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PLASMA LEVELS AND KINETIC DISPOSITION OF 2,4-DINITROPHENOL AND ITS METABOLITES 2-AMINO-4-NITROPHENOL AND 4-AMINO-2-NITROPHENOL IN THE MOUSE

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SUMMARY

Eleven groups of six ICR mice were dosed orally with 22.5 mg/kg 2,4-dinitrophenol. Groups were sacrificed at 0, 0.5, 1, 2, 4, 6, 9, 12, 24, 48, and 96 h post-treatment and plasma was collected for analysis of dinitrophenol, 2-amino-4-nitrophenol, and 4-amino-2-nitrophenol content. Analyses were performed by capillary gas chromatography—mass spectrometry after liquid—liquid extraction of plasma specimens spiked with two internal standards. Quantification was based upon peak-area ratios of base peaks obtained from the three analytes and the trideuterated internal standards 2,4-dinitrophenol and 2-amino-4-nitrophenol. Plasma concentrations for each analyte versus their respective time periods were subjected to pharmacokinetic analysis. Of the two monoamine metabolites, 2-amino-4-nitrophenol was present in the greater amount and had an elimination half-life of 46 h from plasma while that of 4-amino-2-nitrophenol was 26 h.

INTRODUCTION

Authors of several early reports have presented largely qualitative information about in vitro [1, 2] and in vivo [3, 4] metabolism of 2,4-dinitrophenol (2,4-DNP). These authors reported that nitro reduction is the major metabolic pathway for in vitro biotransformation of this compound, involving 81% of the administered dose [2]. Indeed, it has been reported that in isolated liver preparations the monoaminonitrophenol products account for 99% of the reduced metabolites generated from 2,4-DNP. In various studies it has

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been reported that 2-amino-4-nitrophenol (2A4NP) is the predominant reduced metabolite with 4-amino-2-nitrophenol (4A2NP) representing the remainder of the mono-reduced metabolites. Only approximately 1% of the total reduced metabolites were attributed to dinitro reduction and the formation of 2,4-diaminophenol (2,4-DAP).

Our current interest in 2,4-DNP and its metabolism arises from the fact that this compound is a member of the group of chemicals identified by the United States Environmental Protection Agency as priority pollutants. While various aspects of 2,4-DNP toxicity have been well characterized in the past [5], it has been quite recently observed that 4A2NP is a mutagen [6]. The significance of 2,4-DNP metabolism to yield 4A2NP is an unknown factor in the toxicological profile of 2,4-DNP. As a first step toward examining the role of biotransformation in 2,4-DNP toxicity, we have attempted to characterize the kinetics of its reduction to monoamine derivatives in vivo. In order to assess the kinetic dispostion of these phenols it was first necessary to utilize a sensitive and specific method for their analysis. A quantitative analytical technique which simultaneously determines 2,4-DNP, 2A4NP, and 4A2NP concentrations in tissue samples was developed from a method of analyzing 2,4-DNP reported previously by this laboratory [7]. Analytes were extracted from plasma samples along with appropriate trideuterated internal standards by liquid–liquid extraction techniques. Capillary gas–liquid chromatography of underivatized samples combined with multiple-ion detection (MID) mass spectrometry was used for quantitative analysis. The analytical method reported here and the kinetic data derived from this study may stimulate further study of 2,4-DNP toxicity.

EXPERIMENTAL

Subjects

Eleven groups of six ICR mice were used to study the characteristics of 2,4-DNP, 2A4NP, and 4A2NP disposition in plasma. Six animals were used as controls and received vehicle, isotonic sodium bicarbonate (1.4%). The remainder were intubated and dosed with half the 2,4-DNP oral LD_{50} in this species, 22.5 mg/kg [8], dissolved in vehicle such that 100 μl were administered per 10.0 g of mouse body weight. Groups of six mice each were killed by exsanguination at intervals of 0.5, 1, 2, 4, 6, 9, 12, 24, 48, and 96 h post-treatment. Trunk blood (typically 1 to 2 ml) was collected in commercial 3-ml blood collection tubes containing EDTA as anticoagulant (Becton-Dickinson). Samples were immediately placed on ice, then centrifuged at 1500 *g* at 4°C. Plasma was aspirated from the packed cells and stored at −60°C until analysis.

Internal standards

The trideuterated isotopically labeled derivative 2,4-dinitrophenol-3,5,6-d_3 (d_3-2,4-DNP, Merck) was used successfully in previous quantitative analyses of 2,4-DNP in tissues [7]. This compound was utilized in the present study as the internal standard for quantification of both 2,4-DNP and 4A2NP. Under the conditions employed in these analytical procedures, 4A2NP was found

to have appropriate chromatographic behavior to allow use of d_3-2,4-DNP as internal standard for it as well. This was in marked contrast to 2A4NP which was found to exhibit profound differences to 4A2NP in chemical and chromatographic properties. It was necessary to synthesize a stable isotope derivative of 2A4NP for use as an internal standard to accurately quantify this compound. A synthetic route from 2,4-DNP to 2A4NP that proceeds with sufficient yield and excellent product purity has been developed [9]. This synthetic pathway was utilized on a micro scale to produce 2-amino-4-nitrophenol-3,5,6-d_3 (d_3-2A4NP) from commercially available d_3-2,4-DNP.

An 80-mg portion of d_3-2,4-DNP was dissolved in 0.9 ml of distilled water. To this solution were added 160 mg ammonium chloride and 43 μl of 28% ammonium hydroxide. The mixture was heated to 85°C then cooled to 70°C. In five divided portions, 186 mg of fused sodium sulfide were slowly added at intervals to maintain the reaction mixture between 80 to 85°C. Upon completion of sodium sulfide addition the mixture was maintained at 85°C for 15 min longer. The hot slurry was quickly filtered through a heated micro Buchner funnel (1 ml capacity) and the filtrate cooled on ice for 4 h. Crystals of crude product were collected by filtration and pressed dry with filter paper. This material was dissolved in 0.7 ml boiling water and acidified with approximately 43 μl glacial acetic acid. The hot acidified product solution was mixed with approximately 10 mg Norit and filtered while hot. The filtrate was cooled on ice to yield bright yellow crystals of d_3-2A4NP. Upon collection this material was recrystallized from 0.5 ml boiling water, recollected by filtration and dryed in a dessicator overnight. A total of 16.1 mg of pure d_3-2A4NP product was obtained representing a yield of 24%. Mass spectrometric (see Fig. 1) and Fourier transform NMR analysis both indicated d_3-2A4NP purity exceeded 99% (no detectable *m/e* 187 ions and no detectable aromatic protons).

Assay

All specimens and standards prepared from normal human serum were subjected to a liquid—liquid extraction procedure to reduce interferences and concentrate the analytes. Samples of plasma and serum standards were all 100 μl in size and they were diluted with 400 μl of saline. A 100-μl aliquot of distilled water containing 100 ng of d_3-2,4-DNP, plus 100 μl of distilled water containing 5 μg of d_3-2A4NP was added to each specimen. All samples were acidified with 1.0 ml pH 3.0 McIlvaine's citrate buffer [10] and extracted twice with 5 ml of chloroform (Burdick & Jackson Labs.). The pooled organic phase was dryed with 60 mg anhydrous sodium sulfate (Suprapur®, E.M. Reagents), then evaporated under a stream of Zero Grade nitrogen (Air Products) while heating in a sand bath at 120°C. It was found to be crucial to remove the tubes from heat immediately before the final traces of solvent evaporated to prevent losses (via sublimation) of 2,4-DNP and d_3-2,4-DNP. Specimens were reconstituted with 5 μl methanol—pyridine (1:1) immediately prior to injection of 2 μl into the gas chromatograph.

Quantitation of 2,4-DNP, 2A4NP, and 4A2NP was performed using a Finnigan 4000 GC—MS system. The chromatographic separation of the three analytes was achieved on a 15 m × 0.25 mm I.D. DB-5 (1.0 μm film thickness) bonded-phase fused-silica capillary column (J&W Scientific) fitted with a 2-m

fused-silica capillary guard column (Alltech Assoc.). Ultrapure helium was employed as the carrier gas at a flow-rate of 29 cm/sec using splitless injection. The septum was continuously swept with helium at the rate of 10 ml/min, and 30 ml/min was used to purge the injector assembly 48 sec after injection and was maintained throughout the chromatograph cycle. Temperature parameters used for this analysis were: injector 260°C, oven 100°C for 30 sec, then heated at the rate of 6°C/min to 166°C whereupon the rate increased to 15°C/min until the oven reached 260°C which was held for 15 min. The interface chamber and transfer line were held at 260°C through which the end of the column was lead and directly inserted into the ion source assembly of the mass spectrometer. Mass spectrometer data acquisition was performed using electron ionization (ion source temperature, 250°C; emission current, 0.3 mA; electron multiplier voltage, −1400 V; electron energy, 70 V) with MID. The MID mass-to-charge ratios selected corresponded to the base peaks (molecular ions in all cases) of each analyte and the two internal standards. Masses monitored and instrument dwell times used were: *m/e* 154, 200 msec (2A4NP and 4A2NP); *m/e* 157, 100 msec (d_3-2A4NP); *m/e* 184, 100 msec (2,4-DNP); and *m/e* 187, 100 msec (d_3-2,4-DNP).

Peak-area ratios of analyte and internal standard ion peaks were determined for the serum standards containing known amounts of 2,4-DNP, 2A4NP, and 4A2NP. Linear standard curves of concentration versus peak-area ratios resulted from which unknown samples were quantified via interpolation from the regression line. Standard curves were generated each day that a batch of samples was prepared and analyses were performed. The concentration data derived for each analyte in the samples were grouped within their appropriate sampling times post-treatment and calculated mean concentration data were subjected to pharmacokinetic analysis. The ESTRIP program [11], run on a Microproducts minicomputer, was used to generate the best-fitting poly-exponential functions for the three mean concentration versus time data sets obtained in this study. These equations were used to generate curves representing disposition of 2,4-DNP, 2A4NP, and 4A2NP in plasma as a function of time with a Hewlett-Packard 8925A calculator—plotter.

RESULTS

Standards

Complete low resolution mass spectra of d_3-2A4NP, 2A4NP, and 4A2NP are shown in Figs. 1, 2, and 3, respectively. The mass spectrum of 2,4-DNP obtained under similar conditions was presented previously [7]. Each of the phenolic compounds examined in this study is sufficiently stable to yield a molecular ion base peak. Thus the two trideuterated internal standards provide base peaks three mass units greater than their corresponding unlabeled analytes. For quantification, the ratios *m/e* 184 (2,4-DNP)/187 (d_3-2,4-DNP), *m/e* 154 (4A2NP)/187 (d_3-2,4-DNP), and *m/e* 154 (2A4NP)/157 (d_3-2A4NP) were used. for 2,4-DNP, 4A2NP, and 2A4NP concentration calculations, respectively. Consistent utilization of base peaks in MID data acquisition maximized sensitivity in this quantitative assay. Specifically, the 50 ng/ml serum standard gave a 2,4-DNP peak averaging 2565 ion counts, 2A4NP yielded 3574, and 4A2NP

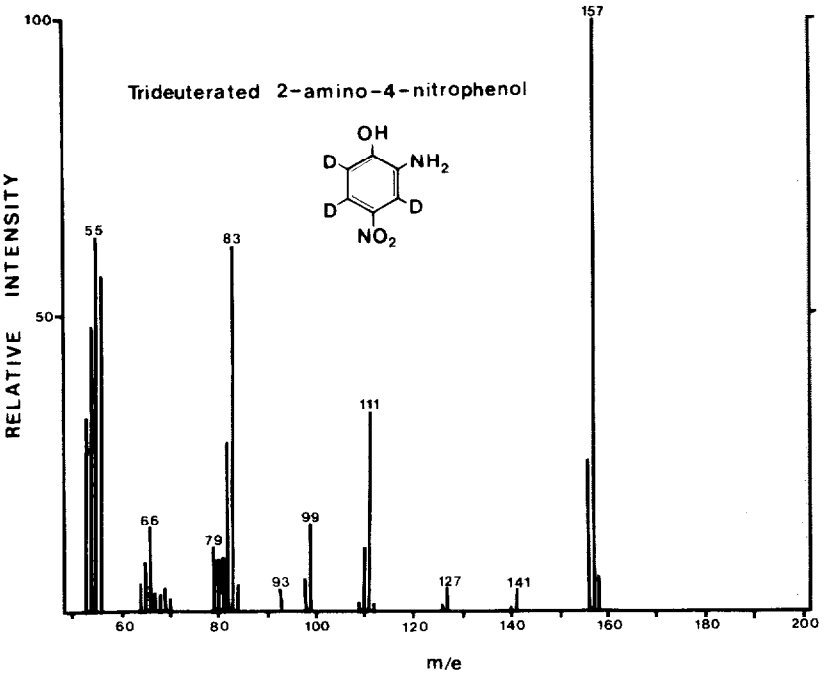

Fig. 1. Electron ionization spectrum of 2-amino-4-nitrophenol-3,5,6-d_3. The parent ion (*m/e* 157) is also the base peak of this compound. This material was synthesized from 2,4-dinitrophenol-3,5,6-d_3 which is absent from the product as demonstrated by the lack of an *m/e* 187 ion.

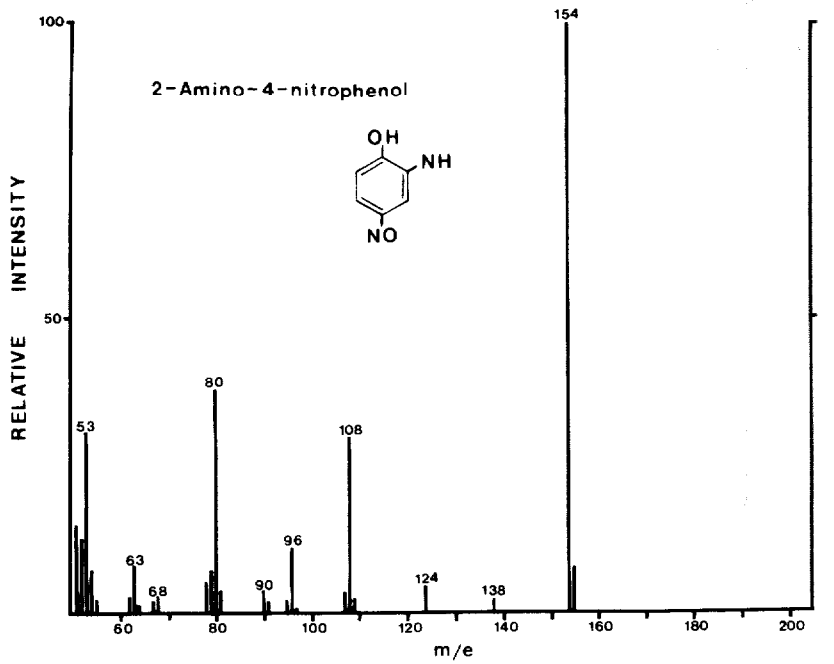

Fig. 2. Electron ionization spectrum of 2-amino-4-nitrophenol. The parent ion (*m/e* 154) is the base peak of this compound and was therefore employed in subsequent MID quantitative analyses.

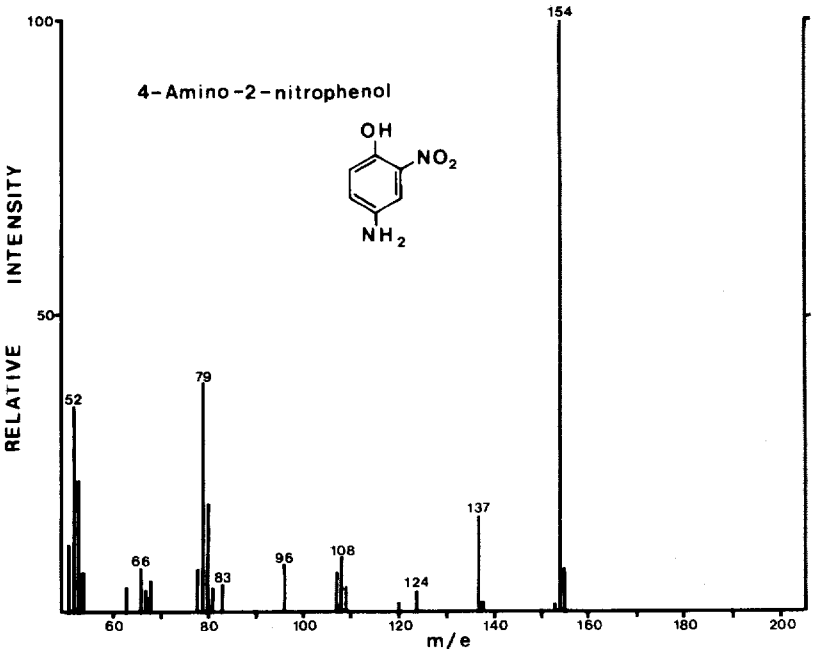

Fig. 3. Electron ionization spectrum of 4-amino-2-nitrophenol. This compound has a common parent ion and base peak at *m/e* 154. Notable differences between the spectrum of this compound and 2A4NP (Fig. 2) involve the *m/e* 108 and 138 peaks.

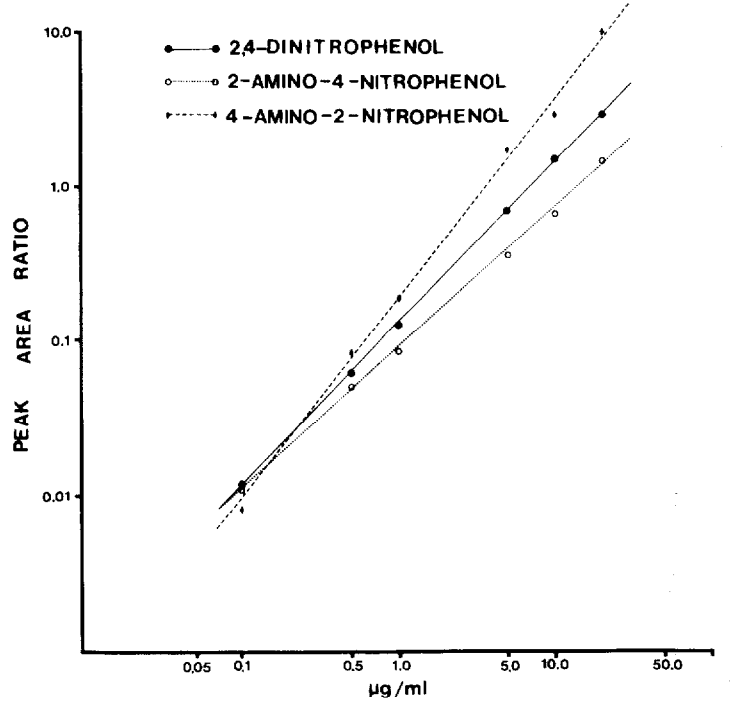

Fig. 4. Means of five standard curves generated from separate batches of samples. The individual curves from which these mean values were prepared were used to quantify 2,4-DNP, 2A4NP and 4A2NP. Standard errors of the mean are not shown as they were typically smaller than the symbols designating each value.

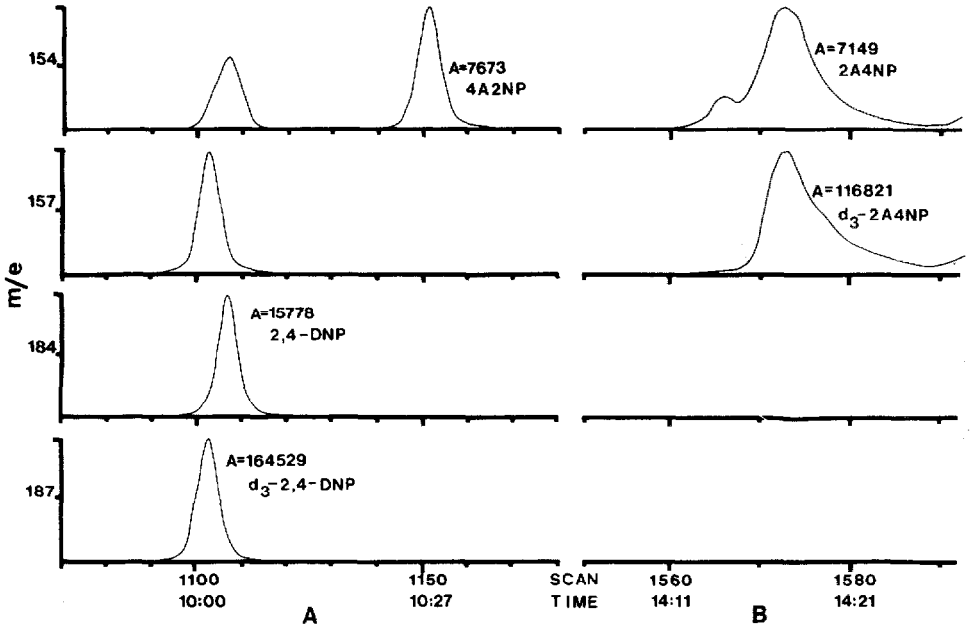

Fig. 5. A composite MID chromatogram of 2,4-DNP, 4A2NP (panel A), and 2A4NP (panel B) with their corresponding standards from a representative 1 μg/ml serum standard. The masses used in MID acquisition represent the base peaks used for quantification. This chromatogram illustrates typical retention times and peak shapes for the analytes and standards. Note that MID data segments shown in panels A and B have different time scales as they were obtained from expansion of remote chromatogram segments.

produced 770 ions. These ion currents were detected by the mass spectrometer electron multiplier and corresponded to areas of the base peaks. Sets of standards were run with each batch of samples in order to quantify that set of specimens. This was done to accommodate variability resulting from extraction, chromatographic column, or instrument changes. When the standards are examined as a group (Fig. 4), adequate reproducibility is evidenced by relatively small standard errors at each data point. Coefficients of variation between five runs for each analyte at the level of 1 μg/ml were 10.4% for 2,4-DNP, 3.5% for 2A4NP, and 20% for 4A2NP. An example of MID chromatogram data obtained from a 1 μg/ml serum standard is shown in Fig. 5. Previous investigation of 2,4-DNP extraction efficiency indicated that it was about 70% [7]. Aqueous solutions of all analytes and deuterated internal standards were found to be stable for more than a month when maintained at room temperature.

Pharmacokinetics

Results of 2,4-DNP, 2A4NP, and 4A2NP concentration measurements were averaged in each of their sampling periods and analyzed with ESTRIP. Employing the quite protracted sampling interval of four days post-treatment allowed inclusion of slow components of disposition processes in this study. Two-compartment open models were found to best represent both 2,4-DNP and 2A4NP disposition from mouse plasma. The kinetic analysis of 4A2NP

TABLE I

SUMMARY OF ESTRIP PHARMACOKINETIC PARAMETERS FOR 2,4-DINITROPHENOL, 2-AMINO-4-NITROPHENOL, AND 4-AMINO-2-NITROPHENOL IN MOUSE PLASMA

Data are based upon the best-fitting exponential equation of the form $C_t = \Sigma_{n=1}^{n=m} A_n \exp(-\beta_n t)$ where β_1 = elimination rate constant, and $\beta_m = k_{abs}$ in other notation systems.

Compound	Subscript (1→m)	Coefficient A	Exponential β	$t_{1/2}$ (h)	F*	r^2**
2,4-DNP	1	3.1	−0.067	10.3	32.9	0.995
	2	123.3	−0.606			
	3	−126.4	−2.980			
2A4NP	1	0.16	−0.015	46.2	13.2	0.982
	2	27.07	−0.318			
	3	−27.23	−8.223			
4A2NP	1	0.16	−0.027	25.7	0.8	0.875
	2	0.58	−0.065			
	3	2.89	−1.247			
	4	−3.63	−4.264			

*F = Sum of squared variances.
**r^2 = Estimate of goodness of fit between calculated function and data values.

concentration versus time data indicated that a three-compartment open model gave the best fit for this metabolite. A summary of the calculated pharmacokinetic parameters is presented in Table I. The best fitting polyexponential functions for the concentration versus time data for each analyte are plotted in Fig. 6. From the fact that 2A4NP and 4A2NP reached their maximum concentrations very rapidly it is apparent that nitro reduction of 2,4-DNP is a very fast reaction. Furthermore this data clearly indicates that 2A4NP is the major mono-reduced metabolite. Based upon calculated areas under the respective concentration versus time curves, approximately 7.9 times as much 2A4NP was formed than 4A2NP. Fully 50% of 2,4-DNP elimination appears to involve direct biotransformation to these two mono-reduced phenols in this study.

DISCUSSION

The data reported here are apparently the first to characterize the concentration versus time relationships of 2,4-DNP and its mono-reduced metabolites in plasma. Unlike many previous studies, the data presented here may be of greater practical usefulness as both oral administration and in vivo metabolism of 2,4-DNP were part of the experimental design. Furthermore, data derived from earlier studies of 2,4-DNP reduced metabolites have been obtained from use of analytical methods such as thin-layer or paper chromatography that preclude precise quantification [1]. Other studies using spectrophotometric [12] or radioisotopic techniques [2] to quantify 2,4-DNP and metabolites may lack necessary specificity. Thus use of high-resolution capillary chromatography coupled to mass spectrometric detection and quantification has

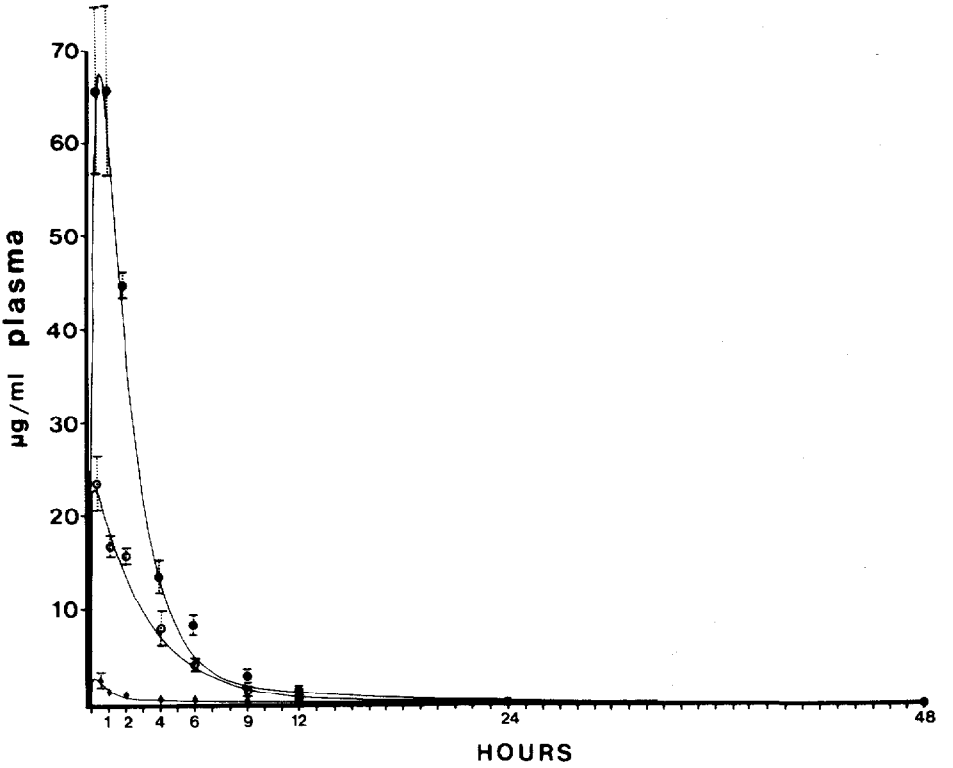

Fig. 6. Computer-plotted curves of 2,4-DNP (•), 2A4NP (○), and 4A2NP (♦) disposition in mouse plasma after 22.5 mg/kg orally. Mean values are plotted for each time period and standard errors are designated by brackets. Areas under the curves were found to be: 2,4-DNP, 207.2 µg h/ml; 2A4NP, 92.1 µg h/ml; 4A2NP, 11.6 µg h/ml. The figure shows the data to the 48-h sample time only to more clearly show curve shape at the early time periods.

provided a potential for marked improvement in selectivity and sensitivity of results reported here.

Use of a third stable isotope-labeled internal standard would fully optimize the analytical procedure for these three compounds. Unfortunately there are no synthetic pathways to produce d_3-4A2NP from d_3-DNP reported in the literature. While the reproducibility of the 4A2NP standards could have been improved upon by use of its deuterated derivative as an internal standard, the use of d_3-2,4-DNP appears to be an adequate means of quantifying this compound.

It was intended to include investigation of the 2,4-DAP metabolite in these kinetic studies but this proved to be impossible under the conditions used in this study. As a quantitatively minor 2,4-DNP metabolite [2], 2,4-DAP is of considerable toxicological interest due to its potential genotoxicity [6]. The difficulty with this compound is the fact that it very readily reacts with oxygen to form reactive quinones which possess unsatisfactory properties for extraction or chromatography on DB-5 columns. Thus characterization of 2,4-DAP kinetics awaits application of suitable sampling techniques, derivatization methods, and chromatographic techniques.

The plasma kinetics of 2,4-DNP observed here are in close agreement to those reported previously [7]. The two mono-reduced metabolites examined in this study show marked kinetic differences in their disposition from plasma. Both monoamines have elimination half-lives considerably longer than their parent 2,4-DNP. Interestingly, 2A4NP, which is produced in great excess over 4A2NP, has an apparent half-life almost twice as long as its isomer.

Quite probably there are other diverse products of 2,4-DNP metabolic processes which remain unidentified and were not examined in this study. Earlier qualitative investigations of 2,4-DNP biotransformation have identified several conjugated metabolites of 2,4-DNP including 2,4-DNP glucuronide [4], 2A4NP ethereal sulfate [4], and 2-amino-4-acetylaminophenol [14]. The majority of metabolic activity involving 2,4-DNP has been attributed by other investigators to hepatic and intestinal microsomal enzymes [3]. The ultimate fate of 2A4NP and 4A2NP may involve subsequent reductions as well as various combinations of conjugations and acetylations. Thus the overall metabolic scheme of 2,4-DNP elimination may involve a large number of intermediates and products whose contribution to its toxicity is unknown. Nevertheless, results of this study indicating that a significant amount of 4A2NP, an identified mutagen, appears in plasma suggests a potential role for this compound in the toxic manifestations of 2,4-DNP. More detailed examination of the kinetics and in vivo toxic effects of 4A2NP would be indicated on the basis of these findings.

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