Nitrogen–Phosphorous Detection of Phencyclidine in Blood Serum

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Abstract □ A method for quantitating phencyclidine in the blood serum of rhesus monkeys with a solvent extraction procedure followed by gas chromatography with nitrogen–phosphorous detection is reported. Phencyclidine was extracted with ether from 0.5 ml of serum (pH 13.5) made basic with 2*M* NaOH, followed by back-extraction into 0.5 *M* sulfuric acid. After the addition of 2 *M* sodium hydroxide, phencyclidine was extracted into a small volume of ether for concentration and injection into the gas chromatograph. The limit of quantitation of phencyclidine in serum was 5 ng/ml. Recovery averaged 51.9 ± 4.3%. Standard curves were linear between 5–50 ng/ml and 100–2000 ng/ml. Comparison between serum and aqueous standards indicated no interference by serum components in the extraction procedure. Pentobarbital, caffeine, and the monohydroxy metabolites of phencyclidine did not interfere with the analysis. This procedure is a rapid and sensitive method for determination of serum phencyclidine levels in animal studies requiring analysis of large numbers of samples.

Keyphrases □ Phencyclidine—analysis using gas chromatography with nitrogen–phosphorous detection, rhesus monkey blood serum □ Gas chromatography—nitrogen–phosphorous detection of phencyclidine in rhesus monkey blood serum □ Drugs of abuse—phencyclidine, analysis using gas chromatography with nitrogen–phosphorus detection, rhesus monkey blood serum

The popularity of phencyclidine [1-(1-phenylcyclohexyl)piperidine] as a drug of abuse has stimulated interest in determination of the drug in body fluids (1, 2). Persons who have ingested phencyclidine (I) often show signs of intoxication even when serum levels are low (10 ng/ml) (3). TLC methods and qualitative, presumptive tests which indicate the presence, but not the concentration, of I in biological matrixes were reported previously (4, 5).

Spectrophotometric quantitation of I after solvent extraction of specimen suffers from the lack of adequate detection limits (4). Homogeneous enzyme immunoassays for I in urine show cross-reactivity with analogs and inactive metabolites of I (6, 7). Radioimmunoassay for I in plasma is sensitive enough for quantitation at plasma levels of ~1 ng/ml (8–10), but some cross-reactivity (2–5%) is seen with hydroxylated metabolites of I in two of these radioimmunoassay procedures (9, 10).

Investigation of an ion-selective electrode for I indicates that potentiometric analysis may provide adequate specificity for the parent drug (11). The reported sensitivity of this electrode is poor compared with radioimmunoassay or gas chromatographic (GC) methods. Preconcentration of sample could possibly lower detection limits; however, this has not been evaluated for use with the phencyclidine-selective electrode.

BACKGROUND

Numerous GLC assays have been used for separation of phencyclidine in biological matrixes. Flame-ionization detection after GC separation of serum extracts cannot provide sufficient sensitivity (4, 12, 13). GC with mass spectrometry lowers detection limits to as little as 5 ng of I/ml in plasma, whole blood, and urine (14–18). Analytical procedures using GC with nitrogen–phosphorous detection provide sensitivity comparable to mass spectrometry (3, 19, 20). However, the solvent extraction procedures reported for sample cleanup prior to separation and subsequent quantitation by nitrogen–phosphorous detection are too laborious for rapid processing of many specimens, or require a larger volume of specimen than is available in studies with small animals.

The analytical procedure reported here combines a rapid extraction procedure for 0.5 ml of blood serum with GC separation and nitrogen–phosphorous detection. This method is particularly useful for analyses in pharmacokinetic studies of phencyclidine in small animals.

EXPERIMENTAL

Apparatus—GC was performed with a gas chromatograph equipped with a nitrogen–phosphorous detector[1]. A 122-cm coiled glass column, 0.2-cm i.d., was silylated with 2% trimethylchlorosilane in hexane and packed with 3% SP-2250-DB on a 100–120 mesh support[2]. The column was conditioned overnight at 250° with a helium flow of 15 ml/min. Gas flows were a helium carrier flow of 25 ml/min and a hydrogen flow of 2.5 ml/min. Air was maintained at a constant pressure of 40 psi. Inlet temperature was 195°, column temperature was 185°, and the detector temperature was 210°. Background current was set at 4×10^{-11} amp.

Reagents—Phencyclidine hydrochloride and two monohydroxy metabolites [4-phenyl-4-piperidinocyclohexanol and 1-(1-phenylcyclohexyl)-4-hydroxypiperidine] were obtained from the National Institute on Drug Abuse. Melting point determinations[3] and electron-impact mass spectrometry[4] were used to confirm identity and purity of the compounds. TLC of phencyclidine produced only one spot on silica gel G, 250-μm plates[5]. Plates were developed with chloroform–ethyl acetate–acetone–triethylamine (38:30:30:2). Spots were visualized by charring with sulfuric acid and heat.

Ketamine hydrochloride[6], caffeine[7], and pentobarbital sodium[8] were used as received. Nanograde ether[9] was distilled in glass each day after

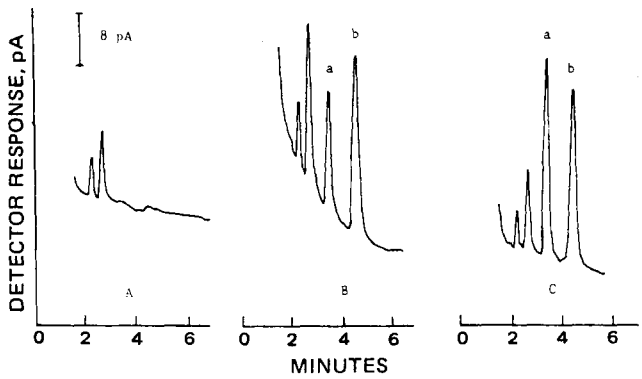

Figure 1—*Chromatograms obtained from extraction and assay of 0.5 ml of blood serum from a rhesus monkey (A), from a 0.5-ml serum sample spiked with 25 ng of phencyclidine and 50 ng of ketamine (B), and from 0.5 ml of serum obtained from a 6.7-kg male rhesus monkey 1.5 hr after intravenous administration of 1.6 mg of phencyclidine/kg (C). Estimated concentration of phencyclidine (a) was 75.4 ng/ml. The concentration of ketamine (b) was 100 ng/ml.*

[1] Tracor model 560 equipped with a model 702 nitrogen–phosphorous detector, Tracor Instruments, Austin, TX 78721.
[2] Supelco, Bellefonte, PA 16823.
[3] Capillary melting point apparatus, Arthur H. Thomas Co., Philadelphia, Pa.
[4] Dupont model 21-491 mass spectrometer.
[5] Analtech, Newark, DE 19711.
[6] Warner-Lambert Co., Ann Arbor, MI 48105.
[7] Sigma Chemical Co., St. Louis, MO 63178.
[8] Robinson, San Francisco, CA 94107.
[9] Mallinckrodt, St. Louis, MO 63147.

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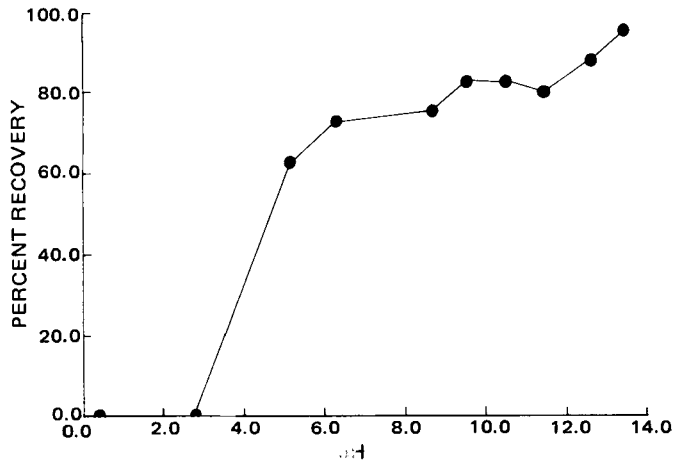

Figure 2—*Recovery of phencyclidine from aqueous solutions of varying pH. Maximum recovery occurs at pH 13.5.*

the addition of aluminum lithium hydride. All other materials were reagent grade.

Procedure—Serum (0.5 ml) obtained from rhesus monkeys was pipetted into a silylated glass tube with a screw cap with an inert liner. For serum phencyclidine concentrations of ~5–20 ng/ml, 50 μl of aqueous ketamine hydrochloride solution (200 ng/ml) was added to each tube as an internal standard. Serum concentrations of 20–100 ng of phencyclidine/ml required 50 μl of aqueous ketamine hydrochloride solution (1 μg/ml), and phencyclidine levels of 100–2000 ng/ml required 50 μl of ketamine solution (10 μg/ml). All stock solutions of phencyclidine and ketamine hydrochloride were prepared with deionized water. Preparation of stock solutions with methanol did not affect extraction and quantitation of phencyclidine and internal standard.

Serum was adjusted to a pH of 13.5 with 0.5 ml of 2 *M* NaOH. Ether (2 ml) was added to each tube. The tubes were placed on a vortex mixer for 60 sec to extract the free bases of phencyclidine and internal standard into the organic phase. The aqueous and organic phases were separated by centrifuging at 3000 rpm for 2 min, and then freezing the aqueous layer in a dry ice–acetone bath. The organic layer was decanted into a second tube containing 0.5 ml of 0.5 *M* H_2SO_4. Phencyclidine and internal standard were back-extracted from the ether into the aqueous layer by vortexing for 30 sec.

Samples were centrifuged at 3000 rpm for 1 min and immersed in a dry ice–acetone bath. The organic layer was discarded, and the aqueous layer was allowed to return to room temperature. The aqueous layer, 0.5 ml of 2 *M* NaOH, and 250 μl of distilled ether were vortexed together for 30 sec and centrifuged for 1 min. After the aqueous phase was frozen in dry ice–acetone, the ether layer was decanted into a conical vial. A small amount of anhydrous potassium carbonate was added to each sample to remove residual moisture. The ether layer was allowed to evaporate to a volume of 10–50 μl. One to five microliters of the sample was injected into the gas chromatograph. After every six injections, a 30-min wait was necessary to allow slow-eluting compounds to be eliminated from the column.

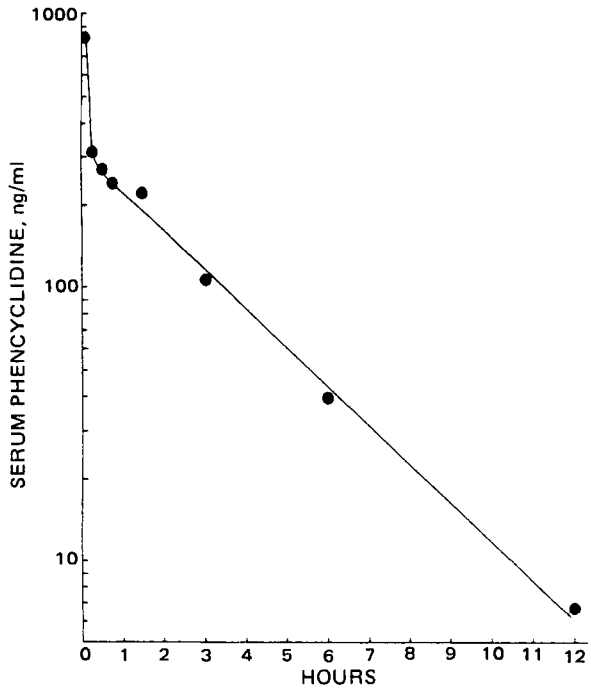

Figure 3—*Semilogarithmic plot of serum levels of phencyclidine in a 5.0-kg male rhesus monkey after administration of a 1.6 mg/kg iv dose.*

Table I—Phencyclidine Recovery from Serum Standards

Phencyclidine Concentration, ng/ml	Mean Recovery[a] ± *SD*, %
4.9	53.7 ± 15.3
9.8	50.5 ± 4.2
48.9	59.5 ± 3.5
97.8	49.1 ± 1.2
489.0	51.2 ± 4.2
978.0	47.5 ± 2.0

[a] Average of six determinations.

A calibration curve was constructed by plotting peak height ratio of phencyclidine to internal standard against the phencyclidine concentration. Serum standards were prepared by addition of appropriate aliquots of aqueous phencyclidine solutions (50 ng/ml, 0.5 μg/ml, or 5 μg/ml) to 0.5 ml of blank serum obtained from dogs or rhesus monkeys. Standard concentrations ranged from 5 to 2000 ng/ml. Standards were extracted and assayed in the same manner as the samples. Standards were also prepared in deionized water and treated identically to serum standards to determine if serum components affected extraction or quantitation of phencyclidine or internal standard.

Recovery of Phencyclidine—Serum standards containing either 4.9, 9.8, 48.9, 97.8, 489.0, or 978.0 ng of phencyclidine/ml and no internal standard were extracted by the same procedure. The final ether extracts were allowed to evaporate to dryness and then reconstituted with 10–100 μl of ethyl acetate containing 2 μg of ketamine/ml. One to five microliters was injected into the gas chromatograph. Peak height ratios of phencyclidine to ketamine were compared with those ratios obtained from ethyl acetate solutions containing 1 or 5 μg of phencyclidine/ml and 2 μg of ketamine/ml.

A profile of phencyclidine recovery *versus* pH of an aqueous solution was constructed. The pH was adjusted with either sulfuric acid or sodium hydroxide solutions. Phencyclidine was present in a concentration of 50 ng/ml. One milliliter of aqueous solution and 2 ml of ether were vortexed for 60 sec and centrifuged at 3000 rpm for 2 min. The ether layer was decanted into a conical vial after freezing the aqueous layer in a dry ice–acetone bath. After evaporation of the ether, samples were reconstituted and treated as already described.

Interference by Other Compounds—Fifty microliters of 1 mg/ml methanolic pentobarbital sodium or caffeine solution was added to 1 ml of deionized water. Methanolic solutions of the two monohydroxy metabolites were used to prepare urine samples containing 1 mg/ml of each metabolite. The resulting samples were extracted and quantitated by the same procedure used for serum to check for interference with phencyclidine or ketamine peaks.

RESULTS AND DISCUSSION

Figure 1 shows representative chromatograms for a serum blank from a male rhesus monkey, a serum blank spiked with phencyclidine, and

Table II—Summary of Linear Regression Analysis of Composite Standard Curves for Phencyclidine in Serum or Water[a]

Standard Curve	Equation	Correlation Coefficient	Standard Error of the Estimate
Serum, 5–50 ng/ml	$y = 0.013x - 0.005$	0.993	0.024
Serum, 100–2000 ng/ml	$y = 0.014x - 0.613$	0.999	0.348
Water, 5–50 ng/ml	$y = 0.014x - 0.005$	0.996	0.020
Water, 100–2000 ng/ml	$y = 0.014x + 0.262$	0.999	0.112

[a] No significant difference between equations for serum and water exists.

Table III—Day-to-Day Coefficient of Variation in Peak Height Ratios for Phencyclidine Serum Standards

Standard Concentration, ng/ml	Number of Determinations	Coefficient of Variation, %
4.9	5	8.3
9.8	5	9.0
14.7	3	6.3
19.6	1	—
48.9	9	4.2
97.8	6	5.5
489.0	5	3.0
685.0	4	8.9
978.0	4	15.7
1958.0	2	8.4

serum obtained 1.5 hr after administration of a 1.6 mg/kg iv dose of phencyclidine to a 6.7-kg male rhesus monkey. Retention times of phencyclidine and internal standard are 3.6 and 4.7 min, respectively. Chromatography of phencyclidine and ketamine at 185° produced good resolution of the two peaks (R = 1.5, 99.7% resolution). Reducing the temperature below 185° produced peak broadening. The lower viscosity of the stationary phase of the SP-2250-DB packing relative to 3% OV-17 allowed operation at lower column temperatures, thus preventing the thermal degradation of phencyclidine to 1-phenylcyclohexene (16). The chromatogram of the serum blank shows two unidentified peaks eluting at 2.3 and 2.7 min. These peaks do not interfere with the analysis. However, small interfering peaks (not evident in this chromatogram) with retention times of 3–5 min were seen with some serum samples. This serum background limited quantitation of phencyclidine in rhesus monkey serum to ~5 ng/ml.

Application of this extraction and quantitation technique to human serum[10] or plasma[11] resulted in a frequent appearance of interfering peaks with retention time of 4–5 min. This interference could not be assigned to pentobarbital, which produced no interfering peaks in the chromatogram, or to caffeine, which produced a peak at 6 min. Extraction and quantitation of the monohydroxy phencyclidine metabolites produced a broad peak at 12 min. Human serum obtained with evacuated collection tubes[12] suitable for trace analysis did not exhibit these large interfering peaks in the chromatogram. This suggests that the interferences seen with serum and plasma extracts might be artifacts of collection or storage. GC–mass spectrometry of extracts containing the interfering substance indicated that it is a basic compound with a probable molecular ion at m/z 86. Further studies are planned to elucidate the structure of this substance.

Hexane and ethyl acetate, as well as ether, were initially evaluated as extracting solvents, since they contained few impurities which interfered with nitrogen–phosphorous detection. However, hexane produced emulsification upon vigorous mixing with serum. The less volatile ethyl acetate (relative to ether) increased the time required to concentrate the final extract.

The percent recovery of phencyclidine from aqueous solutions of varying pH values is shown in Fig. 2. Maximum recovery was 95% at pH 13.5. The single extraction step used in these determinations was not adequate to produce a serum extract free from interference; therefore, phencyclidine was back-extracted into 0.5 M sulfuric acid. Virtually 100% of the phencyclidine should partition into the aqueous layer at this pH (pH < 1) (Fig. 2). Final extraction of phencyclidine from a basified aqueous phase into as small a volume of ether as practical (250 μl) reduced the time required to concentrate the final extract. The average recovery of phencyclidine from serum standards was 51.9 ± 4.3% after the necessary back-extraction steps (Table I).

Linear relationships were found between peak height ratio and phencyclidine serum concentration for concentrations from 5–50 and 100–2000 ng/ml. Above 2000 ng/ml, detector response was no longer linear. The least-squares regression equations are shown in Table II. Statistical analyses (21) indicated no significant difference (p = 0.01) existing between serum and aqueous standard curves at all concentrations. Thus, serum components do not appear to affect the extraction and quantitation procedures. Within-day coefficients of variation in peak height ratios (n = 6) for the 48.9 and 489.0 ng/ml standards were 3.9 and 1.6%, respectively. The day-to-day variation in peak height ratios is presented in Table III. The coefficient of variation is <10% for all but the 978.0 ng/ml standard. The day-to-day coefficient of variation averaged 7.7% over all concentrations.

Serum phencyclidine levels after administration of a 1.6 mg/kg iv dose to a rhesus monkey are shown in Fig. 3. The animal was fasted overnight prior to receiving the dose. After 12 hr, no detectable levels of phencyclidine were present in the serum. The biological half-life, calculated after computer fit of the data to a biexponential equation, was 2.1 hr, which agrees with phencyclidine half-lives in rhesus monkeys reported earlier (14). This rapid extraction procedure, combined with satisfactory detection limits provided by GC with nitrogen–phosphorous detection, is advantageous in animal studies involving numerous small serum samples. In addition, this assay procedure deserves further investigation for clinical use.

[10] Supplied by the Texas State Health Department, Austin, Tex.
[11] Supplied by the Travis County Blood Bank, Austin, Tex.
[12] "Blue-top," siliconized Vacutainer with no additives, Becton-Dickinson, Rutherford, NJ 07070.

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