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Simultaneous determination of cyclohexene oxide and its metabolites in rat plasma and urine by gas chromatography

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Abstract

An assay was developed for the simultaneous measurement of cyclohexene oxide and its metabolites (cyclohexanol, *trans*-cyclohexane-1,2-diol, cyclohexane-1,2-diol-*O*-glucuronide, and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine) in rat urine and plasma using gas chromatography. A mixture of ethyl acetate–acetonitrile (70:30) was used as the extracting solvent for both matrices. This liquid–liquid extraction procedure was followed by the separation of cyclohexene oxide and its metabolites on an HP–FFAP fused-silica capillary column. In order to determine the amount of cyclohexane–1,2-diol-*O*-glucuronide, samples were incubated at 37°C with β-glucuronidase and the amount of cyclohexane-1,2-diol formed from the reaction determined. The extraction efficiencies of cyclohexene oxide and cyclohexanol were greater than 90% both in urine and plasma. However, recovery from the plasma and urine for *trans*-cyclohexane-1,2-diol (60-68%) and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (~76%) were considerably less. Long term stability studies showed that urine samples spiked with cyclohexene oxide and *trans*-cyclohexane-1,2-diol are stable at −20°C for up to 9 weeks. However, plasma samples are only stable for up to 2 weeks under the same conditions. The calibration curves for all analytes were linear over the range of 12.5 to 400 μg/ml and correlation coefficients (r^2) were greater than 0.990. The limit of detection for cyclohexene oxide, cyclohexanol, and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine is 1.56 μg/ml, while the limit of detection for *trans*-cyclohexane-1,2-diol is 3.12 μg/ml. This method has been used for the determination of the disposition and metabolism of cyclohexene oxide, and may be applied in environmental monitoring, as well as in microbiological studies for other epoxide materials. © 1997 Elsevier Science B.V.

Keywords: Cyclohexene oxide; Cyclohexanol; *trans*-Cyclohexane-1,2-diol; Cyclohexane-1,2-diol-*O*-glucuronide; *N*-Acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine

1. Introduction

Cyclohexene oxide (CHO) is an epoxide widely used in organic synthesis, as well as a monomer in photoreactive polymerization of pesticides, pharmaceuticals, perfumery and dyestuff. It is also used extensively as a microbiological substrate and biochemical reagent in metabolic and enzymatic studies [1,2]. Although there are no reports of accidental exposure to humans, there is a potential risk for human health because of its chemical structure (Fig. 1). Many epoxides are potent mutagens or animal carcinogens, and in several short term assays, CHO has been shown to be mutagenic [3,4]. However, up

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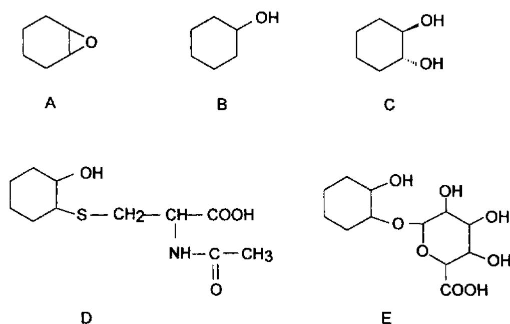

Fig. 1. Structures of (A) cyclohexene oxide, (B) cyclohexanol, (C) *trans*-cyclohexane-1,2-diol, (D) *N*-acetyl-*S*-(2-hydroxy-cyclohexyl)-L-cysteine, and (E) cyclohexane-1,2-diol-*O*-glucuronide.

to this point there are no reports in the literature on occupational exposure to CHO and little is known of its disposition and metabolism [5,6]. It has recently been reported by our laboratory that CHO is rapidly metabolized and eliminated from the body primarily by excretion into the urine [6]. The primary metabolites found in the urine following oral administration are cyclohexane-1,2-diol, and its *O*-glucuronide conjugate. In addition to hydrolysis and conjugation, CHO undergoes nucleophilic attack from glutathione which results in the subsequent formation of *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine. Interestingly, two diastereoisomers of *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine have been identified in the urine following the intraperitonal administration of CHO to rats [7].

Various methods have been reported to measure CHO, but no assay has been developed to determine CHO and its metabolites simultaneously. For example, Colgan et al. [8] reported an HPLC method based on derivatization using a nucleophilic reagent to determine environmental epoxide contaminants. Although quite sensitive, the method is complex and time consuming. Another procedure reported by Kenneth et al. [9,10] utilized gas chromatography for the determination cyclohexene and several of its metabolites (cyclohexanol, cyclohexanone and cyclohexene oxide) in rabbit liver microsomes using an OV-17 capillary column. However, this procedure did not account for any conjugated metabolites formed during the metabolism of CHO. Furthermore, Mraz et al. [11] reported a similar method to analyze cyclohexanone and its metabolites: cyclohexanol; cyclohexane-1,2-diol; and cyclohexane-1,4-diol. Although various methods are available to identify CHO and its potential metabolites individually, no single method has been developed for the simultaneous detection of all these compounds. Therefore, the objective of these studies was to develop a simple gas chromatographic method for the analysis of CHO and its metabolites in biological fluids.

2. Experimental

2.1. Reagents

CHO was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland); *trans*-cyclohexane-1,2-diol (DIOL) was obtained from Pfaltz and Bauer (Waterbury, CT, USA); cyclohexanol (NOL) was purchased from Sigma (St. Louis, MO, USA). All other chemicals used in these studies were of the highest purity.

2.2. Synthesis of n-acetyl-S-(2-hydroxycyclohexyl)-L-cysteine

In order to confirm the presence of *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (MERC) in the urine, a standard was synthesized according to the method of Van Bladeren [5]. Briefly, *N*-acetyl-L-cysteine (5 g, 30 mmol) was dissolved in 75 ml of methanol, sodium methanolate (3.4 g, 60 mmol) was added, and the solution was stirred for 10 min at room temperature. Cyclohexene oxide (6.5 g, 60 mmol) was added to the mixture, and it was refluxed for 3 h in an argon atmosphere. The methanol was evaporated and the residue was resuspended in acidic chloroform (100 ml of chloroform mixed with 5 ml of concentrated HCl). The chloroform layer was separated and crystallized. The melting point of the synthesized standard was 144.5–145.0°C. Furthermore, proton NMR spectra were obtained using a Bruker AM-250 spectrometer (Manming Park, Billerica, MA, USA). The structure of the synthesized standard was consistent with that of the *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine as determined by proton NMR [(250 MHz, DMSO-d_6) (8.30–8.20 (m, 1H, NH), 4.33 (m, 1H, 2CH), 3.25 (m, 1H, CH), 3.10 (m, 1H, CH), 3.05–2.72 (m, 2H, CH2), 1.84 (S,

3H, COCH3), 1.98–1.05 (m, 8H, 4×CH2)]. However, the standard was likely a diastereoisomeric mixture of *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine as indicated by gas chromatography.

2.3. Apparatus and gas chromatography

Gas chromatography was performed on a Hewlett–Packard model 5890 series II gas chromatograph equipped with a flame ionization detector, HP 7673 automatic sampler and HP 7673 splitless injector. Separation and quantification of analytes in plasma and urine utilized an HP-FFAP column (25 m×0.32 mm I.D., 0.52-μm film thickness; Hewlett–Packard, Wilmington, DE, USA). The column temperature was maintained at 50°C for 2 min, then ramped at 7°C/min up to 200°C, where it was maintained for 3 min. The injector and detector temperatures were 200°C and 250°C, respectively. Helium was used as the carrier gas at an inlet pressure of 6.7 psi.

2.4. Preparation, extraction of standard solutions and urine sample treatment

Standard solutions of CHO, NOL, DIOL and MERC (0.1 to 0.8 mg/ml) were made in methanol and were used to determine extraction efficiency. Urine and plasma samples, obtained from male Fischer-344 rats, were spiked with standard solutions of each analyte to obtain final concentrations of 25, 50, 100, 200 and 400 μg/ml. Samples (100 μl) were extracted (two times) with 100 μl of an ethyl acetate–acetonitrile (70:30) mixture. The samples were then centrifuged for 5 min at 12 000 rpm, organic fractions were pooled, and an aliquot (1 μl) was injected onto the gas chromatograph.

Urine samples from rats which received an oral dose (100 mg/kg) of CHO were incubated at 37°C with β-glucuronidase (2000 units/ml urine) type VII A from E. coli (Sigma, St. Louis, MO, USA). Samples were extracted and analyzed by GC as described above. The amount of cyclohexane-1,2-diol-*O*-glucuronide in the urine was calculated by comparing the amount of DIOL detected before and after sample treatment with β-glucuronidase ($DIOL_{\beta\text{-glucuronidase treated}} - DIOL_{untreated}$ = amount of cyclohexane-1,2-diol-*O*-glucuronide).

2.5. Stability of CHO and DIOL in urine and plasma

Since urine and plasma samples are routinely stored at −20°C prior to analysis, the stability of CHO and DIOL in urine and plasma was investigated. Spiked samples of urine and plasma containing CHO and DIOL (50 μg/ml) were divided into six portions. One portion was extracted and assayed immediately, while the remaining five samples were stored at −20°C for 1, 2, 4, 6, and 9 weeks prior to analysis.

2.6. Metabolite identification using mass spectrometry

For metabolite isolation and identification, samples were diluted in 10 m*M* aqueous ammonium hydroxide–acetonitrile (1:1) and then analyzed on a Finnigan TSQ 7000 triple quadrupole mass spectrometer equipped with an electrospray ion source (Finnigan MAT, San Jose, CA, USA). Samples were introduced into the mass spectrometer by flow injection at 0.5 ml/min. Ions with *m/z* values corresponding to putative metabolites were subjected to collision-induced dissociation (CID) with argon gas and the subsequent product ion signals mass analyzed to produce a product ion mass spectrum. Logical fragmentation patterns observed in the resulting MS–MS spectrum provided further evidence as to metabolite identity.

Because of their volatility, samples suspected of containing DIOL and CHO were separated using a Fisons GC-8000 gas chromatograph coupled to a Fisons MD800 quadrupole mass spectrometer (Fisons Instruments, Beverly, MA). Direct on-column injection was used to introduce the extracted samples onto a DB-5-MS capillary column (15 m× 0.25 mm I.D.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). The oven temperature was initially maintained at 60°C for 2 min, then increased at 12°C/min for the next 25 min to a final temperature 300°C, and maintained at 300°C for 8 min. The injector, mass spectrometer source, and interface temperatures were 250°C, 250°C and 275°C, respectively. The mass spectrometer was scanned from *m/z* 50 to 650 in 1 s. The retention time and mass spectra of putative metabolites in the

samples were compared to the mass spectra of authentic standards to verify compound identity.

3. Results and discussion

3.1. Chromatography

Representative gas chromatograms of control urine and plasma samples spiked with CHO, NOL, DIOL, and MERC, as well as plasma and urine samples from male Fischer 344 rats administered CHO intravenously and orally are shown in Fig. 2. CHO was retained on the HP–FFAP column for 7 min, NOL for 11 min, DIOL for 21 min, and MERC for 19 min. Use of this column, which has polar characteristics, significantly aided in the separation of the parent compound and its metabolites. The HP–FFAP column has been previously used to separate other polar compounds such as organic acids, alcohols, aldehydes, and acrylates [12,13].

3.2. Calibration

Standard curves for CHO, NOL, DIOL and MERC were obtained using the least-squares linear regression of the peak area versus the theoretical concentrations. Representative standard curves after extraction of CHO, NOL, DIOL and MERC from plasma and urine are shown in Fig. 3.

3.3. Recovery, accuracy, and precision

The extraction recoveries for CHO, NOL and DIOL from plasma and urine are shown in Table 1 Table 2. Greater than 90% of CHO and NOL were recovered from the urine and plasma. In contrast, only 60 and 68% of the DIOL was extracted from the plasma and urine, respectively. The extraction efficiency of MERC was greater than 76% both in urine and plasma. The lower extraction efficiency for DIOL and MERC may be attributed to their more polar characteristics. Although several solvents were initially tested, ethyl acetate–acetonitrile (70:30) showed maximal extraction efficiency for these four analytes.

Spiked plasma and urine samples (25, 50, 100, 200 and 400 μg/ml each of CHO, NOL and DIOL) were assayed to determine intra- and inter-day precision. The coefficient of variance for intra-day assay for three chemicals were 3.5±2.8% with an accuracy of 98.4±1.2% for the plasma samples, and 2.9±1.8% with an accuracy of 96.4±2.5% for the urine samples. Inter-day assay for three chemicals were 5.0±3.4% with an accuracy of 97.7±1.6% for the plasma samples, and 6.2±4.1% with an accuracy of 96.6±4.1% for the urine samples.

3.4. Linearity and sensitivity

The calibration curves for CHO, NOL, DIOL and MERC in plasma and urine were linear over the range of 25 to 400 μg/ml. The correlation coefficients (r^2) for CHO, NOL, DIOL and MERC were greater than 0.990 both in plasma and urine. In addition, standard curves for CHO and DIOL were found to be linear in lower concentration ranges of 1.56 to 100 μg/ml in plasma sample. The limit of detection for CHO, NOL and MERC was 1.56 μg/ml and 3.12 μg/ml for the DIOL.

3.5. Stability of CHO and DIOL in urine and plasma

Both CHO and DIOL were stable in the urine for up to 9 weeks when stored at −20°C. However, neither of those compounds were stable in plasma to the same extent. DIOL was stable in plasma for up to 4 weeks, while CHO was stable for only 2 weeks of collection. These results suggest that the plasma samples should be analyzed within 2 weeks. The accuracy and extraction efficiency were consistent over several days and a wide range of concentrations. Thus, it appears that the stability of the analytes were dependent on the biological matrix.

3.6. Application

The methods described here were used to measure the urine and plasma concentrations of CHO and its metabolites in a recent disposition and metabolism study [6]. In this study, the blood samples were centrifuged and the plasma layer was removed and stored at −20°C until analysis. As shown in Fig. 4, both CHO and DIOL were detected in the plasma following intravenous administration of CHO. Al-

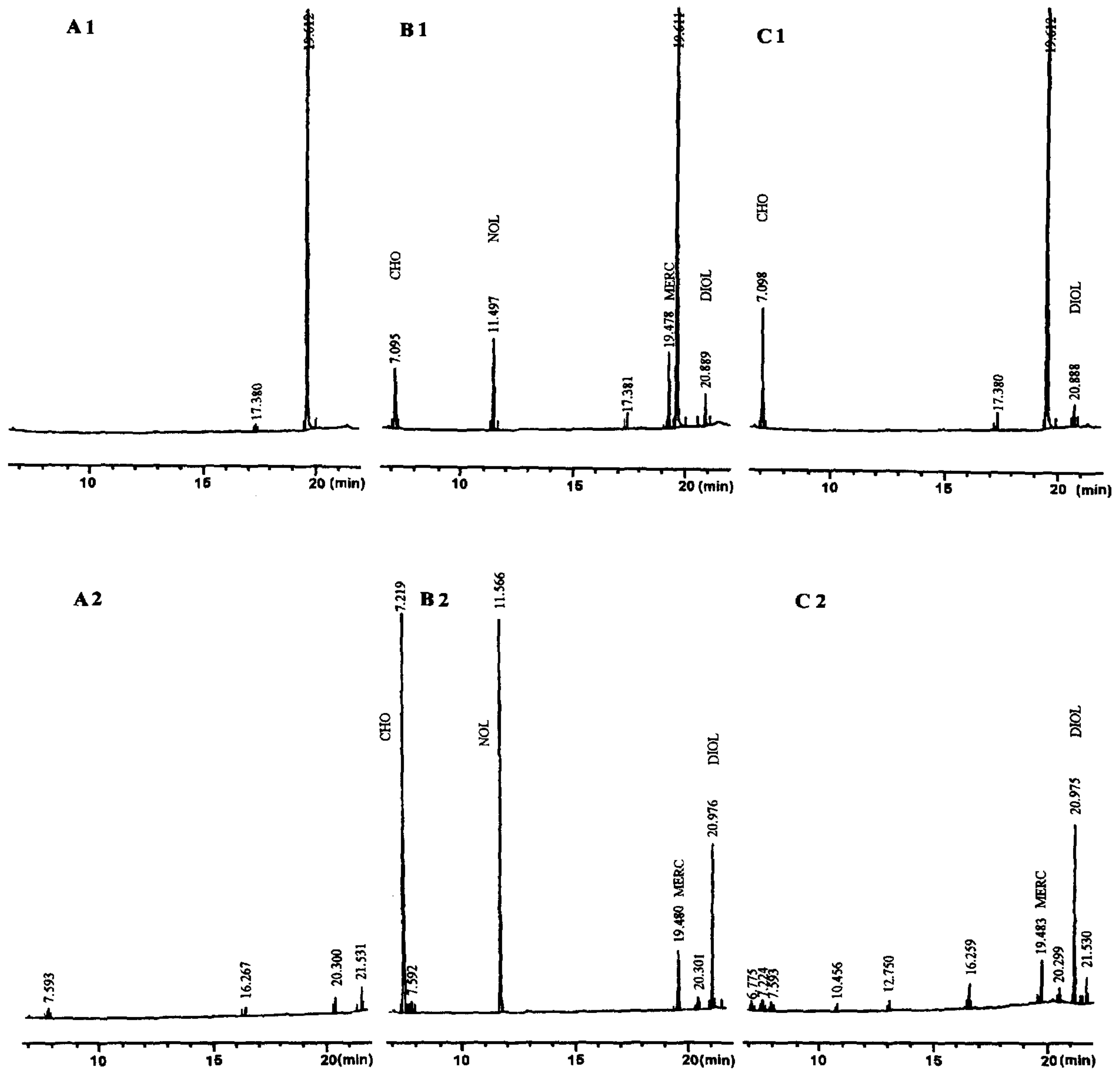

Fig. 2. (A1, A2) Gas chromatograms of a blank rat plasma and urine sample; (B1, B2) gas chromatograms of rat plasma and urine sample spiked with cyclohexene oxide (CHO), cyclohexanol (NOL), *trans*-cyclohexane-1,2-diol (DIOL) and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (25 μg/ml for each compound); (C1) gas chromatogram of a male Fischer 344 rat plasma sample following intravenous administration of cyclohexene oxide (CHO; 50 mg/kg). (C2) gas chromatogram of a male Fischer 344 rat urine sample following single oral dose of cyclohexene oxide (CHO; 100 mg/kg).

though CHO was detected in plasma after intravenous administration, it could not be detected following oral administration. This probably results from its rapid hydrolysis at low pH in gastric fluid, as well as metabolism by rapid intestinal and hepatic tissues.

The primary metabolites excreted in the urine

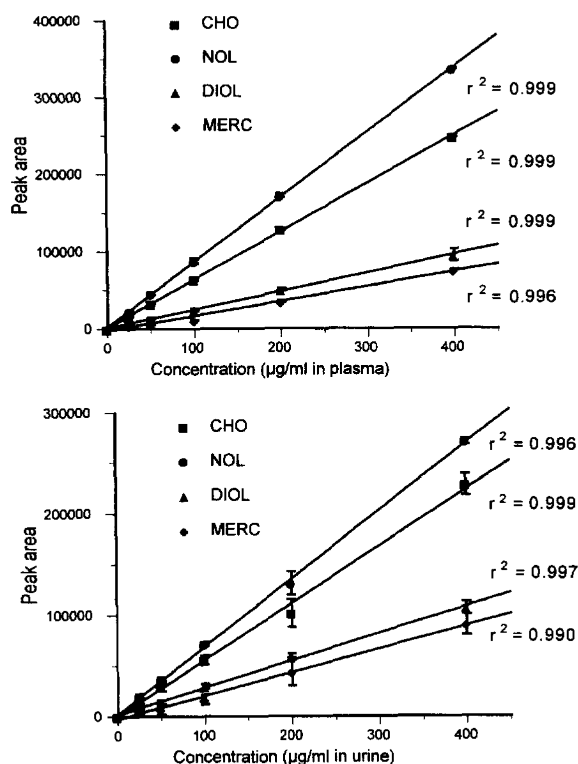

Fig. 3. Standard curves for cyclohexene oxide (CHO), cyclohexanol (NOL), *trans*-cyclohexane-1,2-diol (DIOL) and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (MERC) in male Fischer 344 rat plasma and urine.

were DIOL, cyclohexane-1,2-diol-*O*-glucuronide and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (Fig. 5). In order to determine the amount of cyclohexane-1,2-diol-*O*-glucuronide in the urine, samples were incubated with β-glucuronidase in order to convert this glucuronide conjugate to DIOL. Although not discussed in this manuscript, incubation with sulfatase could be used to determine the presence of cyclohexane-1,2-diol-*O*-sulfate. Interestingly, the metabolism of CHO are similar to those reported for cyclohexanone oxime in the rat [11]. For example, a major urinary metabolite of cyclohexanone oxime was cyclohexane-1,2-diol-*O*-glucuronide. However, cyclohexanol and cyclohexanol-*O*-glucuronide were also detected in the urine of rats given cyclohexanone oxime. These metabolites were not present in the urine or plasma following CHO administration.

The identity of both urinary and blood analytes were determined by either GC–MS or LC–MS/MS. Because of their volatile nature, blood metabolites were analyzed by GC–MS. Chromatographic separation showed two major peaks with mass spectra identical to those of CHO and DIOL. The EI spectrum for *m/z* 98 contained structurally relevant signals at *m/z* 83, 69 and 56, which was identical to the EI mass spectrum of the cyclohexene oxide standard. The EI spectrum for *m/z* 116 also contained structurally relevant signals at *m/z* 98, 83, 70, and 57, and was identical to the EI mass spectrum of the *trans*-cyclohexane-1,2-diol standard (Fig. 6).

As determined by LC–MS/MS, the probable identities of urinary metabolite were deduced from the mass of the $[M-H]^-$ ion and the product ion spectra generated by CID of the $[M-H]^-$ ion (Fig. 7). The predominate metabolite in the urine showed an $[M-H]^-$ ion at *m/z* 291. A major product ion

Table 1
Extraction efficiency of cyclohexene oxide (CHO), cyclohexanol (NOL), *trans*-cyclohexane-1,2-diol (DIOL) and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (MERC) in rat plasma

Concentration (μg/ml)	Recovery (%)			
	CHO	DIOL	NOL	MERC
400	100.4	44.9	102.6	79.7
200	99.2	58.7	109.1	78.2
100	103.4	62.9	109.9	58.9
50	97.0	65.7	105.7	67.3
25	97.2	66.1	108.1	95.8
Mean±S.D.	99.4±2.6	59.7±8.8	108.2±1.8	76.0±14.0

Table 2
Extraction efficiency of cyclohexene oxide (CHO), cyclohexanol (NOL), *trans*-cyclohexane-1,2-diol (DIOL) and *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (MERC) in rat urine

Concentration (μg/ml)	Recovery (%)			
	CHO	DIOL	NOL	MERC
400	102.6	60.9	104.9	95.7
200	84.1	67.8	95.0	97.8
100	89.1	69.1	96.5	85.3
50	91.6	64.7	96.3	75.3
25	92.4	79.4	97.2	85.8
Mean±S.D.	92.0±6.7	68.3±7.0	98.0±3.9	88.0±9.1

was at m/z 99, which represents a loss of 193 mass units characteristic of a glucuronic acid fragment. Furthermore, the overall fragmentation pattern is consistent with a structural assignment as cyclohexane-1,2-diol-*O*-glucuronide. The mass spectrum of the other major metabolite contained a signal corresponding to the $[M-H]^-$ ion at m/z 260 which is consistent with *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine. The major product ion was at m/z 131, which represents an addition of a sulfur to the cyclohexanol ring. Another product ion was at m/z 218, which represents a loss of 42 mass units characteristic of an acyl group. These product ions provide further evidence for the assignment of *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (MERC). Furthermore, the CID spectrum for this metabolite was identical to that of the synthesized standard.

4. Summary

From the gas chromatographic methods developed here, it is possible to simultaneously separate and quantify CHO and its metabolites in both blood and urine. The major obstacle overcome by this study was applying a gas chromatographic method to low molecular weight polar compounds without chemical

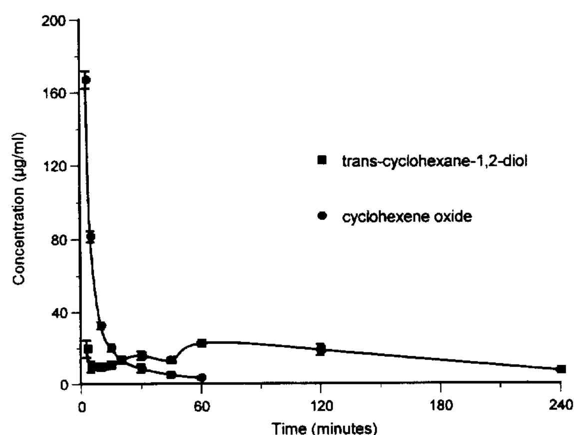

Fig. 4. Concentration of cyclohexene oxide and *trans*-cyclohexane-1,2-diol in male Fischer 344 rat plasma following intravenous administration of cyclohexene oxide (50 mg/kg; Mean±S.D., $n=3$ rats).

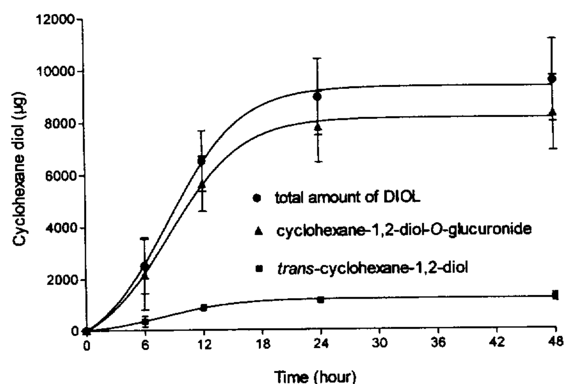

Fig. 5. Cumulative excretion of *trans*-cyclohexane-1,2-diol and cyclohexane-1,2-diol-*O*-glucuronide in urine of male Fischer 344 rats following oral administration of cyclohexene oxide (100 mg/kg; Mean±S.D., $n=4$ rats).

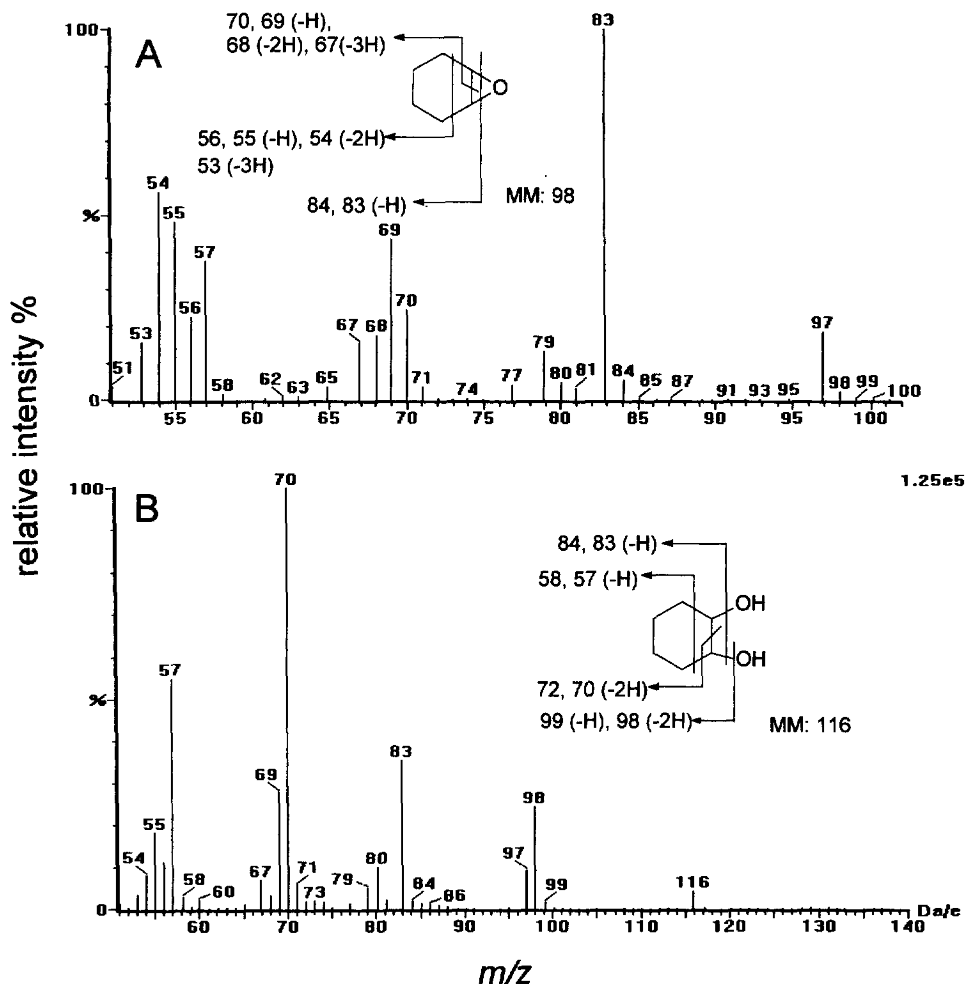

Fig. 6. Representative GC EI mass spectrum of the parent compound cyclohexene oxide (A) at *m/z* 98 obtained from male Fischer 344 rat plasma following intravenous administration of cyclohexene oxide and urinary metabolite cyclohexane-1,2-diol (B) at *m/z* 116 obtained from male Fischer 344 rat urine following oral administration of cyclohexene oxide. Examples of the probable fragmentation patterns are given.

derivatization. Although this method offers a good procedure for monitoring of epoxide materials and their biological metabolites, a limiting factor was the lack of sensitivity, in part due to the column selected. Greater sensitivity for CHO was achieved using a DB-5 capillary column (under similar condition used for the GC–MS analysis), however this system proved unsuitable for separation of the metabolites of CHO. The lack of sensitivity in this procedure did not hinder the utilization of this method in the study of the in vivo metabolism and disposition of CHO [6].

Acknowledgments

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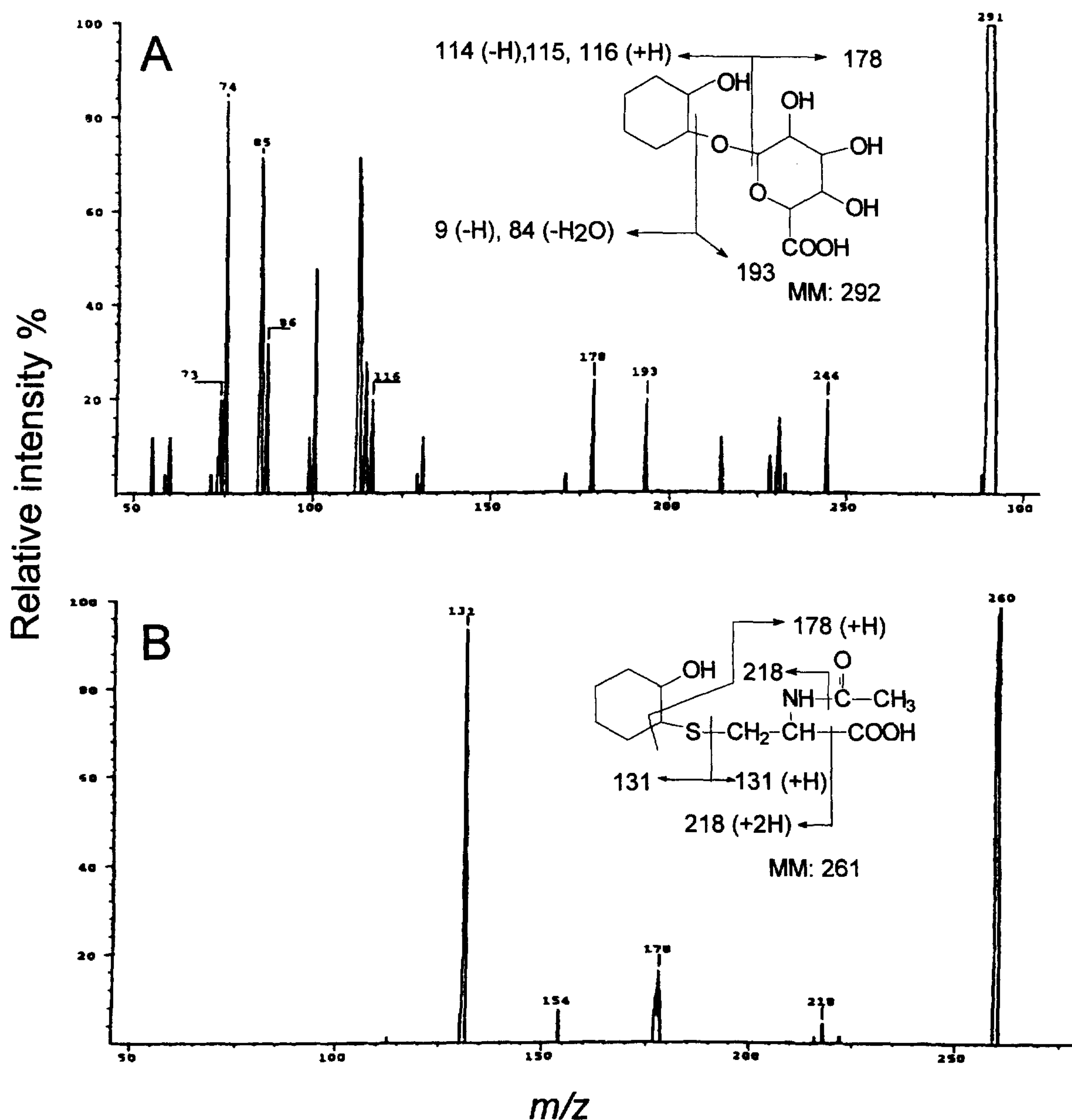

Fig. 7. Representative LC–MS–MS CID mass spectrum of the urinary metabolite cyclohexane-1,2-diol-*O*-glucuronide (A) at *m/z* 291 obtained from the male Fischer 344 rat urine following oral administration of cyclohexene oxide and urinary metabolite *N*-acetyl-*S*-(2-hydroxycyclohexyl)-L-cysteine (B) at *m/z* 260 obtained from the male Fischer 344 rat urine following oral administration of cyclohexene oxide. Examples of the probable fragmentation patterns are given.

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