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## Note

# High-performance liquid chromatographic determination of chemical convulsant pentylenetetrazol in rat serum, cerebrospinal fluid and brain

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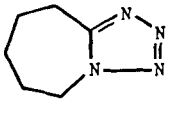
Pentylenetetrazol (pentamethylenetetrazol) (PTZ, 1, Fig. 1) is a chemical convulsant [1] used routinely in epilepsy research to produce convulsions and for laboratory testing of potential anticonvulsant agents [2]. It produces convulsions via its action at the picrotoxin-sensitive site of the benzodiazepine–$\gamma$-aminobutyric acid (GABA) receptor–ionophore complex [3]. Historically, PTZ has been determined in biological fluids by gas chromatographic procedures using either flame ionization detection [4,5] or nitrogen–phosphorus detection (NPD) [6] with the latter mode of detection being most suitable for determining the biophasic concentrations of PTZ in small volumes of biological fluids/tissues [7]. Only recently, while this report was being prepared, has a liquid chromatographic assay for PTZ been published [8]. This procedure involves extraction of PTZ from the biological fluid/tissue prior to chromatography.

This present report describes a simple high-performance liquid chromatographic (HPLC) method for quantitation of PTZ in small volumes of rat serum, cerebrospinal fluid (CSF) and brain without prior extraction of the convulsant. The method uses a commercially available compound as internal standard and



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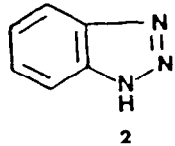

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Fig. 1. Structures of pentylenetetrazol (1) and the internal standard benzotriazole (2).

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has been utilized in preliminary pharmacodynamic study in rats and has general applicability for examining PTZ pharmacodynamics in disease and other physiologic pertubations.

## EXPERIMENTAL

### *Drugs, chemicals and reagents*

PTZ was purchased from Sigma (St. Louis, MO, U.S.A.); benzotriazole, the internal standard (2, Fig. 1), was from Aldrich (Milwaukee, WI, U.S.A.), HPLC-grade acetonitrile was from J.T. Baker (Phillipsburg, NJ, U.S.A.) and potassium phosphate monobasic ($KH_2PO_4$) was from EM Science (Cherry Hill, NJ, U.S.A.).

### *Chromatography*

The HPLC system consisted sequentially of a Waters 510 pump (Waters Assoc., Milford, MA, U.S.A.), a Waters WISP 710B autoinjector, a 5-cm $C_{18}$ guard column and a 25 cm × 4.6 mm I.D., 10-$\mu$m $C_{18}$ Waters $\mu$Bondapak analytical column, a Waters 440 fixed-wavelength detector connected to a Waters extended-wavelength module set as 214 nm (zinc lamp) and a Shimadzu CR3A Chromatopac integrator (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) set at attenuation 5 (32 mV full scale). The column temperature was ambient and the mobile phase, consisting of 7.5% acetonitrile in water containing 0.005 *M* $KH_2PO_4$, was filtered and degassed prior to use. The flow-rate was 1 ml/min.

### *Sample preparation and processing*

Rat serum or CSF (50 $\mu$l) was placed into plastic 1.5-ml Eppendorf tubes (Brinkmann Instruments, Westbury, NY, U.S.A.) followed by addition of 10 $\mu$l of benzotriazole (0.025 mg/ml in acetonitrile) as internal standard and 50 $\mu$l of acetonitrile as protein precipitant for serum samples. The mixtures was vortexed for 15 s and centrifuged at 12 800 *g* for 2 min in an Eppendorf microcentrifuge (Brinkmann Instruments). Of the clear supernatant, 50 $\mu$l (or 25 $\mu$l for CSF) were injected onto the column. To assay PTZ in brain, 0.5 g of tissue from one hemisphere was mixed with 100 $\mu$l of internal standard solution and 1 ml acetonitrile. This mixture was homogenized in a motor-driven tissue homogenizer (Wheaton Instruments, Millville, NJ, U.S.A.), centrifuged and 50 $\mu$l of the supernatant were chromatographed as for serum/CSF. For routine analyses, a calibration curve for PTZ between 50 and 200 $\mu$g/ml or $\mu$g/g was constructed using either drug-free rat serum, CSF or brain tissue.

## RESULTS

PTZ did not show a true UV absorption maximum in the mobile phase used (Fig. 2), hence the detection of PTZ was carried out at 214 nm, the lowest wavelength available using a fixed-wavelength detector. Using the described mobile phase and reversed-phase column, PTZ and internal standard (benzotriazole) had retention times of 11.0 and 13.1 min, respectively. There were no interfering peaks and both these compounds were always well separated from other endog-

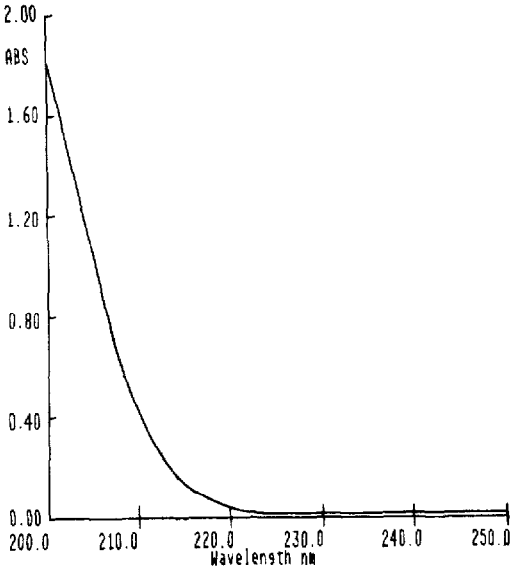


Fig. 2. UV spectra of pentylenetetrazol (100 $\mu$g/ml) in mobile phase consisting of 7.5% (w/v) acetonitrile in water and 0.005 *M* $KH_2PO_4$.

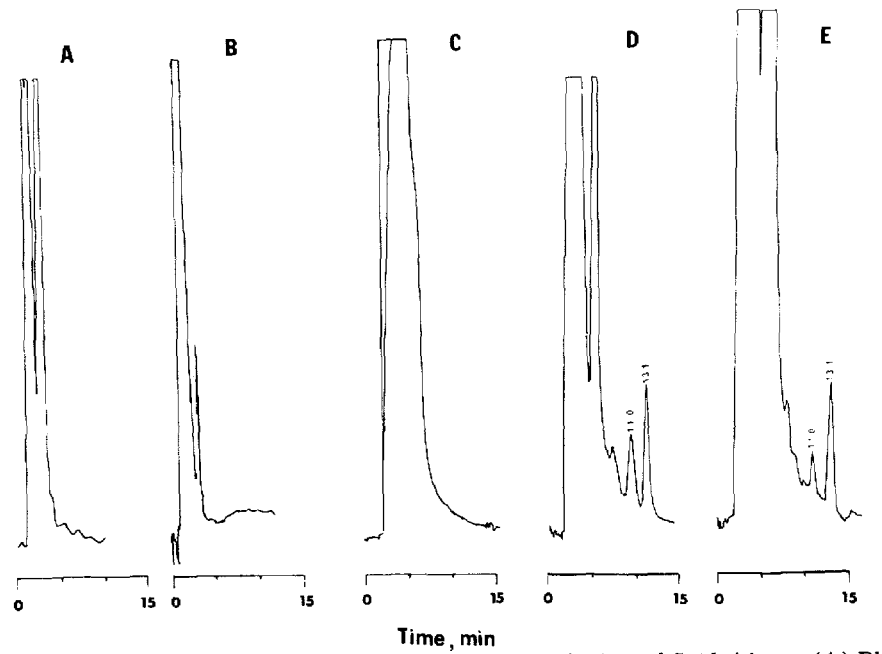


Fig. 3. Chromatograms of acetonitrile-precipitated rat biological fluids/tissue. (A) Blank drug-free serum, (B) CSF and (C) brain, and (D) serum and (E) brain containing 102 $\mu$g/ml and 67 $\mu$g/g PTZ, respectively, from a rat infused with PTZ intravenously to a maximal seizure. PTZ and internal standard, benzotriazole, peaks are at 11.0 and 13.1 min, respectively.

enous peaks from either rat serum, CSF or brain. Fig. 3 shows several chromatograms of either blank rat serum (A), CSF (B) and brain (C) and rat serum (D) or brain (E) when a rat was infused with PTZ to a maximal seizure.

PTZ-to-benzotriazole peak-area ratios were linear with PTZ concentrations from 50 to 200 $\mu$g/ml or $\mu$g/g and linear regression of the data yielded the following equations: $y = 0.004x + 0.0018$ ($r = 0.998$) for serum; $y = 0.004x + 0.0016$ ($r = 0.996$) for CSF; $y = 0.005x + 0.0018$ ($r = 0.998$) for brain. The detection limit of PTZ was 25 $\mu$g/ml using 50 $\mu$l of sample and the coefficient of variation of the assay was 7.8% at 25 $\mu$g/ml and 5.5% at 100 $\mu$g/ml ($n = 4$ at each concentration). The accuracy of the method is greater than 95% at the higher concentration. There were no interferences from other drugs (e.g., anticonvulsant barbiturates like phenobarbital or other antiepileptics like phenytoin or ethosuximide) likely to be used in antiepileptic drug research.

In rats infused intravenously with PTZ to maximal seizure, concentrations (mean $\pm$ S.D., $n = 5$) of PTZ at onset of seizure were $129 \pm 35$ $\mu$g/ml in serum, $102 \pm 21$ $\mu$g/ml in CSF and 85 $\pm$ 24 $\mu$g/g in brain.

## DISCUSSION

Gas chromatographic assays for PTZ with NPD are reproducible and simple especially for quantifying low concentrations encountered during pharmacokinetic studies [7]. However, nitrogen–phosphorus detectors are not available in a number of laboratories (including ours) and due to the ready availablity of HPLC instrumentation in most analytical and research laboratories it was necessary to develop a liquid chromatographic assay for PTZ. The currently developed assay is simple, does not involve prior extraction of PTZ from the biological material and is sensitive enough for quantifying PTZ concentrations that elicit minimal (myoclonic jerks) or maximal (tonic hind limb extention) PTZ seizures. Compared to the previously reported HPLC assay for PTZ [8], however, the current method is not as precise or sensitive but if necessary the sensitivity can be increased further by UV detection at a lower (202 nm) wavelength or by using a larger sample volume and extracting the PTZ as suggested previously [8]. Although the current method uses protein precipitation for sample preparation no problems with column stability have been noted and both the guard and analytical column have not needed replacement after over 200 injections. Routinely, however, the columns are washed with about 20 volumes of water followed by acetonitrile–water (50:50) at the end of each batch of analysis.

During the use of the present assay only one peak corresponding to unchanged (not metabolized) PTZ was noted. Thus the claimed metabolite(s) of PTZ in rats [9,10] were either not present in high enough concentrations in serum (and CNS), were eluted with the solvent front or endogenous biological material or their formation was limited due to use of a short ($\sim$30 min) infusion of PTZ relative to its biological half-life in rats.

In summary, a selective method for the determination of PTZ in rat serum and CNS has been developed using readily available HPLC instrumentation. The procedure permits the pharmacodynamics of PTZ to be determined using small

volumes of biological fluids and thus is suitable for routine studies in small laboratory animals.

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