# GLC Determination of Pentylenetetrazol in Biological Fluids

J. T. STEWART▲ and J. L. STORY*

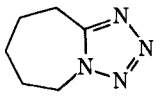
**Abstract** □ A rapid and sensitive GLC method for the determination of pentylenetetrazol was developed. The lower limit of detection is 1 ng./μl. of pentylenetetrazol. The technique involves extraction of drug from water, plasma, urine, and/or whole blood into organic solvent, evaporation of the solvent phase to dryness, and redissolving in water. The aqueous extract is injected onto a 5% polyethylene glycol 20,000 column for quantification. The utility of the procedure is demonstrated by its application to the determination of pentylenetetrazol in mixtures containing various pharmaceuticals as well as to the analysis of blood and urine levels of pentylenetetrazol in drug-treated rabbits.

**Keyphrases** □ Pentylenetetrazol—GLC determination from various samples □ GLC—determination of pentylenetetrazol in various samples

Analytical methodology related to the analysis of drugs in biological fluids is of great importance today. It is essential that the analytical procedures be simple, sensitive, specific, and reproducible. Pentylenetetrazol (I)[1] is widely used in man and animals for anticonvulsant screening. After many years of extensive use, unsatisfactory methods for the determination of submicrogram concentrations of pentylenetetrazol are still being utilized (1–10).

The most sensitive methods for pentylenetetrazol analysis have been GC procedures. Kawamoto (8) used a phenylmethylsilicone[2] column with a thermal conductivity detector. Kolb and Patt (9) separated pentylenetetrazol from a mixture of drugs on a 2.5% phenylmethylsilicone[3], 1% polyamide resin[4] column. Cardini *et al.* (10) employed a 3% polyamide resin[4] column. None of these procedures is sensitive to pentylenetetrazol in nanogram quantities. Recently, Marcucci *et al.* (11) reported a GC method for determining brain levels of pentylenetetrazol which would detect as low as 50 ng.

GC was selected as the analytical method of choice for pentylenetetrazol in this laboratory since the desired sensitivity has been attained with other pharmaceuticals. Numerous qualitative methods for pentylenetetrazol have been developed using paper, thin-layer, and gas chromatography, but attempts to develop sensitive quantitative chromatographic methods have been largely unsatisfactory (3–5, 8–11).



I

[1] Manufactured as Metrazol by Knoll Pharmaceutical Co., Orange, N. J.
[2] DC-550.
[3] SE-52.
[4] Versamide 900.

In this paper, a rapid and sensitive GLC method is presented for the determination of pentylenetetrazol in nanogram quantities from water, plasma, urine, and/or whole blood samples. The analysis of pentylenetetrazol in the presence of other pharmaceuticals is presented, along with the application of the method in the determination of pentylenetetrazol in blood and urine samples from drug-treated rabbits.

## EXPERIMENTAL

**Apparatus**—A gas chromatograph[5] equipped with a differential flame-ionization detector and a recorder[6] was employed. Aluminum columns (3 mm. × 1.83 m.) packed with 5% polyethylene glycol 20,000[7] on 80–100-mesh diatomaceous earth[8] were utilized throughout the study. Helium was used as the carrier gas at a flow rate of 65 ml./min. Injection, column, and detector temperatures were 300, 200, and 265°, respectively. Injections were made into the gas chromatograph with a 10-μl. syringe[9].

**Reagents and Chemicals**—Standard solutions of pentylenetetrazol[10] (200 mcg./ml.) were prepared by dissolving weighed quantities of the powder in distilled water. A $10^{-3}$ *M* solution of procaine hydrochloride[11] in water was employed as the internal standard. All other chemicals used were the highest grades of the commercially available materials.

**Procedures for Determining Pentylenetrazol**—*Method A. Water, Plasma, and Urine Analysis*—To a mixture of 0.5 ml. of sample containing microgram quantities of pentylenetetrazol and 0.5 ml. of phosphate buffer, pH 9.0 (12), in a 10-ml. test tube is added an excess quantity of sodium chloride. The sample is extracted three times with 5-ml. portions of water-saturated ether. During each extraction, samples are mixed for 1 min. on a mixer[12] and centrifuged for 5 min. to separate the layers. The combined ether layers are transferred to another test tube and evaporated to dryness in a 40° water bath. The dried residue in the test tube is redissolved in 0.5 ml. of distilled water containing 0.2 μl. of the procaine hydrochloride solution and rotated vigorously on the mixer in order to extract all of the drug into the water. When nanogram per milliliter levels of pentylenetetrazol are to be detected, it is necessary to redissolve the dried residue in 10–20-μl. quantities of distilled water, taking special precautions that all drug is dissolved. The amount of internal standard is reduced accordingly. One of three microliter samples is then injected into the gas chromatograph for analysis. Pentylenetetrazol concentrations are determined by the relative peak height method (13).

*Method B. Whole Blood Analysis*—Method A is followed except that the original sample in a 10-ml. test tube is extracted once with a 5-ml. portion of benzene. Benzene is used in this extraction to avoid the emulsion formation one encounters with ether extractions of whole blood. Then 4 ml. of the benzene extract is transferred to another 10-ml. test tube, followed by evaporation of the benzene and redissolving the dried residue in water for injection into the gas chromatograph.

***In Vivo* Study**—Subconvulsive doses of 1 mg./kg. body weight of pentylenetetrazol in saline are administered intravenously in the

[5] Model 881, Perkin-Elmer Corp.
[6] Speedomax G, Leeds and Northrup.
[7] Carbowax 20M, Analabs, Inc., Hamden, Conn.
[8] Chromosorb W, Johns-Manville, New York, N. Y.
[9] Hamilton, Co., Whittier, Calif.
[10] Knoll Pharmaceutical Co., Orange, N. J.
[11] Fisher Scientific Co.
[12] Super Mixer, Lab-Line Instruments, Melrose Park, Ill.

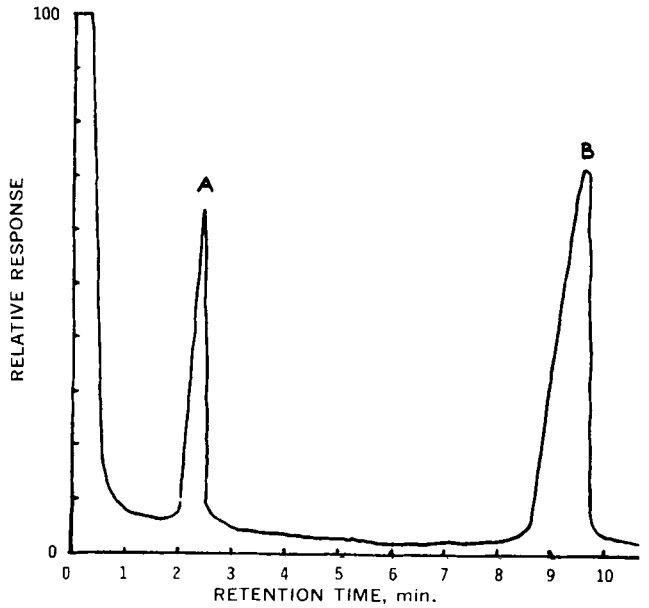



**Figure 1**—*Representative gas chromatogram for pentylenetetrazol on 5% polyethylene glycol 20,000. Key: A, pentylenetetrazol; and B, procaine hydrochloride.*

marginal ear vein of six unanesthetized rabbits[13]. Polyethylene catheters (1.1 mm. i.d.) are inserted into their bladders to collect urine specimens. The rabbits are placed in rabbit boxes, and blood is withdrawn by ear puncture at 15-, 30-, 60-, 90-, 150-, 270-, and 390-min. intervals after injection. The whole blood samples are heparinized and chilled in test tubes at 0.5° before being analyzed using Method B.

## RESULTS AND DISCUSSION

The objective of this study was to develop a sensitive and rapid method for the analysis of pentylenetetrazol in biological materials.

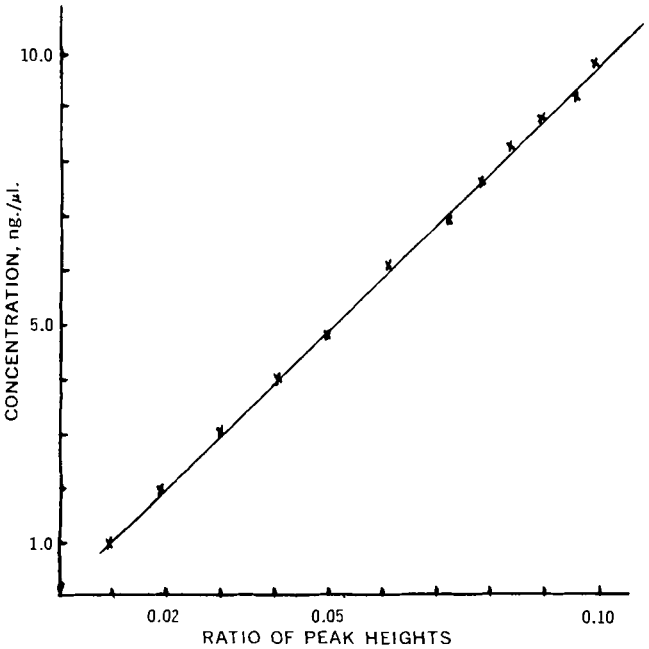



**Figure 2**—*Typical calibration curve for pentylenetetrazol.*

[13] New Zealand white male rabbits (2 kg.), furnished by Cherokee Labs, Atlanta, Ga.

**Table I**—Determination of Pentylenetetrazol–Drug Mixtures for Pentylenetetrazol[a]

| Mixture | Components, Concentration of $10^{-2}$ *M* | Percent Recovery of Pentylenetetrazol[b] |
|---|---|---|
| 1 | Phenobarbital[c]<br>Methamphetamine[d]<br>Pentylenetetrazol | 97.0 |
| 2 | Sulfathiazole[e]<br>Chlorpromazine hydrochloride[e]<br>Pentylenetetrazol | 98.0 |
| 3 | Caffeine[d]<br>Diazepam[f]<br>Pentylenetetrazol | 100.0 |
| 4 | Ephedrine sulfate[g]<br>Aspirin[h]<br>Pentylenetetrazol | 98.0 |
| 5 | Niacin[d]<br>Pentylenetetrazol | 99.0 |

[a] Pentylenetetrazol detected by Method A (see *Experimental* section). [b] Recoveries based on pentylenetetrazol added to drug mixtures before extraction. [c] American Pharmaceutical Co., New York, N. Y. [d] Eastman Kodak Chemicals, Rochester, N. Y. [e] Smith Kline and French Laboratories, Philadelphia, Pa. [f] Hoffmann-La Roche, Nutley, N. J. [g] Ruger Chemical Co., Irvington-on-Hudson, N. Y. [h] Merck and Co., Rahway, N. J.

A major problem in devising a suitable analytical procedure for the drug is its unusual chemical properties. Pentylenetetrazol is freely soluble in both polar and nonpolar solvents. It has a high dipole moment of 6.14 debyes and exhibits tautomerism with aromatic characteristics. It is unreactive partly due to lack of hydrogens on any of the ring nitrogens and full saturation in the other ring. These limit the versatility in measuring low concentrations of pentylenetetrazol by commonly employed methods. The compound possesses no natural fluorescence, color, chromophore, or outstanding functional grouping which would allow the use of fluorometric, colorimetric, UV spectrophotometric, or functional group analysis methods for its estimation.

Severe tailing and peak asymmetry are encountered with microamounts of pentylenetetrazol in most GC systems. A variety of nonpolar to polar liquid stationary phases were used in an attempt to find the optimal conditions for pentylenetetrazol that would give sharp, symmetrical, and reproducible peaks at submicrogram concentrations. Ethylvinylbenzene–divinylbenzene polymer[14] and 5% polyethylene glycol 20,000[7] are the most sensitive phases found. The use of ethylvinylbenzene–divinylbenzene polymer gives better sensitivity than polyethylene glycol 20,000, but it does not afford as good a separation when other drugs are present in samples with pentylenetetrazol. Therefore, polyethylene glycol 20,000 was chosen as the stationary phase of choice since it proved to be a better system for use with biological specimens, which may contain other drugs or chromatographable components.

A representative gas chromatogram for pentylenetetrazol on 5% polyethylene glycol 20,000 is shown in Fig. 1. A typical calibration curve for the drug using ratios of peak heights of pentylenetetrazol to internal standard *versus* concentration of pentylenetetrazol is seen in Fig. 2.

The results of a study to establish optimal conditions for extracting pentylenetetrazol from water, plasma, and/or urine indicate that extraction with ether at pH 9.0 gives the highest percent recoveries (98.4 ± 0.13, 84.3 ± 0.59, and 98.2 ± 0.49%, respectively). Benzene is substituted for ether when whole blood is used because of emulsion formation with ether. The percent recovery of pentylenetetrazol from whole blood with benzene is 84.5 ± 0.61%. Additional benzene extractions do not significantly change these data. Addition of sodium chloride to the aqueous samples is necessary to extract pentylenetetrazol as quantitatively as possible. A preliminary *in vitro* binding experiment performed in this laboratory showed that 8.9–9.0% of pentylenetetrazol is bound to plasma proteins in rabbits (14). This probably accounts for some of the loss of

[14] Porapak Q, Waters Associates, Framingham, Mass.

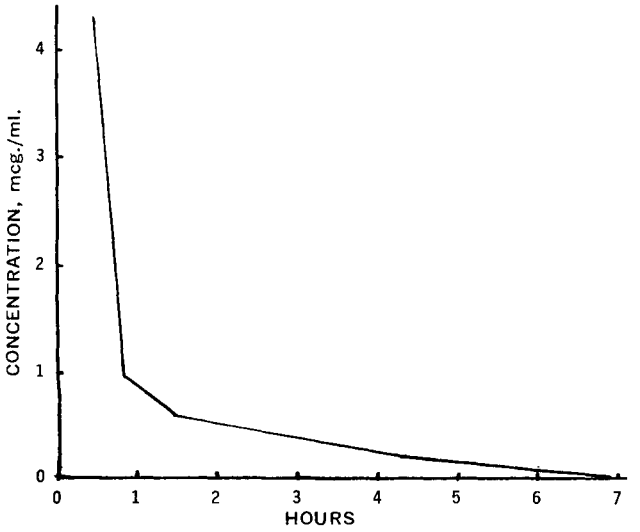



**Figure 3**—*Disappearance of pentylenetetrazol from blood levels of drug-treated rabbits.*

pentylenetetrazol connected with lower percent recoveries from plasma and whole blood.

It is also shown that other drugs, which might be present in biological samples with pentylenetetrazol, do not significantly interfere with the analysis of pentylenetetrazol using the methods described here (Table I).

An *in vivo* study was performed to demonstrate the further application of the procedures. Pentylenetetrazol is injected into rabbits, and blood and urine samples are collected and analyzed. After 6.5 hr., measurable blood levels (25 ng./ml.) are detected by the whole blood extraction method (Method B) as shown in Fig. 3. Excretion of urine by the rabbits was restricted due to renal shutdown, but analysis of urine taken after a 3-hr. period shows levels of 55 ng./ml. of pentylenetetrazol (Method A).

No attempt was made to extract and identify metabolites of pentylenetetrazol; but one consistent unidentifiable peak, having a retention time of 1.8 min. compared to 2.3 min. for pentylenetetrazol, appears on the chromatogram from the rabbit urine extracts. The unidentified peak is sharp and symmetrical and closely approximates the area of the pentylenetetrazol peak. In 1971, Rowles *et al.* (7) demonstrated by the administration to humans of $^{14}C$-labeled pentylenetetrazol that the drug is metabolized and excreted in the urine as unchanged pentylenetetrazol and at least three metabolites. Later in 1971, Ko and Hosein (15) proposed a possible structure for a metabolite of pentylenetetrazol found in human urine. It is said to be the 6-substituted sulfate ester of pentylenetetrazol.

In summary, a rapid and sensitive method involving extraction of pentylenetetrazol from water, plasma, urine, and whole blood and quantification of the extracts by GC was developed. The procedure detects as little as 1 ng./μl. of pentylenetetrazol and can be completed in less than 30 min., with good reproducibility as shown by a relative standard deviation of ±1.1%. The method is relatively free from interferences by other drugs, and the procedure is applicable to nanogram concentrations of pentylenetetrazol in biological fluids.

## REFERENCES

(1) T. E. V. Horsley, *Analyst*, **71**, 308(1946).

(2) L. K. Sharp, *J. Pharm. Pharmacol.*, **4**, 52(1952).

(3) V. K. Macek and J. Vecerkona, *Pharmazie*, **20**, 605(1965).

(4) I. Sunshine, W. W. Fike, and H. Landesman, *J. Forensic Sci.*, **11**, 428(1966).

(5) P. E. Haywood, M. W. Horner, and H. J. Rylance, *Analyst*, **92**, 711(1967).

(6) D. W. Esplin and D. M. Woodbury, *J. Pharmacol. Exp. Ther.*, **118**, 129(1956).

(7) S. G. Rowles, G. S. Born, H. T. Russell, W. V. Kessler, and J. E. Christian, *J. Pharm. Sci.*, **60**, 725(1971).

(8) H. Kawamoto, *Kumanto Med. J.*, **15**, 69(1962).

(9) V. H. Kolb and P. W. Patt, *Arzneim.-Forsch.*, **15**, 924 (1965).

(10) C. Cardini, G. Cavazzutti, and V. Quercia, *Boll. Chim. Farm.*, **109**, 333(1970).

(11) F. Marcucci, M. L. Airoldi, E. Mussini, and S. Garattini, *Eur. J. Pharmacol.*, **16**, 219(1971).

(12) A. Osol, in "Remington's Pharmaceutical Sciences," 13th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1965, p. 245.

(13) S. Dal Nogare and J. S. Juvet, Jr., "Gas-Liquid Chromatography," Interscience, New York, N. Y., 1962, p. 256.

(14) J. T. Stewart and J. L. Story, *J. Pharm. Sci.*, **61**, 652(1972).

(15) G. K. W. Ko and E. A. Hosein, *Can. J. Physiol. Pharmacol.*, **49**, 356(1971).

## ACKNOWLEDGMENTS AND ADDRESSES

Received January 20, 1972, from the *Analytical Laboratory, Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication May 24, 1972.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Houston meeting, April 1972.

Abstracted in part from a thesis submitted by J. L. Story to the University of Georgia in partial fulfillment of the Master of Science degree requirements.

* Fellow, American Foundation for Pharmaceutical Education, 1970–1971.

▲ To whom inquiries should be directed.