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Excretion of formaldehyde, malondialdehyde, acetaldehyde and acetone in the urine of rats in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, paraquat, endrin and carbon tetrachloride

M. A. Shara, P. H. Dickson, D. Bagchi and S. J. Stohs*

School of Pharmacy and Allied Health Professions, Creighton University Health Sciences Center, California at 24th Street, Omaha, NE 68178 (USA)

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ABSTRACT

Formaldehyde (FA), acetaldehyde (ACT), malondialdehyde (MDA) and acetone (ACON) were simultaneously identified in urine, and their excretion quantitated in response to chemically induced oxidative stress. Urine samples of female Sprague–Dawley rats were collected over dry ice and derivatized with 2,4-dinitrophenylhydrazine. The hydrazones of the four lipid metabolic products were quantitated by high-performance liquid chromatography on a Waters 10-μm μ-Bondapak C_{18} column. The identities of FA, ACT, MDA and ACON in urine were confirmed by gas chromatography–mass spectrometry. An oxidative stress was induced by orally administering 100 μg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 75 mg/kg paraquat, 6 mg/kg endrin or 2.5 ml/kg carbon tetrachloride to rats. Urinary excretion of FA, ACT, MDA and ACON increased relative to control animals 24 h after treatment with all xenobiotics. The system has wide-spread applicability to the investigation of altered lipid metabolism in disease states and exposure to environmental pollutants.

INTRODUCTION

The peroxidation of membrane lipids is associated with a wide variety of toxicological effects, including decreased membrane fluidity and function, impaired mitochondrial and Golgi apparatus functions, inhibition of enzymes associated with various organelles including the endoplasmic reticulum, and impaired calcium homeostasis [1,2]. In many human diseases, membrane damage often occurs in an organ or tissue, which provokes lipid peroxidation and accelerates the disorder [3]. When lipid peroxides and peroxidation products accumulate, they leak from the organ or tissue into the bloodstream and may be excreted in the urine [1,3]. Lipid peroxidation reflects the interaction between molecular oxygen and polyunsaturated fatty acids, resulting in the oxidative deterioration of the latter with the production of various breakdown products including alcohols, aldehydes, ketones and ethers [4,5].

The detection of lipid peroxidation products in the urine provides a non-invasive method of assessing lipid metabolism and oxidative stress. Ekstrom *et al.* [6] reported the detection of urinary malondialdehyde (MDA) after derivatization with 2,4-dinitrophenylhydrazine (DNPH), and separation of the adducts by high-performance liquid chromatography (HPLC). Identification of the hydrazone derivative of MDA was based on HPLC retention time. Ekstrom *et al.* [7] also confirmed the identity of the MDA hydrazone standard by means of mass spectrometry (MS). However, the identity of the MDA hydrazone

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from urine which had been derivatized with DNPH was not confirmed by MS. Furthermore, no attempt was made to identify any of the other peaks which could be separated by HPLC. In the present study, four lipid metabolites have been identified in the urine of rats by HPLC and gas chromatography–mass spectrometry (GC–MS), and the effect of free radical-induced cell injury by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), paraquat, endrin and carbon tetrachloride on the excretion of these metabolites has been examined.

EXPERIMENTAL

Animals

Female Sprague–Dawley rats, weighing 140–160 g (Sasco, Omaha, NE, USA), were used in these studies. All animals were acclimated three to five days prior to use. TCDD was obtained from the Chemical Resource Program, National Cancer Institute (Bethesda, MD, USA). TCDD was dissolved in corn oil containing 10% acetone, and was administered intragastrically at a single dose of 100 μg/kg [8]. The herbicide paraquat was dissolved in water and given orally at a single dose of 75 mg/kg [1,9]. The chlorinated cyclodiene insecticide endrin [10] and carbon tetrachloride [1,11] were dissolved in corn oil and administered orally at single doses of 6.0 mg/kg and 2.5 ml/kg, respectively. Control animals received the corresponding vehicles.

Urine collection

Rats were placed in metabolism cages (Nalgene, Rochester, NY, USA) for urine collection between 21.75 and 26.25 h after treatment. During urine collection, the animals were allowed free access to tap water but received no food. The urine-collecting vessels were positioned over styrofoam containers filled with dry ice which permitted the collection of urine in the frozen state over the 4.5-h period. The collected urine was also free from contamination of food particles since the animals received no food during the urine collection period.

Derivatization and extraction of lipid metabolites

DNPH was used as the derivatizing agent in the identification and quantitation of urinary metabolites. DNPH (310 mg) was dissolved in 100 ml of 2 *M* hydrochloric acid to make the derivatizing reagent. In 50-ml screw-capped PTFE-lined tubes, 1.0-ml aliquots of urine, 8.8 ml of water and 0.20 ml (3.13 μmol) of DNPH reagent were mixed, followed by the addition of 20 ml of pentane. The tubes were intermittently shaken for 30 min, and the organic phases were removed. The aqueous phases were extracted with additional 20-ml aliquots of pentane. The pentane extracts were combined, evaporated under a stream of nitrogen in a 37°C water bath and reconstituted in 0.40 ml of acetonitrile. A 20-μl aliquot of each sample was injected onto the HPLC column and the peaks were isocratically eluted as described below.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 510 pump (Milford, MA, USA), a Model U6K Waters loop injector, a Waters μ-Bondapak C_{18} (10 μm particle size, 125 Å, 30 cm × 3.9 mm I.D.) reversed-phase column fitted with a Rainin RP-18, 5-μm OD-GU pre-column (Rainin, Woburn, MA, USA), a Waters Model 484 tunable absorbance detector and a Fisher Recordall (Series 5000) strip chart recorder. The acetonitrile–water (49:51, v/v) mobile phase was filtered through a Rainin Nylon-66 membrane filter (0.45 μm pore size), degassed using a Millipore filtration kit (Rainin) and pumped at a flow-rate of 1 ml/min. The detector was set at a wavelength of 330 nm and 0.01 absorbance units full scale (a.u.f.s.). The chart recorder speed was 0.25 cm/min. Following injection of a sample, the isocratic elution was carried out for 40 min.

Hydrazone standards

Synthetic hydrazone derivatives were prepared by reacting 30 ml of DNPH stock solution with an excess (1–3 mmol) of formaldehyde (FA), acetaldehyde (ACT), MDA or acetone (ACON). The reaction proceeded rapidly at room temper-

ature. The precipitated hydrazones were filtered, dried and recrystallized from methanol. Solutions containing 50 ng/μl of the four synthetic hydrazones were prepared and chromatographed as described above. Urine samples were spiked with known amounts of each of the synthetic hydrazones to identify and/or confirm the urinary hydrazones by HPLC co-elution.

Acetonitrile solutions (100 μg/ml) of the four synthetic hydrazones were prepared, and a UV–visible scan between 500 and 300 nm was obtained in a Perkin-Elmer Lambda 6 spectrophotometer in order to determine the absorption maxima for the four hydrazones.

To calculate the percentage extraction recoveries, 0, 0.5, 1, 2, 3 or 5 μg of each of the synthetic hydrazones of FA, ACT, MDA and ACON were added to 1.0-ml urine samples obtained from control animals during a single collection. The same extraction procedure described above was applied and the percent recoveries were calculated.

Gas chromatography–mass spectrometry

In order to determine the identity of lipid excretion products in the urine, GC–MS analyses were performed. The GC–MS system consisted of a Hewlett-Packard Model 5890 gas chromatograph (Fullerton, CA, USA) with a 15 m × 0.32 mm I.D. capillary column 0.25 μm film thickness (Supelco SPB-5, Bellefonte, PA, USA) which was connected directly to the mass spectrometer via a heated transfer line. The transfer line temperature was maintained at 250°C. The carrier gas was helium at an average linear velocity of 65.8 cm/s, and the injector temperature was 230°C. The injector was operated in the splitless mode. A temperature program was used which consisted of a starting temperature of 75°C which was increased to 175°C at increments of 25°C/min. Between 175 and 200°C the temperature was increased at a rate of 5°C/min, and finally to 300°C at increments of 25°C/min. The mass spectrometer was a Finnigan-MAT Model 50 B quadrupole instrument (Palo Alto, CA, USA) in combination with an INCOS data system. The instrument was set on electron ionization mode. The ion source temperature was 180°C, and the ionization energy was 70 eV. The system was coupled to a Data General Model DG 10 computer (Southboro, MA, USA) and a Printronix Model MVP printer (Irvine, CA, USA).

For GC–MS analysis, the four hydrazone derivatives of FA, ACT, MDA and ACON were dissolved in chloroform (50 ng/μl). Similarly, hydrazine-derivatized urine samples were reconstituted in chloroform. Samples (2 μl) of standards and extracts were injected onto the GC–MS system.

Following the full-spectrum identification of each of the hydrazones, a selective ion monitoring (SIM) program was prepared, and additional spectra were obtained in the SIM mode.

Statistical methods

Significance between pairs of mean values was determined by Student's t-test. A $P < 0.05$ was considered significant for all analyses.

RESULTS

Identification of urinary lipid metabolites

Utilizing HPLC and GC co-elution methods as well as MS techniques, MDA, FA, ACT and ACON were identified as urinary lipid metabolites.

UV–visible scan studies indicated that the absorption maxima of the four sythetic hydrazones of MDA, FA, ACT and ACON were 307, 349, 359 and 362 nm, respectively. Therefore, 330 nm was routinely used to monitor these compounds by HPLC. The extraction recoveries of the MDA, FA, ACT and ACON hydrazones were 74, 78, 88 and 91%, respectively, based on studies involving the addition of known amounts of the hydrazone derivatives to control urine.

Fig. 1A depicts a typical HPLC profile of the four hydrazone standards, while Fig. 1B and C contain representative HPLC profiles form urine of control and TCDD-treated rats, respectively. Fig. 2A is a typical GC–MS elution profile of the hydrazones of standard FA, ACT, MDA and ACON. The retention times for the standards exactly corresponded with the chromatographic

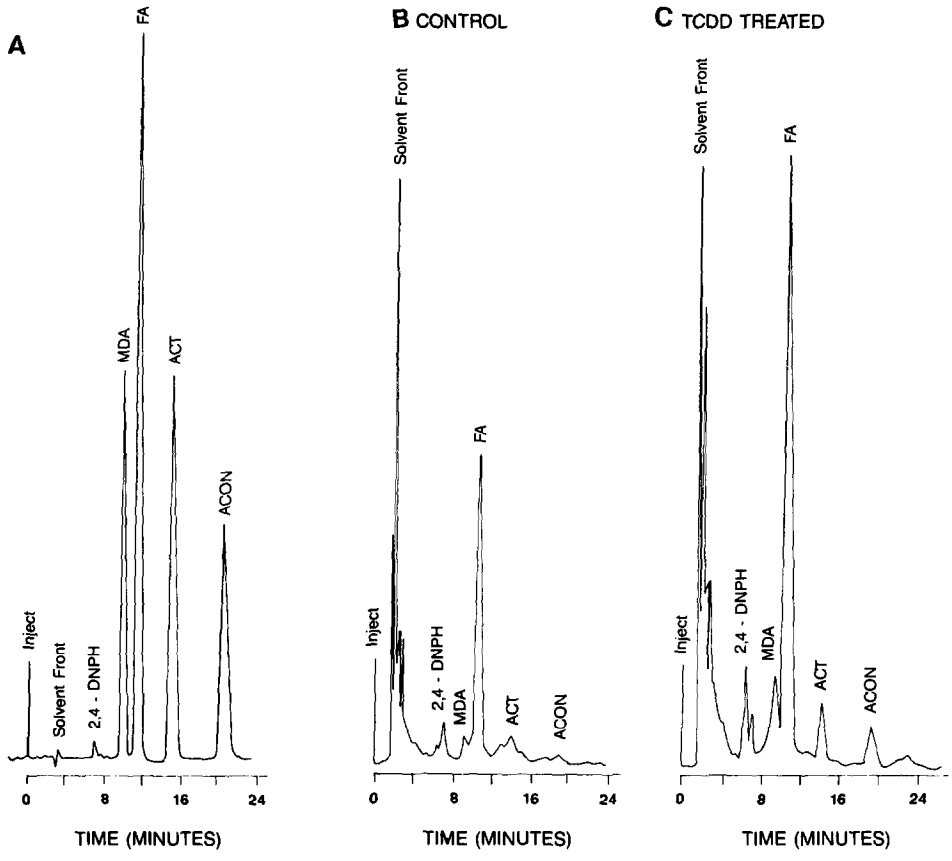

Fig. 1. Chromatograms of DNPH derivatives of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON). (A) Standards; (B) urine from control animals; (C) urine from TCDD-treated animals. A 20-μl volume of each sample was injected.

peaks for extracts of urine, and co-elution occurred when the standards were added to the urine samples for both HPLC and GC. Urine samples were also spiked with synthetic hydrazone samples to identify and confirm the urinary hydrazones by co-elution.

The order of elution of the four hydrazones upon GC was similar but not identical to that of the HPLC. Fig. 2B is a typical full-scanning GC–MS profile of the hydrazone derivatives extracted from a urine sample. There are two notable differences as compared to the HPLC profile. The FA derivative (5.4 min) eluted first upon GC, and the ACON derivative (7.4 min) eluted last. The order of GC elution of MDA and FA was reversed in contrast to the order observed on HPLC. In addition, the ACT derivative exhibited two peaks (6.3 and 6.6 min), corresponding to its *syn* and *anti* isomers which were separated by GC but not by HPLC (Figs. 1A and 2A).

The MS data for the four lipid metabolites are presented in Figs. 3–6. The molecular ions 210, 234, 224 and 238 of the synthetic hydrazones of FA, MDA, ACT and ACON, respectively, were identified (Figs. 3A, 4A, 5A and 6A). The same molecular ions were demonstrated in extracts of urine samples following GC–MS (Figs. 3B, 4B, 5B and 6B). It should be noted that the MS of urinary MDA (Fig. 4B) also contains peaks of FA since the GC separation of the MDA and FA was incomplete. However, the results clearly show that FA, MDA, ACT and ACON were excreted in the urine of rats.

SIM was also used to provide further confir-

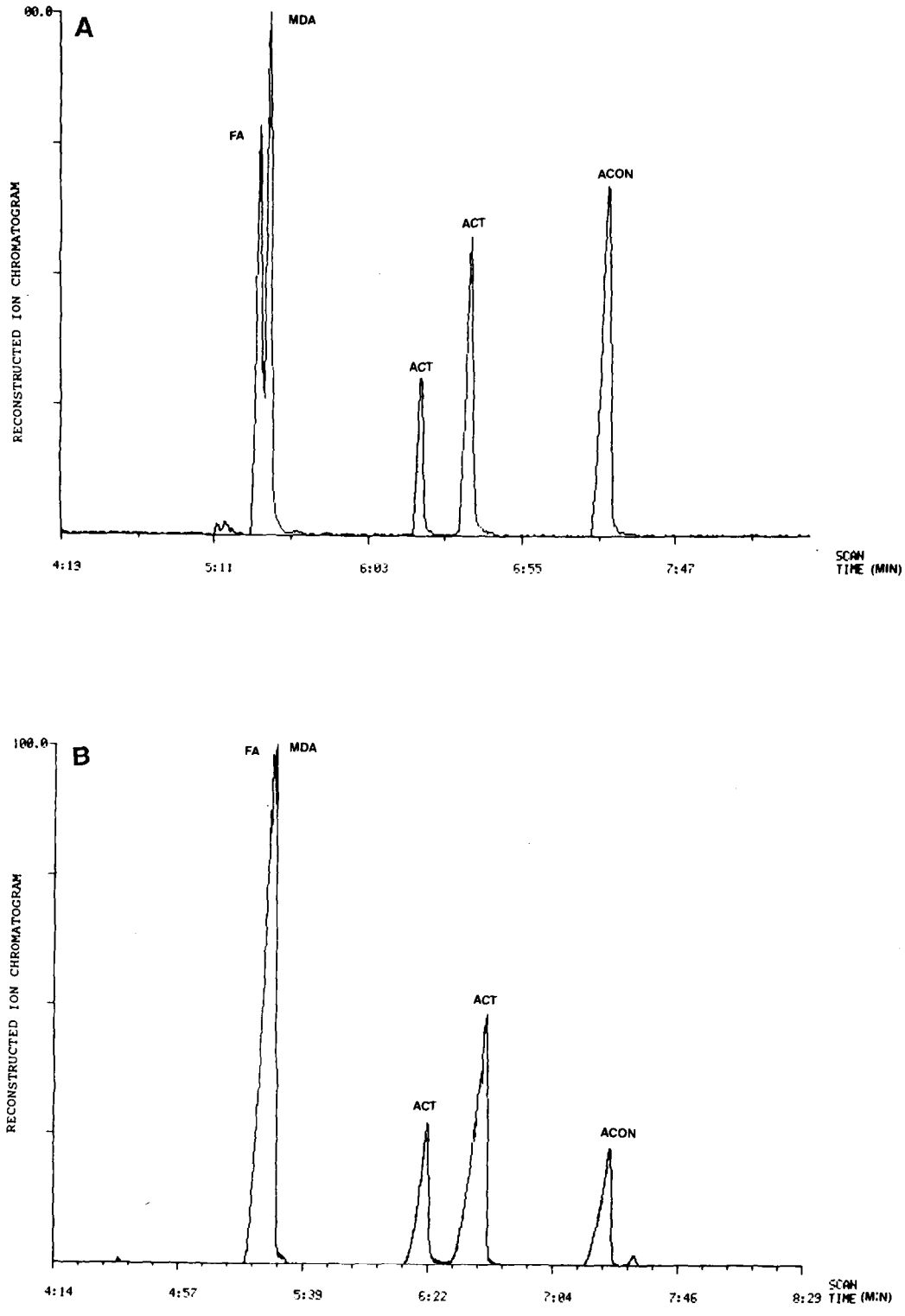

Fig. 2. (A) Full-scanning GC–MS of DNPH derivatives of formaldehyde (FA), malondialdehyde (MDA), acetaldehyde (ACT) and acetone (ACON) standards; (B) full-scanning GC–MS of rat urine.

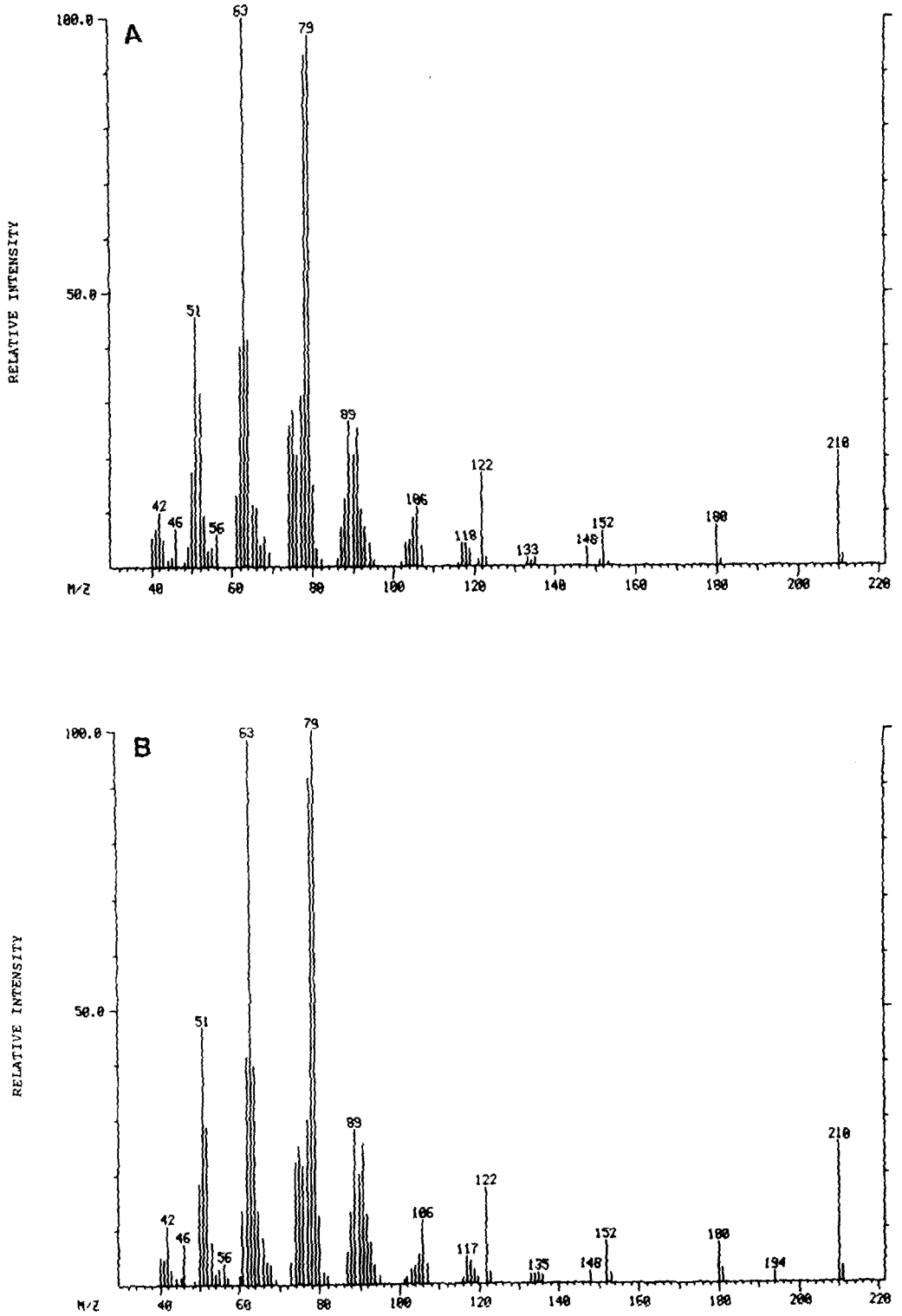

Fig. 3. (A) Mass spectrum of DNPH derivative of formaldehyde standard; (B) mass spectrum of DNPH-derivatized formaldehy rat urine.

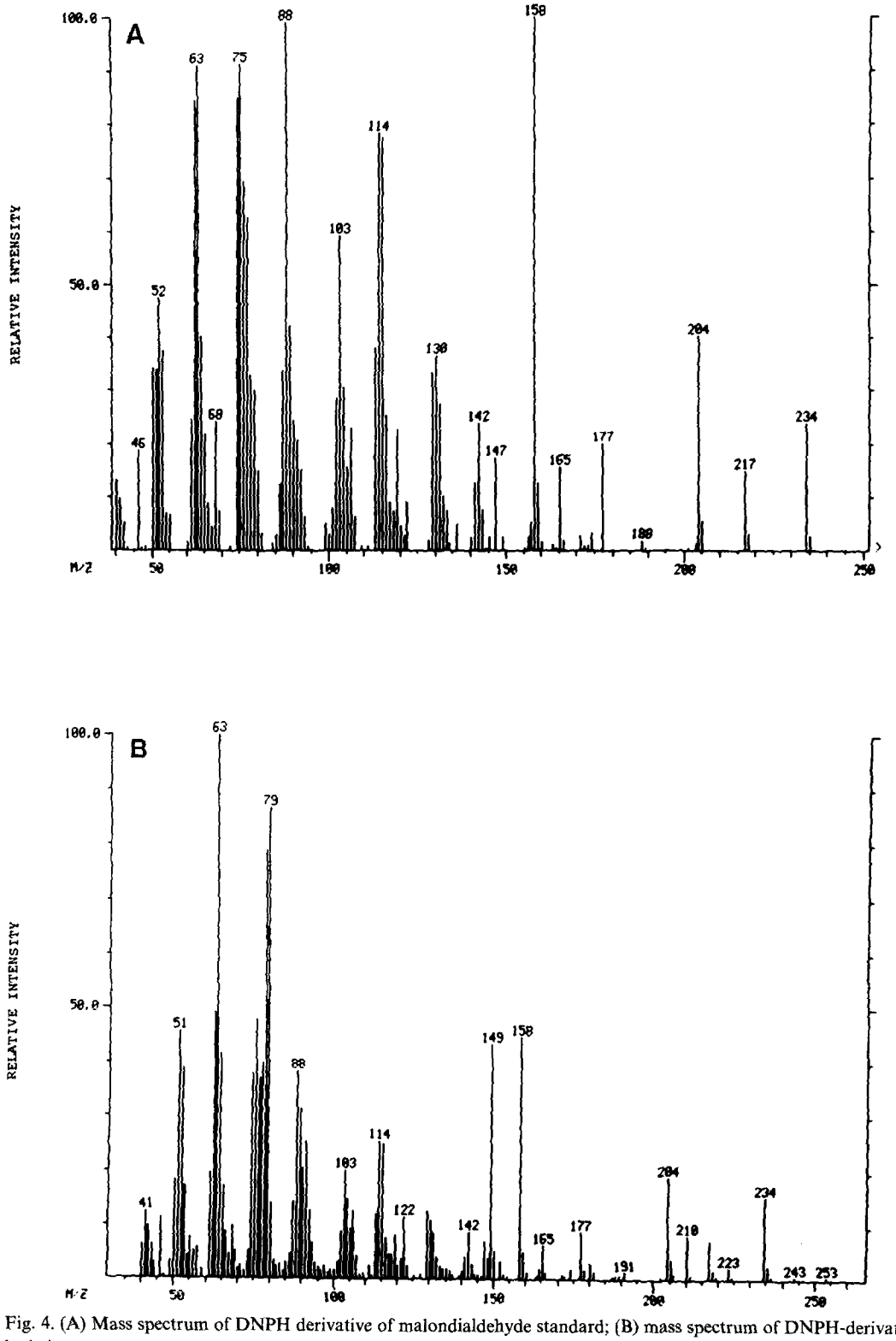

Fig. 4. (A) Mass spectrum of DNPH derivative of malondialdehyde standard; (B) mass spectrum of DNPH-derivatized malondialdehyde in rat urine.

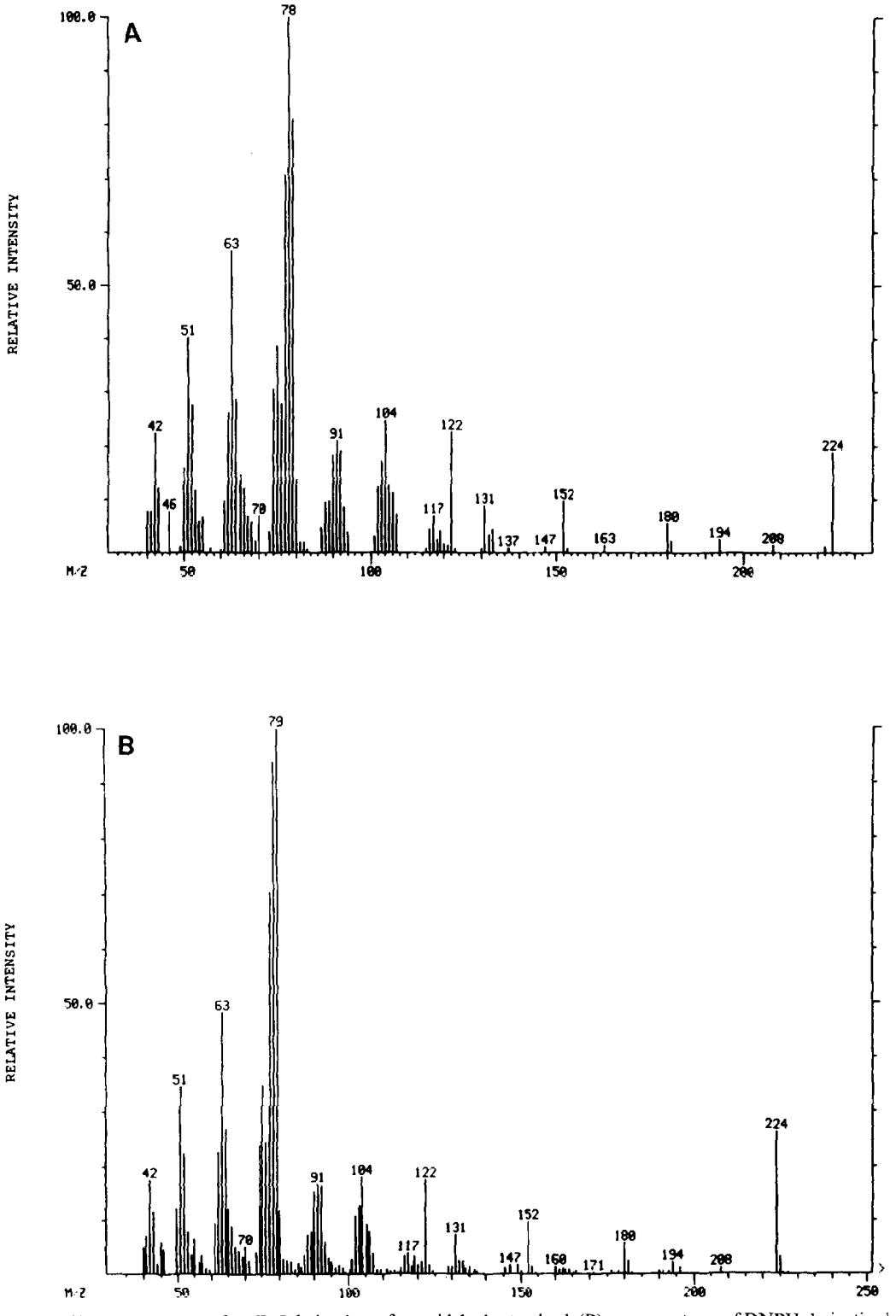

Fig. 5. (A) Mass spectrum of DNPH derivatives of acetaldehyde standard; (B) mass spectrum of DNPH-derivatized acetaldehyde in rat urine.

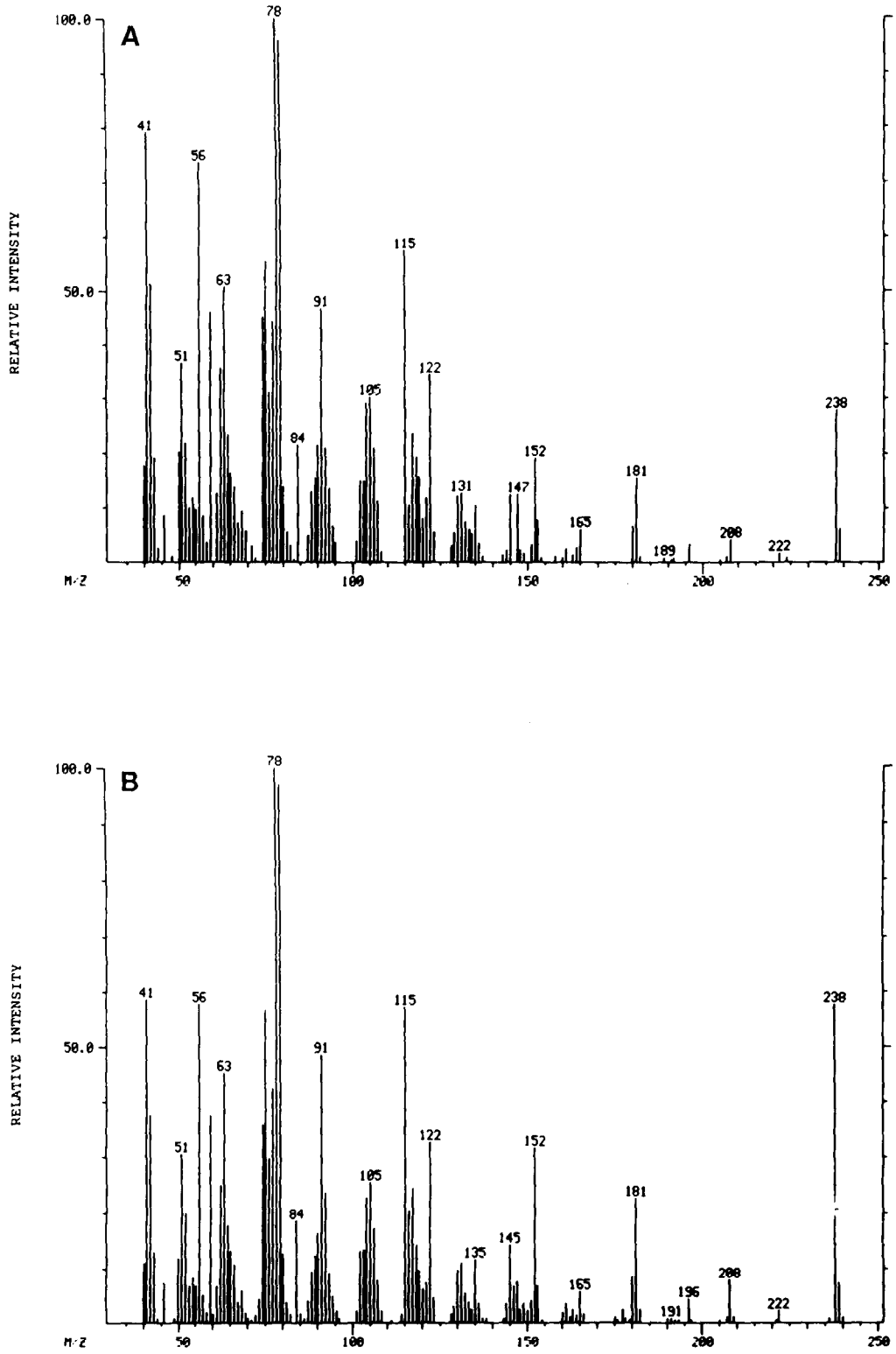

Fig. 6. (A) Mass spectrum of DNPH derivative of acetone standard; (B) mass spectrum of DNPH-derivatized acetone in rat urine.

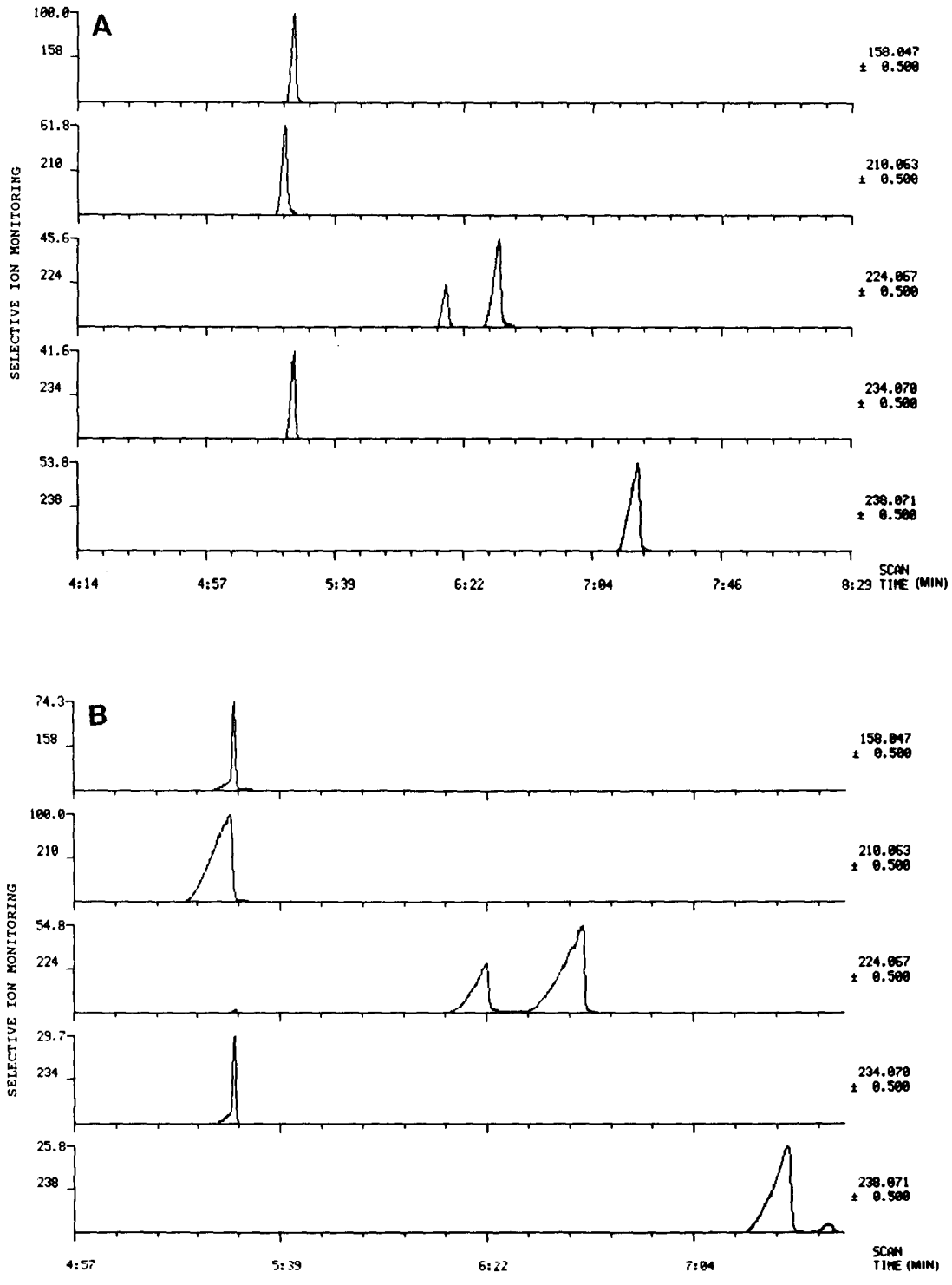

Fig. 7. (A) Selective ion monitoring for the DNPH derivative of malondialdehyde (158, 234), formaldehyde (210), acetaldehyde (224) and acetone (238) standards; (B) selective ion monitoring of the DNPH derivatives of malondialdehyde (158, 234), formaldehyde (210), acetaldehyde (224) and acetone (238) from rat urine.

mation of the identity of the four lipid metabolites in urine. Ion chromatograms for a mixture of the hydrazones of the four reference standards and an extract of urine are presented in Fig. 7A and B, respectively. The ions which were selected in this display mode are 158 and 234 for MDA, 210 for FA, 224 for ACT and 238 for ACON. A comparison of Fig. 7A and B clearly demonstrates that the mass ions produced by the four standards were associated with the components of urine that were separated by GC. The results provide further confirmation of the identities of the four lipid metabolites in the urine of rats.

Construction of calibration curves

Calibration curves for each of the synthetic hydrazones, namely MDA, FA, ACT and ACON, were generated. The concentrations for each standard which were injected and chromatographed were 0.5, 1, 2, 4, 10, 20 nmol/ml for MDA hydrazone, 5, 10, 20, 40, 60, 100 nmol/ml for FA hydrazone and 1, 2, 5, 10, 25 and 50 nmol/ml of ACT and ACON hydrazone standards. Each dilution was injected and chromatographed in triplicate. The concentrations of each standard, MDA, FA, ACT and ACON hydrazones, were plotted against the peak height obtained. Peak heights were directly proportional to the amount of hydrazone injected. In each case, the calibration line was linear, with all points having a very small standard deviation. The r values were 0.9917 for MDA, 0.9867 for FA, 0.9798 for ACT and 0.9853 for ACON.

Quantitation of urinary lipid metabolites

GC is not ideal for quantitation because of poor peak shape of the hydrazone derivatives, but it is sufficient for identification by GC–MS. The hydrazone samples are well chromatographed by HPLC and thus were used for quantitation. The accuracy of the HPLC quantitation technique was determined by standard addition technique. Addition of even 5 pmol of any of these hydrazone derivatives was accurately reflected in the peak heights. The limits of detection for MDA, FA, ACT and ACON were 2, 0.5, 0.3 and 0.2 pmol injected, respectively.

Previous studies by Ekstrom *et al.* [7] have quantitated the urinary excretion of MDA by HPLC following treatment with hydroquinone and chloroform. No other urinary lipid peroxidation products were examined or identified. However, several large peaks eluting downfield from the MDA peak are readily observable on the HPLC profile published by Ekstrom *et al.* [7] but were not identified.

The results in Table I provide quantitative data for control, and TCDD-, paraquat-, endrin- and carbon tetrachloride-treated animals 24 h post-treatment. The results are presented as nmol/kg

TABLE I

EXCRETION OF MALONDIALDEHYDE, FORMALDEHYDE, ACETALDEHYDE AND ACETONE BY RATS TREATED WITH ENDRIN, CARBON TETRACHLORIDE, PARAQUAT AND TCDD

Each value is the mean of four animals. Urine was collected from 21.75 h to 26.25 h post-treatment (4.5 h).

Excretion product	Excretion (nmol/kg body weight per 4.5 h)				
	Control	Endrin-treated (6 mg/kg)	Carbon tetrachloride-treated (2.5 ml/kg)	Paraquat-treated (75 mg/kg)	TCDD-treated (100 μg/kg)
Malondialdehyde	1.45 ± 0.23	3.67 ± 0.31[a]	1.94 ± 0.26[a]	3.69 ± 0.35[a]	1.77 ± 0.16
Formaldehyde	32.87 ± 2.11	70.60 ± 4.81[a]	43.56 ± 3.52[a]	73.61 ± 5.91[a]	49.31 ± 3.58[a]
Acetaldehyde	1.77 ± 0.28	3.41 ± 0.29[a]	2.31 ± 0.32[a]	7.25 ± 0.54[a]	2.49 ± 0.20[a]
Acetone	2.81 ± 0.31	7.92 ± 0.47[a]	5.71 ± 0.57[a]	30.01 ± 2.13[a]	5.29 ± 0.63[a]

[a] $P < 0.05$ with respect to the control group.

body weight per 4.5 h. As can be seen, these four toxicants produced varying increases in the excretion of MDA, FA, ACT and ACON as compared to the results for control animals. Of the four metabolites, FA was excreted in the greatest amount. The greatest increase (10.7-fold) in the excretion of the four metabolites occurred with ACON in response to paraquat. Paraquat administration also resulted in a 2.4-fold increase in ACT excretion, while paraquat and endrin produced 2.2- and 2.1-fold increases in FA excretion.

DISCUSSION

FA, ACT, MDA and ACON have been identified and quantitated in the urine of rats employing a single HPLC system. Furthermore, four toxicants with apparently different mechanisms of free radical-induced cell injury [1,8,10] all enhance the excretion of these four lipid metabolites (Table I). Direct comparisons of urinary lipid metabolites in response to the four toxicants cannot be made since the effects are dependent upon dose and the differing toxicokinetics of each xenobiotic. Furthermore, urine collections were made at only a single interval.

Previous studies have identified MDA in various biological matrices by means of diverse chromatographic techniques. Draper *et al.* [12] applied HPLC procedures to thiobarbituric acid-derivatized urine samples and were able to identify low levels of MDA after an acid hydrolysis procedure was applied. Alterations in free MDA levels in the urine of rats treated with the herbicide paraquat have been reported by Tomita *et al.* [9]. Paraquat is known for its redox cycling and ability to induce an oxidative stress [9].

Ekstrom *et al.* [6] reported the detection of urinary MDA after derivatizing with DNPH and separating the adducts by HPLC procedures. The identity of the MDA hydrazone was confirmed only by retention time. Later, Ekstrom *et al.* [7] confirmed the identity of the MDA hydrazone derivative standard by means of MS. However, the identity of MDA from urine which had been derivatized with DNPH was not confirmed by MS. Furthermore, no attempt was made to identify any of the other peaks clearly present on the UV–visible trace of the chromatograph.

Serum MDA has been identified by Kawai *et al.* [13] using similar HPLC techniques to those of Ekstrom *et al.* [7]. while Largilliere and Melancon [14] determined free MDA in plasma by HPLC. Lee and Csallany [15] have assessed free MDA in rat liver by HPLC. Tomita *et al.* [16] reported a method for the determination of urinary MDA by means of GC and electron-capture detection utilizing pentafluorophenylhydrazine as the derivatizing agent. Poli *et al.* [17] identified MDA, 4-hydroxynonenal, propanol, butanone and hexanal in liver utilizing a combination of thin-layer chromatography and HPLC techniques. However, FA, ACT and ACON were not identified.

Free ACT in blood has been determined as the DNPH derivative by HPLC [18]. Formaldehyde and ACT have been identified in urban air [19] and industrial surfactants [20] as their DNPH derivatives by HPLC. However, these procedures have not been applied to biological samples.

The sources of the four lipid metabolites which have been identified are not entirely clear. The increases in these products may be due to either lipid peroxidation or β-oxidation. Dhanakoti and Draper [11] demonstrated that urinary MDA excretion was enhanced following administration of the known liver toxin carbon tetrachloride and the free radical generating anthracycline antineoplastic antibiotic adriamycin. Furthermore, the fate of radiolabeled MDA administered to rats was examined. MDA appeared to be extensively metabolized to acetate and carbon dioxide. Based on these observations, the urinary acetaldehyde identified in this study may arise from the breakdown of MDA which is formed due to lipid peroxidation. Previous studies have shown that TCDD [20], paraquat [1], carbon tetrachloride [1] and endrin [10] produce marked increases in the formation of MDA and other thiobarbituric acid reactive substances in the liver.

The enhanced formation of ACON in response to disease states such as diabetes as a consequence of enhanced β-oxidation is also well known [21]. Winters *et al.* [22] reported that rat

liver microsomes metabolized glycerol to FA. Glycerol is a product of the metabolism of triglycerides by adipose tissue and other tissues that possess the enzyme that activates glycerol, namely, glycerol kinase. Liver and brown tissue are known to have high glycerol kinase levels [22]. Other possible sources of FA might include the breakdown of MDA to acetate or ACT and a one carbon fragment [11], and/or the cleavage of a one carbon fragment from acetoacetic acid with the formation of ACON.

The HPLC methodology used in conjunction with the application of GC–MS provides conclusive identification and quantitation of MDA, FA, ACT and ACON excretion in the urine of rats. Furthermore, an increased excretion of these lipid metabolites occurs in response to free radical-induced cell injury. The methodology is simple and requires relatively little work-up time. The preparation of a 1.0-ml urine aliquot can be completed in less than 2 h including the elution by HPLC. Furthermore, excellent reproducibility and sensitivity are achieved. The detection limit is approximately 50 pmol for any of the four lipid metabolites in a 1.0-ml urine aliquot in a 20-μl injection volume. Numerous applications of this procedure exist for the study of exposure to environmental pollutants as well as altered lipid metabolism in various disease states.

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