Quantitative GLC Determination of Pentylenetetrazol in Biological Fluids

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Abstract □ A quantitative, sensitive, and specific GLC method was developed for the determination of pentylenetetrazol in water, plasma, and urine. The assay involves a single extraction of the sample into chloroform followed by centrifugation, evaporation, and chromatography. The method for pentylenetetrazol is reproducible, and the sensitivity limit of the assay is 0.5 μg of pentylenetetrazol/ml of biological fluid using a 2-ml sample. This method has a sensitivity sufficient to detect human plasma levels after therapeutic clinical doses and was successfully applied to monitor complete plasma level profiles of this drug in dogs. The data indicate that this drug is very rapidly absorbed following an oral dose, and the half-life of the drug in plasma is approximately 1 hr.

Keyphrases □ Pentylenetetrazol—GLC analysis in plasma and urine □ CNS stimulants—pentylenetetrazol, GLC analysis in biological fluids □ GLC—analysis, pentylenetetrazol in plasma and urine

Pentylenetetrazol[1] is being used for various clinical purposes in humans and animals. The main use in humans is as a central nervous system (CNS) stimulant for the therapeutic management of chronic mental depression in geriatric patients. Although several GC methods (1–3) and other techniques (4, 5) have been described for the determination of pentylenetetrazol in biological samples, complete blood level profiles of this drug have not been reported. One reported GC method (3) involves extraction of pentylenetetrazol from aqueous samples with ether or benzene. However, its usefulness in quantitative determinations of the drug in plasma samples was limited by low extraction recoveries and inadequate reproducibility.

To evaluate the absorption and elimination of this drug in the body, a sensitive and specific method of drug analysis was needed. This report describes a sensitive and specific GLC method for the quantitative determination of pentylenetetrazol in biological fluids.

EXPERIMENTAL

Chemicals—Pentylenetetrazol[1] and the calibration marker, hexamethylenamine[2], were used as received. Spectrophotometric grade chloroform[3] and absolute ethanol[4] USP were employed.

Instrumentation—Chromatographic determinations were made with a gas chromatograph[5] equipped with a dual flame-ionization detector and a recorder[6]. The chromatographic column was a 0.3-cm (0.125-in.) o.d. stainless steel column, 1.8 m (6 ft) in length, packed with 10% UC-W98 on Diatoport S (80–100 mesh)[7].

[1] Metrazol, Knoll Pharmaceutical Co., Orange, N.J.
[2] Wm. P. Poythress & Co., Richmond, Va.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] U.S. Industrial Chemicals Co., New York, N.Y.
[5] Varian Aerograph model 1800.
[6] Bristol's Co., Waterbury, Conn.
[7] Hewlett-Packard, Avondale, Pa.

Table I—Partition Coefficients of Pentylenetetrazol between Water and Various Organic Solvents at 22°

Organic Solvents	Partition Coefficient[a], Solvent Drug Concentration/ Water Drug Concentration
Ether	0.11
Carbon tetrachloride	0.25
n-Octanol	1.37
Benzene	2.25
Chloroform	23.4

[a] Each value is an average of three experiments.

The column was conditioned for 24 hr at the operating settings after each chromatographic determination. The operating conditions were: column oven temperature, 195°; injection tube temperature, 250°; and detector block temperature, 255°. Compressed nitrogen was used as a carrier gas at the flow rate of 60 ml/min. The flow rates of hydrogen and compressed air were maintained for optimal results.

Extraction Procedure—Place a 2-ml plasma or urine sample in a Teflon-lined screw-capped centrifuge tube (15 ml) and add 12 ml of water-saturated chloroform. When more than a 2-ml sample is used, the volume of chloroform is at least six times greater than the volume of the sample. The sample is then extracted by mixing for at least 5 hr in a rotating apparatus[8], followed by centrifugation at 2000 rpm for 10 min.

About 8 ml of the filtered chloroform layer is transferred to a centrifuge tube and evaporated to dryness in a water bath at 80°. The wall of the centrifuge tube is washed down with 0.5 ml of anhydrous ether, followed by complete evaporation at room temperature. The residue is then redissolved in 10–20 μl of ethanol containing hexamethylenamine (1-μg/μl solution). One microliter of ethanol, containing an unknown amount of pentylenetetrazol and 1 μg of hexamethylenamine, is injected into the chromatograph. Hexamethylenamine is used as a calibration marker due to its stability, solubility, and adequate retention time.

Standard Calibration Curve for Pentylenetetrazol Standards—Known quantities of pentylenetetrazol are dissolved in ethanol containing hexamethylenamine (1 μg/μl) to obtain pentylenetetrazol concentrations over a 0.2–4.0-μg/μl range. When 1-μl aliquots of these standard solutions are injected into the column, the samples contain varying amounts of pentylenetetrazol per microgram of the calibration marker. The calibration curve is then obtained by plotting the known concentrations of pentylenetetrazol against the corresponding peak height ratios. The peak height ratios are obtained by dividing the peak height due to the hexamethylenamine calibration marker into the peak height of the pentylenetetrazol standard.

Calibration Curve of Pentylenetetrazol Standards Analyzed in Human Plasma—Known amounts of pentylenetetrazol (5–30 μg) are added to 2-ml aliquots of control human plasma, and the drug is then extracted in the same manner as described in *Extraction Procedure*. After evaporation of the solvent extracts, the residues are dissolved in 10-μl aliquots of ethanol containing 1 μg/μl of hexamethylenamine. Following the injection of 1 μl of each sample into the chromatograph, a calibration curve for pentylenetetrazol standards analyzed in plasma is made as previously described.

[8] Menold, Lester, Pa.

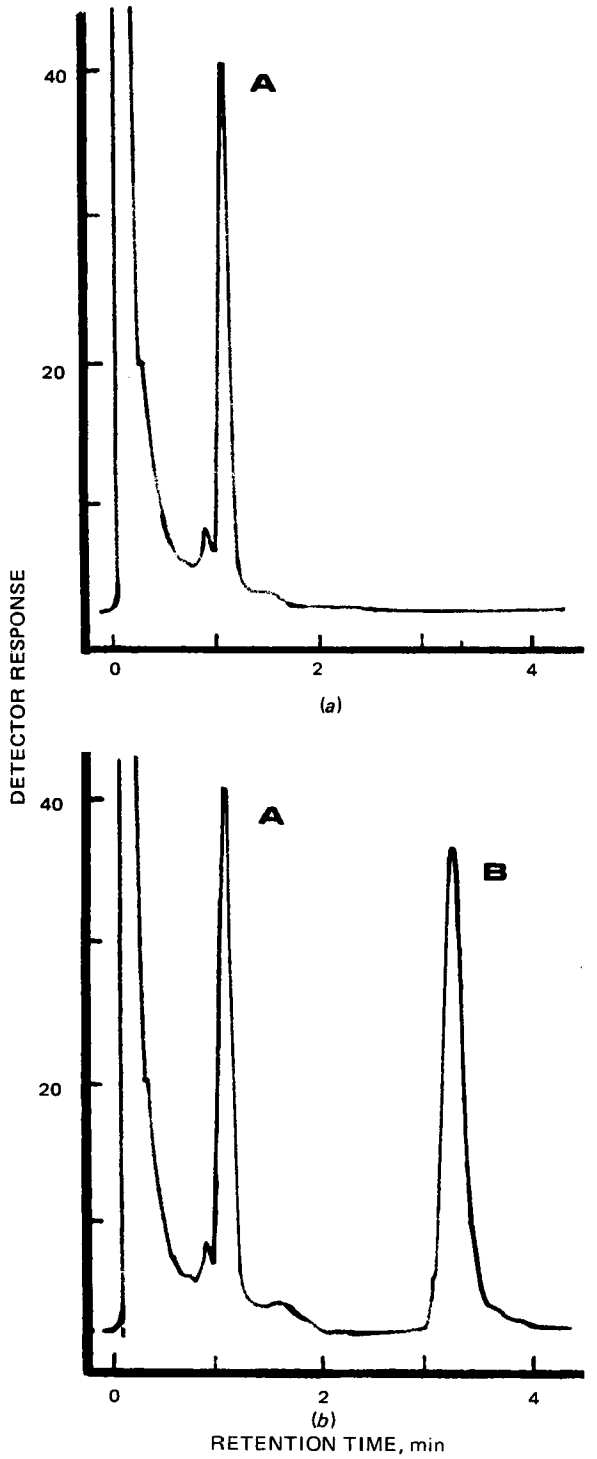

Figure 1—*Gas–liquid chromatograms of:* (a) *dog plasma extracts of control plasma and* (b) *plasma extract containing pentylenetetrazol. Key: A, hexamethylenamine; and B, pentylenetetrazol.*

Drug Recovery Studies—Known quantities of pentylenetetrazol (2–30 μg) were added to 2 ml of dog control plasma. The samples were thoroughly mixed and processed in the same manner as the unknown samples. The pentylenetetrazol in each sample was determined chromatographically by injecting a 1-μl aliquot of the ethanol containing the dissolved residue into the column and measuring the peak height ratios.

Calculations—The concentration of pentylenetetrazol (micrograms per milliliter) in unknown plasma or urine samples is determined as follows:

$$\text{drug concentration (micrograms per milliliter)} = \frac{\text{slope of calibration curve} \times \text{peak height ratio} \times \text{microliters of ethanolic solution} \times \text{milliliters of chloroform used for extraction}}{\text{milliliters of chloroform taken for evaporation} \times \text{milliliters of plasma or urine sample}} \quad \text{(Eq. 1)}$$

Drug Partition Studies—To determine the best solvent for the extraction of pentylenetetrazol from biological samples, the partition properties of this drug between water and various organic solvents were determined at room temperature. Ten milliliters of pentylenetetrazol in water (10 mg/ml) and an equal volume of a water-saturated organic solvent were mixed in a centrifuge tube with a Teflon-lined screw-cap by a rotating apparatus for 20 hr. After equilibrium, 5 mg of hexamethylenamine was dissolved in 5 ml of the aqueous layer, and 1 μl of this solution was directly injected into the chromatograph. Peak height ratios were measured for the determination of pentylenetetrazol in each water layer. The partition coefficients are expressed as the ratio of the drug concentration in the organic layer to the drug concentration in the water.

Drug Plasma Level Studies—To demonstrate the utility of this method, the pentylenetetrazol dose (15 mg/kg) was intravenously administered to one dog (11 kg) and orally administered to another dog (9 kg). Blood samples (4 ml) were withdrawn from the cephalic vein of the front leg using a heparinized in-dwelling catheter at various time intervals after dosing. Blood samples were centrifuged, and the plasma was transferred for extraction. During the experiment, dogs were anesthetized with pentobarbital sodium. Urine samples were collected for the chromatographic examination.

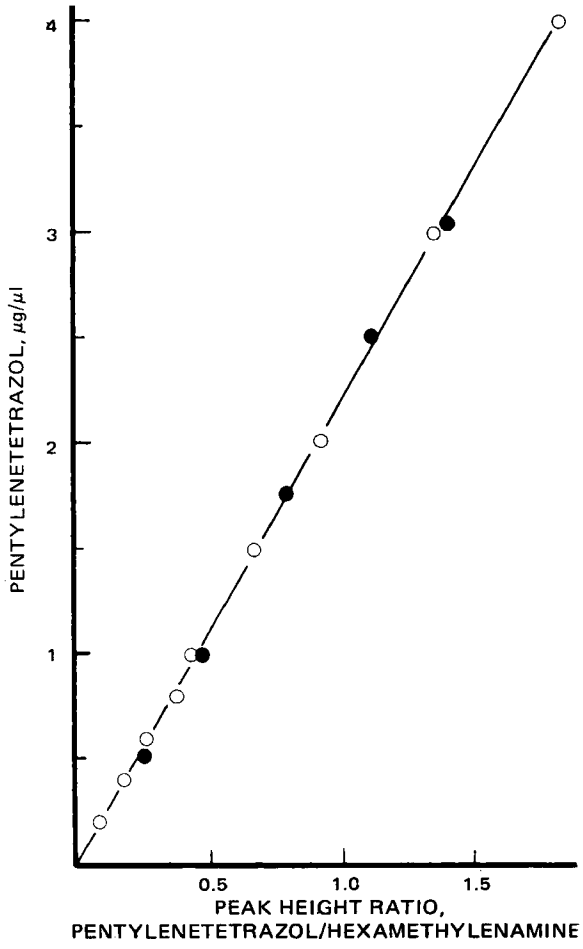

Figure 2—*Standard calibration curve for pentylenetetrazol to the calibration marker in ethanol (○), and for pentylenetetrazol extracted from human plasma (●).*

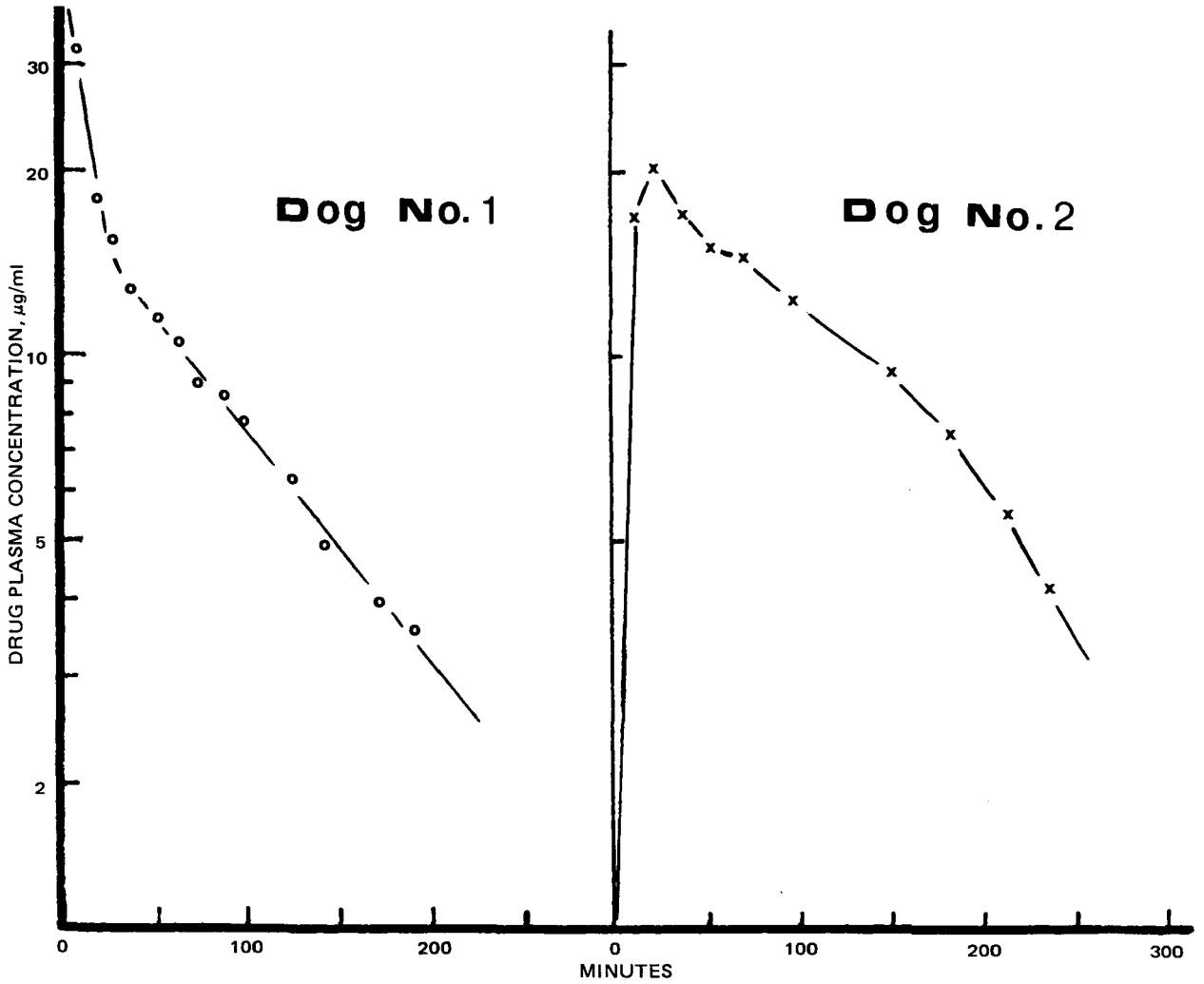

Figure 3—*Plasma concentrations of pentylenetrazol following the rapid intravenous administration of a 15-mg/kg dose in Dog 1 (11 kg) and following the oral administration of a 15-mg/kg dose in Dog 2 (9 kg).*

Human blood samples were obtained from three patients (57–68 years of age) regularly taking pentylenetetrazol tablets. This experiment was performed to test the sensitivity of the proposed technique for detecting drug plasma levels in patients after subconvulsive therapeutic doses.

RESULTS AND DISCUSSION

Drug Partition Studies—Since pentylenetetrazol has a high solubility in water as well as in most organic solvents, it was necessary to determine the partition properties of this compound (Table I). The large partition coefficient of pentylenetetrazol in the chloroform layer led to the use of chloroform in the extraction. The partition coefficient of this drug in chloroform is about 200 times greater than in ether, although these two solvents have similar dielectric constants (6). This difference may be due to the formation of a molecular interaction between pentylenetetrazol and chloroform. After equilibrium, the concentration of pentylenetetrazol in the water layer was determined by direct injection of an aliquot of the aqueous phase into the chromatograph and determination of the peak height ratio of the drug to the calibration marker.

Table II—Recoveries of Pentylenetetrazol Standards Added to Control Biological Fluids of the Dog

Pentylenetetrazol Added, μg/ml		Recovery of Pentylenetetrazol[a], %	
		Plasma	Urine
1		105.0	96.0
2		105.0	—
3		96.7	103.0
5		98.0	—
7		107.1	102.5
9		105.6	98.0
11		98.2	—
13		—	96.5
15		93.4	95.1
	Mean	101.1	98.5
	± *SD*	5.1	3.4

[a] Each percent recovery of pentylenetetrazol represents the results from a single sample subjected to the complete pentylenetetrazol analytical method.

Chromatography—Figure 1 shows typical chromatograms for a control plasma extract in which the calibration marker was added and for a pentylenetetrazol standard added to a control plasma sample and analyzed by this procedure using the calibration marker. Both peaks are sharp and symmetrical, and the chromatographic time for each determination is relatively short. Analysis of many control samples of plasma showed no interfering peaks with retention times similar to pentylenetetrazol (3.2 min) or hexamethylenamine (1.1 min).

Calibration Curves—As shown in Fig. 2, a linear relationship exists for known concentrations of pentylenetetrazol over a 0.2–4-μg/μl range when plotted against the corresponding peak ratios. An identical linear line is also obtained for the analysis of drug standards added to control samples by plotting the concentrations of pentylenetetrazol added to control plasma samples against the peak height ratio as described under *Experimental*. This correlation indicates a high efficiency of extraction and reproducibility of the analytical method for pentylenetetrazol.

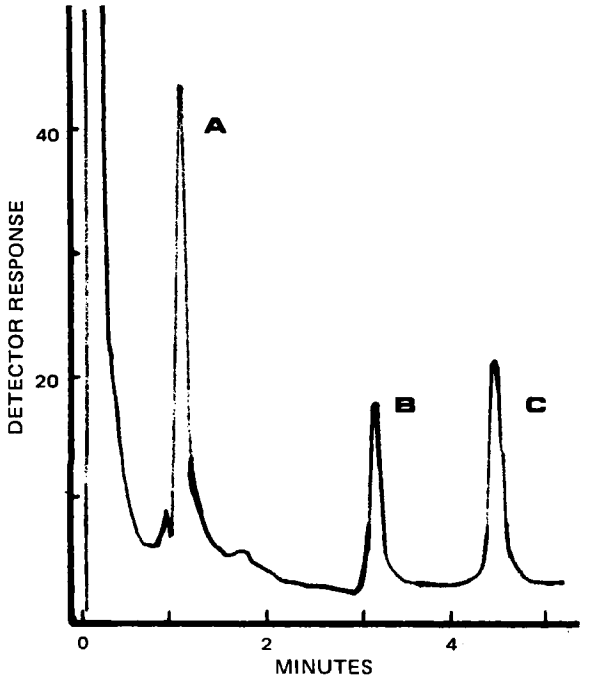

Figure 4—*Gas–liquid chromatogram of dog plasma extracts. Key: A, calibration marker; B, pentylenetetrazol; and C, unidentified metabolite.*

Drug Recovery—Results for recovery experiments of pentylenetetrazol standards added to control dog plasma and urine are shown in Table II. The data indicate that recovery of the drug from dog plasma is essentially quantitative in the 1–15-μg/ml range using a 2-ml plasma sample (mean ± *SD* = 101.1 ± 5.1%). Actual concentrations of the drug in dog plasma samples were also in this concentration range.

The average recovery of the drug added to control dog urine (2 ml) was 98.5 ± 3.4%. Because of its high partition property into chloroform, almost complete recovery of the drug was achieved after a single extraction process. The mean recoveries of the drug from control human plasma (95.8 ± 4.4%) and urine samples (97.0 ± 3.9%) were also quantitative.

Drug Plasma Level Studies—The described analytical method for pentylenetetrazol was used in the determination of plasma levels of pentylenetetrazol in dogs following rapid intravenous and oral administrations of a 15-mg/kg dose. Plasma profiles after the rapid intravenous injection (Fig. 3) indicate the applicability of the two-compartment pharmacokinetic model proposed previously (7, 8). The half-life of the drug in the plasma of one dog (male, 11 kg) was about 1 hr.

The drug plasma levels after oral administration of the aqueous solution (135 mg in 20 ml of water) to the other dog (female, 9 kg) indicate that GI absorption of this drug was very rapid and efficient. After the maximal drug peak, which occurred in the plasma at about 25 min (Fig. 3), the plasma levels declined slowly and then more rapidly. The initial slow decline was due to the simultaneous absorption and elimination process.

Table III—Plasma Concentrations of Pentylenetetrazol in Human Plasma Samples

Subject	Dose, mg	Sample Time, hr Postdose	Concentration, μg/ml
A	100	3	1.86
B	100	5	1.45
C	100	1.25	3.1

Detection of Pentylenetetrazol in Human Plasma—Human plasma samples were obtained from three patients given therapeutic oral doses of the drug. These plasma samples (4 ml) were then analyzed by the described method (Table III). The chromatographic results indicate that the method is sufficiently sensitive to detect the plasma levels of pentylenetetrazol after therapeutic doses of the drug.

Detection of Unidentified Metabolite—During a chromatographic analysis of plasma extracts from drug-treated dogs, a new peak due to a drug metabolite with a retention time of 4.5 min was consistently observed (Fig. 4). The size of the peak due to the metabolite in the plasma reached a maximum after about 1 hr and decreased slowly thereafter. Analysis of urine samples for the drug also showed the peak due to the metabolite at the identical retention time (4.5 min). No peak with this retention time was seen in the control plasma or urine samples. Extensive metabolism of this drug was previously reported (4, 9).

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ACKNOWLEDGMENTS AND ADDRESSES

Received November 13, 1974, from the *School of Pharmacy, University of Georgia, Athens, GA 30602*

Accepted for publication March 12, 1975.

The authors thank Dr. Jerry L. Curtis at the Central State Hospital, Milledgeville, Ga., for supplying the human plasma samples of pentylenetetrazol.

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