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Effects of Physical Binding of *o*-Nitroanisole with Feed upon Its Systemic Availability in Male F344 Rats

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Physical binding of test chemicals to feed often occurs in dose formulations prepared for uses in toxicology studies. The tenacity of this binding appears to increase with storage time of the feed formulations. The effects of this binding on systemic bioavailability are unknown. o-Nitroanisole (ONA) which is a typical example of this phenomenon, has been widely used in the dye industry as a dyestuff intermediate (Kirk-Othmer, 1963). Because of potential worker exposure, its chronic toxicity and carcinogenicity was evaluated by the National Toxicology Program in a 2-year bioassay with ONA in dosed feed formulations. Routine analysis of ONA concentrations in feed using a standard extraction procedure showed that the apparent concentration decreased with storage time. However, after the aged ONA feed samples were digested with strong acid (8.3 N HCl) , the determined concentrations were the same as those obtained from freshly prepared feed formulations. This finding was indicative of physical binding between ONA and feed components.

In this paper, we report the results of a study in which two groups of male F344 rats were dosed for 7 consecutive days with freshly prepared ONA feed formulations or 30 day aged ONA feed formulations followed by an 18 hour urine collection. To evaluate the systemic availability the urinary concentrations of ONA's main metabolites, free and conjugated o-nitrophenol (ONP), as well as the levels of creatinine were determined . The influence of ONA physical binding upon its systemic bioavailability was evaluated by comparing the total (free and conjugated) ONP urinary excretion between the two groups.

MATERIALS AND METHODS

o-Nitroanisole(lot #1517AM/04) was obtained from Aldrich Chemical Co. and the purity was found to be 99%. m-Dinitrobenzene (Lot # 107F-5011) was obtained from Sigma Co. and was used as the internal standard for rat urine ONP analysis. o-Nitrophenol (Lot #88-75-5) was obtained from Aldrich Chemical Company Inc. and used as a reference standard. HPLC grade acetonitrile (lot # A998-4, Fisher Solvents), reagent grade ethyl ether (lot # E138-1, Fisher Solvents) and reagent

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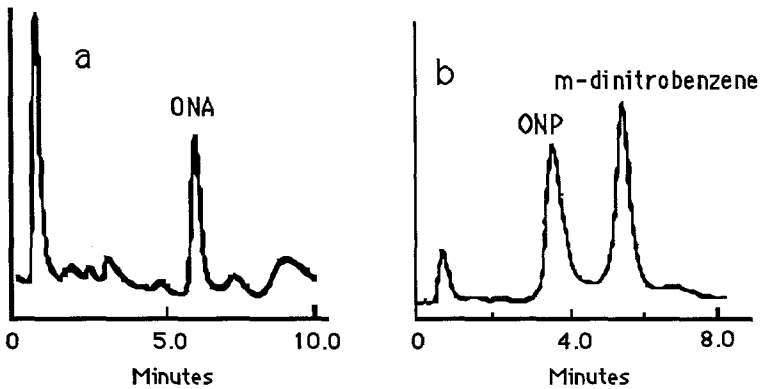

Figure 1. HPLC chromatogram of a: ONA in feed extract (0.25 mg ONA/g feed); b: ONP in rat urine (0.20 mg ONP/ml rat urine).

grade petroleum ether (b.p. 30-60 °C, lot # AU246, Burdick & Jackson) were used to extract ONA from feed. One β-glucuronidase and sulfatase mixture (lot # 73F-7185-1, Sigma) was used for treatment of all rat urine samples to convert conjugated ONP to free ONP.

A Perkin-Elmer Series 4 Liquid Chromatograph equipped with a Perkin-Elmer ISS-100 autosampler and a Hewlett Packard 1040A diode array detector was used. The column was a C8 Whatman PartiSphere column (lot 100933, Whatman Inc.) and the guard column was an Adsorbosphere C8 guard column cartridge (lot # 7049, Alltech Associates, Inc.). The elution phase was water:acetonitrile (70/30) at a flowrate of 1.5 ml/min. The detector wavelengths were 331 and 278 nm for ONA and ONP, respectively. The injection volumes were 50 and 20 µl for ONA and ONP, respectively.

Dosed feed was formulated in two steps: In the first step, a pre-mix was prepared by adding an appropriate amount of ONA to approximate 50 g NIH-07 feed and blending by spatula, then more feed was blended to the pre-mix until a third of the required total feed was added. In the second step, the pre-mix was blended with the remaining feed for 20 minutes using a Patterson-Kelly-Cross-Shell Blender with the intensifier on for 5 minutes.

For confirming the concentration of ONA in dosed feed, an aliquot (3 g) of the feed formulation was digested with concentrated hydrochloric acid (8.3N, 12 ml) at 75 °C for 45 minutes and then ONA was extracted from the digested feed three times with 13 ml aliquot of an ethyl ether and petroleum ether mixture (1:1). After transferring the solvent to a new tube, the solvent was evaporated under a stream of nitrogen gas. The oily residue was dissolved with 0.4 ml corn oil and ONA was extracted with 15 ml acetonitrile. A 3 ml aliquot of acetonitrile was diluted with 8 ml of water:acetonitrile (1:1) and filtered with a 0.45 µ Acrodisc filter into the HPLC autosampler vial. A typical HPLC chromatogram is presented in Fig. 1a. The concentration of ONA in feed samples were determined using spiked standards which

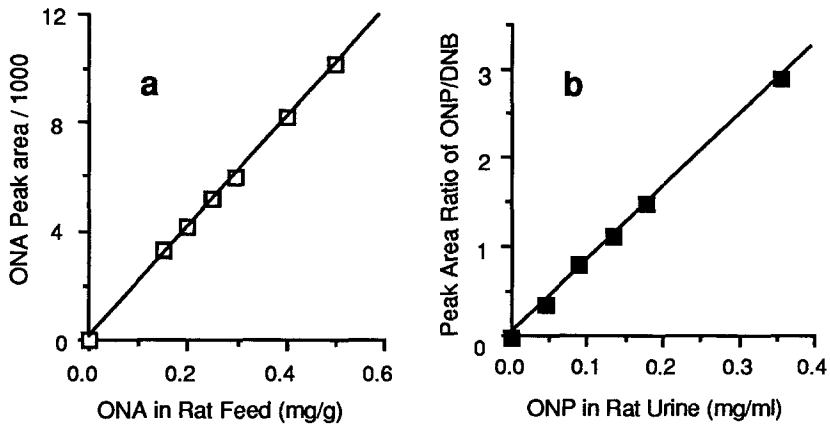

Figure 2. HPLC standard curve of a: ONA in spiked feed, b: ONP in spiked rat urine with m-dinitrobenzene as internal standard.

were prepared by adding an aliquot of one of two ONA ethanol stock solutions directly into control feed. A typical standard curve is presented in Fig. 2a.

For analysis of total ONP in rat urine, 3 ml aliquots of rat urine were mixed with 17 ml of 0.2M acetate buffer (pH=5.0) in a glass vial. To each vial, 350 units of β-glucuronidase and 15 units of sulfatase were added and incubated for 3 days at 37 ^{o}C under nitrogen. Five ml of the incubated solution were loaded onto a C8 disposable extraction column (J.T.Baker Inc. Phillipsburg, NJ) and eluted with 1 ml of acetonitrile. The eluates were then mixed with 1 ml of a 0.08 mg/ml solution of m-dinitrobenzene in water:acetonitrile (70:30) and injected directly onto the HPLC column. A typical HPLC chromatogram is shown in Fig.1b. The standards were prepared by spiking aliquots of a 0.5 mg/ml of ONP water solution into control rat urine and treating the standards as above. The standard curve was obtained by the plotting peak area ratios of ONP and m-dinitrobenzene against the ONP concentration (see Fig. 2b). Urine creatinine was quantitated by a Monarch 2000 System (Instrumentation Laboratory Co., Lexington, Massachusetts) with commercial IL test kits.

Eight week old male Fisher 344 rats (Charles River Breeding Laboratories, Raleigh, NC) were quarantined for two week prior to treatment. Control diet was made available for three hours each day (11 am to 2 pm) while water was provided *ad libitum*. After the quarantine period, the animals were weighed, tatooed and randomized by weight into two groups (A and B) of 22 rats each for a crossover design study. Each group was dosed daily for 3 hrs (11am to 2 pm) for seven consecutive days with 0.25 mg ONA /g NIH-07 feed, which had either been freshly prepared or stored for 30 days at room temperature. On day 7, an 18 hr urine sample was collected from each rat. Concentrations of total ONP and creatinine were determined. After the urine collection the rats were fed with control diet for three days and then the dose groups were crossed and the rats treated for an ad-

ditional seven days following the same dosing regimen. Again, an 18 hr urine sample was collected from each rat and was analyzed for total ONP and creatinine.

The mean body weight and the average daily feed consumption as well as the total urinary excretion of ONP of the two dose groups were analyzed using a Student t-test with 0.05 as the level of significance (Huntsberger *et al*, 1977).

RESULTS AND DISCUSSION

Physical binding of ONA to NIH-07 feed was first observed during stability studies of the dosed feed at levels used in the subchronic toxicology studies (Table 1). In those studies which utilized an acetonitrile solvent extraction step for quantitation of ONA levels, the experimentally determined concentrations in aged dosed feed samples were much lower than the theoretical concentrations. However, when the samples were extracted with acetonitrile to which hydrochloric acid was added, the determined concentrations approached the theoretical values. This finding raised the concern about the effect of physical binding on the systemic availability of ONA in rodents dosed with feed formulations which would be held for up to four weeks before being used in the bioassay.

Table 1 Physical binding of ONA to NIH-07 Feed

Storage days	HCl(%)*	Found/Theoretical**
0	0.0	100
7	0.0	74
7	0.8	80
7	2.0	89

*percent of HCl in acetonitrile extract solution.
**theoretical ONA concentration was 40 mg/g.

In the current study we selected a dosed feed concentration of 0.25 mg/g which was similar to those used in the NTP's 2 year chronic study. This concentration was chosen because it was felt that the effect on systemic availability may be more pronounced at the lower dose levels used in the chronic study. The methodology used in this current study for the analysis of ONA dosed feed utilized a rigorous acid digestion step prior to an extraction and quantitation by HPLC. The method was validated over the range of 0.15 to 0.50 mg/g using freshly spiked feed standards. The percent relative standard deviation (RSD) for the low and high standards were 2.7 and 3%, respectively, and the relative error ranged from -7.8% to +12%. The standard curve was linear over this concentration range with a correlation coefficient of 0.9960. These results showed that the method was performing satisfactorily.

The homogeneity of the blended ONA dosed feed was confirmed by taking an aliquot for analysis from each of three blender points. The determined concentration

Table 2. ONA Concentration in Fresh Dosed Feed Formulations*

Days	Found (mg/g ± SD)	Days	Found (mg/g± SD)
1	0.240 ± 0.008	11	0.240 ± 0.003
2	0.254 ± 0.010	12	0.245 ± 0.003
3	0.246 ± 0.012	13	0.250 ± 0.003
4	0.263 ± 0.008	14	0.245 ± 0.004
5	0.258 ± 0.004	15	0.241 ± 0.007
6	0.259 ± 0.009	16	0.251 ± 0.006
7	0.252 ± 0.011	17	0.256 ± 0.007

*Feed formulations were prepared, analyzed and used for dosing rats daily. On day 8,9,10, all rats were fed with control feed, on day 11 the animals were crossed over.

Table 3. ONA Concentration in Aged Dosed Feed Formulations*

Batch	Storage days	found (mg/g ± SD)	Found/Theoretical** (%)
1st batch	0	0.257 ± 0.009	103
	29	0.240 ± 0.007	96
2nd batch	0	0.240 ± 0.005	96
	29	0.240 ± 0.012	96

*Batches were used after 29 days of storage, batch 1 was used on study days 1 through 7 and batch 2 was used on study days 11 through 17.
**Theoretical ONA concentration was 0.25 mg/g.

was 0.257 mg/g with a percent relative standard deviation of 3.1%. This is within the acceptable limit of 5% which has been established by our laboratory. Daily analysis of the freshly prepared ONA dosed feed and aged ONA dosed feed is listed in Tables 2 and 3, respectively. The average concentration of ONA in blends prepared over the 14 days was found to be approximately 0.25 mg/g with a relative standard deviation of 3% which is within the expected precision of the analysis method. Aged feed concentrations varied between 0.24 and 0.257 mg/g. Note that the results demonstrate that the physical binding phenomenon was overcome by the use of the acid digestion step.

The metabolism of ONA has been previously studied (Miller *et al.*, 1985, Moldeus, *et al.*, 1976). It has been shown that biotransformation occurs rapidly after administration to rats, and approximately 71-78% of the dose was excreted into urine within 24 hrs. The major metabolites of ONA in rat urine were reported to be ONP-sulate (63%) and ONP-glucuronide (11%) and a small percentage (1.5%) was excreted as free ONP. Based on these finding, it was decided that a determination of total ONP in rat urine would be a good indicator of bioavailability. A method for

Table 4. Absolute amount of total ONP and creatinine excreted in rat urine during an 18 hr collection period after dosing with ONA feed formulations for 7 days using a crossover design*.

week	Feed	Total ONP (mg)	Creatinine (mg)
1st	Fresh	2.7±0.7	6.5±0.8
	Aged	2.6±0.6	5.7±0.8
2nd	Fresh	2.2±0.6	6.8±1.0
	Aged	2.4±0.5	6.9±1.0

*data are presented as mean±SD for 22 rats.

determination of total ONP in rat urine was developed and validated over the concentration range of 0.045 to 0.357 mg/ml, the percent RSD for the high and low standards were 4.8 and 6%, respectively. The relative error ranged from -4.8% to +7.5%. The standard curve was linear over this range with a correlation coefficient of 0.9955.

No significant differences in the rat mean body weight between the two groups were noted before or after crossover (Fig.3). The daily feed consumption data (Fig. 4) also showed that no statistical difference existed between the two groups. Therefore, it may be concluded that neither formulation had any adverse effects on the test animals.

The absolute amount of ONP found in urine during an 18 hr collection period is shown in Table 4. Since there were no statistical differences in the absolute amount of ONP in the urine between fresh and aged feed formulation groups in both the first and second weeks, the systemic availability of ONA from fresh and aged feed formulations was the same. When the average excretion of total ONP was expressed as a ratio of ONP/creatinine (Fig. 5), again no significant differences were found between the fresh and aged feed formulation groups. However, there was a significant decrease ($p<0.05$) of ONP/creatinine ratio between the first and second week groups. This may have be caused by damaged liver and/or kidney function, which has been documented in a NTP subchronic toxicity study of ONA (unpublished results). Although the intake of ONA in the second week was larger than in the first week due to the increased feed consumption, there was no apparent increase in output of ONP in urine. Therefore, it seems that the metabolism was affected by continuous low dose exposure to ONA.

The fact that no differences were found in the extent of bioavailability of ONA from fresh and aged feed is reassuring since the phenomenon of apparent decreases in dosed feed concentrations has been observed in other toxicology studies (Dux *et al*, 1983, Rollheiser *et al.*, 1979). In these cases, as in the case of ONA, the severity of the extraction method had to be increased to achieve the same recovery of analyte

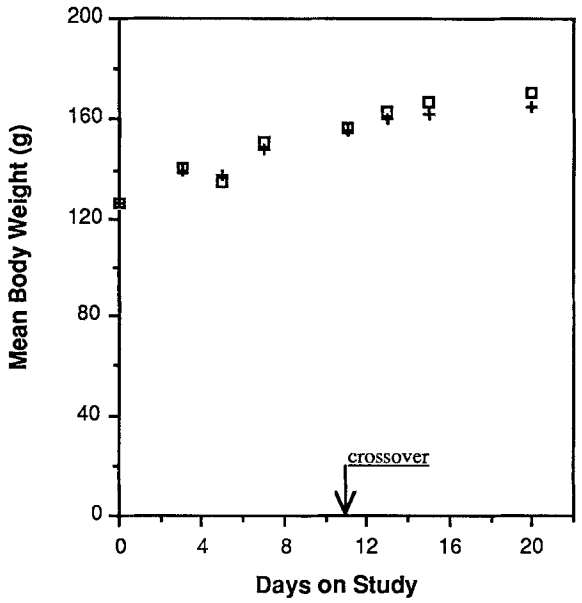

Figure 3. Growth curves for male F344 rats administered fresh or aged ONA feed formulation in a crossover design. ❑ Group A, 1st week fresh feed, 2nd week aged feed, † Group B, 1st week aged feed, 2nd week fresh feed.

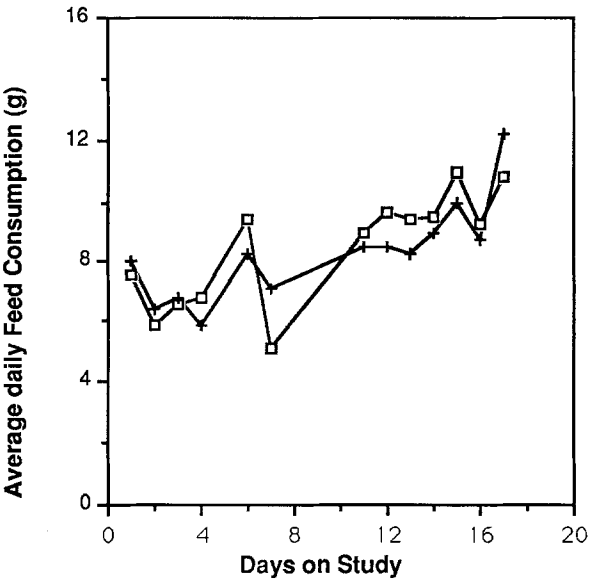

Figure 4. Average feed consumption for male F344 rats administered fresh or aged ONA feed formulation in a crossover design. ❑ Group A, 1st week fresh feed, 2nd week aged feed, † Group B, 1st week aged feed, 2nd week fresh feed.

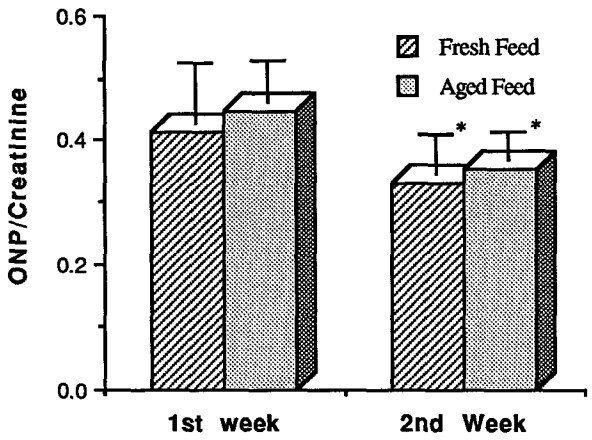

Figure 5. Average 18 hr urinary excretion of total ONP corrected for creatinine excretion after dosing with fresh or aged ONA feed formulation in male F344 rats. *P<0.05 compared to first week.

from aged dosed feed formulations as was found for freshly prepared formulation. It can be concluded from this study that the physical binding of ONA in feed formulations does not affect its systemic availability in toxicology studies.

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