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ACKNOWLEDGMENTS

Abstracted in part from a thesis submitted by E. E. Linn to Wayne State University in partial fulfillment of the Master of Science degree requirements.

GLC Determination of Nanogram Quantities of a New Analgesic, Nefopam, in Human Plasma

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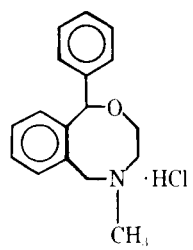
Abstract □ A sensitive and specific method was developed for the quantitative GLC determination of plasma nefopam levels. The method includes a multiple-step solvent extraction of the analgesic drug and the internal mass standard, orphenadrine. The accuracy, expressed as the relative error, was −4, 6, 6, and 4% at 20, 40, 70, and 130 ng/ml, respectively. The precision, expressed as relative standard deviation, was 17, 7, 3, and 5% at these same concentrations, respectively. Quantitation of nefopam in human plasma is possible down to 20 ng/ml with a 2-ml plasma sample; the sensitivity can be increased by using larger plasma samples. The method was applied successfully to the determination of plasma nefopam levels in humans in pharmacokinetic studies at therapeutic doses.

Keyphrases □ Nefopam—GLC analysis in plasma □ GLC—analysis, nefopam in plasma □ Analgesics—nefopam, GLC analysis in plasma

Nefopam[1], 3,4,5,6-tetrahydro-5-methyl-1-phenyl-1*H*-2,5-benzoxazocine hydrochloride (I), is a member of a new class of analgesics with a unique heterocyclic structure. It was originally synthesized by Klohs *et al.* (1) and was introduced as an analgesic drug in 1975 in Mexico and in 1976 in the Federal Republic of Germany. It is currently in the late stages of clinical testing in the United States, and a number of studies concerning the analgesic properties of I were reported[2] (2–5). *In vitro* metabolic data were published (6).

A prerequisite for *in vivo* metabolic and pharmacokinetic studies is a sensitive and specific assay. Since the recommended therapeutic dose of this basic drug for humans is relatively low, the plasma I concentrations are in the low nanogram per milliliter range. This report describes a sensitive and specific GLC method for the accurate determination of small amounts of I in human plasma. The method has been used to follow plasma pharmacokinetics of I. Another similar GLC method recently was published by a group collaborating with this laboratory (7).



I

EXPERIMENTAL

Reagents—Ether was freshly distilled each day; all other reagents were analytical reagent grade. A *d,l*-mixture of I was used, and the ^{14}C-I (^{14}C-label in the 1- and 6-positions of the oxazocine ring; radiochemical purity >99%) employed for recovery studies was also the racemate. The internal standard (orphenadrine, II) and I were dissolved in methanol. Aqueous solutions of 0.1 *N* HCl and 0.1 *N* NaOH were prepared in distilled water. Carbon disulfide was diluted with methanol to give a final concentration of 2% methanol.

Blank Plasma—Human plasma was obtained from volunteers who had fasted overnight and had not been on any medication for the previous week.

Apparatus—GLC was carried out on a chromatograph[3] with a hydrogen flame-ionization detector. Glass columns (182 cm × 2 mm i.d.) were rinsed with methanol and acetone and dried. They were packed with 3% 100–120-mesh cyclohexanedimethyl succinate on Gas Chrom Q[4]. The column oven was operated at 221° while the injection port and detector were operated at 225 and 260°, respectively. The accuracy and sensitivity of the assay were enhanced markedly by use of a digital electronic integrator[5] for calculating the relative detector response.

For the maximal detector response of I, the flow rates were set as follows: helium carrier gas, 40 ml/min; hydrogen, 40 ml/min; and air, 250 ml/min. The recorder chart speed was 1.25 cm/min. The integrator was operated under the following conditions: noise suppression, 2; recorder presentation, 20; slope sensitivity, 0.03 (up slope and down slope); baseline reset, 0; area threshold, 100; front shoulder, off; and rear shoulder, 1000 mv.

Standard Solutions—The internal standard solution contained 10

[1] Riker Laboratories, St. Paul, Minn.
[2] In Ref. 2, blood level data should read nanogram instead of microgram.
[3] Fisher Victoreen model 4400, equipped with a Varactor electrometer.
[4] Applied Science Laboratories, State College, Pa.
[5] Hewlett-Packard model 3370 B.

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Table I—Extraction Recovery of ^{14}C-Nefopam from Human Plasma

Nefopam Added, ng/ml	Nefopam Recovered[a], ng/ml	Recovery, %
10	10.40	104.0
10	10.60	106.0
10	10.33	103.3
30	30.70	102.5
30	30.78	102.6
30	30.99	103.3
100	99.5	99.5
100	101.2	101.2
100	108.9	108.9
300	279.6	93.2
300	272.1	90.7
300	274.2	91.4
		Mean ± *SD* 100.6 ± 5.8

[a] After correcting for aliquot factor (× 0.64).

Table II—Accuracy and Precision of Nefopam Analysis in Human Plasma by GLC

Nefopam Added to Human Plasma, ng/ml	Nefopam Detected, ng/ml		Difference
20.0	19.9		−0.1
	21.6		1.6
	13.5		−6.5
	21.2		1.2
	19.8		−0.2
	Mean ± *SD*	19.2 ± 3.3	Mean error −0.8
	RSD	17%	Relative error −4%
40.0	41.3		1.3
	39.2		−0.8
	42.1		2.1
	42.8		2.8
	47.1		7.1
	Mean ± *SD*	42.5 ± 2.9	Mean error 2.5
	RSD	7%	Relative error 6%
70.0	77.8		7.8
	77.2		7.2
	71.7		1.7
	73.9		3.9
	73.4		3.4
	Mean ± *SD*	74.8 ± 2.6	Mean error 4.8
	RSD	3%	Relative error 6%
130.0	126.1		−3.9
	131.1		1.1
	143.7		13.7
	133.5		3.5
	141.2		11.2
	Mean ± *SD*	135.1 ± 7.3	Mean error 5.1
	RSD	5%	Relative error 4%

μg of orphenadrine (II)/ml of methanol. The standard solution of I contained 1 μg/ml.

Extraction of I from Plasma—To a 16 × 125-mm culture tube were added 2 ml of experimental plasma and 0.20 ml of methanol containing 2 μg of internal standard. Along with the experimental samples, 40, 80, 140, and 260 ng of I in methanol were added to 2 ml of blank plasma. Then 3.0 ml of 0.1 *N* NaOH and 5.0 ml of freshly distilled ether were added. The tubes were shaken for 10 min on a mechanical shaker[6] and centrifuged[7] for 5 min at about 1000×*g*. Then 4.0 ml of the top (ether) layer was transferred to a clean culture tube, 3.0 ml of 0.1 *N* HCl was added, and the tubes were shaken for 5 min and centrifuged for 2 min.

The ether was aspirated and discarded, and the aqueous phase was washed with another 5.0 ml of ether. To the washed aqueous phase were added 3.5 ml of 0.1 *N* NaOH and 5.0 ml of ether. The tube was shaken for 10 min and centrifuged for 5 min. Then 4.5 ml of the ether layer was transferred to a 12-ml conical extraction tube and concentrated down under nitrogen at 50° to a volume of about 100 μl. The final evaporation of ether down to dryness was controlled carefully, and immediately thereafter the residue was dissolved in 10 μl of methanolic carbon disulfide. Then 4.0-μl aliquots of the methanolic carbon disulfide solution were injected into the gas chromatograph.

All values are in terms of nefopam hydrochloride equivalents.

Recovery Tracer Study—Radiolabeled ^{14}C-nefopam was employed to investigate the extraction efficiency of the method. The labeled drug was added to samples, and the described method was carried out. Samples taken at various steps during extraction were analyzed for ^{14}C-content by liquid scintillation counting[8]. Corrections for quenching were made by an external standard method.

***In Vivo* Study**—An *in vivo* evaluation provided information about the applicability and limitations of the method for analysis of human plasma samples. Subjects received single oral doses of 60 mg of I in two different tablet formulations. Subjects fasted overnight and for 4 hr after drug administration. Blood samples were drawn into heparinized containers[9] at 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 hr after drug administration. Red blood cells were separated immediately by centrifugation, and the plasma was removed and stored at −20° until assayed.

RESULTS

Recovery—Chemically, I is a tertiary amine, and attempts were directed toward optimal recovery of the free base from plasma. An extraction recovery study with ether and ^{14}C-labeled I was conducted at four concentrations: 10, 30, 100, and 300 ng of I/ml. Excellent recovery

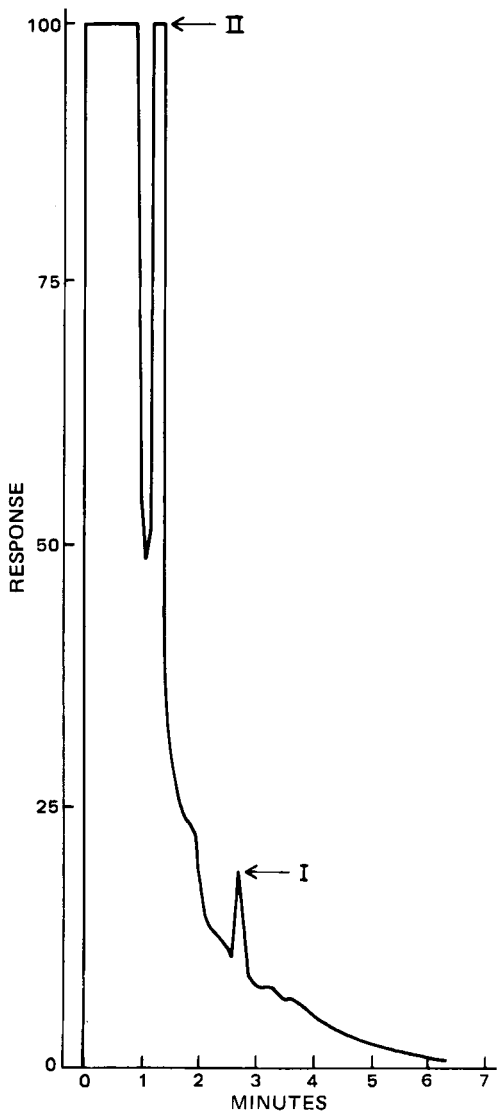

Figure 1—*Chromatogram tracing from human plasma extract.*

[6] Precision Scientific Co. model 65855.
[7] International Equipment Co. model XED, explosion proof.
[8] Model 3380 liquid scintillation spectrometer, Packard Instrument Co.
[9] Vacutainers.

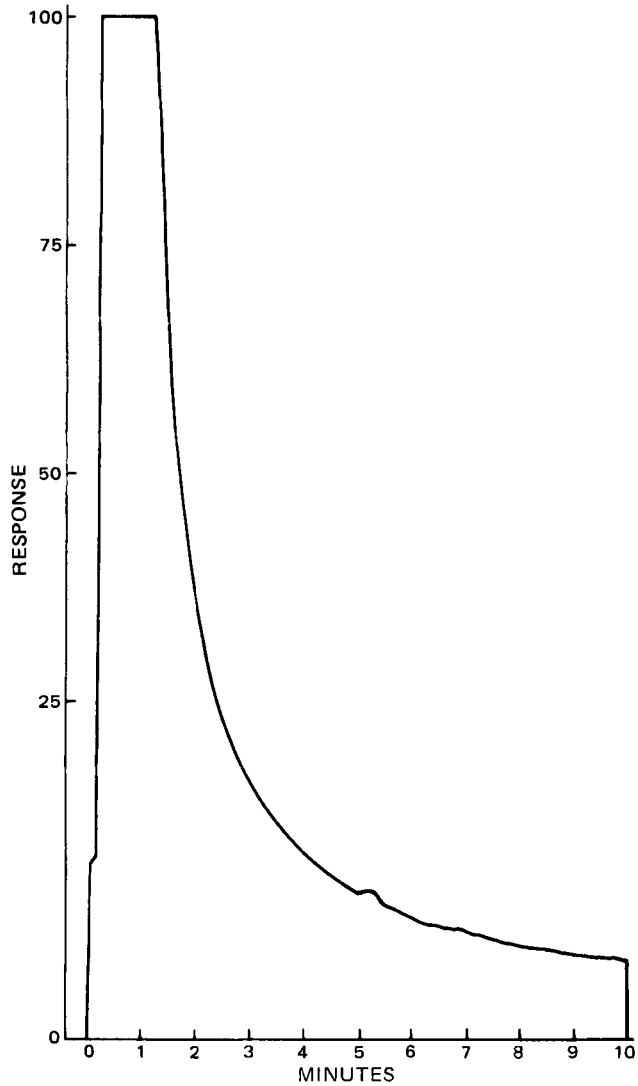

Figure 2—*Typical chromatogram tracing of a blank human plasma extract.*

was obtained (Table I) over this concentration range; the mean extraction recovery from plasma was 100.6% with a standard deviation of 5.8% (Table I). These results indicate that quantitative extraction from human plasma was achieved over a concentration range of 10–300 ng/ml, which covers the concentrations found in most biological samples analyzed so far from pharmacokinetic studies of I.

At lower nanogram per milliliter concentrations, problems were encountered initially in obtaining acceptable recovery of I. The drug was sometimes adsorbed to the surface of the glass tubes, from which it could be recovered by addition of an excess of other amines in solution. To keep the loss of I to a minimum during extraction, the amount of the internal standard added was in large excess compared to I in plasma samples. Orphenadrine was chosen as the mass internal standard. Methanolic solutions of I and the internal standard, when kept refrigerated, were stable for several months. The ether must be freshly distilled on each analysis day to avoid interfering peaks.

Accuracy and Precision—The accuracy of the method was checked by carrying samples at 20, 40, 70, and 130 ng/ml in replicates of five through the entire method; concentrations were calculated from a pooled standard curve constructed by linear regression (Table II). The mean detected concentrations were 19.2, 42.5, 74.8, and 135.1 ng/ml, respectively. The standard deviations were 3.3, 2.9, 2.6, and 7.3 and the relative standard deviations were 17, 7, 3, and 5%, respectively.

The accuracy of this method is indicated by the small mean error between the detected and theoretical values. The mean errors were −0.8, 2.5, 4.8, and 5.1 for 20, 40, 70, and 130 ng/ml, respectively. The corresponding relative errors were −4, 6, 6, and 4%.

Chromatographic Separation—Some packings (OV-17 and OV-1) did not give the desired separation or result in enough detector response at low nanogram per milliliter concentrations of I. The cyclohexanedimethyl succinate packing gave good chromatographic separation plus considerably improved detector response (Fig. 1). Pure I in methanol gave discernible peaks in absolute amounts as low as 100 pg under the conditions described.

When plasma extracts in chloroform, ether, or methyl acetate were injected into the chromatograph, interference from the solvent peak tracing decreased the sensitivity considerably. The use of methanolic carbon disulfide as the final solvent largely eliminated this problem, because it gave only minimal detector response. The 2% methanol content was necessary to dissolve the residue of I completely.

Specificity—*Interference from Endogenous Materials in Plasma*—There was no significant interference with I or the internal standard by any endogenous materials in normal human plasma. None of the predose samples from a human study demonstrated any significant GLC peaks corresponding to I or the internal standard. There was an extremely small peak close to the internal standard, but it contributed no more than 1% in area to the internal standard. Correction for this insignificant interference was not included in the routine calculation of the unknown. A typical GLC tracing of blank human plasma extract is shown in Fig. 2.

Interference from Metabolites of I—One metabolite of I found in urine, desmethylnefopam (III), did not interfere. The GLC tracing following injection of ≈ 400 ng of III did not give any discernible peak. The lack of interference by III was further supported by the report that carbon disulfide decomposes primary and secondary amines (8). There exist no GLC data on other likely metabolites. Since metabolites of I are likely to be more polar if also present in the plasma, they would, theoretically, be less readily extractable and require longer elution times than I under the present GLC operating conditions.

Human Plasma Standard Curves—Several standard curves covering the 10–130-ng/ml range were generated over 1 year. Linearity extended, however, up to 1500 ng/ml. Standard curves constructed by linear regression fitting indicated an excellent linear relationship between response and drug concentration present in the 10–130-ng/ml range. The sensitivity of the assay was 20 ng/ml with a 2-ml sample. Coefficients of correlation of individual curves were always better than 0.99. A pooled standard curve (n = 12) yielded a correlation coefficient of 0.9998, and the good linear fit was indicated by the coefficient of determination, r^2 = 0.9996.

Human Plasma Analysis for I—Typical plasma I levels in a healthy subject (selected from determinations in 24 individual subjects after a 60-mg po dose) are given in Fig. 3. The curve shown was created by a single determination per time point. A peak plasma level of 99 ng/ml was

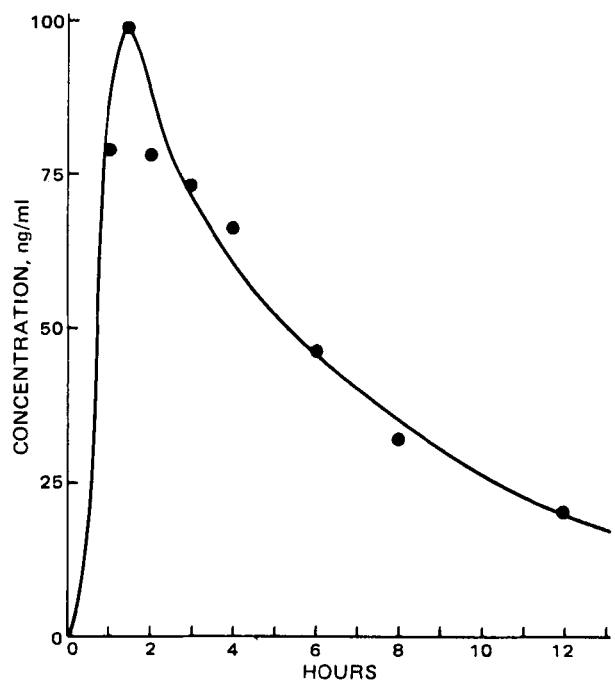

Figure 3—*Plasma levels of nefopam in one human subject following a single oral dose of 60 mg of nefopam hydrochloride.*

reached 60 min postdosing, and the decrease in plasma level between 3 and 12 hr was not a simple monoexponential decline. These results indicate that the method is applicable for determination of plasma concentrations of I in humans at this therapeutic dose.

DISCUSSION

The results demonstrate that GLC with a flame-ionization detector is very effective for the determination of the lipophilic drug, I, in biological fluids in the low nanogram per milliliter concentration range. Most of the endogenous material and one metabolite of I did not interfere. A minor interference peak from a blank human plasma extract was sometimes observed, but its amount was insignificant (<1% of the internal standard peak).

The lower limit of sensitivity is 20 ng/ml with a 2-ml plasma sample; however, the sensitivity can be improved to 10 ng/ml by employing larger plasma samples.

Both the accuracy and the precision of the method are good. The 20-ng/ml value shown in Table II has a larger relative standard deviation than the other three. One of the five values is approximately 60% lower than the mean of the other four values. If the 13.5-ng/ml value had been excluded, the relative standard error would have been 6%. This value is well within the range of the other relative standard errors. The 13.5-ng/ml value was probably due to random error. Since this analytical method is intended for samples from biological experiments, the levels of precision and accuracy are more than adequate. For 32 samples, including six standards, the total extraction and analysis time is 8 hr. Close to 500 human plasma samples have been analyzed successfully by this method over 1 year.

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ACKNOWLEDGMENTS

The authors thank W. J. Hammar and J. E. Bunker for the synthesis of ^{14}C-nefopam and S. F. Chang for valuable discussions. They also acknowledge the early GLC work by A. R. Hansen and G. Graham and participation in the clinical blood level study by Dr. D. T. Calderwood and Dr. B. J. Baltes.

Differential Pulse Polarographic Determination of Clorazepate Monopotassium and Dipotassium

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Received March 3, 1978, from the *Analytical Research and Development Department, Endo Laboratories, Inc., Subsidiary of E. I. du Pont de Nemours & Co., Inc., Garden City, NY 11530.* Accepted for publication May 1, 1978.

Abstract □ Polarographic investigation of clorazepate monopotassium and dipotassium showed two cathodic waves at about −1.28 and −1.66 v. The cathodic wave associated with clorazepate monopotassium or dipotassium at about −1.66 v was a pH-independent, diffusion-controlled wave. This wave was used to develop a specific stability-indicating procedure for clorazepate monopotassium and dipotassium in the presence of their degradation products, namely, nordiazepam, 2-amino-5-chlorobenzophenone, and glycine. The method involves a 10^{-2} *M* LiOH–10^{-1} *M* LiCl extraction of the active ingredient from the formulation, filtration, dilution with the same supporting electrolyte, and then use of the standard addition technique for drug quantitation in capsules. Typical formulation excipients did not interfere with the analysis. Accuracy and precision of the procedure were 99.55 ± 0.68%.

Keyphrases □ Clorazepate monopotassium and dipotassium—differential pulse polarographic analyses in pharmaceutical preparations □ Polarography, differential pulse—analyses, clorazepate monopotassium and dipotassium in pharmaceutical preparations □ Tranquilizers—clorazepate monopotassium and dipotassium, differential pulse polarographic analyses in pharmaceutical preparations

Several methods have been reported for the quantitative analysis of clorazepate monopotassium (I) and dipotassium (II) and their capsule formulations, including UV spectrophotometry (1), fluorometry (2), colorimetry (3), potentiometry (4), GLC (2, 5), TLC (1), and high-pressure liquid chromatography (6). One method utilized ac and differential pulse polarography for the assay of clorazepate dipotassium in its capsules (7). In this polarographic procedure, the clorazepate dipotassium was dissolved in an acetate buffer containing 10% dimethylformamide to give *N*-desmethyldiazepam after 10 min. Then quantitation was achieved through measurement of the reducible double bond C=N moiety.

The reported procedures lack specificity and, consequently, cannot be used for stability studies. In this study, differential pulse polarography was applied to achieve a specific stability-indicating procedure for the analysis of clorazepate monopotassium or dipotassium in capsule formulations.

EXPERIMENTAL

Apparatus—A polarograph[1] was equipped with a 4.0 *N* saturated calomel fiber junction reference electrode, a dropping mercury electrode at 40 cm (62 cm corrected), a platinum wire auxiliary electrode, and a drop timer[2]. An *x–y* recorder[3] was attached to the polarograph.

Reagents and Solutions—All chemicals were reagent grade[4]. The potency and purity of clorazepate monopotassium and dipotassium reference standards[5] were 94.27 and 99.77%, respectively, as ascertained by the manufacturers' physicochemical and analytical procedures.

Working standards contained 40.0 and 51.0 mg of I and II/100 ml, respectively.

The supporting electrolyte was 10^{-2} *M* LiOH–10^{-1} *M* LiCl.

[1] Model 174, Princeton Applied Research, Princeton, N.J.
[2] Model 170, Princeton Applied Research, Princeton, N.J.
[3] Model 2000, Houston Co., Houston, Tex.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] Abbott Laboratories, North Chicago, Ill.

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