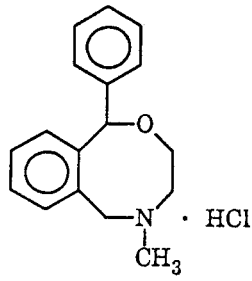

FIGURE 1 Structure of Nefopam Hydrochloride

analgesia with an analgesic potency lying in the range of 0.2 to 0.6 times that of morphine sulfate but without the propensity for addiction and respiratory depression[1]. Currently, it is comprehensively used in hospitals for the treatment of a wide range of painful conditions and has been relatively well tolerated by most patients.

For more detail documenting its pharmacokinetic properties, there had been many reports of application to analyze the drug content in human fluid such as GLC[2], GC-FID[3], HPLC-UV[4] and HPLC-ECD[5] methodologies. Some of them show relatively high sensitivity, precision and accuracy. However, these analytical methods generally require a cumbersome extraction protocol and are also time consuming. Besides, HPLC-UV method is simpler and more suitable for routine practice in the laboratory and hospital but the low selectivity of conventional UV techniques near 200 nm leads to the difficulty of determining nefopam in plasma.

In this paper, a simple high-performance liquid chromatographic (HPLC) fluorimetric detection method has been developed to eliminate these drawbacks and enables the rapid determination of plasma nefopam concentration. Moreover, an advanced photodiode-array UV detector in HPLC system also has been used to describe the peak purity and qualitation in this method. In our laboratory, the method has been successfully applied to an in vivo iontophoresis study.

MATERIALS AND METHODS

Chemicals and Reagents

Nefopam hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.) Glycine, Sodium hydroxide and dibasic potassium hydrogen phosphate (E. Merck, Darmstadt, F.R.G.) were used to prepare different pH buffers. Sodium propanesulfonate was purchased from TCI (Tokyo, Japan). Hipersolv-grade cyclohexane for liquid-liquid extraction was from BDH (Poole, U.K.). Acetonitrile (E. Merck, Darmstadt, F.R.G.) was HPLC grade and was used as the mobile phase and de-protein solvent. Milli-Q water was prepared through a Milli-RO 60 water purification system (Millipore, Bedford, MA, U.S.A.).

Chromatography

HPLC was performed with a Shimadzu liquid chromatographic system (Shimadzu Corp., Kyoto, Japan) with a Model LC-6A pump, a Model SIL-9A autosampler, a Model RF-551 spectrofluorometric detector set at the high sensitivity (Ex 284nm, Em 302nm) and a Model C-R6A CHROMATOPAC integrator. An additional Model SPD-M6A photodiode-array detector (PAD) for assay validation was placed in series between the column and spectrofluorometric detector. Nefopam was eluted isocratically with the mixture of acetonitrile and 0.05M phosphate buffer (pH=3.0) (20:80) containing 0.02M sodium propanesulfonate salt through a NOVA-PAK C_{18} analytical column (5μm, 3.9*150 mm) at a flow rate of 1.0 mL/min. Peak areas were integrated and recorded by the C-R6A CHROMATOPAC integrator. The PAD data were stored and processed by a 386DX personal computer (Aveen, Taipei, Taiwan) provided with a chromatographic software (Shimadzu). Mobile phase always filtered through a 0.22 μm millipore filter followed by degassing.

Sample Preparation

Blood bank plasma for the preparation was provided by the Tri-Service General Hospital (Taipei, Taiwan). Other blood samples were drawn into

heparinized tubes, plasma was separated by centrifugation and stored at -20°C until assay.

Standard curve in plasma: 0.5 mL of plasma containing varied amount of nefopam with a range of 0.1 to 2.0 μg/mL were prepared in centrifuge tubes, then 0.5 mL of acetonitrile was added. The mixture was thoroughly mixed and centrifuged at 3000 rpm for 15 minutes. Following centrifugation the upper layer was filtrated by 0.45 μm millipore filter and injected into the HPLC for analysis.

De-protein management: A 0.5-mL spiked sample of plasma was placed into a 20*15 mm centrifuge tube containing 0.5 mL of acetonitrile and mixed thoroughly, then centrifuged at 3000 rpm for 15 minutes. The final solution was filtrated by 0.45 μm millipore filter and injected into the HPLC for analysis.

Liquid-liquid extraction: In order to compare with conventional cumbersome method, a liquid-liquid extraction management was also taken place. Following Liu's method[4], a 2.0-mL spiked sample of plasma was alkalinized by 200.0 μL of 1.0M glycine buffer (pH 11.0) and extracted by cyclohexane (6.0 mL). Then through acidified (0.1M HCl, 3.0mL) and alkalinized (0.5 M NaOH, 1.0mL) manipulation, the final extract in cyclohexane (6.0mL) was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100.0μL of mobile phase for injection into the HPLC column.

In vivo iontophoresis study

An in-vivo evaluation provides information about the applicability of the method for analysis of rat plasma samples. In this study 6-8 week-aged female hairless mouse (Animal Center, Tri-Service General Hospital, Taipei, Taiwan) were fixed in consciousness, then 1.5% nefopam gel through a self-synthesis polymer membrane was applied on the skin of abdomen. Nefopam within the gel administered into the body was based on the iontophoresis impulse and another group was only based on the passive transmission used as the control. Blood samples were collected by the direct heart transfixion at the 0.5, 1.0, 2.0 and 3.0

hours postdose. Plasma were separated by the centrifugation and stored in -20°C until analysis

RESULTS AND DISCUSSION

Precision, accuracy and sensitivity

The highly selective and simple procedure described in this paper gives a clear separation of nefopam in blank and spiked samples. (Fig 2). Its efficacy demonstrated the two advantages: (1) the elution monitored by fluorescence detection allowing the direct assay of nefopam content without interference from other components of the assay plasma. (2) no requirement of compound modification and extremely simple de-protein management achieving the rapid determination of plasma nefopam.

The precision and linearity of the HPLC method were examined. The repetitive analyses (n=3) of the nefopam standard solution (0.1-2.0 μg/mL) in plasma gave a corresponding coefficient of variation (both intra- and inter-day coefficients of variation less than 10%) (Table 1). The retention time of nefopam in the HPLC system was 10.30 min. The C.V. (n=15) of the retention times was 0.5%.

The calibration graph of peak area versus nefopam concentration showed excellent linearity over the range 0.1-2 μg/mL(Fig. 3). The detection limit was 0.5 ng (signal-to-noise ratio=3).

Photodiode-array detection validation

For additional validating this assay method, HPLC of nefopam spiked with plasma was carried out with the on line photodiode-array detector to provide the spectrum, the absorbance ratio and the criteria for assessing the purity of peaks. Data on the peak purity of spiked plasma was obtained by comparing the spectra in the ascending, apex and descending portion of nefopam containing peak. Spectrum

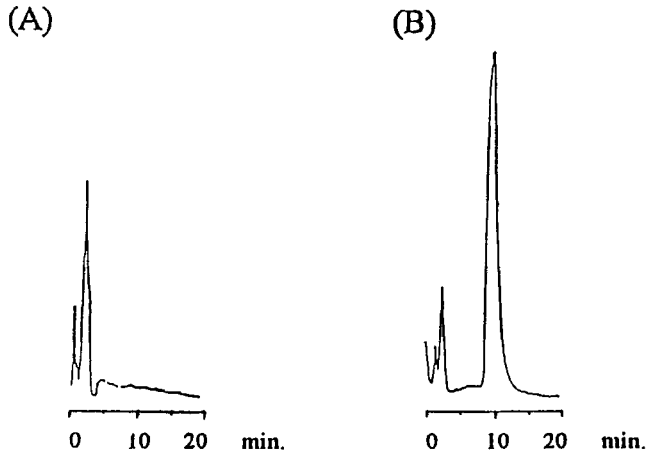

FIGURE 2 Chromatograms of (A) blank plasma, (B) plasma spiked with nefopam (0.4μg/mL).

TABLE 1 Intra- and Inter-day Standard Curve Data of Nefopam in Plasma

Concentration	Intra-day		Inter-day	
(μg/mL)	Found (μg/mL)	C.V. (%)	Found (μg/mL)	C.V. (%)
0.1	0.12	8.41	0.09	5.78
0.2	0.25	4.41	0.20	7.21
0.4	0.41	2.92	0.39	3.44
0.8	0.86	3.27	0.83	1.31
1.6	1.59	2.46	1.64	2.52
2.0	2.12	1.60	1.98	3.17

n=3

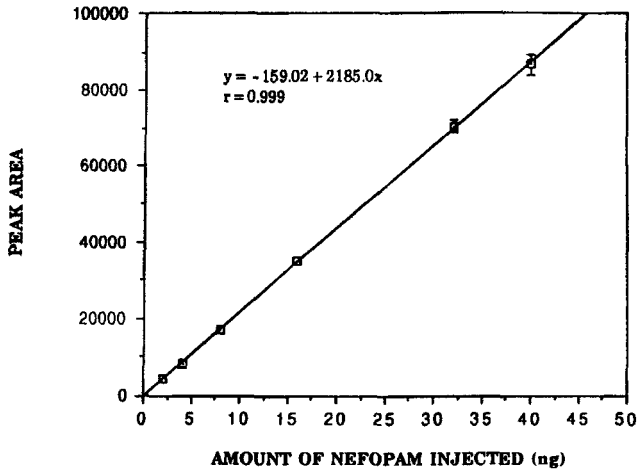

FIGURE 3 A plot of concentration vs peak area of nefopam in plasma.

of the three portions from 195 to 380 nm were superimposable, indicating the absence of impurities and that the corresponding purity index was 0.9998 (Fig 4). In this identification, the retention time and absorbance spectra were also taken into account. Different wavelength chromatograms in the time window were normalized and overlaid. Homogeneity of chromatograms showed no co-eluted interference and a high quality of the separation.

De-protein and liquid-liquid extraction

Table 2 summarized the recoveries of the nefopam from human plasma by ACN de-protein and liquid-liquid extraction at 100, 10 and 1 μg/mL concentration. Triplicate at each concentration gave overall recoveries (Mean ± S.D.) of 94.79 ± 0.92 and 94.31 ± 0.75, respectively. The fact that there was no significant difference between the two methods reveals that high selectivity of fluorescence determination could effectively eliminate the complexity in the sample preparation.

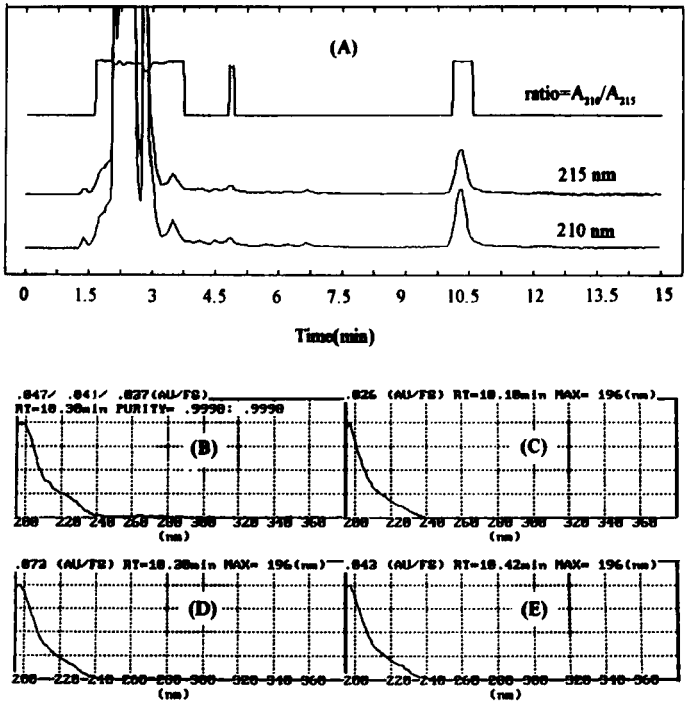

FIGURE 4 (A) Chromatograms of plasma spiked with nefopam (0.4 μg/mL) detected by diode-array detector at 210 and 215 nm. (B) Overlay spectra of nefopam for assessing peak purity. (C), (D),(E) UV spectra acquired in the ascending, apex and descending portion in the range of 195-380 nm.

In vivo iontophoresis study

A plasma concentration-time curve presented in Fig. 5 clearly depicted the iontophoresis efficacy. In comparison with the control group, applying current in skin increased the penetration of nefopam from gel up to seven fold after 3 hours postdose. The significant effect of penetration increment appeared after a half hour. Large scale studies were undertaken in our laboratory applying this analytical method.

TABLE 2 Recoveries of Nefopam from Human Plasma by Different Management

Nefopam	De-protein		Liquid-liquid extraction	
Added (μg/mL)	Found (μg/mL)	Recovery (%)	Found (μg/mL)	Recovery (%)
100	95.24	95.2	93.61	93.6
	95.82	95.8	92.97	93.0
	94.57	94.6	94.62	94.6
10	9.42	94.2	9.43	94.3
	9.53	95.3	9.49	94.9
	9.35	93.5	9.52	95.2
1	0.94	94.6	0.94	94.1
	0.93	93.7	0.95	95.2
	0.96	96.2	0.93	93.9
Mean ± S.D.		94.79 ± 0.92		94.31 ± 0.75

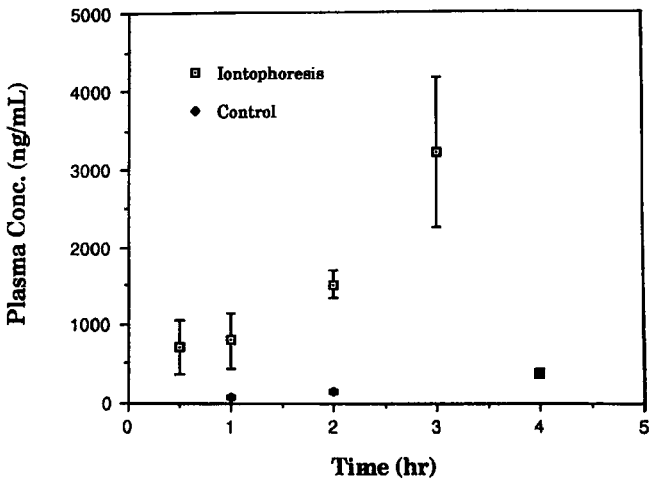

FIGURE 5 Comparison of the plasma nefopam concentration-time profiles in iontophoresis and control groups.

Conclusion

In summary, the fluorimetric method described here is simple, highly selective, specific and routinely useful for the quantitative analysis of nefopam in plasma. Moreover, it is convenient and can be readily incorporated into existing laboratory HPLC system as a novel method.

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Received: December 6, 1993
Accepted: January 14, 1994