Journal of Chromatography, 226 (1981) 79—89
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 998

QUANTITATIVE DETERMINATION OF NEFOPAM IN HUMAN PLASMA, SALIVA AND CEREBROSPINAL FLUID BY GAS—LIQUID CHROMATOGRAPHY USING A NITROGEN-SELECTIVE DETECTOR

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(First received March 17th, 1981; revised manuscript received June 15th, 1981)

SUMMARY

A sensitive and selective gas—liquid chromatographic method for the determination of nefopam in human plasma, saliva and cerebrospinal fluid has been developed. The method includes the selective extraction of nefopam and the internal standard, orphenadrine, from biological fluids by a double extraction procedure. The extracted nefopam and internal standard are analyzed by a gas chromatograph equipped with a 3% OV-17 glass column and a nitrogen—phosphorus flame ionization detector (NPFID) operated in the nitrogen mode. The detector provides the needed high sensitivity and also selectivity due to the inherent characteristics of NPFID to discriminate against non-nitrogen containing materials. Five nanograms nefopam per ml plasma or saliva are routinely quantitated with a 1-ml sample or as little as 2 ng per ml cerebrospinal fluid with a 3-ml sample. The intra-day reproducibilities, expressed as the relative standard deviation, are 5, 2 and 3% at 10, 35 and 75 ng/ml plasma levels, respectively. The accuracies expressed by relative error at these levels are 12, −4 and −2%, respectively. The inter-day reproducibility is demonstrated by the small relative standard deviation, 2%, of the slopes from ten plasma standard curves run on ten different days. In various clinical studies in humans the method has been successfully applied to the study of single-dose pharmacokinetics of nefopam and the monitoring of nefopam concentrations in saliva and cerebrospinal fluids.

INTRODUCTION

Nefopam, 3,4,5,6-tetrahydro-5-methyl-1-phenyl-1H-2,5-benzoxazoxine hydrochloride, also Acupan® or Ajan®, (I), is a new analgesic having a unique heterocyclic structure. The compound, first synthesized by Klohs et al. [1], was introduced in Mexico in 1975 and the German Federal Republic in 1976. Recently, it has entered the market in the United Kingdom, Belgium, and some South American countries and was recently approved for marketing in France.

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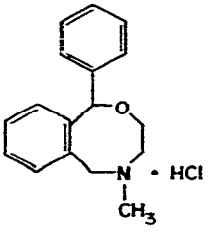
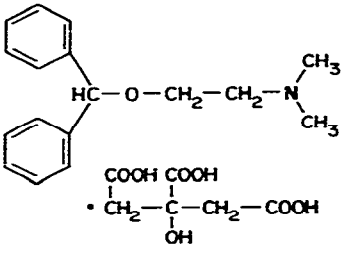
A number of studies concerning the analgesic properties of I have been reported [2—6].



I
(Nefopam)



Internal Standard
(Orphenadrine Citrate)

Prior to in vivo metabolic studies it was necessary to establish a sensitive, chemically specific assay to follow the physical translocation and chemical biotransformation of I. The recommended therapeutic dose of this basic drug is low, and the initial studies in humans indicated that the plasma concentrations of I are usually in the low ng/ml range. Two gas—liquid chromatographic (GLC) methods for the determination of I in plasma have been reported [7, 8]. These methods involve detection of I in plasma by the flame ionization detector. One method has a sensitivity of 20 ng/ml with a 2-ml sample and the second method 10 ng/ml with a 5-ml sample. The aim of the present investigation was to improve the sensitivity and expand the method to other biological fluids such as saliva and cerebrospinal fluid, thus providing a tool for broader investigation of the pharmacokinetics of I at the lower concentrations present at later time periods post dosing as well as in other biological fluids. Because I is a nitrogen-containing compound, and there are many literature reports citing the use of a nitrogen-selective detector for plasma drug quantitation [9—13], it seemed probable that the use of a nitrogen-selective detector could increase the sensitivity of an assay method for I. This paper presents the results of this investigation with a nitrogen-specific detector. A similar GLC method using the same approach was developed concurrently by a group collaborating with this laboratory [14].

EXPERIMENTAL

Reagents

All solvents were Nanograde® and all reagents were analytical reagent grade. Aqueous solutions of 0.1 *N* and 0.5 *N* sodium hydroxide, and 0.1 *N* hydrochloric acid were prepared in distilled water.

Blank plasma

Human plasma was obtained from volunteers who had fasted overnight and had not been on any medication for the previous week. The donors were also asked to refrain from caffeine-containing food and beverages for at least 24 h prior to donating blood.

Gas chromatography

A Hewlett-Packard Model 5840A gas chromatograph equipped with a Model 18847A dual nitrogen—phosphorus flame ionization detector was used. The glass column (180 cm × 2 mm I.D.) was silanized and packed with 3% OV-17 on 80—100 mesh Chromosorb W HP and conditioned overnight at 240°C.

The operating conditions were: injection port temperature, 225°C; detector temperature, 300°C; oven temperature, 190°C isothermal; carrier gas (helium) flow-rate, 30 ml/min; hydrogen flow-rate, 3 ml/min; air flow-rate, 80 ml/min. The detector voltage (d.c.) was set at 16—18 V depending on the age of the bead. An oven temperature program was maintained following each injection: 8.5 min isothermal heating at 190°C, then 30°C/min from 190—240°C and 3 min isothermal heating at 240°C. A 4-min period for cooling and stabilization was maintained between injections. Under these conditions typical retention times of I and the internal standard were 7.6 and 4.5 min, respectively.

Standard solutions

All stock solutions of I and the internal standard were made in methanol. Standard solutions containing 100, 50, 25, 10 and 5 ng of I (free base) per 0.1 ml were made by diluting a 10 μg/ml primary standard solution. The 10 μg/ml stock solution of orphenadrine was diluted to give an internal standard solution of 40 ng (free base) per 0.1 ml.

Extraction of I

Plasma. To a 15 × 125 mm culture tube with a polyethylene-lined screw cap add 1 ml of experimental plasma and 0.1 ml of methanol. Along with the samples, prepare six standards in blank plasma by adding 0, 5, 10, 25, 50, and 100 ng of I in 0.1 ml of methanol to 1 ml of blank plasma. Add 0.1 ml of internal standard solution, 3 ml of 0.1 *N* sodium hydroxide and 5 ml of benzene in that order to all tubes. The tubes are shaken for 10 min on a reciprocal mechanical shaker (horizontal position) and centrifuged for 5 min at 1670 *g*. Then 4.5 ml of the top (benzene) layer are transferred to a tube containing 3 ml of 0.1 *N* hydrochloric acid, and the tubes are shaken for 10 min and centrifuged for 5 min.

The benzene is aspirated and discarded, and 1 ml of 0.5 *N* sodium hydroxide and 5 ml of benzene are added to each tube, and the tubes are shaken for 10 min and centrifuged for 5 min. Then 4.5 ml of the benzene layer are transferred to a 12-ml conical extraction tube and evaporated to dryness at 60°C under a nitrogen stream. The residue is redissolved in 100 μl of methanol and mixed for 10 sec with a vortex mixer. Then 10-μl aliquots of each sample are injected into the gas chromatograph. The benzene can be replaced with toluene in this procedure.

Cerebrospinal fluid. Because of the extremely low concentrations of I in the cerebrospinal fluid (CSF), 3 ml of CSF are generally analyzed. When less than 3 ml of CSF sample is available, distilled water is added to make up the total volume of 3 ml. The extraction and analytical procedures are the same as that for plasma.

Saliva. One milliliter or less saliva sample is analyzed in the same manner as plasma; when less than 1 ml is used, distilled water is added to make up the total volume to 1 ml.

Calculation

The area of each I and internal standard peak was determined by an HP Model 18850A integrating GLC terminal.

Area ratios between I standards and the internal standard were plotted against concentration of I. A straight line was fitted by the least-squares method, and its slope and intercept at the area ratio axis were determined. The mathematical expression of the standard curve is:

$$\text{area ratio} = A\ (\text{I concentration}) + B \tag{1}$$

where A is the slope of the line, and B is the intercept of the line at the area ratio axis. Unknown samples were calculated from the following equation:

$$\text{I concentration in unknown sample} = \frac{\text{area ratio} - B}{A} \tag{2}$$

RESULTS AND DISCUSSION

Chromatography

Baseline separation of I and the internal standard was achieved using the OV-17 column, with no significant interference with I or the internal standard by any endogenous material present in normal human plasma. Typical GLC tracings of extracts from blank human plasma, plasma from human blood dosed with I, and authentic I and internal standard are shown in Fig. 1. The retention times, under the experimental conditions are 7.6 and 4.5 min for I and the internal standard, respectively. The reason for needing this apparent over-separation is that caffeine (retention time = 5.7 min) is eluted between I and the internal standard. In a clinical study where caffeine-containing food or beverages could not be controlled, the size of the caffeine peak varied widely depending on the time of ingestion of caffeine-containing food and beverages and the time of blood sampling. When the caffeine peak is extremely large, it can affect the accuracy of measurement of I at the 5 ng/ml level. Since there are some materials which are eluted substantially slower than I, in order to avoid interference with the next sample by these memory peaks, the column temperature is programmed up to drive off these materials quickly before the next injection.

Limited tests with toluene as the solvent for extraction show identical results in comparison with the standard procedure in which benzene is the solvent. Thus, benzene can be substituted by toluene in this method to eliminate the potential exposure of the analyst to any possible benzene toxicity. In the work of Schuppan et al. [7], diethyl ether was used for extraction. The use of benzene or toluene in the current procedure eliminates the extra precautions taken for the potential explosion of diethyl ether.

At the last step of the extraction procedure where the extract of I was evaporated to dryness and methanol was added to recover I before injection, it is important to add enough methanol to assure the total recovery of I. Incomplete recovery or excessive methanol (diluting the sample) will result in decreased sensitivity, since a smaller sample is presented to the detector. By using ^{14}C-labelled I, it was determined that 100 μl of methanol gives total re-

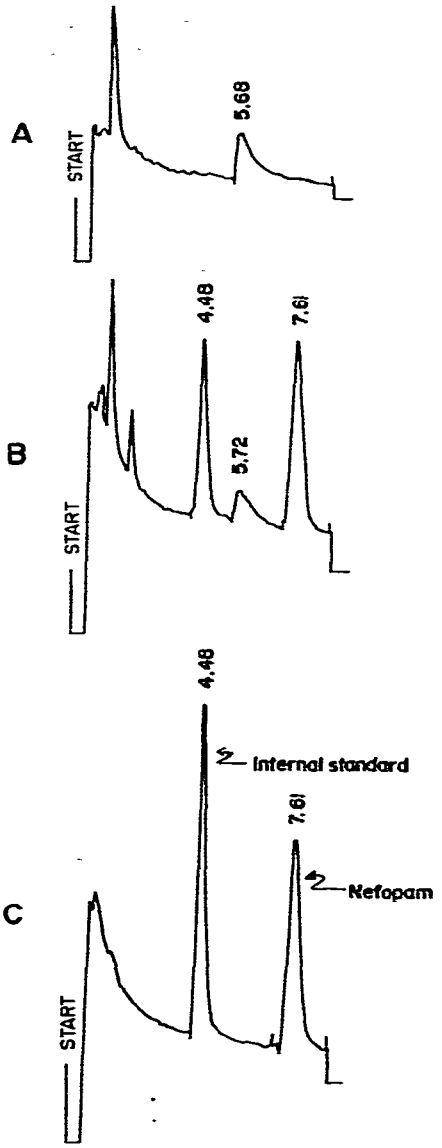


Fig. 1. Chromatograms from human plasma. (A) Blank plasma; (B) plasma of subject dosed with nefopam with the internal standard added, nefopam, 67 ng/ml, internal standard, 40 ng/ml; (C) mixture of authentic nefopam and internal standard, amount injected, nefopam, 5 ng, internal standard, 4 ng.

covery of I under the described experimental conditions.

It was also observed that there is some solvent effect on the nefopam response. For the same amount of nefopam injected in different volumes of solvent, the area responses are different; the larger the solvent volume, the lesser the area per ng of I. Also, for the same solution, when various volumes are injected the resulting responses are not directly proportional; the larger the volume the lesser the response per ng of I. Thus, it is strongly advisable that a

fixed volume of all I standards and unknowns is analyzed to eliminate the potential solvent effect. In our standard procedure a relatively large volume of the sample, 10 μl, is injected because the increase in overall sensitivity with the relatively large amount of I injected more than offsets the loss of response due to the solvent effect.

Selectivity

Two known metabolites of I, desmethyl nefopam and nefopam N-oxide were investigated for possible interference with this method. Even though the direct injection of the N-oxide gave a peak at the retention time of I, presumably due to the conversion of the N-oxide to I on the column, when as much as 100 ng of N-oxide was added to the blank human plasma and extracted following the general procedure, no discernible peak appeared at the retention time of I. N-Oxide, a relatively neutral compound, is most likely left behind during the extraction procedure. Desmethyl nefopam is eluted more slowly than I. Furthermore, when 100 ng of desmethyl nefopam were added to the blank human plasma and treated similarly, only a small peak was detected, probably due to extensive adsorption of the secondary amine on the column. The small response and the longer retention of desmethyl nefopam do not constitute an interference with I.

Extraction recovery

The extraction recovery of I from plasma was determined by a slightly modified extraction procedure in which the internal standard was added after the extraction was completed. The standard curve for this particular experiment was obtained by analyzing various concentrations of I with internal standard present, but without extraction. The extraction recovery check from human plasma was done in triplicate at five concentrations: 5, 10, 25, 50, and 100 ng/ml. The recovery obtained over this concentration range is 87—105% (Table I).

TABLE I

EXTRACTION RECOVERY OF I FROM HUMAN PLASMA (n = 3)

I added (ng/ml)	I detected (mean ± S.D., ng/ml)	Mean recovery (%)
5	4.3 ± 0.3	87
10	10.5 ± 0.4	105
25	23.7 ± 5.2	95
50	52.5 ± 7.1	105
100	97.2 ± 4.1	97

The extraction recovery variations between replicates are relatively wide in some instances. This further emphasizes the importance of both the selection of an internal standard having similar physical properties to I and addition of the internal standard directly to the plasma. The occasional lack of constant extraction recovery of I can be compensated for by the use of orphenadrine as an internal standard; thus, quantitative analysis can be assured.

Sensitivity

Five ng of I per ml of plasma are routinely quantified with a 1-ml sample. The sensitivity (defined as least amount quantifiable) for detection of I in plasma can be further increased by the use of larger samples. However, based on our experience with data obtained following a single therapeutic dose, a 5 ng/ml sensitivity is adequate. For CSF, because of the low concentration of I, a multiple-ml sample has to be used. A sensitivity of 2 ng/ml CSF with a 3-ml sample can be realized. For saliva, the concentration of I in most of the samples analyzed is higher than its corresponding plasma level; therefore a sensitivity of 5 ng/ml with a 1-ml sample is sufficient. In order to quantitate nefopam at 5 and 10 ng/ml levels, the d.c. voltage of the nitrogen detector has to be optimized. However, too high a d.c. voltage will shorten the life span of the alkali bead. Some preliminary data indicated that at low concentration levels, the use of peak height ratios for calibration is slightly better than our current routine method involving area ratios.

Linearity

Six or seven single standards (0—100 ng) were run daily with the unknown samples. The mathematical expression of the least-squares line is $Y = 0.0229$ (I) $- 0.0280$. The correlation coefficient is 0.9995 and the coefficient of determination is 0.9990 indicating good linear proportionality between concentration of I and detector response.

Precision and accuracy

The intra-day precision and accuracy of this method were checked by carrying samples at three concentration levels (10, 35, and 75 ng/ml) in replicates of five through the entire procedure. The detected concentrations were calculated from a standard curve constructed from duplicate standards. The precision was determined by comparing the results between the five replicate samples at each concentration level with the mean and expressed as the relative standard deviation (Table II). The mean detected concentrations were 11.4, 33.6, and 73.7 ng/ml, respectively. The standard deviations were 0.6, 0.7, and 2, and the relative standard deviations were 5, 2, and 3%, respectively.

The accuracy of this method is indicated by the small mean error between the detected and actual values for the samples described above. The mean errors were 1.4, −1.4, and −1.3 ng/ml for the 10, 35, and 75 ng/ml concentrations, respectively. The corresponding relative errors were 12, −4, and −2%. Since this method is intended to analyze samples from biological studies, the precision and accuracy levels are more than adequate.

Ten standard curves for human plasma covering a 5—100 ng/ml range were generated during the analysis of plasma samples from a metabolic study in humans over a period of 19 days. The slope for each standard curve is tabulated in Table III. The slopes ranged from 223×10^{-4} to 237×10^{-4} with a mean and standard deviation of $(230 \pm 5) \times 10^{-4}$. The day-to-day reproducibility of the human plasma standard curve is excellent as indicated by the very small (2%) relative standard deviation of the slopes.

TABLE II

PRECISION AND ACCURACY OF DETERMINATION OF I (n = 5)

I added (ng/ml)	I detected (mean ± S.D., ng/ml)	Relative S.D. (%)	Mean error (ng/ml)	Relative error (%)
10	11.4 ± 0.6	5	1.4	12
35	33.6 ± 0.7	2	−1.4	−4
75	73.7 ± 2	3	−1.3	−2

TABLE III

SLOPES OF THE LINEAR REGRESSION LINES OF THE PLASMA STANDARD CURVES FROM TEN DIFFERENT DAYS

The ten standard curves covered a 5—100 ng/ml range. Mean ± S.D. = 230 ± 5; relative S.D. = 2%.

Day	Slope × 10^4
1	230
2	233
6	236
7	229
8	229
9	236
13	237
14	225
16	226
19	223

Application of the method

Plasma. This method has been extensively applied to the study of single-dose plasma pharmacokinetics in normal, healthy human volunteers, and the monitoring of plasma levels of I in patients following a repeated dosing regimen. One example of these applications is described here.

A normal, healthy human volunteer was given a single, intramuscular (deltoid muscle), 30-mg dose of I. Ten-ml heparinized blood samples were taken before medication and 1, 2, 3, 5, 7, 9, 11, 13, 15, 24, 36, and 48 h after dosing. The plasma levels of I in these samples were determined by the method described above. The resulting plasma concentrations of I vs. time after dose were plotted on a log—linear scale (Fig. 2). The highest plasma levels were detected 1 h post dosing (first sampling time) indicating the rapid absorption of I from the injection site. The plasma levels of I from 1 to 15 h decline monoexponentially with an estimated plasma half-life ($t_{½}$) of 4.3 h. The last quantifiable time point ($\geqslant$ 5 ng/ml) was at 15 h, which is about four half-lives. Thus, the method is more than adequate for the measurement of the plasma pharmacokinetics of nefopam in humans following a single 30-mg intramuscular dose.

Saliva. For certain drugs plasma to saliva concentrations remain constant over a wide concentration range [15] and thus, the saliva concentration reflects the plasma concentration. In this situation, saliva level measurement provides a

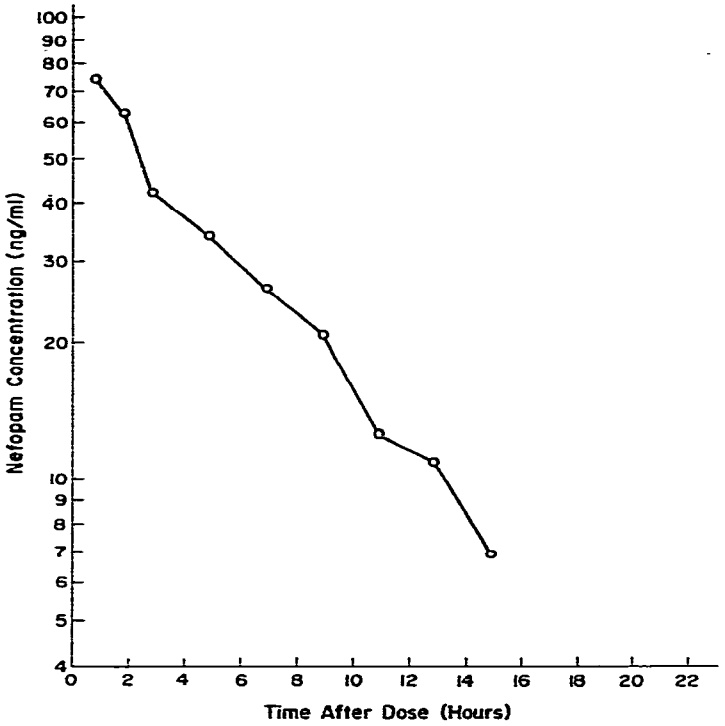


Fig. 2. Plasma nefopam levels in a normal healthy human volunteer following a single intramuscular 30-mg dose of nefopam.

non-invasive means for monitoring drug levels in the body. I was investigated to determine if such a constant plasma—saliva relationship exists. In the intramuscular study described above, saliva samples were also taken before medication and at 1, 2, and 3 h. Saliva samples were obtained by paraffin stimulation and expectoration into a disposable sputum-collecting cup. The first two expectorations were discarded and then a 3-min saliva collection was taken. It was demonstrated that a standard curve (5—100 ng/ml) in saliva is identical to that in distilled water, and in all subsequent saliva analyses, a standard curve of I in distilled water was used for the calibration of unknowns. Saliva samples from eight subjects were analyzed. The results are shown in Table IV. The results indicated that I is present in saliva and in most of the samples the saliva/plasma ratios were larger than one. However, they were variable. Thus, the saliva concentration of I does not reflect the plasma levels accurately, and at best approximates the plasma levels.

Cerebrospinal fluid. CSF was obtained from patients with pain due to neurological disease; these patients had been treated with I, 60 mg tid orally for three weeks [17]. The CSF was taken for diagnostic purposes and part of the sample was analyzed for I. Because of the difficulties in obtaining blank CSF for the daily standard curves, it was first demonstrated with a limited amount of CSF that the I standard curve in CSF was identical to that in distilled water, and then during each routine analysis run of unknowns, a distilled water standard curve was used to calibrate the concentrations of I in

TABLE IV

PLASMA AND SALIVA CONCENTRATIONS OF I IN HUMANS FOLLOWING A SINGLE INTRAMUSCULAR 30-mg DOSE OF I

Subject No.	Sampling time (h post dosing)	Plasma (ng/ml)	Saliva (ng/ml)	Saliva/plasma
1	1	74	108	1.5
	2	62	96	1.6
	3	42	109	2.6
2	1	75	176	2.4
	2	50	85	1.7
	3	54	51	0.9
4	1	43	216	5
	2	33	105	3.2
	3	32	79	2.5
8	1	89	361	4.1
	2	53	213	4.0
	3	53	169	3.2
3	1	60	290	4.8
	2	59	148	2.5
	3	58	90	1.6
5	1	46	107	2.3
	2	37	60	1.6
	3	28	27	1.0
6	1	49	113	2.3
	2	34	37	1.1
	3	28	25	0.9
7	1	46	70	1.5
	2	27	36	1.3
	3	25	27	1.1

the CSF. The detailed results of the CSF and plasma concentration relationship are being prepared for publication [17]. In general the CSF concentration of nefopam in humans is very low. These CSF concentrations generally were in the range of expected unbound plasma nefopam concentrations.

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