Journal of Chromatography, 276 (1983) 77—84
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1724

ANALYSIS AND KINETICS OF 2,4-DINITROPHENOL IN TISSUES BY CAPILLARY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(First received January 5th, 1983; revised manuscript received March 4th, 1983)

SUMMARY

Five groups of six ICR mice were orally dosed with 22.5 mg/kg 2,4-dinitrophenol. Groups were sacrificed at 1, 3, 6, 12, and 24 h post treatment, and serum, liver, and kidney tissues were collected for analysis of dinitrophenol content. Quantitation was performed via a capillary gas chromatography—mass spectrometry technique after liquid—liquid extraction of biological specimens spiked with a trideuterated dinitrophenol internal standard. Concentration versus time data for each tissue were subjected to pharmacokinetic analysis. Similar two-compartment open models were found to characterize most phases of the disposition of this compound. The kidney appears to maintain a more persistent low concentration of 2,4-dinitrophenol.

INTRODUCTION

The compound 2,4-dinitrophenol (2,4-DNP) [51-28-5] possesses a number of well characterized biological activities [1] and toxicological properties [2]. This compound is a member of the original group of compounds identified by the United States Environmental Protection Agency as priority pollutants. While many toxicological properties of 2,4-DNP are known, its potential for more subtle genotoxic or reproductive effects remains inadequately studied [3, 4]. There is a single report in the literature describing a teratogenic interaction between insulin and 2,4-DNP [5]. Furthermore, the specific influence of pharmacokinetic factors upon reproductive toxicity is an area of developing interest in the field of toxicology. Thus, our interest in 2,4-DNP kinetics has arisen from the proposition that this factor plays a significant role in reproductive toxicity.

As is the case with most environmental contaminants, little is known about the biological fate of 2,4-DNP in vitro [6, 7], and an early study of in vivo

0378-4347/83/$03.00

kinetics has utilized rather non-specific and insensitive photometric techniques [8]. We have developed a sensitive and specific quantitative analysis for 2,4-DNP utilizing capillary gas chromatography coupled with mass spectrometry (GC—MS). This method employs a liquid—liquid extraction procedure which is applicable to biological tissue specimens. A study of the kinetic disposition of 2,4-DNP in serum, liver, and kidney tissues in the mouse was undertaken utilizing concentration data obtained with the analytical method described in this report. The kinetic data derived from this study may be of practical use in further toxicological investigations upon 2,4-DNP.

EXPERIMENTAL

Subjects

Thirty-six ICR mice were used in this study. Six animals were used as controls and 30 were orally dosed via intubation with one half the reported oral dose LD_{50} of 2,4-DNP in this species [9]. The dose was 22.5 mg/kg and it was administered as a solution in isotonic $NaHCO_3$ (1.4%) such that 150 μl of the preparation were used per 10.0 g of mouse body weight. Groups of six mice each were killed by exsanguination at intervals of 1, 3, 6, 12, and 24 h post treatment. Blood was collected, allowed to clot, and centrifuged to obtain small volumes of serum. In addition, liver and kidney tissues were collected. Serum and all tissues were stored at −20° C until analysis.

Assay

All specimens and standards were subjected to a liquid—liquid extraction procedure to clean up the samples prior to chromatography. Specimens of serum ranging in volume from 100 to 300 μl were diluted to a final volume of 1.0 ml with saline. A 100-μl aliquot of distilled water containing 1.0 μg of 2,4-dinitrophenol-3,5,6-d_3 (d_3-2,4-DNP, Merck) was added as a stable isotope internal standard to all serum samples and to standards prepared in normal human serum. Liver and kidney samples were mechanically homogenized (1:20, w/v) in saline. Homogenate (0.5 ml) was added to 0.5 ml saline to yield 25 mg tissue per 1 ml total aqueous tissue preparation in each tube. Tissue 2,4-DNP standards were prepared by adding known amounts of analyte to homogenates of control (untreated) mouse tissues. These tissue homogenates, specimens and standards, also received 100 μl of d_3-2,4-DNP internal standard solution. All samples were acidified with 1.0 ml McIlvaine's citrate buffer (pH 3.0) [10] and extracted twice with 5 ml diethyl ether (Burdick & Jackson Labs., double distilled in house before use). The organic phase was counter-extracted with two 1-ml volumes of freshly prepared 5% aqueous $NaHCO_3$. The resulting aqueous phase was acidified with 250 μl of 6 *N* HCl and re-extracted twice into 5 ml diethyl ether. The organic phase was dried with 60 mg anhydrous sodium sulfate (Suprapur®, E.M. Reagents). Following removal to a clean conical tube, the diethyl ether was evaporated away at 60° C under a stream of Zero Grade nitrogen (Air Products). Samples were reconstituted with 10 μl benzene (Burdick & Jackson Labs.) and a 1.0-μl aliquot was taken for injection into the gas chromatograph.

Quantitation of 2,4-DNP was performed using a Finnigan 4000 GC–MS

system. Chromatographic separation of 2,4-DNP was achieved on a 30 m × 0.25 mm I.D. DB-5 (1.0 μm thick film) bonded phase fused silica capillary column (J&W Scientific) with helium carrier at a flow-rate of 29 cm/sec using splitless injection. Conditions required for this separation were: injector 220°C, oven 70°C for 30 sec then programmed to 210°C at 8°C/min. At 30 sec post injection, the injector was vented with a helium flow-rate of 32 ml/min to eliminate excess solvent vapors. The septum was swept with 13 ml/min helium. The interface chamber was maintained at 250°C and the capillary column was led from the oven, through the interface, and directly 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, 0.30 mA; electron multiplier voltage, −1490 V; electron energy, 70 V) and multiple ion detection (MID) for the base peak (also molecular ion) of 2,4-DNP (*m/z* 184) and d_3-2,4-DNP (*m/z* 187) with an instrument dwell time of 0.1 sec for each mass.

Peak area ratios of analyte and internal standard ion peaks (*m/z* 184/187) were determined for the standards containing known amounts of 2,4-DNP. Linear standard curves of concentration vs. peak area ratios resulted by which unknown samples were quantified via interpolation from the regression line. Standard curves were generated each day analyses were performed using the appropriate sample matrix for the samples in question. The concentration data derived from each mouse tissue were grouped within each sampling time post treatment and then subjected to pharmacokinetic data analysis. The ESTRIP

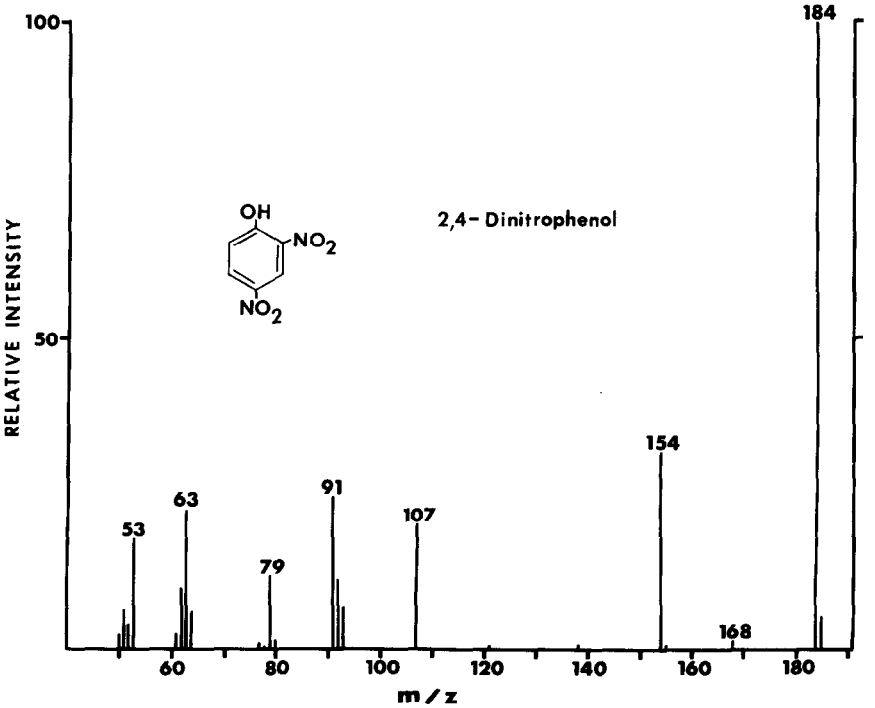

Fig. 1. Electron ionization spectrum of 2,4-dinitrophenol. The parent ion (*m/z* 184) is also the base peak of this compound. Trideuterated 2,4-DNP also yields a molecular ion (*m/z* 187) that is the base peak.

program [11], run on a Microproducts minicomputer, was used to generate the best fitting polyexponential function for the concentration vs. time data obtained in this study. These equations were used to generate the curves representing mean disposition of 2,4-DNP in all three tissues as a function of time with a Hewlett-Packard 8925A calculator and plotter.

RESULTS

Standards

A complete low-resolution mass spectrum of 2,4-DNP is shown in Fig. 1 to illustrate the suitability of molecular ion monitoring in this assay. The compound is sufficiently stable to yield a molecular ion base peak. Thus the base peak from the trideuterated internal standard is conveniently three mass units greater than that of the analyte. Sets of standards run with each batch of samples were used to quantitate that group of specimens. These daily batches of standards were intended to accommodate any variability resulting from instrumental parameter changes or column degradation. When all standard runs for each tissue are examined as a group (Fig. 2), quite good reproducibility is evidenced by small coefficients of variation at each data point. Key coefficients

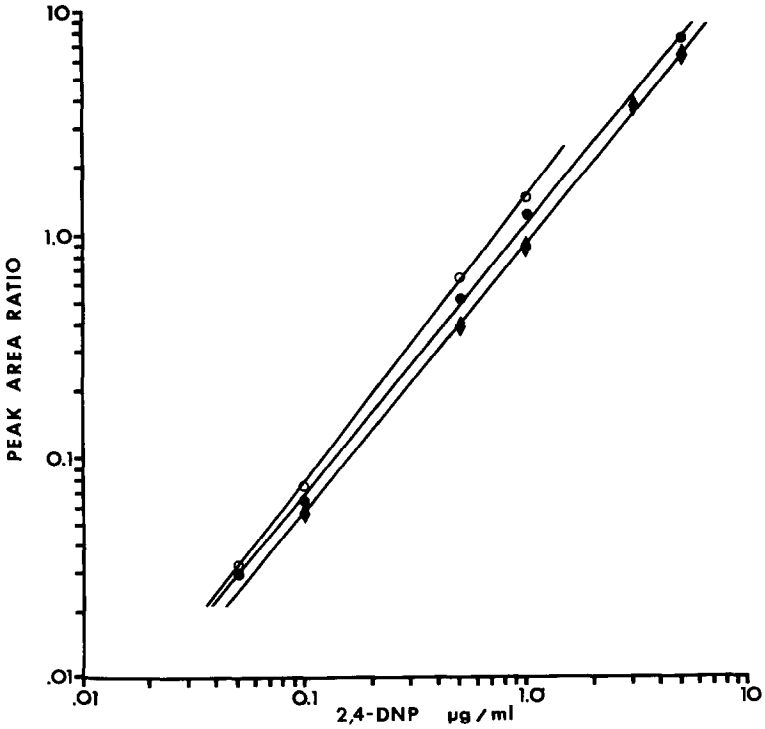

Fig. 2. Means of standard curves which were generated during each daily analysis and used to quantitate 2,4-DNP in serum, liver, and kidney tissues. Peak area ratios of 2,4-DNP and d_3-2,4-DNP base peaks were similar in the different tissue matrices. Standard errors of the mean are not shown as the errors were smaller than the symbols designating each mean value. The method exhibited satisfactory day-to-day reproducibility for all three tissues. Serum, $n = 5$ (♦); kidney, $n = 5$ (●); liver, $n = 4$ (○).

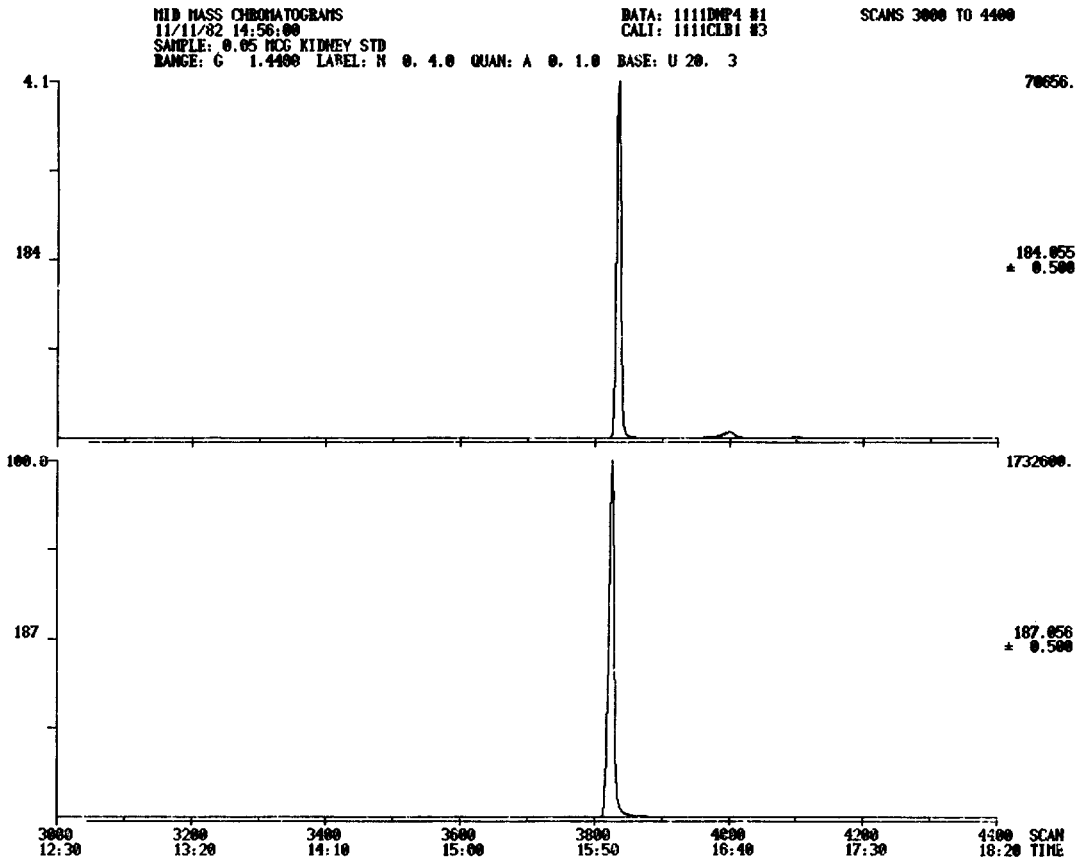

Fig. 3. Base peak mass chromatograms of 2,4-dinitrophenol and the trideuterated 2,4-dinitrophenol-3,5,6-d_3 internal standard obtained from a 50-ng (in 25 mg) kidney tissue standard. The retention time for 2,4-DNP was 16 min under the conditions employed in this assay and this standard gave a molecular ion signal of 70,656 ion counts. Note how this acidic compound exhibits little peak tailing on the DB-5 capillary column.

of variation were as follows: 1 μg/ml serum, 3.5%; 1 μg per 25 mg liver, 2.5%; 1 μg per 25 mg kidney, 10.0%. This analytical technique is also characterized by high sensitivity. Fig. 3 represents a typical ion chromatogram obtained from the 50-ng (in 25 mg tissue) kidney standard. The very high ion count (70,656) derived from this technique is suitable for other applications requiring much greater sensitivity or employing smaller samples of biological material. The method appears to be at least an order of magnitude more sensitive than required for this study. Examination of extraction efficiency of standards indicated that the double extractions used throughout this study yielded about 70% recovery of 2,4-DNP. Aqueous solutions of 2,4-DNP and d_3-2,4-DNP were found to be stable for more than a month while maintained at 4°C.

Pharmacokinetics

Results of the 2,4-DNP concentration measurements for each tissue were averaged in each sampling time period and analyzed with ESTRIP. With the minimal number of sampling intervals used in this study, a two-compartment open model was found to best represent the disposition of 2,4-DNP in all tissues. The summary of calculated kinetic parameters is presented in Table I. It is apparent from the quite similar half-times for absorption, distribution, and elimination (except in kidney), observed in these three tissues that rapid 2,4-DNP exchange occurs between these sites. Major differences are seen in the coefficients of the exponential equations indicating different relative peak concentrations of 2,4-DNP among the tissues. In Fig. 4 we have plotted the best fitting functions for the concentration vs. time data for each tissue. Here it is very clear that the primary difference among the serum, liver, and kidney kinetics is the maximum concentration attained and the uniquely slow elimination from the kidney. Curve shape is obviously similar with calculated peak concentrations occurring at the same time, approximately 1 h post treatment.

TABLE I

SUMMARY OF ESTRIP PHARMACOKINETIC PARAMETERS FOR 2,4-DINITROPHENOL IN THE MOUSE

Data are based upon the best fitting triexponential equation of the form $C_t = A_3\exp(-B_3t) + A_2\exp(-B_2t) + A_1\exp(-B_1t)$ where $B_1 = \beta$; $B_2 = \alpha$; and $B_3 = k_{abs}$ in other notation.

Tissue	Subscript	Coefficient (A)	Exponential (B)	$t_{1/2}$ (h)	F*	r^2**
Serum	1	2.821	0.09	7.70	6.81	0.997
	2	165.300	0.57	1.20		
	3	−168.100	1.39	0.50		
Liver	1	0.213	0.08	8.70	1.77	0.974
	2	44.410	0.69	1.00		
	3	−44.620	1.17	0.59		
Kidney	1	0.173	$9.1 \cdot 10^{-3}$	76.20	2.87	0.971
	2	46.410	0.61	1.14		
	3	−46.590	1.11	0.62		

*F = sum of squared variances.
**r^2 = estimate of goodness of fit between calculated function and data.

DISCUSSION

The description of 2,4-DNP kinetics reported here is apparently the first such data based upon a highly sensitive and specific GC—MS analytical technique. Many years ago a spectrophotometric technique was developed for quantitating 2,4-DNP [6]. This relatively nonspecific method was used in an elegant kinetic study of disposition in serum and ocular tissues of the duck and rabbit [8]. In that study of the relationship between kinetics and cataractogenic potential of 2,4-DNP, clearance from duck serum was found to be a biexponential process with rate constants similar to those reported here in the mouse. Various other analytical techniques have also been applied to the

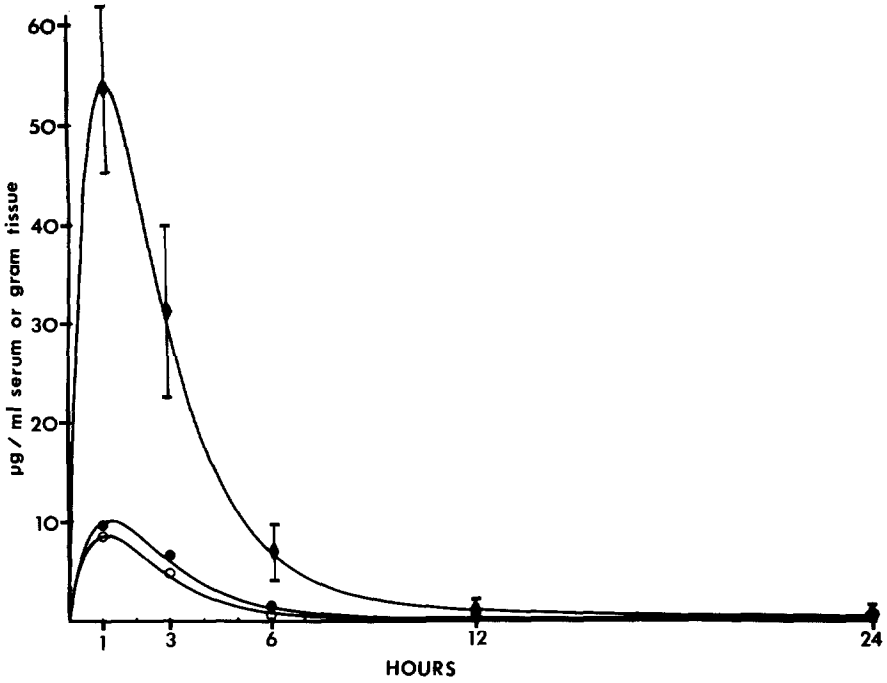

Fig. 4. Computer-plotted curves of 2,4-dinitrophenol disposition in mouse serum (♦), liver (○), and kidney (●) after oral administration of 22.5 mg/kg. Mean values are plotted for each time period and standard errors for the serum data are designated by brackets. Liver and kidney S.E.M. values were too small to be plotted. It is apparent that the major difference among these curves is their magnitude.

qualitative determination of 2,4-DNP. An extremely sensitive bioluminescent assay has been reported quite recently [12]. Also, a high-performance liquid chromatographic technique has been described which is very sensitive [13]. While these techniques are very capable of quantitating trace amounts of 2,4-DNP, their selectivity may be questionable when dealing with complex biological materials. These methods have not been applied to the assessment of 2,4-DNP kinetics in tissues.

Prior to the development of capillary columns with covalently bonded liquid phases, gas—liquid chromatography of polar compounds had been difficult. Derivatization of 2,4-DNP in industrial effluents has been attempted to improve gas—liquid chromatographic separation, but this was reported to result in little improvement [14]. Under the conditions used in this analytical technique, excellent chromatographic resolution was attained with the very acidic 2,4-DNP molecule in underivatized condition. We had originally hoped to apply a technique similar to this analysis to quantify selected metabolites of 2,4-DNP. The nitro-reduced metabolites are of particular interest as they have been identified in vitro [7], and the 4-amino-2-nitrophenol species has been identified as a potential carcinogen [15]. Technical difficulties associated with extraction of the metabolites have to date foiled attempts to analyze these amino derivatives in biological samples in our laboratory. Pure 4-amino-2-nitrophenol does elute from the DB-5 capillary column (data not shown)

under the conditions employed for 2,4-DNP analysis as do acetylated derivatives of 2,4-DNP, 4-amino-2-nitrophenol and 2-amino-4-nitrophenol. Future work with reduced metabolites may utilize such acylated derivatives.

The pharmacokinetics reported here must be viewed as preliminary data. Since a two-compartment open model seems to fit observed concentration vs. time data in all tissues examined, additional time points should be utilized to more accurately characterize each phase of disposition. Ideally, at least four data points should be employed in defining each exponential term of the kinetic function. The merit of this study and, indeed, the major point of interest lie in the fact that parallel samples were obtained and that the results indicate a quite close correspondence in concentrations among these tissues at all sampling times. The very close similarities in absorption and distribution may well result from the fact that 2,4-DNP is soluble in both lipids and water. The apparent persistence of 2,4-DNP in the kidney tissues ($t_{1/4}$ 76 h) may be a result of tissue binding of this compound, since protein binding is a recognized phenomenon with this material [8]. While 2,4-DNP binding sites have been assumed to exhibit uniform affinity, our data suggest that there may exist some differences among tissues.

ACKNOWLEDGEMENTS

This work was supported in part by Biomedical Research Development Grant 1-S08-RR-09171-03. The authors wish to thank Teresa Garland for typing the manuscript.

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