Parathion Exposure Studies. A Gas Chromatographic Method for the Determination of Low Levels of p-Nitrophenol in Human and Animal Urine

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In conjunction with a current project designed to determine the correlation between exposure of rats to parathion and the urinary excretion of diethylphosphate and diethylphosphorothionate metabolites (SHAFIK et al. 1971), it was desirable to determine and compare p-nitrophenol (PNP) metabolite excretion rates and patterns. The commonly used colorimetric method (ELLIOTT et al. 1960) permits determination of as little as 0.1 ppm of PNP but requires large (100 ml) urine samples at the low levels. In addition, high background interference is observed at low levels and the procedure lacks specificity. A gas chromatographic procedure (CRANMER 1970) involving on-column derivatization of PNP to the trimethyl silyl ether permits determination of 0.05 ppm PNP in human urine. To obtain this sensitivity in rat urine, cleanup is required to remove interfering compounds. However, the silyl ether derivative proved to be unstable in a silica gel cleanup system used routinely in our laboratory. Hence, a derivatization method had to be devised which would permit cleanup of the rat urine extract prior to injection into a gas chromatograph equipped with an electron capture detector. The formation of a stable derivative which could be subjected to silica gel chromatography, gas chromatography, isolation for mass spectrometric identification and other desired manipulations was our goal.

METHOD

Reagents and Apparatus

p-nitrophenol (PNP) standard, recrystallized from benzene and stored in amber bottles at 0°C.

silica gel, Woelm Activity Grade I, Waters Associates, Inc., Framingham, Mass. Activate for at least 24 hours at 135°C., prior to use. Partially deactivate by adding 3% (w/w) water to a portion in a glass-stoppered Erlenmeyer flask, mix and allow to equilibrate for 2-4 hours with periodic shaking.

sodium sulfate, washed with hot methanol followed by benzene and dried overnight at 135°C.

N-alkyl-N'-nitro-N-nitrosoguanidine, where alkyl group is ethyl, propyl, iso-butyl, butyl, iso-amyl, amyl or hexyl; Aldrich Chemical Co.

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diazoalkane, prepared by method of STANLEY (1966). Increase amount of nitrosoguanidine precursor by 0.15 g. for each additional methylene group in the homologous series. Diazoethane must be prepared in hexane, but all higher diazoalkanes should be prepared in ethyl ether because of the low solubility of these compounds in hexane. Use a well-ventilated hood when handling these reagents and store unused portions in tightly closed vials in the freezer.

The nitrosoguanidine precursors are potent carcinogens. Diazoethane should be prepared fresh daily to minimize background interference when working at levels of 0.5 ppm or less.

Concentrator tubes - 25 ml, graduated, glass-stoppered, ℨ 19/22 Kontes K-570050.

Condensers - 200 mm jacket length, ℨ 19/22. Kontes K-286810.

Centrifuge tubes - 13 ml and 45 ml, graduated, glass-stoppered. Kontes K-410550.

Chromatography columns - size 22. Kontes K-420100.

Micro Tek MT 220 gas chromatograph - equipped with tritium foil electron capture (EC) detector and glass U-shaped column, 6 ft x 1/4" packed with 4% SE-30/6% QF-1 on 60/80 mesh Gas-Chrom Q. Operating temperatures: inlet, 200°C, transfer line 225°C; detector 205°C; column, 175°C. Nitrogen flow rate: 80 ml/min. A glass lined off-column injection system was used for all analyses.

Analytical Procedure:

Standard curves: Prepare a standard solution by dissolving 10 mg., accurately weighed, of PNP in 5 ml. ethyl acetate and dilute to 100 ml with hexane. Pipette a 1 ml. aliquot containing 100 µg PNP into a 13 ml. centrifuge tube and alkylate by adding about 2 ml diazoethane.

Mix and allow to stand unstoppered for 20 minutes, then remove excess diazoethane by bubbling nitrogen through the solution, adding hexane if necessary to maintain a solvent volume of at least 1 ml. Dilute the solution to 10 ml with hexane and make appropriate dilutions from this standard to give a series of solutions with concentrations of 0.02, 0.06, 0.1, 0.3 and 0.5 µg/ml of PNP. Inject 5 µl of each standard solution into the gas chromatograph corresponding to 0.1, 0.3, 0.5, 1.5 and 2.5 ng PNP. The response of the EC detector in our instrument was linear over this range.

PNP in rat urine:

Parathion exposure: Pairs of male Sprague-Dawley rats weighing 265-275 g were housed in stainless steel metabolism cages.

For 12 successive days each rat was dosed by gavage with 1/10th or 1/100th LD_{50} parathion in peanut oil (104 µg or 10.4 µg respectively). Urine was collected in 24 hour increments prior to, during, and for several days after exposure. The urine was frozen until analysis and was centrifuged before using to remove solids.

Extraction and alkylation:

Pipette an aliquot of urine (1-5 ml., depending upon level of PNP expected or estimated to be present) into a 25 ml. concentrator tube. Check pH to insure that urine is not acidic, and neutralize with aqueous NaOH if necessary. Extract with two 5 ml portions of diethyl ether, centrifuging to break the emulsion which forms. Discard the ether extract and evaporate traces of ether remaining on the aqueous layer with a stream of nitrogen. Add conc. HCL in a ratio of 1 part acid to 4 parts urine, attach a stoppered condenser to the tube. Provide circulating ice water to the condenser and place tube in a boiling water bath for 1 hour, taking care that pressure does not loosen the stopper. At the end of hydrolysis cool the tube and rinse the condenser with a few ml. of water. Rinse with a volume of benzene equal to about twice the aqueous volume and remove condenser. Stopper the tube and mix contents on a Vortex mixer for 1 minute, centrifuge, and transfer the organic layer to a 45 ml. centrifuge tube. Extract the aqueous layer with an additional volume of benzene and add this to the first extract. Evaporate to about 5 ml. with a stream of nitrogen in a 40°C water bath. Alkylate the entire concentrate or a suitable aliquot with 4 ml diazoethane.

Allow the solution to stand for 20 minutes, then remove excess reagent by bubbling nitrogen through the solution while maintaining a solvent volume of at least 1 ml. Carefully concentrate to 0.5 ml under a gentle stream of nitrogen, and dilute to 5 ml. with hexane.

Cleanup:

Prepare a silica gel column by adding 1 g silica gel (3% w/w water) to a chromatography column plugged with glass wool. Top with 1 g sodium sulfate and pre-wash with 10 ml. hexane. Discard the wash. Add the alkylated extract to the column. Rinse the tube with 3 ml. of hexane and add this to the column. Elute with 8 ml of 40% benzene in hexane discarding all eluates to this point. Place a receiver under the column and continue elution with 10 ml of 60% benzene in hexane. This fraction contains the ethyl ether derivative of PNP which is then quantitated by gas chromatography.

Results and Discussion

The results of studies in which known amounts of PNP were added to rat and human urine and carried through the entire procedure are shown in Table I.

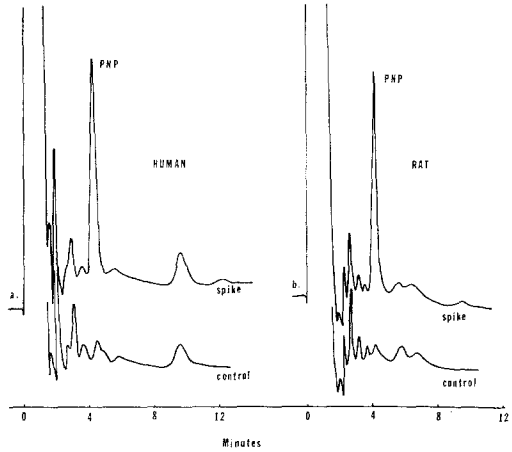

Fig. 1. a) Gas chromatogram of extract of human urine fortified at 0.02 ppm of PNP.

b) Gas chromatogram of extract of rat urine fortified at 0.05 ppm of PNP.

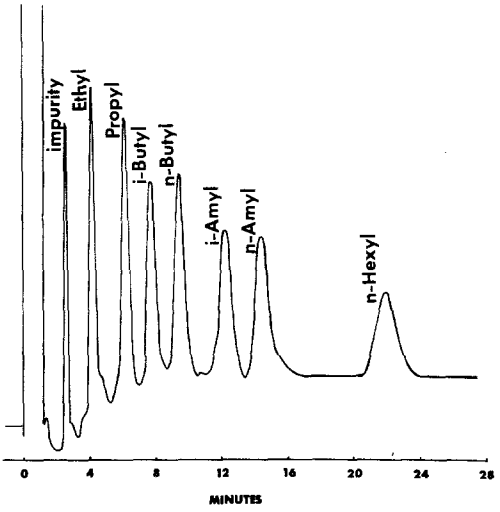

Fig. 2 Gas chromatogram of a mixture of ethers of PNP, each representing 2 ng. of the free phenol.

Table I. Recovery of PNP from Rat and Human Urine

ppm added	Percent recovered* Rat	Human
0.50	94	--
0.10	85	97
0.050	88	96
0.020	--	97

*Average of three determinations

At the lowest levels in both rat and human urine, an interfering peak with a retention time the same as PNP-ether became noticeable and prevented accurate quantitation of levels lower than those reported. Figures 1 a and 1 b show chromatograms obtained from human and rat urine samples fortified at 0.02 ppm and 0.05 ppm, respectively, and their corresponding controls after cleanup by the method described.

With the availability of nitrosoguanidine precursors of the higher diazoalkanes, it is possible to prepare a series of higher molecular weight ethers of PNP. These may be used to provide further confirmation of the identity of PNP. Their preparation and cleanup are the same as that of the ethyl ether, and they appear to be reasonably stable. Figure 2 shows the resolution of a mixture of ethers of PNP, each representing 2 ng of the free phenol.

The urine of rats exposed to parathion was analyzed by the method described above. In Figure 3, the rate of excretion of PNP is compared to that of total alkyl phosphates by a method previously described, (SHAFIK et al. 1971). The excretion pattern of PNP differs considerably from that of the alkyl phosphates in the multiple exposure experiment. During the first few days of exposure, the PNP excretion rate was more than twice as great as that of the alkyl phosphates, reaching a maximum at about the third day. After the third day of exposure, PNP excretion began to decline steadily until after 10 days it was lower than that of the alkyl phosphates. By comparison, the rate of excretion of alkyl phosphates remained essentially constant throughout the entire 12 days of exposure. Neither metabolite was detected in urine 2 days after cessation of exposure.

It is evident that in poisoning cases (acute exposure) urinary PNP is a good index of exposure to parathion, and it has been used successfully as such (ATTERBERRY et al. 1971; ELLIOTT, et al. 1960; and WALDMAN et al. 1954). Although it appears to be less reliable as an index to chronic parathion exposure, the presence of PNP in urine can still serve as confirmation of parathion expo-

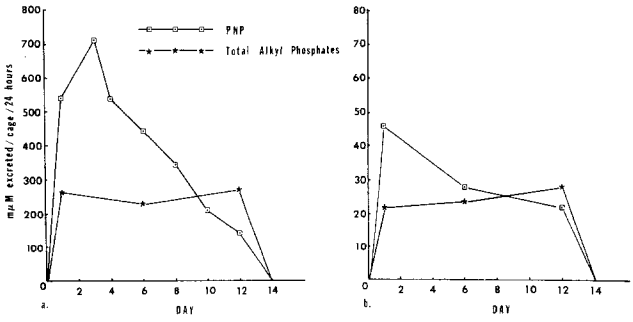

Fig. 3 Excretion of metabolites of parathion fed at
a) 0.1 LD_{50} b) 0.01 LD_{50}
The animals were dosed beginning on day 0 and daily through day 11.

sure. When used in conjunction with alkyl phosphate analysis, it can serve as a valuable tool in epidemiological investigations to differentiate between exposure to methyl parathion, ethyl parathion, and EPN.

Summary

A method for the determination of low levels of PNP in rat and human urine was developed. The method involves acid hydrolysis of urine, extraction with benzene, derivatization of PNP with diazoethane, silica gel column chromatographic cleanup and gas chromatographic determination of the ethyl ether of PNP using an electron capture detector. Recoveries of fortified rat and human urine samples averaged in the range of 85-97%. The limits of detectability were 0.05 ppm for rat urine and 0.02 ppm for human urine. The method was applied to the analysis of PNP in urine of rats exposed to 0.1 and 0.01 LD_{50} of parathion.

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