Determination of p-Nitrophenol in Human Urine

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The level of p-nitrophenol (PNP) in urine has been used successfully as an indicator of exposure to methyl parathion, ethyl parathion and ethyl p-nitrophenyl phenylphosphonothioate (EPN) (1-3). This report describes a simple, rapid gas chromatographic method which is sensitive to 50 ppb of PNP in urine and requires less than two hours for analysis. The method utilizes hydrolysis and extraction techniques similar to those of the method of Elliott et al. (2), but differs in sample clean-up and in the use of a gas chromatographic determinative procedure.

MATERIALS AND METHODS

Apparatus*: The following glassware was used (all ♁ 14/20): evaporative condenser columns (Kontes K-569251), graduated test tubes (K-288250), and condensers (K-282200).

The heating block used was a Thermoline 20-tube capacity dry bath at 120 °C, and the circulating pump was a Fisher suction pump.

A Micro-Tek MT-220 gas chromatograph, equipped with a tritium or nickel-63 electron-capture detector, was operated at the following temperatures: inlet, 200°C; column, 140°C; detector, 215°C for tritium or 250 °C for nickel-63. The chromatographic columns were glass U-tubes, 6' X 1/4", packed with 5% DC-200 on 80-100 mesh Gas Chrom Q, or with 1.5% OV-17 + 1.95% QF-1 on 100-120 mesh Chrom W.

Reagents: Hydrochloric acid, concentrated, analytical reagent; sodium hydroxide, 0.1 N and 20% solutions; pHydrion paper, ranges 1-2 and 11-12.5; benzene and diethyl ether, Nanograde solvents or equivalent; sodium sulfate, anhydrous granules; and hexamethyldisilizane (HMDS), 20:80 (v/v) in hexane.

Standards: PNP standards prepared at concentrations of 2, 10, 25, 50, and 100 pg PNP/μl in HMDS-hexane (10:90 v/v).

Procedure

Hydrolysis, Cleanup, and Extraction: A sample of urine (e.g., 2.7 ml) is placed in a 15 ml ground glass test tube with ♁ joint compatible with a stoppered water-cooled condenser. For each 0.9 ml of urine, 0.1 ml of concentrated HCl is added, and ground glass-stoppered, ice water-cooled condensers are fitted to the test tubes. The mixture is gently boiled for one hour in a dry bath heating block. Caution: During the boiling, the condensers must be stoppered and cooled with constantly circulating ice cold water. After one hour the hydrolyzed urine is cooled and the condenser is rinsed with 2 ml of 0.1 N NaOH. The cooled hydrolyzed urine is adjusted to a pH of 11 or higher (with approximately 0.4 ml of 20% NaOH solution). Since this adjustment need not be absolute, a drop of urine on a strip of narrow range pHydrion paper is sufficient for pH de-

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termination. The alkaline urine is extracted two times with 5 ml of benzene-diethyl ether (80:20 v/v) and the extracts are discarded. The urine is reacidified with concentrated HCl to pH 2 or lower and extracted with 5.4 ml of benzene-diethyl ether. The benzene-diethyl ether extract of the acid urine is removed with a disposable pipette and dried by the addition of granular sodium sulfate. A 1 ml aliquot of the dried extract is pipetted into a 5 ml ground glass-stoppered test tube and 1.0 ml of HMDS in hexane (20:80 v/v) is added.

Gas Chromatographic Determination: The gas chromatographic determinative step involves on-column silanization of PNP to the less polar and more volatile trimethylsilyl ether (PNP-TMS). A portion, preferably 5 µl, of the dried benzene-hexane-diethyl ether extract containing the PNP and HMDS (10% v/v) is injected into a gas chromatograph equipped with an electron-capture detector and either a 5% DC-200 or 1.5% OV-17 + 1.95% QF-1 column.

RESULTS

Recovery: Recovery efficiencies were determined by dividing non-hydrolyzed urine samples into two equal portions and adding a known amount of PNP to one sample. Both urine samples were carried through the complete procedure and analyzed by gas chromatography. Recoveries were 92-100% over the concentration range shown in Table I.

Precision: The precision of the method was evaluated by comparing the variation obtained from six replicated analyses of four urine samples with different PNP concentrations (Table II).

TABLE I

Recovery of PNP Added to Human Urine (ppb)

PNP Present in Urine	PNP Added to Urine	PNP Recovered	% Recovery
0	25	24	96
0	100	94	94
0	500	485	97
55	25	74	92
74	25	95	95
111	25	130	95
163	100	245	93
295	100	395	100
420	500	885	96
1210	500	1600	94
1210	2500	3600	97

TABLE II

Comparison of 6 Replicate Analyses
of Urine Samples (ppb)

Sample No.	Mean	Range	Standard Deviation
1	55	53-56	1.1
2	111	106-114	2.6
3	300	280-315	13.4
4	415	400-440	15.0

DISCUSSION

Hydrolysis: The findings reported herein are consistent with those of Elliott et al. (2). A ratio of 90 to 10 by volume of urine to concentrated hydrochloric acid with a one hour reflux produced maximum results. Losses of PNP are observed if the condensers are not chilled with ice water and stoppered.

Clean-up: Compounds which are acidic and are extracted from strongly acid aqueous solutions by benzene-diethyl ether, but are not extracted from strongly alkaline aqueous solutions, will be included in the final extract. Certain weak acids, as well as neutral and basic compounds, will be excluded.

Extraction: Recoveries of the extraction and cleanup steps were 92-100%. Elliott et al. (2) reported recoveries of 70-75% for known amounts of PNP. The increased recovery is probably due to the fewer manipulations and the exclusion of the step in the procedure of Elliott et al. (2) which partitions PNP into acetonitrile from a 20% sodium hydroxide solution.

Gas Chromatography: The gas chromatography of PNP involves the conversion of the free phenol to the more volatile, less polar trimethylsilyl ether (PNP-TMS). This conversion is accomplished after the solution containing PNP and HMDS is injected into the gas chromatographic column. A similar procedure was reported by Esposito (4) for the conversion of fatty acids, carboxylic acids, and polyhydric alcohols to trimethylsilyl ethers.

Since reactive HMDS is injected directly onto the column, liquid phases with reactive hydrogens cannot be used. Several silicone liquid phases including DC-200, QF-1, OV-1, OV-17, and OV-210 have been found to give good results. All the Apiezon greases have been found to be suitable.

The response of the electron capture detector to PNP-TMS is approximately equal to that obtained for lindane, the gamma isomer of BHT. This high affinity for capturing electrons reduces the analytical problems due to gas chromatography of interfering substances which have been carried through the procedure. HMDS elutes as a solvent front and does not produce interfering peaks.

Solid supports used in the preparation of columns for the pro-

cedure should be silanized. It may be necessary in some instances to condition the column by injecting 5 μl of a 100 pg/μl solution of PNP in HMDS-hexane (10: 90 v/v) until a constant peak height is obtained for PNP-TMS.

Columns should be examined to determine that all the PNP injected is converted to PNP-TMS. This is accomplished by injecting HMDS-hexane (10:90 v/v) without PNP. If a PNP peak is detected, the column has adsorbed PNP and requires further conditioning with HMDS.

SUMMARY

A rapid, sensitive gas chromatographic method for the determination of PNP in urine at levels as low as 50 ppb is described. The method involves an acid hydrolysis, cleanup, selective extraction, and on-column formation of the trimethylsilyl ether derivative of PNP.

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