Electron-Capture Gas Chromatographic Determination of 2-*sec*-Butyl-4,6-dinitrophenol (DNBP) Residues in Feed, Tissue, and Excreta

Thomas R. Edgerton* and Robert F. Moseman

Gas chromatographic methods for determining the herbicide DNBP (2-*sec*-butyl-4,6-dinitrophenol) in feed, blood, urine, feces, and tissues are presented. After extraction the sample was reacted with diazomethane to produce the methyl ether of DNBP prior to analysis by electron-capture gas chromatography. An acid alumina column cleanup was developed to remove interferences from the sample extracts. Average recoveries of greater than 85% were obtained from tissues, excreta, and feed fortified with known amounts of DNBP. Because of apparent binding or salt formation, methodology for freshly fortified and aged feed are also presented. Residue levels of DNBP from rats fed at two feeding levels are also presented.

DNBP (2-*sec*-butyl-4,6-dinitrophenol) was developed by Dow Chemical Company in 1940. The compound is a general contact herbicide used on a variety of crops for control of seedling weeds and grasses.

There is currently no FDA approved method for the analysis of DNBP residues. McKellar (1971) determined residues of DNBP and 2-amino-6-*sec*-butyl-4-nitrophenol, a possible metabolite, in milk and cream of cows fed DNBP. This was accomplished by acid hydrolysis of the milk and cream, extraction with ethyl ether, methylation with diazomethane reagent, and cleanup on an anionic alumina column. Recoveries of 80 and 98% for milk and cream, respectively, were reported. Yip and Howard (1968) determined DNBP residues on fruits and vegetables by extraction of the sample with chloroform, methylation with diazomethane, and gas–liquid chromatographic analysis of the resulting derivative. Recoveries of greater than 80% were obtained with samples fortified at the 0.2 ppm level. Guardigli et al. (1971) determined DNBP residues on various crops by thin-layer chromatography. The samples were extracted with acetone and cleaned up by alkaline hydrolysis and liquid–liquid partitioning prior to analysis.

This paper will describe a selective and quantitative method for the determination of DNBP residues in feed and rat adipose tissue, brain, liver, blood, urine, and feces.

The first step in the analytical method for urine, blood, feces, and liver consisted of acid hydrolysis to free the phenol from conjugates. The freed phenol was extracted into an organic solvent, and for liver the crude extract was cleaned up by liquid–liquid partitioning. The cleaned-up extract was then methylated with diazomethane, passed through a 3% deactivated acid-alumina column, and analyzed by electron-capture gas chromatography. Brain and adipose tissue were extracted with organic solvent and carried through partitioning and column cleanup. No hydrolysis was performed. Feed was extracted with an organic solvent, methylated, and analyzed directly by EC–GC with no column cleanup.

MATERIALS AND METHODS

Experimental. Eighteen Sherman rats weighing about 250 g each were housed in individual conventional cages having free access to water and feed. The animals were conditioned to their environment before the feeding study began.

U.S. Environmental Protection Agency, Health Effects Research Laboratory, Research Triangle Park, North Carolina 27711.

After the conditioning period the DNBP was incorporated into the feed ration as follows: One part DNBP (Technical) in acetone dissolved in two parts peanut oil was dripped into pre-mix chow and placed in a large Baker's Mixer. The concentrate chow contained 2000 ppm DNBP. Aliquots were weighed and diluted with chow to make final concentrations of 50 and 200 ppm DNBP.

Six rats were continued on a DNBP-free diet while six rats were fed 50 ppm for 138 days and six rats fed 200 ppm for same length of time. Three groups of four rats each were placed in metabolism cages for collection of urine and feces for the overnight period immediately prior to sacrifice. During this period all rats were fed plain chow. All were sacrificed after 138 days with two rats from each diet level used for tissue analysis. All tissue samples were stored in scintillation vials and frozen immediately after sacrifice. Feces and urine were stored in specimen bottles and frozen prior to analysis.

Apparatus. Tracor, MT-220, gas chromatograph equipped with a nickel-63 electron-capture detector, was operated in the pulsed linearized mode. A Borosilicate glass column (1.8 m × 4 mm i.d.) was packed with 80/100 mesh Gas-Chrom Q coated with 5% OV-210. The column was operated at 200–205 °C with 5% methane in argon at a flow rate of 60–80 mL/min. Detector, inlet, and transfer line temperatures were 250, 235, and 220 °C, respectively.

Reagents and Materials. Anhydrous, granular, sodium sulfate was Soxhlet extracted for 4 h with hexane and oven dried at 130 °C.

Acid alumina, Brockman Activity I (Fisher Scientific), was dried for 24 h at 130 °C and stored in a desiccator. Three grams of deactivated acid alumina was freshly prepared for each chromatographic column by adding 90 μL of benzene-extracted distilled water, mixing, and allowing 1–2 h for equilibration.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) should be handled carefully since it is a known carcinogen.

All solvents were pesticide quality or equivalent.

DNBP analytical reference standard, 99%, was obtained from the EPA Pesticide Repository, Research Triangle Park, N.C.

Methylating Reagent. Potassium hydroxide (2.3 g) was dissolved in 2.3 mL of distilled water in a 125-mL Erlenmeyer flask and cooled to room temperature. Twenty-five milliliters of ethyl ether was then added and the flask was cooled in a refrigerator. The following step was carried out in a glove box or well-ventilated hood. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (1.5 g) was added in small portions to the flask with vigorous shaking. The

ether layer was decanted into a scintillation vial and stored in a freezer (Stanley, 1966).

Preparation of Standard Solution. An analytical standard of DNBP was prepared in benzene and stored at −15 °C in a brown glass bottle. A solution of DNBP in benzene was methylated by adding 5 mL of diazomethane reagent in a well-ventilated hood (Howard and Yip, 1971). (CAUTION: Diazomethane is toxic and may be explosive under certain conditions.) The methylated standard was allowed to stand for 30 min before EC–GC analysis.

Chromatography of DNBP on Acid Alumina. Three grams of deactivated acid alumina was weighed into a beaker and enough hexane added to form a slurry. This slurry was then poured into a prewetted size 22-9 Chromaflex column (Kontes 420530) to which a glass wool plug and 10 mm of anhydrous Na_2SO_4 had been added. After the alumina settled, another 15 mm of anhydrous Na_2SO_4 was added. The column was prerinsed with 20 mL of hexane. When the hexane level reached the top of the Na_2SO_4 layer, a methylated DNBP standard or sample extract in 0.2 mL of hexane was placed on top of the column. Quantitative transfer of the sample was accomplished with three 0.5-mL rinsings with hexane. An additional 18.5 mL of hexane and 20 mL of 10% benzene in hexane was eluted through the column and discarded. Methylated DNBP was eluted from the column with 10 mL of 60% benzene in hexane.

Analysis of Feed, Tissue, and Excreta. *Feed.* A 2-g sample of freshly fortified feed was weighed into a 100-mL round-bottom centrifuge tube and extracted with a Willems Polytron tissue homogenizer (Johnsen and Starr, 1972) with 25 mL of benzene for approximately 2 min. After extraction, the sample was centrifuged and the supernatant layer transferred through anhydrous Na_2SO_4 into a 100-mL volumetric flask. The extraction was repeated twice more, combining the extracts and adjusting the volume to 100 mL. An aliquot was methylated and analyzed by EC–GC.

For feed samples which aged for more than 1 day after fortification a different extraction procedure was used. One gram of feed was weighed into a 150-mm screw-cap culture tube and 7 mL of 6 N HCl was added. The tube was sealed and placed in a boiling water bath for 1 h with periodic shaking. The sample was extracted twice for 1 h each on a mechanical rotator at 30–50 rpm using two 5-mL portions of 1:1 (v/v) ethyl ether–hexane. The sample was centrifuged after each extraction, and the extracts were combined. The sample was concentrated under a gentle stream of N_2 to 0.2–0.5 mL and methylated for 1 h with 5 mL of diazomethane reagent. For samples containing high levels of DNBP, no column cleanup was performed.

Adipose Tissue. Approximately 500 mg of tissue was extracted with three 3-mL portions of acetonitrile in a Duall Tissue Grinder. The sample was centrifuged after each extraction and the supernatant layers were combined in a 40-mL screw-cap centrifuge tube. The extract was partitioned with two 5-mL portions of hexane and centrifuged after each extraction, and the hexane layer was discarded. Twenty-five milliliters of acidic (pH 2) 2% aqueous Na_2SO_4 was added to the acetonitrile and subsequently extracted with two 5-mL portions of hexane. The hexane extracts were combined, concentrated to a volume of 0.3–0.5 mL under a gentle stream of nitrogen, and methylated with 5 mL of diazomethane reagent. The methylated extract was allowed to stand for 1 h before column cleanup and analysis.

Brain. Approximately 500 mg of tissue was extracted with three 3-mL portions of 1:1 (v/v) chloroform–methanol in a Duall Tissue Grinder. The sample was centrifuged, and the supernatant layers were combined in a 40-mL screw-cap centrifuge tube. The extract was evaporated under a stream of N_2 just to dryness. Nine milliliters of acetonitrile was added and carried through the same liquid–liquid partitioning described for adipose tissue. Methylation was allowed to proceed overnight prior to column cleanup.

Liver. Approximately 500 mg of tissue was homogenized with 5 mL of 6 N HCl in a Duall Tissue Grinder. The homogenate was transferred to a 150-mm screw-cap culture tube, sealed, and placed in a boiling water bath for 1 h with periodic shaking. The hydrolzyed liver homogenate was extracted twice for 1 h each on a mechanical rotator at 30–50 rpm using two 5-mL portions of hexane. The sample was centrifuged, and the extracts were combined in a 40-mL screw-cap centrifuge tube. The hexane fraction was extracted with two 5-mL portions of acetonitrile, combining the acetonitrile fractions in a 40-mL screw-cap centrifuge tube. The sample extract was carried through the same liquid–liquid partitioning and methylation as for brain.

Blood. One gram of oxalated whole blood was weighed into a 125-mm screw-cap culture tube and 3 mL of 6 N HCl was added. The tube was sealed and placed in a boiling water bath for 1 h with periodic shaking. The sample was extracted twice for 1 h each on a mechanical rotator at 30–50 rpm using 5-mL portions of 1:1 (v/v) ethyl ether–hexane. The sample was centrifuged, and the combined extracts were carried through the same concentration and methylation procedure as for fat. Since DNBP levels exceeded 10 ppm, column cleanup was found to be unnecessary in this study.

Urine. Two milliliters of urine were transferred to a 125-mm screw-cap culture tube and hydrolyzed with 0.4 mL of concentrated HCl. Extraction, concentration, and methylation were performed as previously described for blood.

Feces. One gram of dried, pulverized feces was weighed into a 150-mm screw-cap culture tube. Seven milliliters of 6 N HCl was added to the tube, shaken, and allowed to stand until frothing ceased. Hydrolysis, extraction, partitioning, methylation, and column cleanup followed the previously described method for liver. Methylation was completed in 1 h.

RESULTS AND DISCUSSION

As shown in Table I, recoveries of DNBP from fortified feed, rat tissues, and excreta averaged better than 85%. Method sensitivity is estimated at 0.1 ppm for tissue samples and excreta.

Table II lists recoveries of various levels of methylated DNBP standards through the acid alumina column. An upper limit of 10 μg of DNBP methyl ether was established as the maximum load for satisfactory column cleanup. At levels above 10 μg of DNBP methyl ether, the column exhibited a marked tailing effect. Due to the high concentration levels of DNBP in blood and feed from the feeding study, no alumina column cleanup was required.

Complete conversion of a DNBP standard to its methyl ether was accomplished in 30 min. However, a longer methylation time was required for sample extracts. Liver and brain extracts were allowed to stand overnight while adipose tissue, blood, feed, feces, and urine samples showed quantitative methylation in 1 h. Methylation efficiency for DNBP in liver extracts is shown in Table III.

At pH 6, 60% of the DNBP could be extracted from aqueous Na_2SO_4 with hexane. At pH 2 the recovery was found to be 95%. As a result, all aqueous Na_2SO_4 was

Table I. Recoveries of DNBP from Fortified Feed, Tissue, and Excreta

Sample	ppm added	No. of determinations	% range	Av % recov	% SD
Feed[a]	50	3	97-99	98	±0.6
	100	3	97-98	98	±0.7
	200	3	95-97	96	±1.5
Adipose	0.1	4	84-92	88	±3.5
	0.3	4	89-101	93	±5.3
	1.0	4	90-94	92	±2.0
Brain	0.1	4	81-88	83	±3.0
	0.3	4	84-87	86	±1.6
	1.0	4	83-88	86	±1.8
Liver	0.1	4	81-88	85	±2.3
	0.3	4	79-88	82	±4.4
	1.0	4	80-86	83	±2.7
Blood	0.1	4	83-89	85	±2.8
	1.0	4	82-90	87	±4.3
	10.0	3	85-92	88	±3.6
	30.0	3	86-92	89	±3.1
Urine	0.1	4	86-92	88	±3.0
	1.0	4	86-90	88	±2.0
	10.0	3	91-95	93	±2.3
	30.0	3	88-94	91	±2.5
Feces	0.1	4	83-89	85	±3.1
	1.0	4	82-87	85	±2.6
	10.0	3	85-95	90	±5.2
	30.0	3	91-94	93	±1.4

[a] Freshly fortified.

Table II. Typical Recoveries of DNBP from Acid Alumina Column

DNBP added, μg	DNBP recovered, μg	% recov
0.05	0.049	98
0.10	0.097	97
0.50	0.47	94
1.00	0.96	96
5.00	4.74	95
10.00	9.57	96
20.00	13.60	68
30.00	18.32	61

Table III. Methylation Efficiency[a]

Time, min	DNBP, ng
15	105
30	180
60	670
240	947
1080	960

[a] 500-mg equivalent liver extract with 1000 ng of DNBP added; 5 mL of diazomethane reagent.

acidified to pH 2 with HCl prior to use.

The choice of extracting solvent for brain was found to be critical. Various solvents plus mixtures of solvents were tried. Acetonitrile extraction yielded variable recoveries of 20–70%. Acetone, methanol, hexane, and petroleum ether plus various other solvent mixtures showed similar recovery variations. A mixture of 1:1 (v/v) chloroform–methanol was found to give the most reproducible recoveries. Acid hydrolysis of brain and adipose tissue showed no appreciable increase of DNBP from the feeding study samples. In fact, this additional step led to increased clean-up difficulties and lower overall recoveries. Figure 1 illustrates a representative chromatogram of adipose tissue from recovery studies following liquid–liquid partitioning and alumina column cleanup.

The length of extraction time for hydrolyzed liver, blood, urine, and feces was also critical. Various extraction times were attempted. At 5 and 30 min on a mechanical rotator

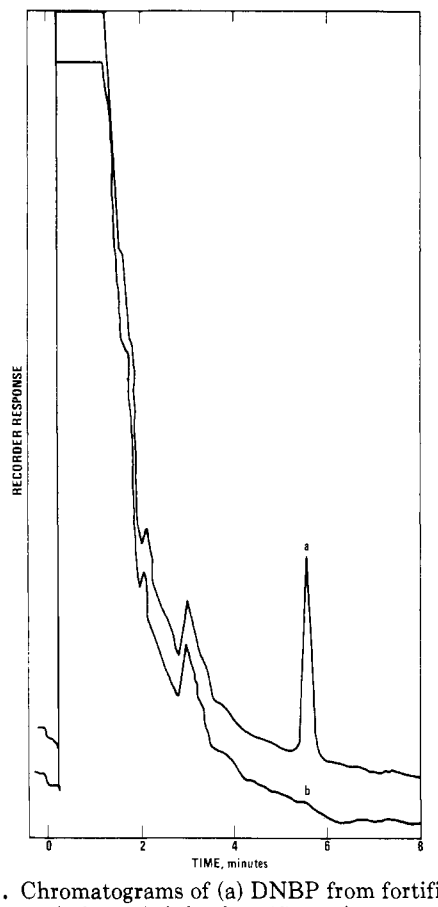

Figure 1. Chromatograms of (a) DNBP from fortified adipose tissue extract (1.0 ppm); injection, 15 mg tissue equivalent; (b) control adipose; injection 15 mg tissue equivalent. Column: 5% OV-210 on 80/100 mesh Gas-Chrom Q; oven temperature 205 °C; 5% methane in argon; flow rate 60 mL/min.

Table IV. Recovery of DNBP from Fortified Feed

Time, days	DNBP, μg added[a]	DNBP recovered, μg	Apparent % loss
0	50	48.8	
2	50	35.5	28
7	50	33.0	33
90	50	12.5	75
180 (hydrolysis)	50	46.8	

[a] Ten grams of feed with 500 μg of DNBP added. Exposed to ambient lab conditions for entire length of study.

at 30–50 rpm recoveries for the above tissues were 30 and 60%, respectively. One-hour extraction yielded an average recovery of better than 85%. Figure 2 illustrates a representative chromatogram of a liver extract from a recovery study following acid hydrolysis and alumina column cleanup.

Difficulty was encountered in the analysis of aged feed fortified with DNBP using Polytron extraction and organic solvents. Based on the communication of Dekker and Selling (1975) it was initially assumed that solvent extraction of feed without hydrolysis was sufficient. Polytron extraction was adequate for freshly fortified feed. Feed samples from the DNBP feeding study showed losses of 18% at the 50 ppm level and 19% at the 200 ppm level 25 days after fortification.

In view of these results a control experiment was done. Fortification of the feed was accomplished by pipetting a volume of benzene containing 500 μg of DNBP onto 10 g of feed. The sample was tumbled for adequate mixing and allowed to dry. The fortified feed was analyzed after

Figure 2. Chromatograms of (a) DNBP from fortified liver extract (1.0 ppm); injection, 20 mg tissue equivalent; (b) control liver; injection, 20 mg tissue equivalent. Column: 5% OV-210 on 80/100 mesh Gas-Chrom Q; oven temperature 205 °C; 5% methane in argon; flow rate 60 mL/min.

Table V. Stability of DNBP Standard

Time, h	DNBP,[a] μg	% loss
0	10.0	0
3	8.2	18
18	8.1	19
36	7.8	22
72	7.3	27

[a] 10 μg/mL standard stored in clear glass bottle. Exposed to ambient lab lighting.

drying (time 0) and monitored over a 90-day period. Results of this control experiment are shown in Table IV. Recoveries of fortified feed with acid hydrolysis averaged 92%.

A stability study with analytical standard was then initiated to determine the effect of ambient conditions on dilute DNBP standards. A dilute standard was stored on a bench top in a clear glass bottle and brown glass bottle with analyses performed at various time intervals on each standard. Table V shows the loss of DNBP in the clear glass bottle. The standard stored in the brown glass bottle showed no apparent loss over the same time interval.

Table VI. DNBP Residues from Feeding Study

Sample	50 ppm[a] feeding level[b] (ppm found)	200 ppm[a] feeding level[b] (ppm found)	Control
Adipose	0.88	3.46	<0.1
Brain	0.70	1.16	<0.1
Liver	0.29	1.06	<0.1
Blood	15.4[c]	39.7	<0.1
Urine	0.99[c]	1.01[c]	<0.1
Feces	2.07[c]	1.73[c]	<0.1

[a] Actual DNBP based on 80% technical was 40 and 160 ppm. [b] Average two determinations, two samples. [c] Average two determinations, one sample pooled urine and feces.

Because of this finding, all standards of unmethylated DNBP were kept in brown glass bottles.

DNBP fortification levels in feed based on 80% technical active ingredient were 40 and 160 ppm. Analysis of tissues and excreta from the DNBP feeding study are listed in Table VI. Adipose tissue and liver increased proportionally with the increase of DNBP in feed. Blood exhibited a threefold increase from the 40 to 160 ppm level. Amounts of DNBP in brain, feces, and urine did not appear to be dose related.

SUMMARY

Detailed methodology for determination of DNBP residues in feed, tissues, and excreta have been presented. Extraction of aged feed by the Polytron homogenizer was found to be inadequate. Acid hydrolysis of aged feed was a more efficient extraction process. A highly efficient method of hydrolysis of feed, liver, blood, urine, and feces has been presented. An acid alumina column cleanup prior to EC–GC analysis was sufficient for removal of extraneous substances in the media investigated at levels less than 10 ppm DNBP. Recoveries of greater than 85% were found in fortified feed, tissue, and excreta.

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