Confirmation of Paranitrophenol as a Human Urinary Metabolite at the Nanogram Level

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A process is described for confirmation of the presence of paranitrophenol as a human urinary metabolite. Paranitrophenol is determined as *p*-ethoxynitrobenzene (Shafik et al., 1973). An extract containing *p*-ethoxynitrobenzene in benzene or hexane is reduced with aqueous chromous chloride to *p*-phenetidine, which is then converted to the amide by reacting with heptafluorobutyric anhydride. Heptafluorobutryl-*p*-phenetide is determined by gas chromatographic separation on an OV-1 column using a ^{63}Ni detector. The method was applied to urine samples known to contain PNP and to urine samples determined to have PNP present at 10 ppb concentration or more. All tests confirmed the presence of PNP.

Paranitrophenol (PNP) as a human urinary metabolite is detected and measured by the multiphenol method (Shafik et al., 1973) down to levels of ten nanograms/milliliter. The method measures PNP as *p*-ethoxynitrobenzene. Analyses using this method have been conducted for a number of years to determine the extent of PNP residues in the general population.

Confirmational analysis of PNP as a human urinary metabolite has been lacking. Certain other phenolic compounds and urinary metabolites are readily confirmed by use of the Hall electroconductivity detector (HECD) in the halogen mode (Morgan, personal communication, 1977). These would include 3,5,6-trichloropyridinol from Dursban (Lorsban or chloropyrifos); 2,4,5 trichlorophenol from Gardona, lindane, 2,4,5-T, or silvex, and pentachlorophenol.

Confirmational methods for some nitro-containing pesticides have been shown to be effective at the part per million level by reduction of the nitro group to an amino group (Lawrence et al., 1977) with detection of the amino group by the Coulson conductivity detector. The procedure made use of chromous chloride as a reducing agent (Forbes et al., 1975) which has been shown to reduce a number of organophosphorus insecticides containing nitro groups. Reduction of a nitro group to an amine group has been observed to result in a loss of sensitivity when using electron-capture detection and the electroconductivity detector in the nitrogen mode as used by Lawrence is an improved alternate method of detection. The sensitivity of electroconductivity detection is somewhat dependent on the number of nitrogen atoms in a molecule and the basicity of the conductivity solvent (Hall, 1974, 1976). An attempt to use the Hall electroconductivity detector (HECD) in the nitrogen mode for measuring concentrations of *p*-phenetidine solutions found that the lower detectable limit was 2.5 ng. The HECD in the nitrogen mode proved to be less sensitive than electron capture by a factor of 20 when measuring a fluorinated derivative.

In the work described herein, a confirmation procedure was developed by making use of a combination of reactions. Chromous chloride reduction of the nitro group to an amine group (Lawrence et al., 1977) was followed by an adaptation of a method for formation of the amide (Bradway and Shafik, 1977) using heptafluorobutyric anhydride. The procedure has been tested on human urine samples known to contain PNP resulting from feeding studies with parathion and it has been used to confirm the presence of PNP as determined by the multiphenol procedure (Shafik et al., 1973).

EXPERIMENTAL SECTION

Apparatus. A Tracor Microtek 220 gas–liquid chromatograph equipped with a ^{63}Ni electron-capture detector was fitted with a borosilicate glass column $^1/_4$ in. o.d. × 4 mm i.d. × 6 ft long. The column was packed with 3% OV-1 on Chromosorb W-HP (80–100 mesh) and conditioned as described in the Manual of Analytical methods for the Analysis of Pesticide Residues in Human Environmental Samples (Thompson, 1974). Conditions for operating the chromatograph include: nitrogen flow, 90 mL/min; column temperature, 148 °C; inlet temperature, 210 °C; detector temperature, 280 °C.

Reagents. Chromous chloride was purchased as an aqueous solution, approximately 1 N from Fisher Scientific Company; heptafluorobutyric anhydride, bp 108–110 °C, was used as purchased from Aldrich Chemical Company; *p*-phenetidine, 98% pure, was purchased from Aldrich Chemical Company; benzene and other solvents used in the multiphenol procedure were distilled in glass by Burdick and Jackson. Sodium hydroxide and sodium sulfate were analytical grade reagents. Paranitrophenol, mp 112–114 °C (Eastman Chemical Co.) standard solutions were prepared in hexane with suitable dilutions.

CHEMICAL METHOD

Place a 1-mL aliquot of the solution to be tested from the Shafik multiphenol procedure containing from 10 to 200 ng of *p*-ethoxynitrobenzene in a 15-mL screwcap-type centrifuge tube and pass a slow stream of nitrogen through it for 1 min to deaerate the sample. If a significant amount of solvent evaporates, add fresh solvent to maintain 1 mL volume. Using a 1-mL syringe equipped with a 12-cm needle, transfer 0.5 mL of chromous chloride solution to the tube. Cap and shake vigorously for 1 min. Then add 5 mL of 3 N NaOH solution, shake the tube gently, and centrifuge for 5 min at 1500 rpm.

Transfer the top organic layer with a disposable pipet to a clean glass stoppered centrifuge tube. Remove any inadvertently transferred aqueous portion by adding a small quantity of hexane-washed anhydrous sodium sulfate and allow the tube and contents to stand for about 5 min. Transfer to a similar clean glass stoppered centrifuge tube, add 10 μL of heptafluorobutyric anhydride, mix, and allow the solution to stand at room temperature for 45 min. After this period add 1 mL of water, mix well, and allow the layers to separate. Inject from 1 to 5 μL of the organic

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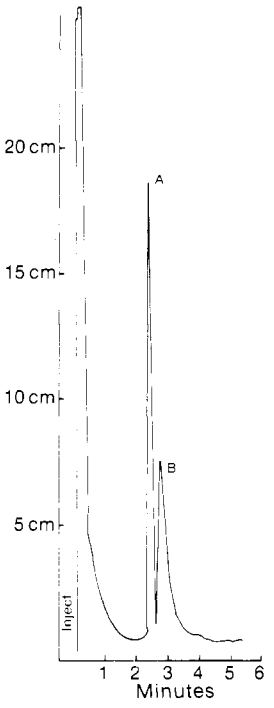

Figure 1. Emerging peaks of (A) heptafluorobutryl-*p*-phenetide (B) *p*-ethoxynitrobenzene (from the Shafik procedure) OV-1 column at 148 °C, 90 mL/min nitrogen flow, attenuation 10 × 8.

layer into the gas chromatograph.

The procedure will yield a qualitative estimation of the presence of heptafluorobutryl-*p*-phenetide and may be compared to a reference peak of the heptafluorobutryl derivative of *p*-phenetidine.

RESULTS AND DISCUSSION

Figure 1 demonstrates the shift in retention time which occurs on an OV-1 chromatographic column when *p*-ethoxynitrobenzene (peak B) is reduced and amidated to form heptafluorobutyryl-*p*-phenetide (peak A). Initial trials were made using a 5% 1,4-butanediol succinate column on Chromosorb W. However, suitable separations were not obtained. Under the conditions proposed for chromatographing, it was determined that heptafluorobutryl-*p*-phenetide would yield 40% of full-scale deflection at 10 × 8 sensitivity when 25 pg was injected. This allows confirmation of PNP at the same level as determined by the Shafik multiphenol procedure. The sensitivity of the heptafluorobutryl group suggests that the *p*-phenetide may be measured reliably at the 10 pg/μL level.

Figures 2 and 3 may be compared. Figure 2 represents the normal chromatographic response of a solution of mixed phenolic standards when chromatographed as the ethoxyl derivative prepared in the Shafik multiphenol procedure and separated on 3% OV-1 column. Figure 3 shows the chromatographic response after submitting a mixture of the same standards to the reduction–derivatization procedure. *p*-Ethoxynitrobenzene is converted to heptafluorobutryl-*p*-phenetide and is represented in Figure 3 as HFBA derivative. The transformation appears to be quantitative since no peak appears where one previously observed *p*-ethoxynitrobenzene. An unknown peak does appear in Figure 3 and appears in all chromatograms after the reduction step. However, it does not interfere with the HFBA derivative peak. The increased sensitivity of the heptafluoro group to electron capture as compared to the nitro group is seen by comparing Figures 2 and 3. Ordinarily we have noticed the heptafluoro group to be about

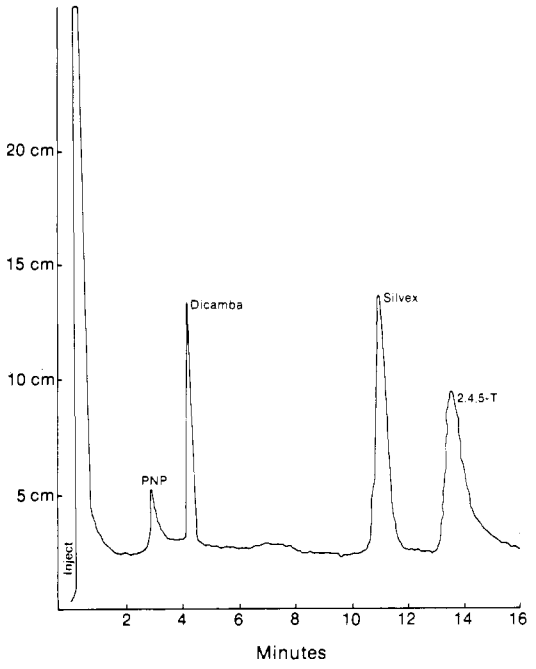

Figure 2. Electron-capture detection of 60–80% benzene in hexane fraction in Shafik multiphenol procedure prepared as ethoxyl derivatives. Standard solutions in picograms/microliter; PNP, 35; dicamba, 15; silvex, 30; 2,4,5-T, 35. Two microliters injected on OV-1 column at 148 °C, 90 mL/min nitrogen, attenuation 10 × 8.

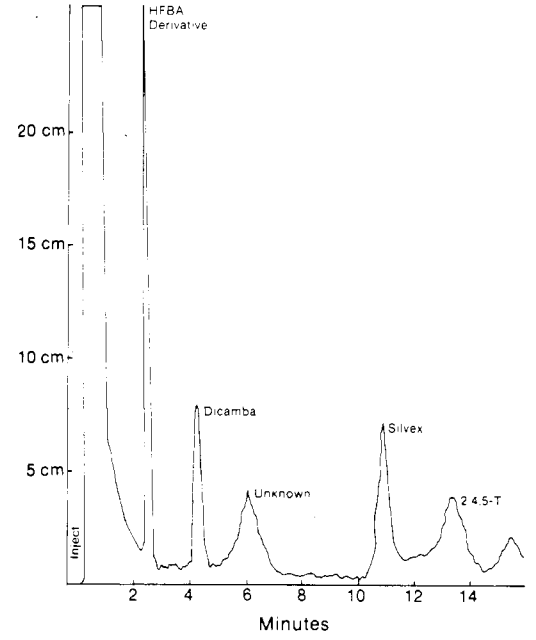

Figure 3. One milliliter of four pesticide standard solutions reacted with aqueous chromous chloride and derivatized with HFBA to form the amide. Column conditions same as in Figure 2. One microliter injected.

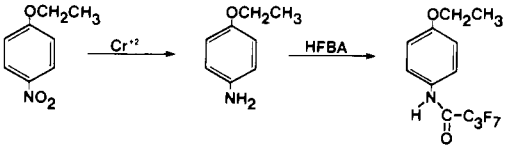

Figure 4. Reduction–derivatization of *p*-ethoxynitrobenzene.

15 or more times as responsive as a nitro group in the same type of compound.

Figure 4 shows the chemical steps involved in forming the heptafluorobutryl-*p*-phenetide derivative.

Table I. Confirmation of Paranitrophenol[a] as Heptafluorobutryl-*p*-phenetide in Urine from Parathion Exposed Subjects and from the General U.S. Population

type of sample	PNP,[a] ng/mL	rel. peak height[d] of hepta-fluoro-butryl-*p*-phenetide
blank	0	none
general U.S. population[c] 1	12	3
general U.S. population 2	21	5
general U.S. population 3	26	6
parathion exposed[b] 1	18	22
parathion exposed 2	21	27
parathion exposed 3	25	30
parathion exposed 4	32	40
parathion exposed 5	67	103

[a] Determined by Shafik (1973) procedure. [b] Samples from Morgan et al. (1977). [c] Samples provided by R. W. Kutz, Acting Chief, Environmental Monitoring Branch, Office of Pesticide Programs, Environmental Protection Agency. [d] Chart divisions observed using OV-1 column; 148 °C column temperature; 90 mL/min nitrogen flow; sensitivity 10 × 8; 4 μL injected.

Table I shows the results of applying the method to urine samples obtained from human subjects with parathion exposure and from the general U.S. population. Morgan et al. (1977) conducted experiments by feeding measured quantities of methyl and ethyl parathion to human subjects and subsequently examining the urine for nitrophenolic and alkyl phosphate metabolites. The multiphenol method of Shafik was applied and the amount of the PNP in the urine was determined. These samples had been kept frozen and were used to test the method of confirmation described herein. Table I shows that all "parathion exposed" samples had confirming peaks of heptafluorobutryl-*p*-phenetide.

Likewise, in Table I, are shown the results of testing the method with urine samples from the general U.S. population found to have measurable PNP residues. Confirming peaks of heptafluorobutryl-*p*-phenetide were also obtained from the general population samples although the peak heights were lower than for a similar amount of PNP in parathion exposed subjects. Positive confirmation was obtained. The general population samples are a portion of a series collected by the U.S. Public Health Service as a part of a Health and Nutrition Examination Survey conducted jointly with the Environmental Protection Agency. Table I also demonstrates that samples with as low as 12 ng/mL of PNP are confirmed by the method. The lowest limit of detection required is presently related only to the lower limit of detection of PNP by the multiphenol method which is about 10 ng/mL. No attempt was made to quantitate the method.

Detection of PNP as a urinary metabolite may be related to pesticide exposure. If PNP residues in humans should result from exposure to the pesticides, parathion, or EPN, it would mean a very significant amount of these pesticides are carried through the food chain or by some other means. Direct exposure of the general population to parathion or EPN would seem to be unlikely.

Aside from the fact that the method confirmed the presence of PNP residues in all samples, it is interesting to see that PNP residues are present in some segment of the human population not known to be exposed to pesticides. The measurement and confirmation of PNP residues leads to interesting questions as to the source of the PNP. Should the residue result from pesticide exposure, it would mean significant carryover from insecticide and acaracide usage. Other sources such as dyes, industrial chemicals, and flavorants must be considered.

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