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Bioactivation of 4-Methylphenol (p-cresol) via CYP-mediated Aromatic Oxidation in Human Liver Microsomes

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Abbreviations:

HLM, human liver microsomes; GSX, ϒ-glutamyl-cystein-glycin-^{13}C2-^{15}N; MS, mass spectrometry; CID: collision induced dissociation; multiple reaction monitoring: MRM; NL: neutral loss MS scan.

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Abstract

It has previously been proposed that 4-methylphenol (p-cresol) is metabolically activated by oxidation of the methyl group to form a reactive quinone methide. In present study a new metabolism pathway is elucidated in human liver microsomes. Oxidation of the aromatic ring leads to formation of 4-methyl-*ortho*-hydroquinone that is further oxidized to a reactive intermediate 4-methyl-*ortho*-benzoquinone. This bioactivation pathway is fully supported by the following observations: 1), one major and two minor GSH adducts were detected in microsomal incubations of p-cresol in the presence of glutathione; 2), a major metabolite of p-cresol was identified as 4-methyl-*ortho*-hydroquinone in microsomal incubations; 3), the same GSH adducts were detected in microsomal incubations of 4-methyl-*ortho*-hydroquinone; 4), the same GSH adducts were chemically synthesized by oxidizing 4-methyl-*ortho*-hydroquinone followed by the addition of GSH, and the major conjugate was identified by LC/MS/MS and NMR as 3-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone. In addition, it was found that 4-hydroxybenzylalcohol, a major metabolite derived from oxidation of the methyl group in liver microsomes, was further converted to 4-hydroxybenzaldehyde. In-vitro studies also revealed that bioactivation of p-cresol was mediated by multiple CYPs, but 2D6, 2E1 and 1A2 are most active enzymes for formation of quinone methide, 4-methyl-*ortho*-benzoquinone and 4-hydroxybenzaldehyde, respectively. Implications of the newly identified reactive metabolite in p-cresol-induced toxicity remain to be investigated in the future.

Introduction

4-Methylphenol (p-cresol) is a natural product present in many foods, crude oil and coal tar, and is also detected in animal and human urine. In addition to its industrial uses, p-cresol is also used as an antiseptic and disinfectant because of its bactericidal and fungicidal properties. As a metabolite of toluene, p-cresol is a known toxin associated with toxicity of its precursor molecule. Ingestion of p-cresol can cause death (Monma-Ohtaki et al., 2002), and acute exposure can lead to a number of toxic effects such as uraemia (De Smet et al., 2003) and hepatotoxicity (Kamijo et al., 2003). Presumably, uraemic toxicity of p-cresol is resulted from its high serum protein-binding affinity. Because p-cresol competes with drugs for protein binding, it can substantially enhance the toxic effect of uremia (Lesaffer et al., 2001). In-vitro studies have suggested a potential association between hepatotoxicity and metabolic activation of p-cresol (Thompson et al., 1994; Thompson et al., 1995), although such a relationship remains to be established in vivo.

In rat liver microsomal incubations, it has been found that 4-hydroxybenzylalcohol is the primary metabolite of p-cresol (Sato et al., 1956). In-vitro studies using rat liver microsomes have also demonstrated that p-cresol is oxidized to form a quinone methide intermediate that can be detected as a stable conjugate when glutathione (GSH) is present as a trapping agent in incubations (Thompson et al., 1995). The quinone methide is a highly reactive electrophile, which can alkylate cellular proteins and nucleic acids, and thus potentially lead to toxic effects (Monks et al., 2002). However, a similar study has not been conducted using human liver microsomes.

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In the present study we investigated the metabolism and bioactivation of p-cresol in human liver microsomes. Structural analysis of both stable and reactive metabolites revealed that, in addition to oxidation of the methyl group, oxidation of the benzene ring can lead to formation of 4-methyl-ortho-hydroquinone that is further oxidized to a reactive intermediate as 4-methyl-*ortho*-benzoquinone. In addition, it was found that, in microsomal incubations, 4-hydroxybenzylalcohol derived from p-cresol is further oxidized to 4-hydroxybenzaldehyde.

Materials and Methods

Materials.

Reagents and solvents used in the current study were of the highest possible grade available. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): p-cresol, 4-methylphenol-2,3,5,6-d_4, 4-methyl-*ortho*-hydroquinone, 4-hydroxybenzylalcohol, 4-hydroxybenzaldehyde, 3,4,5,6-tetrachloro-[1,2]benzoquinone, glutathione, β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate and glucose-6-phosphate dehydrogenase. Human and rat liver microsomes, and *Supersomes* containing cDNA-expressed CYPs were obtained from Gentest Corp. (Woburn, MA). Stable-isotope labeled glutathione [GSX, ϒ-glutamyl-cystein-glycin-$^{13}C2$-^{15}N] was obtained from Cambridge Isotope Laboratories (Andover, MA), and isotopic purity was 94% estimated by the supplier using nuclear magnet resonance (NMR).

Instrumentation.

Mass spectrometry analyses were performed on a Micromass (Manchester, UK) *Quattro Micro* triple quadrupole mass spectrometer. NMR spectra were obtained on a

Bruker DPX 300 NMR spectrometer, and chemical shifts of H^1 were expressed relative to tetramethylsilane.

Microsomal Incubations and Stable Isotope Trapping of Reactive Metabolites.

All incubations were performed at 37^{o}C in a water bath as previously described (Yan et al., 2002). p-Cresol or its derivative was individually mixed with human or rat liver microsomal protein in 50 mM potassium phosphate buffer (pH 7.4). After a 5-min preincubation at 37^{o}C, reactions were initiated by the addition of a NADPH generating system to give a final volume of 1.0 ml. The final reaction mixture contained p-cresol at a desired concentration (10-200 μM), 1 mg/ml microsomal protein, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase and 3.3 mM magnesium chloride. After a 30-min incubation, reactions were terminated by the addition of 150 μl of trichloroacetic acid (10%). Samples were centrifuged at 10000 g for 15 min at 4 ^{o}C to pellet the precipitated protein, and supernatants were subjected to LC-MS/MS for direct analysis of metabolites.

To trap reactive metabolites formed in microsomal incubations, 1 mM GSH or a mixture of GSH and GSX (1:1) were added to reaction mixtures (Yan et al., 2004). Reactions were initiated by the addition of the NADPH regeneration solution, and incubations were performed for 60 min.

To assess formation of GSH conjugates by individual CYPs, incubations were performed similarly, except that liver microsomes were substituted by *Supersomes* that contained individual enzymes at a final concentration of 100 pmoles/ml. To analyze the metabolite formed by individual CYPs, GSH was omitted in the incubations. Resulting samples were processed as described above, and analyzed by LC-MS/MS.

Synthesis of 3-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone.

4-Methyl-*ortho*-hydroquinone (0.62g) in 10 ml diethyl ether was converted to 4-methyl-[1,2]benzoquinone by a slow addition to a diethyl ether solution of 3,4,5,6-tetrachloro-[1,2]benzoquinone (1.34 g) at -5 to 0 °C (Carlson et al., 1985). The resulting reaction mixture was treated with glutathione (1.54 g) *in situ* at 0 °C and followed by the addition of 20 ml H_2O and 10 ml THF. After stirring for 24 hours at room temperature, the reaction mixture was diluted with 20 ml H_2O. The aqueous layer was washed with 30 ml diethyl ether and lyophilized to give a crude solid product (2.01 g). The crude solid product was analyzed by LC-MS/MS for GSH adducts. A total amount of 50 mg crude product was subjected to purification by a reversed phase HPLC to provide 28 mg of the GSH adduct. MS, *m/z* 429.9 (MH^+); ^{1}H-NMR (CD_3OD): δ 2.17 (s, 3H); 2.14-2.23 (m, 2 H); 2.46-2.59 (m, 2H); 3.06 (dd, *J* = 13.8, 8.8 Hz, 1H); 3.30 (m, 1H); 3.86 (s, 2H); 4.02 (t, *J* = 6.6 Hz, 1H); 4.47 (m, 1H); 6.60 (br d, *J* = 1.6 Hz, 1H); 6.70 (br d, *J* = 1.6 Hz, 1H); ^{13}C-NMR (CD_3OD): δ 174.5, 173.0, 172.7, 171.7, 146.4, 144.6, 130.8, 126.3, 120.4, 117.6, 54.6, 53.7, 41.8, 37.2, 32.5, 27.1, 20.7.

LC-MS/MS Analyses.

LC-MS/MS analyses were performed on a Micromass (Manchester, UK) *Quattro Micro* triple quadrupole mass spectrometer that was interfaced to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). The ESI ion source was operated in either the positive ion mode for analyzing GSH adducts or the negative mode for detecting metabolites of p-cresol, and experimental parameters were set as follows: capillary voltage 3.2 kV, source temperature 120 ^{o}C, desolvation temperature 300 ^{o}C,

sample cone voltage 22 V. Data were processed using the *Masslynx* version 4.0 software from Micromass.

For rapid profiling of GSH adducts, samples were first subjected to chromatographic separations on an Agilent Zorbax SB C18 column (2.1 x 50 mm), and eluents were introduced to the triple quadrupole mass spectrometer that was operated in the neutral loss scanning mode detecting all protonated molecules losing 129 Da under collision-induced dissociation (CID). The starting mobile phase consisted of 95% water (0.5% acetic acid), and the metabolites were eluted using a single gradient of 95% water to 95% acetonitrile over 7 min at a flow rate of 0.25 ml/min. At 7 min, the column was flushed with 95% acetonitrile for 2 min before re-equilibration at initial conditions. LC-MS/MS analyses were carried out on 30-μl aliquots of samples. Mass spectra collected in the neutral loss scanning mode (NL) were obtained by scanning over the range *m/z* 350-600 in 2s.

For subsequent structural characterization of metabolites and GSH adducts, an Agilent Zorbax SB C18 column (2.1 x 150 mm) was used for chromatographic separations. A 20-min LC run was performed to separate metabolites: the starting mobile phase consisted of 95% water (0.5% acetic acid), and the metabolites were eluted using a single gradient of 95% water to 95% acetonitrile over 15 min at a flow rate of 0.25 ml/min. At 15 min, the column was flushed with 95% acetonitrile for 2 min before re-equilibration at initial conditions.

To relatively compare the level of GSH adducts and metabolites formed in incubations, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using collision energy at 20 eV. MRM transitions used are *m/z* 414→179

for glutathionyl-4-methylphenol, *m/z* 430→301 for 4-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone, and *m/z* 121→92 for 4-hydroxybenzaldehyde, respectively.

Results

Stable isotope trapping and detection of reactive metabolites of p-cresol.

To facilitate screening of all reactive metabolites derived from p-cresol, incubations were performed with human liver microsomes in the presence of both natural and stable isotope-labeled glutathione (Yan et al., 2004). Reactive metabolites were trapped to form both natural and stable-isotope-labeled GSH adducts. Samples were subjected to LC/MS/MS NL scan detecting protonated molecules that lost 129 Da upon CID. Because glutathione was triply labeled at glycine, formed GSH adducts were positively identified by examining a unique isotopic doublet of a mass difference of 3 Da. (Yan et al., 2004). As shown in Figure 1A, although several peaks showed responses to the NL scanning for 129 Da, four components, GA at 3.20 min, GB at 3.96 min, GC at 4.2 min and GD at 3.75 min, exhibited the expected isotopic doublet, respectively. Both GC and GD appeared as small shoulder peaks of GB under current chromatographic conditions (LC column, C18, 2.1 x 50 mm), but were well resolved on a longer column (C18, 2.1 x 150 mm) (data not shown). The protonated molecule of GA displayed an isotopic doublet at *m/z* 414 and 417 (Figure 1B), suggesting formation of a GSH adduct likely resulting from the quinone methide intermediate (Thompson et al., 1995). Interestingly, GB, GC and GD exhibited an identical isotopic doublet at *m/z* 430 and 433 (Figure 1C-E), suggesting formation of three additional isomeric GSH adducts. No GSH adducts were detected when either p-cresol or the NADPH regenerating system was omitted from incubations. These results suggested that GSH adducts were formed from

reactive metabolites of p-cresol via CYP-mediated metabolic pathways. In addition, all four adducts (GA-D) were detected in both human and rat liver microsomes at different concentrations of p-cresol (10 – 200 μM), which indicated that formation of GSH adducts was not dependent upon substrate concentrations.

MS Characterization of GSH Adducts.

Tandem MS analyses were performed to characterize the structure of individual adducts. As shown in Figure 2A, CID MS/MS spectrum of the MH^+ at *m/z* 414 gave product ions at *m/z* 339 and 285, apparently resulting from neutral losses of glycine (75 Da) and ϒ-glutamate (129 Da), respectively, which further confirmed the presence of a GSH moiety in the metabolite; the product ion at *m/z* 107 was apparently derived from losing the GSH moiety of the conjugate.

As shown in Figure 2B-D, tandem MS spectra of GB, GC and GD were virtually indistinguishable. Neutral losses of glycine and ϒ-glutamate were observed for all three metabolites, giving rise of product ions at *m/z* 355 and 301 respectively, which confirmed that GB, GC and GD were isomeric GSH adducts.

To further elucidate the structure of GSH adducts, 4-methylphenol-2,3,5,6-d_4 was used in microsomal incubations in the presence of GSH. NL MS/MS analysis revealed that precursor ions of GA appeared at *m/z* 418 (Figure 3A). A mass shift of 4 Da observed for GA indicated that the glutathione moiety is attached to the methyl group, which is consistent with glutathionyl-4-methylphenol previously proposed by others (Thompson et al., 1995). A mass shift of 2 Da was observed for GB, GC and GD (Figure 3B-D), suggesting that both the GSH moiety and hydroxyl group were on the aromatic ring. Therefore, it was concluded that those adducts (GB, GC and GD) were not

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derived from hydroxylation of GA. Otherwise, a mass shift of 3 Da would be detected for those three conjugates.

Identification of stable and reactive metabolites of p-cresol in incubations.

It can be envisioned that p-cresol was first converted to 4-methyl-*ortho*-hydroquinone that was further oxidized to a reactive intermediate 4-methyl-*ortho*-benzoquinone, resulting in formation of isomeric adducts in the presence of GSH in incubations. To further confirm this bioactivation pathway, microsomal incubations of p-cresol were performed in the absence of GSH. LC-MS/MS analysis of microsomal samples detected two metabolites (M1 and M2) showing molecular ions ($[M-H]^-$) at *m/z* 123 Da (Figure 4A). M1 eluted at 7.8 min (Figure 4B) was identified by tandem MS as 4-hydroxybenzylalcohol. As shown in Figure 4C, molecular ion ($[M-H]^-$) at *m/z* 123 lost a water to give product ions at *m/z* 105 that was subsequently fragmented to generate product ions at *m/z* 77 ($C_6H_5^-$). The identity of M1 was further confirmed by comparing the LC-MS/MS characteristics of the reference compound.

The tandem MS spectrum of the metabolite M2 (RT 9.6 min) is shown in Figure 4D, which is consistent with 4-methyl-*ortho*-hydroquinone. Deprotonated molecules ($[M-H]^-$) at *m/z* 123 underwent consecutive losses of the methyl and hydroxyl group generated product ions at *m/z* 108 and *m/z* 91, respectively. Product ions at *m/z* 65 were apparently resulted from a loss of the diol moiety, which were the key fragment for positively assigning M2 as 4-methyl-*ortho*-hydroquinone. Retention time and mass spectral characteristics of M2 were identical to that of synthetic 4-methyl-*ortho*-hydroquinone.

Another major metabolite M3 ($[M-H]^-$, *m/z* 121, RT 9.35 min) was detected in the same microsomal incubations (Figure 5A). As seen in CID-MS/MS spectrum of M3 (Figure 5B), the most abundant product ions appeared at *m/z* 92, apparently resulting from a loss of CHO, which suggested that M3 is 4-hydroxybenzaldehyde. LC-MS/MS analysis of the reference compound further confirmed the structure of M3.

Formation of GSH Adducts by 4-Methyl- *ortho*-hydroquinone.

Two experiments were conducted to further verify that 4-methyl-*ortho*-hydroquinone was the precursor molecule for GB, GC and GD. First, 4-methyl-*ortho*-hydroquinone was incubated with human liver microsomes in the presence of GSH to generate corresponding conjugates (Fig. 6B); second, 4-methyl-*ortho*-hydroquinone was used as the starting compound to chemically synthesize GSH adducts (Fig. 6C). As shown in Figure 6, three GSH adducts were generated by both methods, which exhibited identical LC-MS/MS characteristics comparing to those formed in microsomal incubations of p-cresol (Fig. 6A).

The major conjugate was unambiguously identified as 3-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone by comparing ^{1}H-NMR spectra in the aromatic region of the GSH conjugate and 4-methyl-*ortho*-hydroquinone. For 4-methyl-*ortho*-hydroquinone, couplings between two aromatic protons at *meta*-positions (H^a and H^b) were observed, with a coupling constant of 1.6 Hz (Fig. 7A). NMR spectrum of the conjugate is shown in Figure 7B, which clearly suggests that two aromatic protons are *meta*-positioned. In addition, high resolution NMR revealed weak couplings between the aromatic protons and the aliphatic protons on the methyl group (data not shown). The results clearly indicated that the GSH moiety is at the C-3 position. If the GSH moiety is at the C-4

position, the *meta*-couplings would not be detected. Apparently, 6-(glutathione-S-yl)-5-methyl-benzene-1, 2-diol would exhibit two doublet peaks (J = 7.5 Hz), due to the coupling between two *ortho* aromatic protons (H^b and H^c). The addition of glutathione to 4-methyl-[1,2]benzoquinone could result in three adducts, 3-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone, 4-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone and 6-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone. Two minor adducts were not characterized by NMR because of low abundance and difficulty in isolation. Presumably, they are 4-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone and 6-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone, respectively.

Metabolite Formation in Incubations with Recombinant CYPs.

The formation of glutathionyl-4-methylphenol (GA), 3-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone (GB) and 4-hydroxybenzaldehyde (4HBZD) was investigated in microsomes derived from insect cell expressed recombinant human CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively. Both glutathionyl-4-methylphenol and 3-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone were detected in GSH-fortified incubations with all CYP enzymes tested. Similarly, 4-hydroxybenzaldehyde was also detected in all incubations without GSH. As seen in Table 1, individual isozymes showed different enzymatic activity. At the same enzyme concentration (100 pmoles/ml), CYP2D6 was the most active enzyme catalyzing formation of glutathionyl-4-methylphenol (GA), which was followed by CYP2C19, 1A2, 1A1 and 2E1, and enzymatic activity of CYP2C9 and 3A4 was relatively low. CYP2E1 and 1A2 were the most active in formation of 3-(glutathione-S-yl)-5-methyl-*ortho*-

hydroquinone (GB), 2-diol and 4-hydroxybenzaldehyde (4HBZD) respectively, whereas enzymatic activity of other isozymes was significantly lower.

Discussion

Although it has been recognized that p-cresol is metabolically activated to form a reactive quinone methide intermediate (Thompson et al., 1995), the proof of the reactive metabolite as a causative agent for p-cresol-induced toxicity still remains elusive. It is essential to completely understand bioactivation pathways of p-cresol in order to comprehensively address the toxicity mechanism.

In this study we have investigated bioactivation of p-cresol in human liver microsomes fortified with NADPH, in attempt to elucidate metabolic pathways leading to formation of reactive intermediates. In addition to the GSH adduct (GA) derived from the quinone methide intermediate, three new GSH adducts (GB, GC and GD) were detected using stable-isotope GSH trapping in combination with LC-MS/MS analysis of incubations of p-cresol with human liver microsomes. The major GSH adduct GB was identified as 3-(glutathione-S-yl)-5-methyl-*ortho*-hydroquinone by tandem MS in combination of NMR analysis of the synthetic conjugate.

Based on our results, a new bioactivation mechanism of p-cresol has been proposed. As shown in Scheme I, there are two metabolic pathways responsible for bioactivation of p-cresol. As previously proposed by others (Thompson et al., 1995), oxidation of the methyl group leads to formation of a quinone methide intermediate that is trapped by GSH to form glutathionyl-4-methylphenol (GA) in microsomal incubations. Formation of the quinone methide intermediate is mediated by several CYPs which include 2D6, 2C19, 1A2, 1A1 and 2E1. The newly identified pathway is aromatic

oxidation leading to formation of 4-methyl-*ortho*-hydroquinone that is further oxidized to 4-methyl-[1,2]benzoquinone. The o-quinone intermediate could be trapped in microsomal incubations by GSH to form three adducts, but 3-(glutathione-S-yl)-5-methyl *ortho*-hydroquinone is the predominant conjugate. This bioactivation pathway was further confirmed by incubating 4-methyl-*ortho*-hydroquinone with human microsomes to generate the same conjugates. In the presence of GSH as a trapping agent, three adducts were detected, which exhibited identical chromatographic and MS characteristics of conjugates formed by p-cresol. The aromatic oxidation pathway is primarily mediated by CYP2E1, and also to a less extent by other CYPs such as 1A1, 1A2 and 2D6. Additionally, two new metabolites, 4-methyl-*ortho*-hydroquinone and 4-hydroxybenzaldehyde, were identified in microsomal incubations of p-cresol using LC-MS/MS. It is apparent that 4-hydroxybenzaldehyde is the oxidation metabolite of 4-hydroxybenzylalcohol, the primary metabolite of p-cresol (Scheme I). Formation of 4-hydroxybenzaldehyde is primarily catalyzed by 1A2, and other isozymes such as 1A1 and 2D6 are also active.

It has been well established that substituted phenols such as p-cresol could be oxidized to reactive quinones by tyrosinase, a copper monooxygenase that catalyzes oxygenation of phenols to catechols and subsequently to the corresponding o-quinones (Solomon et al., 1996). It is reasonable to expect that p-cresol can also be oxidized by CYPs to 4-methyl-*ortho*-hydroquinone that is subsequently converted to a reactive o-quinone intermediate. This bioactivation pathway has not been reported in previous studies using rat liver microsomes (Thompson et al., 1994; Thompson et al., 1995). The reason why this metabolic pathway was not identified previously by other investigators is

not clear. Species difference is not a factor since the same GSH adducts were detected in both rat and human liver microsomes. Different analytical methods could be a likely explanation. Alternatively, it is possible that this bioactivation pathway is less dominant comparing to the quinine-methide pathway (Thompson et al., 1994; Thompson et al., 1995).

Quinones are known as a class of toxicological intermediates, which could cause a variety of toxic efforts such as acute cytotoxicity, immunotoxicity and carcinogenesis (Bolton et al., 2000). As highly redox active molecules, quinones can redox cycle with their semiquinone radicals, leading to formation of reactive oxygen species (ROS). As Michael receptors, quinones can alkylate cellular nucleophiles such as proteins and DNA. More recent studies have also demonstrated that some quinones and hydroquinones can poison Topoisomerase II (Lindsey et al., 2005; Bender et al., 2004). Considering that 4-methyl-*ortho*-benzoquinone is abundant in microsomal incubations of p-cresol, toxicological significance of its reactive metabolite should not be completely ignored. For example, as a highly redox active molecule, 4-methyl-*ortho*-benzoquinone might potentially play an important role in inhibition of liver mitochondria respiration caused by p-cresol (Kitagawa et al., 2001). It has been shown that microsomal activation of p-cresol led to oxidative DNA damage (Murata et al., 1999) and formation of DNA adducts (Gaikwad et al., 2001; Gaikwad et al., 2003), but the structures of formed DNA adducts has not been elucidated at present. The quinone methide generated from aliphatic oxidation of p-cresol has been proposed as the reactive metabolite leading to formation of DNA adduct (Thompson et al., 1995). Our present results raised another possibility that, similar to quinone methide, 4-methyl-*ortho*-benzoquinone may also play an important

role in DNA adduct formation. For instance, estrogens such as equilenin (Zhang et al., 2001) and 17β-estradiol (Bradlow et al., 1986) formed DNA adducts via quinone intermediates. Future structural characterization of DNA adducts derived from p-cresol would provide new insights on the corresponding role of the quinone methide and *ortho*-quinone in covalent modification of nucleic acids.

Apparently, a quantitative comparison of the quinone methide and *ortho*-quinone formed in microsomal incubations would be very helpful for understanding how these two pathways may be involved in potential toxicity of p-cresol. However, such a direct comparison is not experimentally feasible due to instability of those metabolites. An alternative is to measure the level of their stable adducts. But one should realize that, since two reactive intermediates might have different reactivity with GSH, the amount of each GSH adduct formed in incubations may not necessarily be proportional to the level of their corresponding reactive metabolite. It should be noted that aldehydes may also be reactive and could covalently modify proteins and nucleic acids (Hecht et al., 2001; Kaminskas et al., 2004). The reactivity of 4-hydroxybenzylaldehyde, a major metabolite of p-cresol, remains to be determined.

In conclusion, 4-hydroxybenzaldehyde, 4-methyl-*ortho*-hydroquinone and its corresponding GSH adducts were identified in incubations of p-cresol with human liver microsomes. Consequently, a new bioactivation pathway is rationalized: aromatic oxidation of p-cresol leads to the formation of 4-methyl-*ortho*-hydroquinone that is further oxidized to a reactive intermediate 4-methyl-*ortho*-benzoquinone. Given the fact that benzoquinones are known causative agents in the toxicity of many toxicants (Monks et al., 2002), the present finding is of significance in understanding the toxicology

mechanism of p-cresol, and more studies are necessary in the future to further examine the possible role of 4-hydroxybenzaldehyde and 4-methyl-*ortho*-hydroquinone in p-cresol-induced covalent modifications of proteins and DNAs.

References

Bradlow, H. L., Hershcopf, R., Martucci, C., Fishman, J. (1986) 16 alpha-hydroxylation of estradiol: a possible risk marker for breast cancer. *Annals New York Acad. Sci.* **464**, 138-151.

Bender, R. P., Lindsey, R. H. J., Burden, D. A., Osheroff, N. (2004) N-Acetyl-p-benzoquinone Imine, the Toxic Metabolite of Acetaminophen, Is a Topoisomerase II Poison. *Biochemistry* **43**, 3731-3739.

Bolton, J. L., Trush, M. A., Penning, T. M., Dryhurst, G., Monks, T. J. (2000) Role of quinones in toxicology. *Chem. Res. Toxicol.* **13**, 135-160.

Carlson, B. W.; Miller, L. L. (1985) Mechanism of the oxidation of NADH by quinines, energetics of one-electron and hydride routes. *J. Am. Chem. Soc.* **107**, 479-485.

De Smet, R., Van Kaer, J., Van Vlem, B., De Cubber, A., Brunet, P., Lameire, N. and Vanholder, R. (2003) Toxicity of free p-cresol: A prospective and cross-sectional analysis. *Clin. Chem.* **49**, 470-478.

Gaikwad, N. W., Bodell, W. J. (2003) Formation of DNA adducts in HL-60 cells treated with the toluene metabolite p-cresol: a potential biomarker for toluene exposure. *Chem.-Biol. Interact.* **145**, 149-158.

Gaikwad, N. W., Bodell, W. J. (2001) Formation of DNA adducts by microsomal and peroxidase activation of p-cresol: role of quinone methide in DNA adduct formation. *Chem.-Biol. Interact.* **138**, 217-229.

Hecht, S. S., McIntee, E. J., Wang, M. (2001) New DNA adducts of crotonaldehyde and acetaldehyde. *Toxicology* **166**, 31-36.

Kamijo, Y., Soma, K., Kokuto, M., Ohbu, M., Fuke, C. and Ohwada, T. (2003) Hepatocellular injury with hyperaminotransferasemia after cresol ingestion. *Arch. Path. & Lab. Med.* **127**, 364-366.

Kaminskas, L. M., Pyke, S. M., Burcham, P. C. (2004) Strong protein adduct trapping accompanies abolition of acrolein-mediated hepatotoxicity by hydralazine in mice. *J. Pharmacol. Exp. Ther.* **310**, 1003-1010.

Kitagawa, A. (2001) Effects of cresols (o-, m-, and p-isomers) on the bioenergetic system in isolated rat liver mitochondria. *Drug and Chem. Toxicol.* **24**, 39-47.

Lesaffer, G., De Smet, R., D'heuvaert, T., Belpairea, F. M., Lameire, N., Vanholder, R. (2001) Kinetics of the protein-bound, lipophilic, uremic toxin p-cresol in healthy rats. *Life Sci.* **69**, 2237-2248.

Lindsey, R. H. J., Bender, R. P., Osheroff, N. (2005) Effects of Benzene Metabolites on DNA Cleavage Mediated by Human Topoisomerase IIα: 1,4-Hydroquinone Is a Topoisomerase II Poison. *Chem. Res. Toxicol.* **18**, 761-770.

Monks, T. J., Jones, D. C. (2002) The metabolism and toxicity of quinones, quinonimines, quinone methides, and quinone-thioethers. *Curr. Drug Metab.* **3**, 425-438.

Monma-Ohtaki, J., Maeno, Y., Nagao, M., Iwasa, M., Koyama, H., Isobe, I., Seko-Nakamura, Y., Tsuchimochi, T. and Matsumoto, T. (2002) An autopsy case of poisoning by massive absorption of cresol a short time before death. *Forensic Sci. International* **126**, 77-81.

Murata, M., Tsujikawa, M., Kawanishi, S. (1999) Oxidative DNA damage by minor metabolites of toluene may lead to carcinogenesis and reproductive dysfunction. *Biochem. Biophy. Res. Comm.* **261**, 478-483.

Sato, T., Suzuki, T., Fukuyama, T., Yoshikawa, H. (1956) Conjugation of S35-sulfate with phenolic compounds. III. Metabolism of p-cresol, p-hydroxybenzaldehyde, and p-hydroxybenzoic acid in rat liver. *J. Biochem.* **43** 413-420.

Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. (1996) Multicopper Oxidases and Oxygenases. *Chemical Reviews* **96**, 2563-2605.

Thompson, D. C., Perera, K., Fisher, R., Brendel, K. (1994) Cresol isomers: comparison of toxic potency in rat liver slices. *Toxicol. and Appl. Pharmacol.* **125**, 51-58.

Thompson, D. C., Perera, K., London, R. (1995) Quinone Methide Formation from Para Isomers of Methylphenol (Cresol), Ethylphenol, and Isopropylphenol: Relationship to Toxicity. *Chem. Res. Toxicol.* **8**, 55-60.

Yan, Z., Caldwell, G.W., Wu, W., McKown, L., Rafferty, B., Jones, W., Masucci. J. A. (2002) *In vitro* identification of metabolic pathways and cytochrome P450 isoforms involved in the metabolism of etoperidone. *Xenobiotica* **32**, 949-962.

Yan, Z., Caldwell, G. W. (2004) Stable-isotope trapping and high throughput screenings of reactive metabolites using the isotope MS signature. *Anal. Chem.* **76**, 6835-6847.

Zhang, F., Swanson, S. M., van Breemen, R. B., Liu, X., Yang, Y., Gu, C., Bolton, J. L. (2001) Equine estrogen metabolite 4-hydroxyequilenin induces DNA damage in the rat mammary tissues: formation of single-strand breaks, apurinic sites, stable adducts, and oxidized bases. *Chem. Res. Toxicol.* **14**, 1654-1659.

Legends

Figure 1. LC/MS/MS detection of GSH adducts derived from p-cresol by constant scanning for neutral losses of 129 Da. (A), HPLC chromatogram of p-cresol-GSH adducts; (B), NL-MS/MS spectrum of GA; (C), NL-MS/MS spectrum of GB; (D), NL-MS/MS spectrum of GC; and (E), NL-MS/MS spectrum of GD. Asterisk marked false responses to NL scan.

Figure 2. CID-MS/MS spectra of adducts GA (A), GB (B), GC (C) and GD (D).

Figure 3. CID-NL-MS/MS spectra of GSH adducts derived from p-cresol-d4. Three adducts were detected, which corresponded to natural conjugates GA (A), GB (B), GC (C) and GD (D).

Figure 4. LC-MS/MS analysis of M1 and M2 derived from p-cresol in HLM incubations. (A), HPLC chromatogram of molecular ions ($[M-H]^-$) at *m/z* 123; (B), expanded A to show metabolite M1; (C), CID-MS/MS spectrum of M1; (D), CID MS/MS spectrum of M2.

Figure 5. LC-MS/MS analysis of M3 derived from p-cresol in HLM incubations. (A), HPLC chromatogram of molecular ions ($[M-H]^-$ *m/z* 121); (B), CID-MS/MS spectrum of the metabolite M3 at *m/z* 121.

Figure 6. LC-MS/MS analysis of GSH adducts prepared by microsomal incubations of p-cresol (A) and 4-methyl-benzene-1, 2-diol (B), and by chemical synthesis (C).

Figure 7. NMR spectra of 4-methyl-ortho-hydroquinone (top) and the synthetic GSH adduct (bottom).

Scheme I. Bioactivation pathways of p-cresol in human liver microsomes.

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Table 1. GSH adducts and 4-hydroxybenzaldehyde formed by individual CYPs.

CYP	Level of GA[1,*]	Level of GB[2,*]	Level of 4-HBZD[3,*]
1A1	52	28	16
1A2	67	20	100
2C9	14	1	1
2C19	80	5	14
2D6	100	23	35
2E1	26	100	11
3A4	6	19	1

[1], GA: glutathionyl-4-methylphenol; [2], GB: 3-(glutathione-S-yl)-5-methyl-benzene-1, 2-diol; [3], 4-HBZD: 4-hydroxybenzaldehyde.

***** Values are expressed as percentages relative to the highest level.

Scheme 1

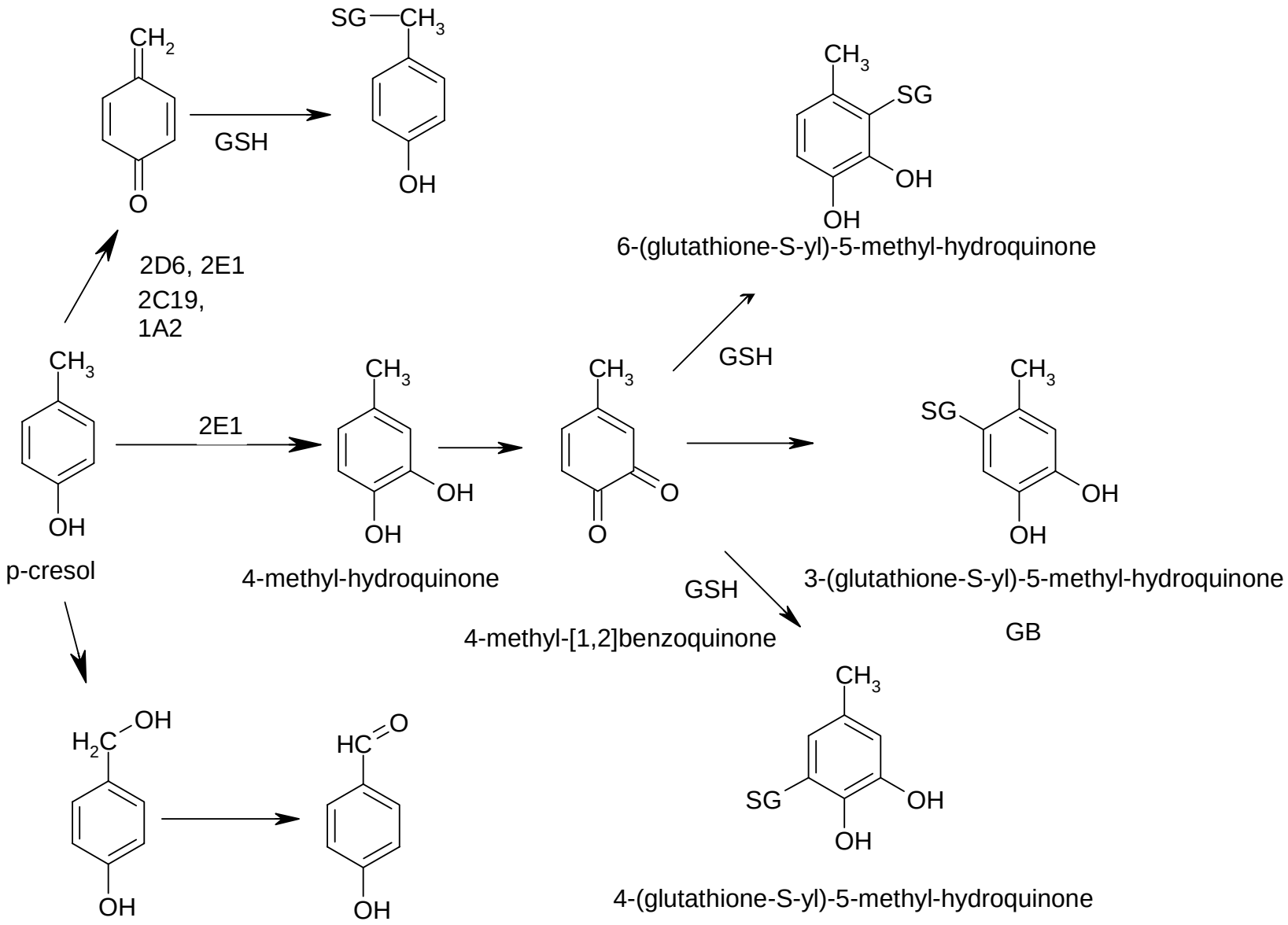


Fig. 1 A, B

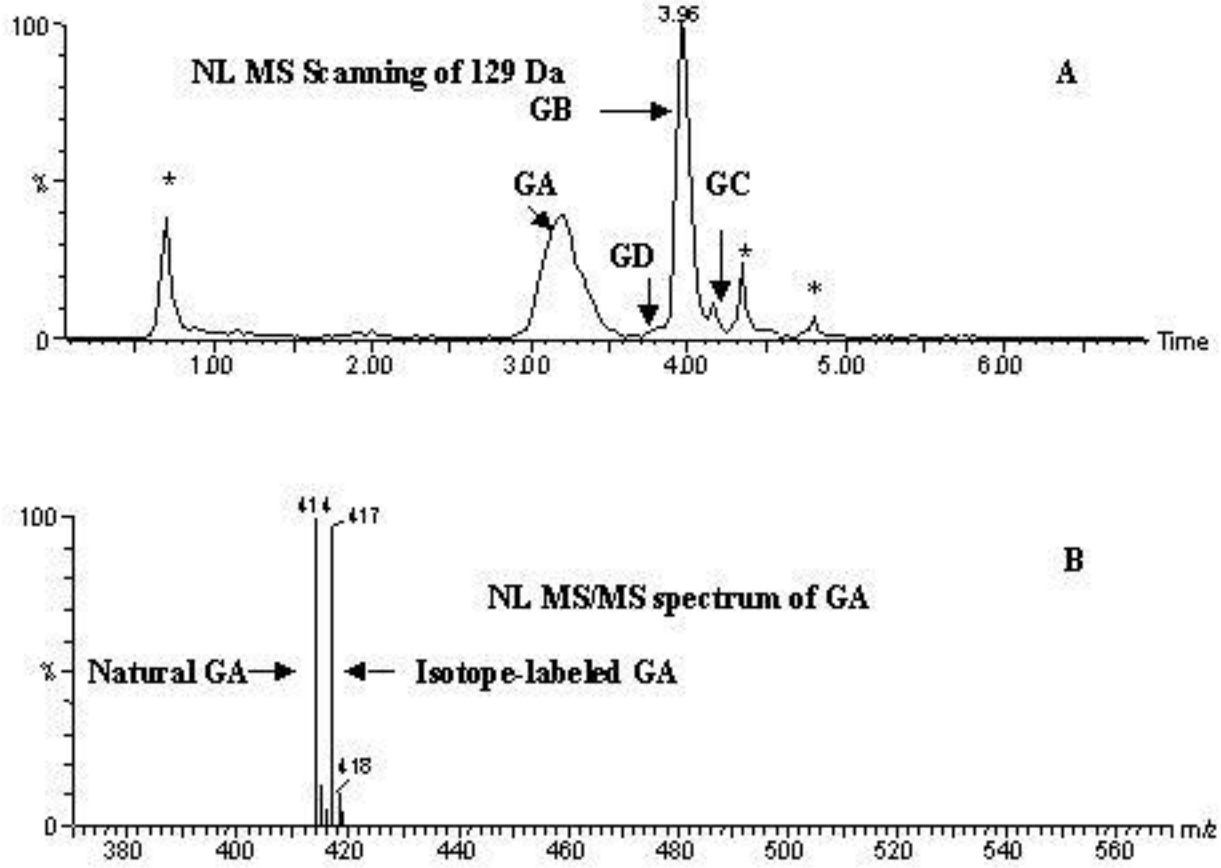


Fig. 1-C&D

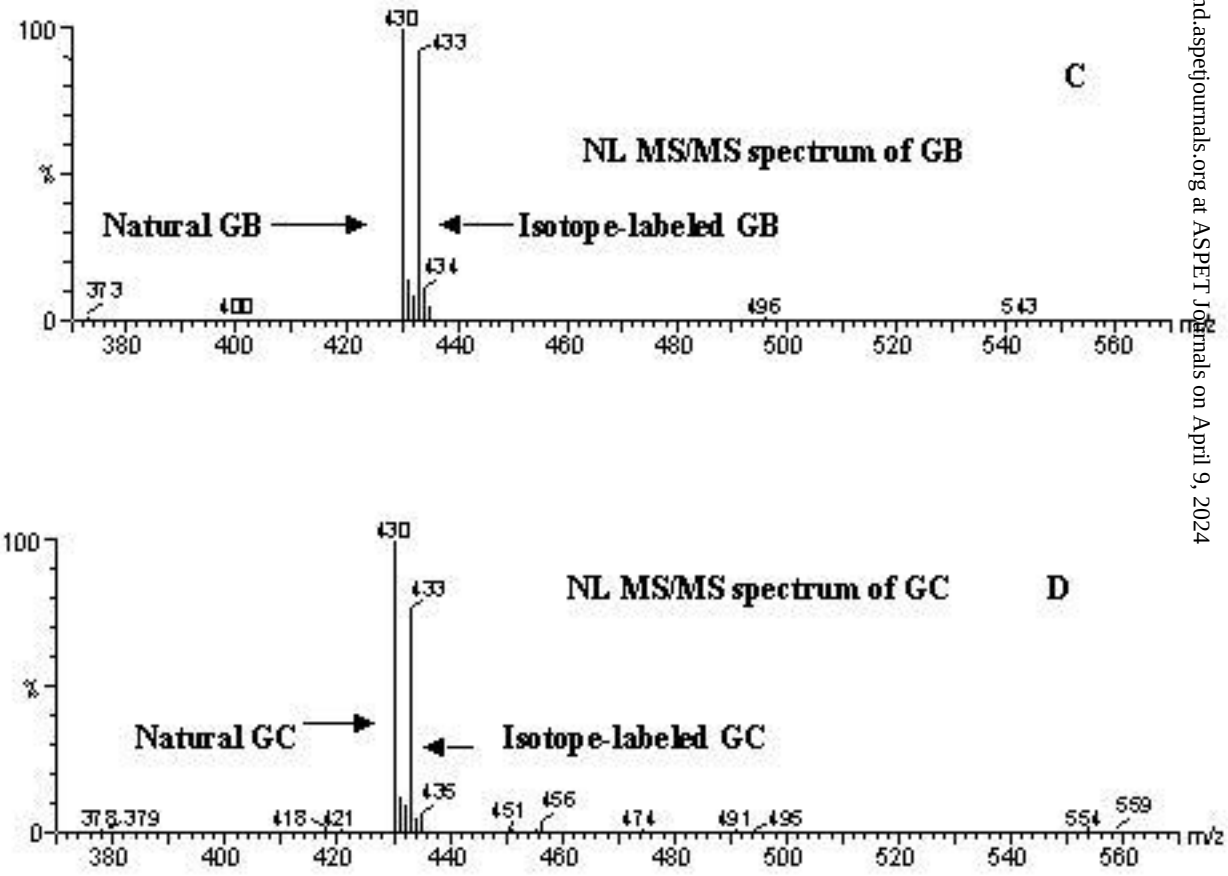


Figure 1E

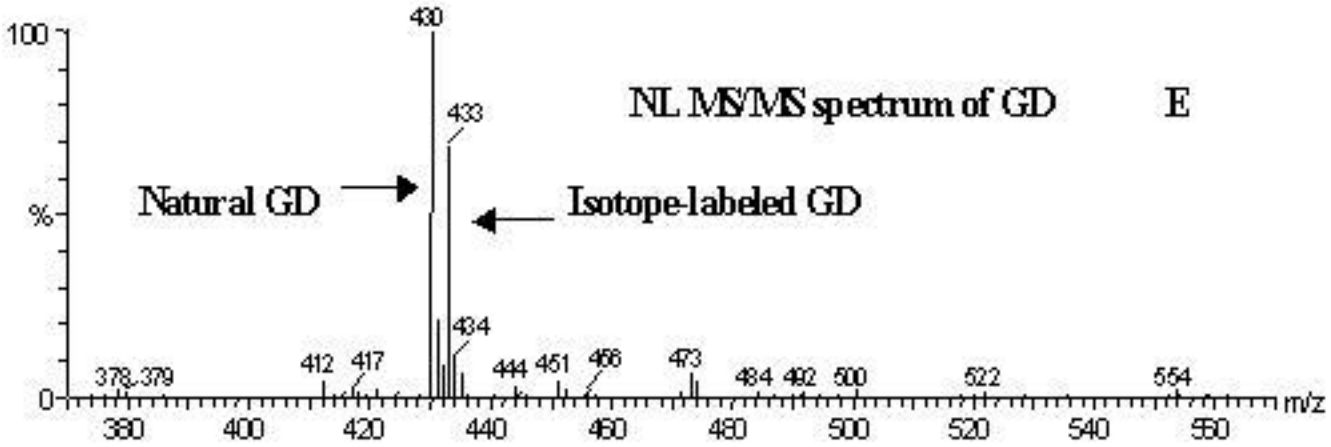


Fig.2-A&B

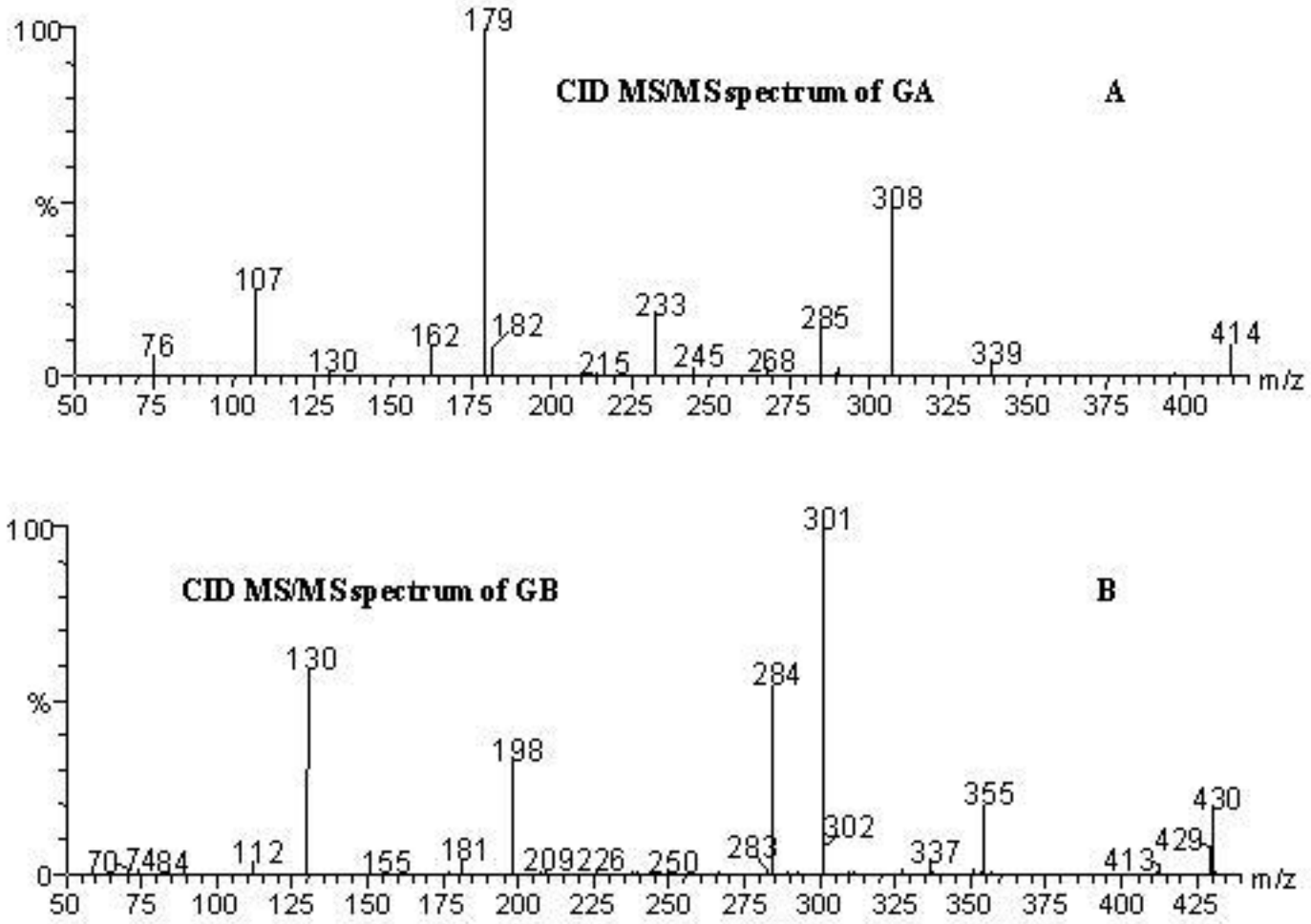
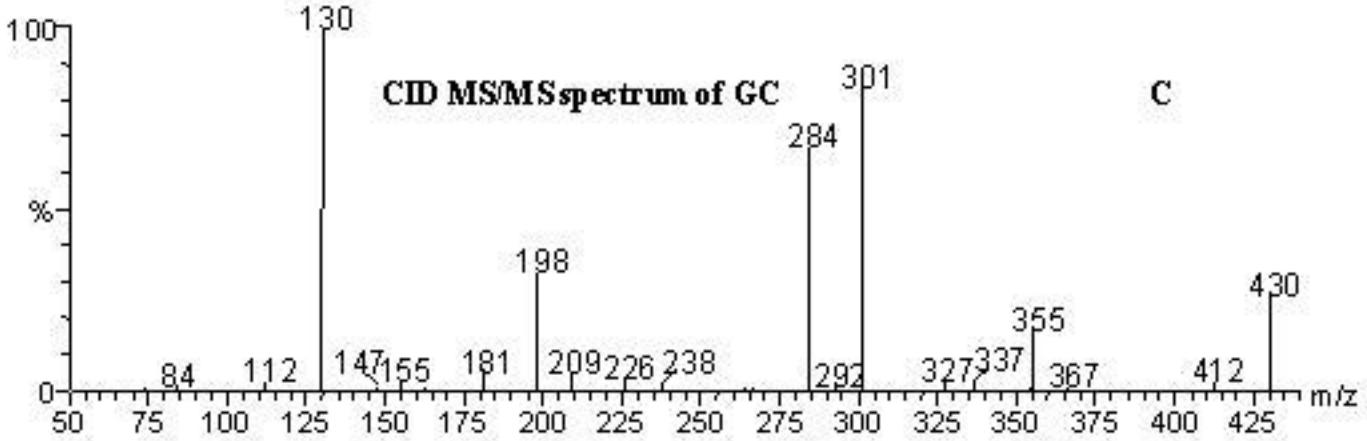


Fig.2-C&D



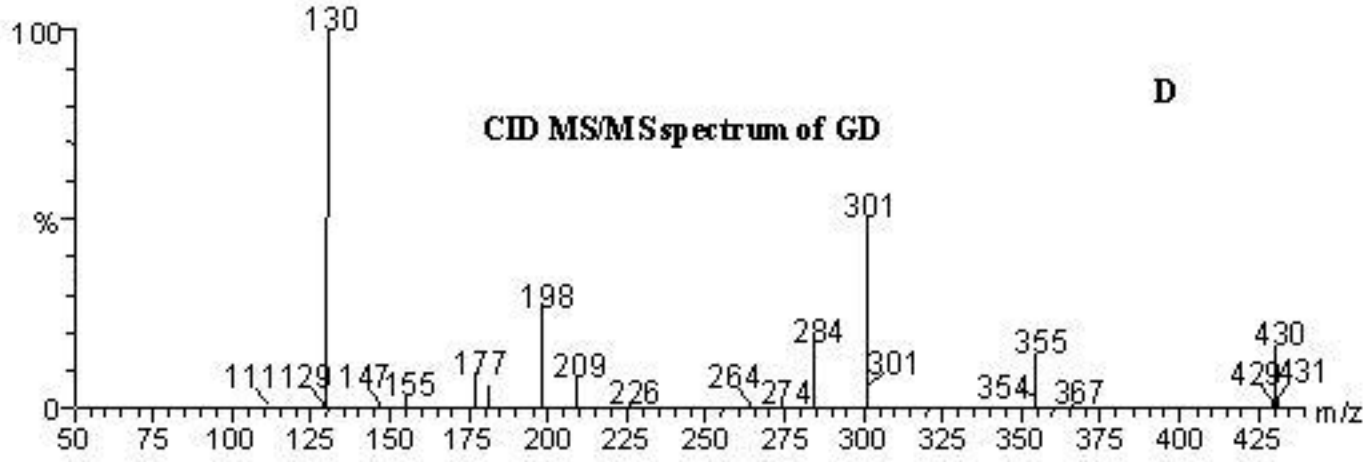
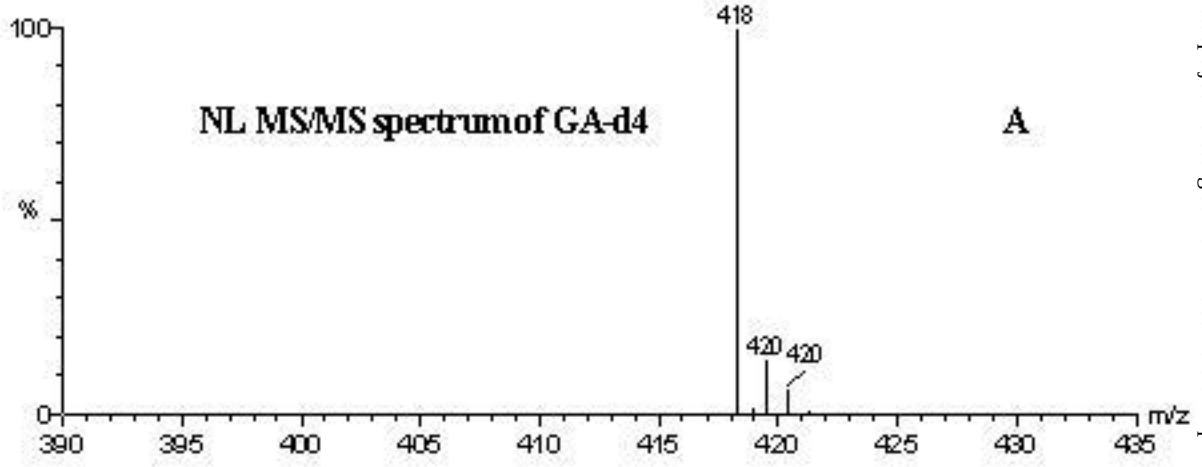


Figure 3A&B



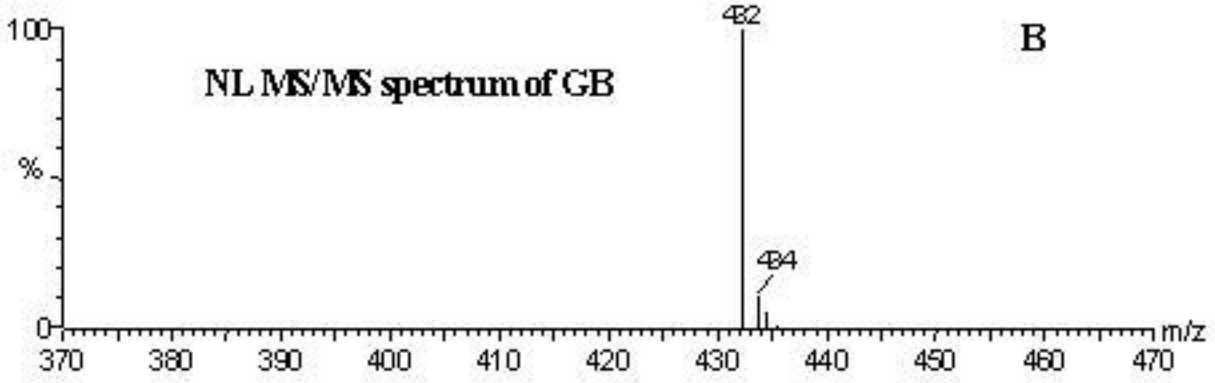


Fig. 3-C&D

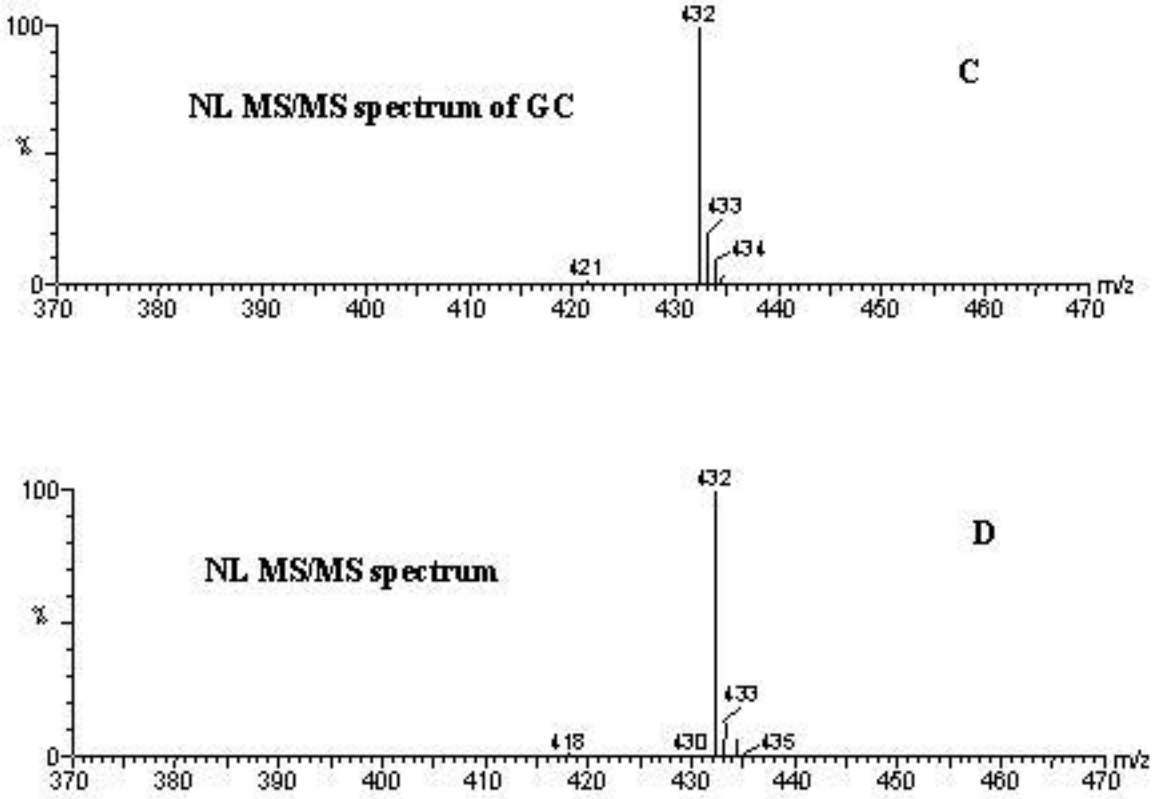


Figure 4 A&B

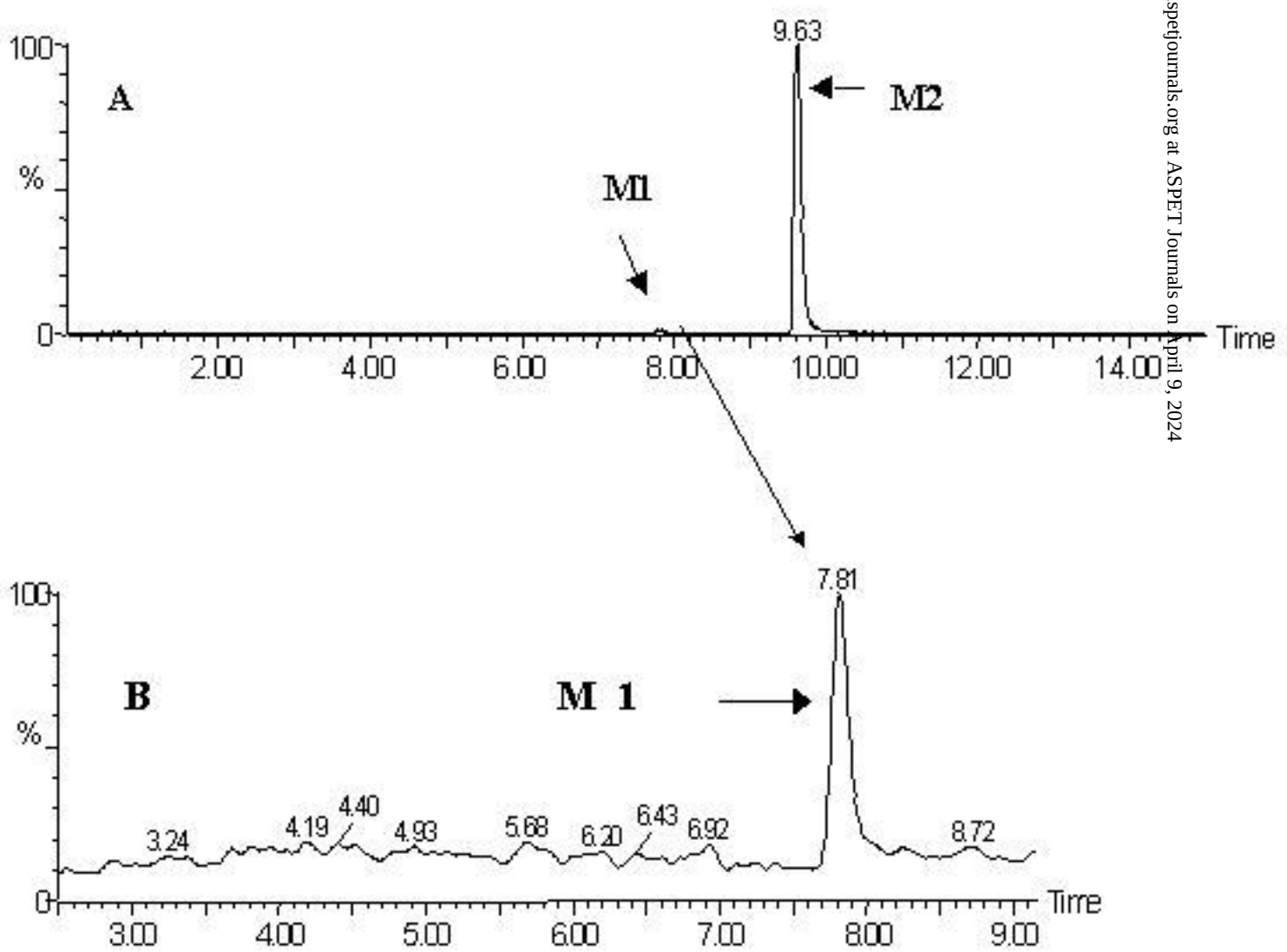
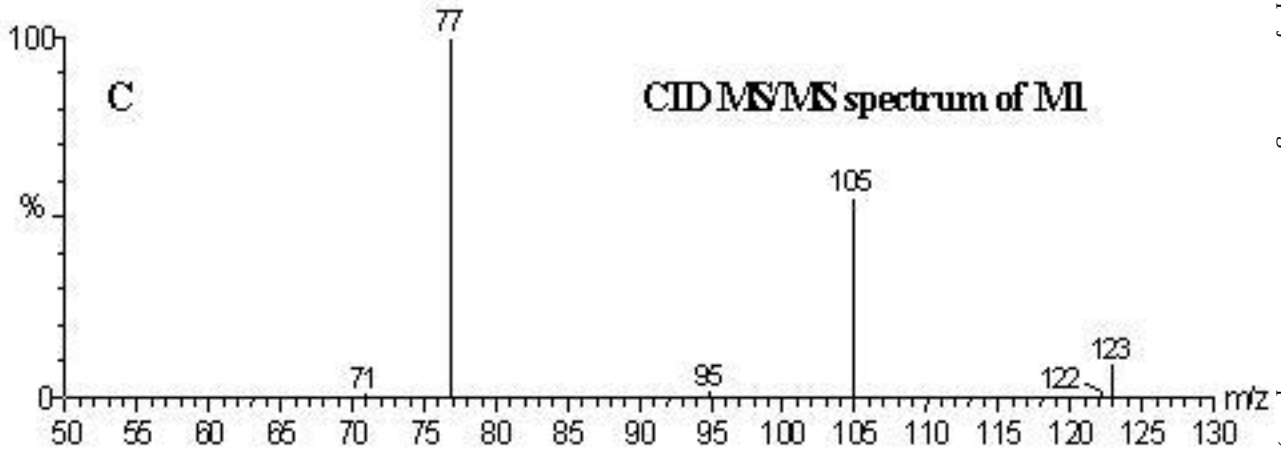


Figure 4 C&D



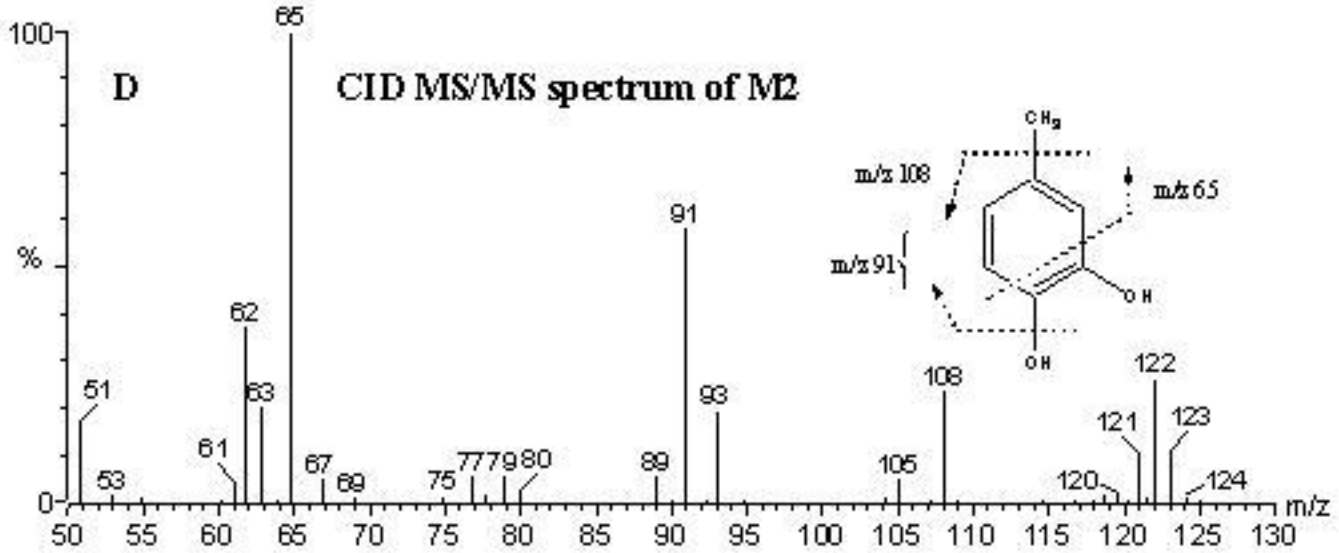


Fig.5-A&B

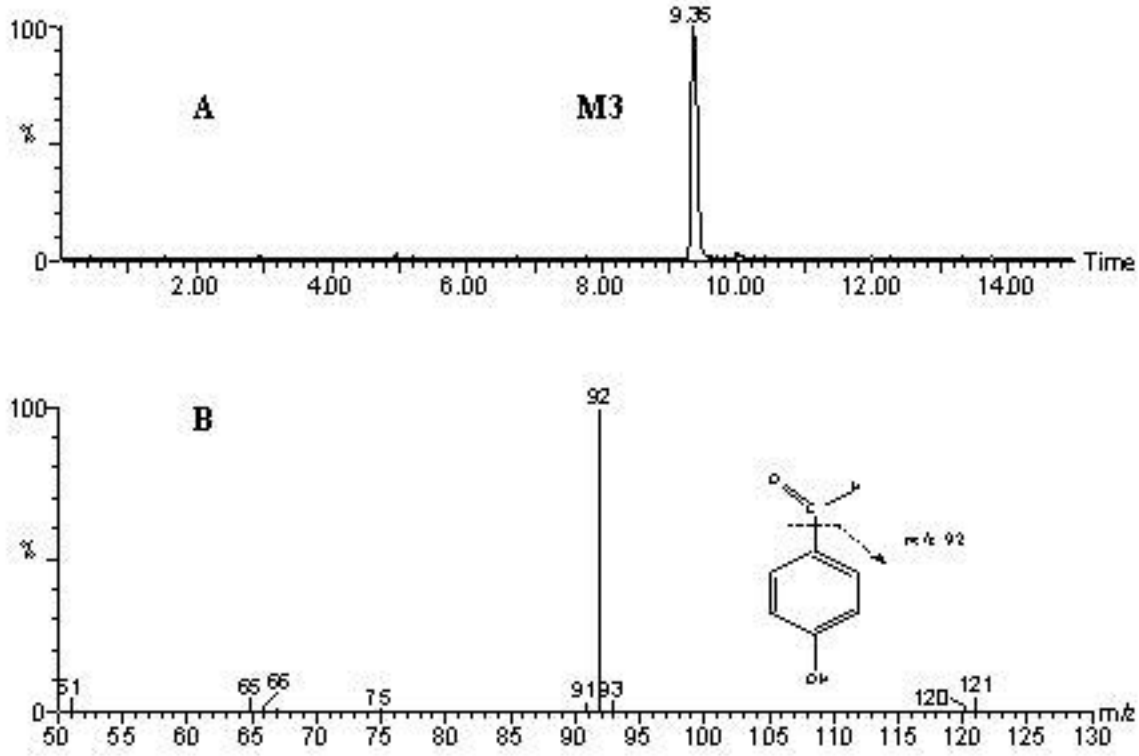


Figure 6 A&B

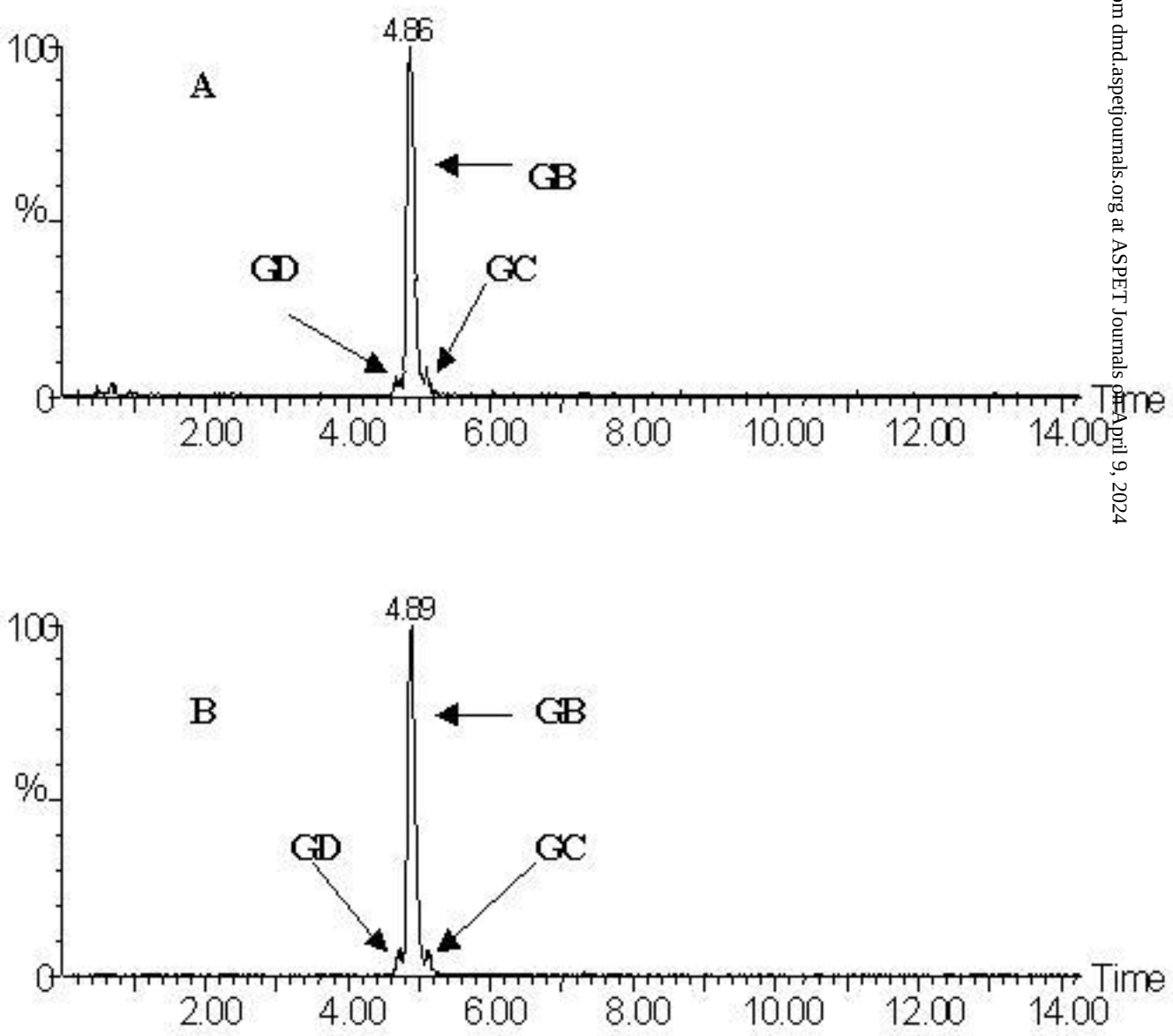


Figure 6 C

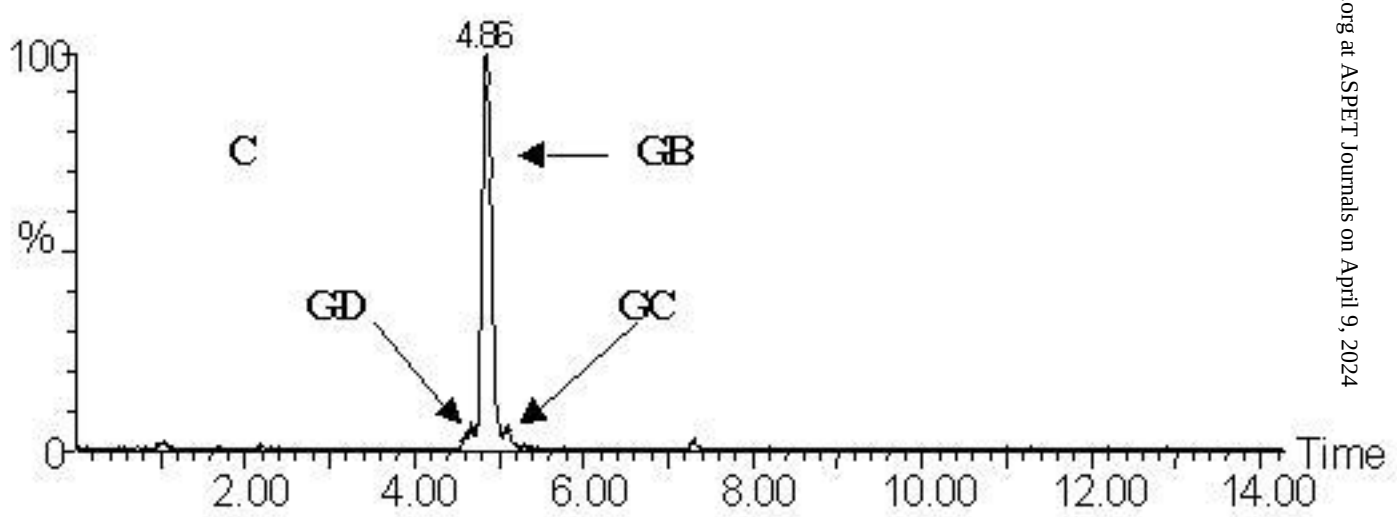


Figure 7