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EVIDENCE FOR THE FORMATION OF A KNOWN TOXIN, P-CRESOL, FROM MENTHOFURAN

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SUMMARY: Menthofuran (II, 4,5,6,7-tetrahydro-3,6-dimethyl benzofuran), the proximate toxin of R-(+)-pulegone (I), was administered orally to rats (200 mg/kg of body weight/day) for three days and the urinary metabolites were investigated. Among the several metabolites formed, two of them viz. 4-Hydroxy-4-methyl-2-cyclohexenone (VII) and p-cresol (VIII) were indentified. In support of the formation of these metabolites, it has been demonstrated that phenobarbital induced rat liver microsomes readily convert 4-methyl-2-cyclohexenone (V) to 4-hydroxy-4-methyl-2-cyclohexenone (VII) and p-cresol (VIII) in the presence of NADPH and O_2. Possible mechanism for the formation of these two metabolites (VII, VIII) from menthofuran (II) has been proposed.

INTRODUCTION: R-(+)-Pulegone (I), a monoterpene ketone, the major constituent of pennyroyal oil from Mentha pulegium, has been shown to be both hepatotoxic and pneumotoxic (1-3). The toxicity results in internal bleeding, pulmonary edema, centrilobular necrosis and cardiovascular arrest (4,5). Earlier studies have demonstrated that menthofuran (II) as one of the metabolites of R-(+)-pulegone (I) both in vivo and in vitro (6,7). Menthofuran (II) has been estimated to be responsible for atleast half of the hepatocellular necrosis caused by pulegone (I) (8). Recent studies have indicated that an unsaturated γ-ketoaldehyde (III) derived from R-(+)-pulegone (I) is the ultimate chemically reactive metabolite (9). The same metabolite (III) has also been shown to be formed when

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menthofuran (II) was incubated with rat liver microsomes in the presence of NADPH and O_2 (10). It has been proposed earlier that there exists atleast two pathways that independently produce toxins from R-(+)-pulegone (I): (i) formation of an unsaturated γ-ketoaldehyde (III) via methofuran and (ii) formation of reactive metabolite(s) of unknown structure (11). This suggests that the contributing factors to the overall toxicity mediated by R-(+)-pulegone are still not clear.

In this communication we present evidence for the formation of 4-hydroxy-4-methyl-2-cyclohexenone (VII, keto alcohol) and p-cresol (VIII), a known toxin from menthofuran (II). Besides, the biochemical basis for the formation of p-cresol (VIII) is also presented.

METHODS

Chemicals: Menthofuran (II), a generous gift from Firmenich, Geneva, Switzerland, was purified by column chromatography on neutral alumina using hexane as the solvent.

Animals and dosing: Adult male rats (IISc. strain) weighing 160-180 g fed ad libitum were used in these studies. Menthofuran (II) was administered orally (200 mg/kg of body weight/day) by gastric intubation as a suspension in 1% methyl cellulose solution (2 ml) for 3 days. Control animals were given only the vehicle. Urine was collected in bottles at 0-4^{o}C. Pretreatment of rats with phenobarbital (PB) was carried out as reported earlier (10).

Extraction and isolation of metabolites: Extraction of urinary metabolites and their separation into acidic and non-acidic metabolites were carried out as reported earlier (6). After removing the acidic metabolites, the remaining fraction was subjected to chromatography on a silica gel column using 5-20% ethylacetate in hexane.

Synthesis: 4-Methyl-2-cyclohexenone (V) and 4-hydroxy-4-methyl-2-cyclohexenone (VII) were synthesized as previously reported (12,13).

Preparation of microsomes: PB-induced rat liver microsomes were prepared as reported earlier (10). Protein determinations were conducted by the method of Lowry et. al. (14).

Microsomal incubations: Microsomal protein (2 mg/ml) was incubated in the presence of $NADP^+$ (0.5 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit), $MgCl_2$ (10 mM), 4-methyl-2-cyclohexenone (V, 2 mM in 30 µl of

acetone) and Tris.Hcl (0.1M, pH 7.4) in a total volume of 5.0 ml. The reaction was initiated by the addition of NADPH generating system and the mixture incubated aerobically in a rotary shaker for 30 min at 37°C. The reaction was terminated by the addition of saturated $Ba(OH)_2$ and $ZnSO_4$(0.25 M). The precipitated protein was removed by centrifugation. The supernatant was extracted with methylene chloride, dried and analysed by GC-MS.

GC-MS analysis: Analysis was carried out as reported earlier (10). Capillary column (50 m x 0.25 mm) containing 10% QF_1, on chromosorb-w was used for the effective separation of metabolites.

Chromatographic procedures: TLC was performed on silica gel G-coated plates (0.25 mm) developed with hexane/ethyl acetate (80:20, v/v) as the solvent system.

RESULTS AND DISCUSSION: TLC analysis of the non-acidic metabolites showed the presence of two major (R_F 0.47 and 0.42) and three minor (R_F 0.85, 0.25 and 0.12) metabolites which were absent in control urine extract. This fraction (200 mg) was subjected to column chromatography over silica gel (20 g) and two of the metabolites with R_F 0.47 and 0.12 were identified. The metabolite with R_F 0.47 was eluted from the column with hexane/ethyl acetate (19:1) and identified as p-cresol (VIII) on the basis of various spectral analyses [IR (liquid film): γ max 3400, 1512, 1455, 1236, 1101 and 810 cm^{-1}; NMR($CDCl_3$):δ7.02, 6.72 (4H, two doublets, aromatic protons), 5.56 (1H, s, hydroxyl proton), and 2.28(3H,s,methyl protons); GC-MS: m/z 108 (M^+), 107 (M^+-1), 90(M^+-H_2O) and 79 (M^+-CHO)]. This was further confirmed by comparing the IR, NMR and mass spectra with that of authentic p-cresol (VIII).

The metabolite with R_F 0.12 could not be isolated in the pure form. However, when the fraction containing this metabolite was subjected to GC-MS analysis, the fragmentation pattern [m/z 125 (M^+-1), 111 (M^+-15), 97 (M^+-29), 83 (M^+-43), 69(M^+-57) and 57 (M^+-69)] corresponded well with that of authentic 4-hydroxy-4-methyl-2-cyclohexenone (VII, Fig.1).

When PB induced rat liver microsomes were incubated with 4-methyl-2-cyclohexenone (V) in the presence of NADPH and O_2,

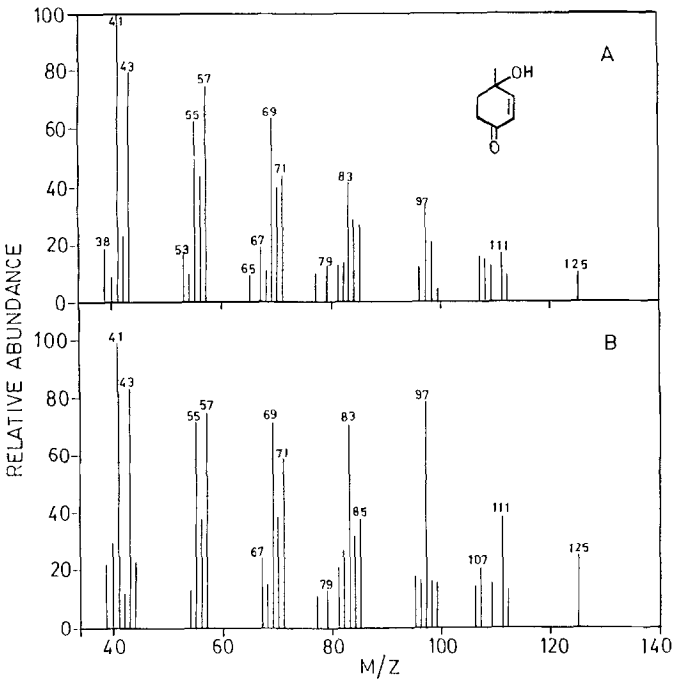

Fig.1. Electron impact mass spectra of 4-hydroxy-4-methyl-cyclohexenone (VII); A (authentic), B (enzymatically formed).

and the assay mixture worked up as described under Methods, the methylene chloride extract of the assay mixture upon TLC analysis indicated the presence of one major and one minor metabolite with 0.12 and 0.47 as R_F values, respectively. The GC-MS analysis of the major metabolite (R_F 0.12) gave fragmentation pattern similar to that of authentic 4 hydroxy-4-methyl-2-cyclohexenone (VII) (Fig.1) and the minor metabolite corresponded to that of p-cresol (VIII). It was also observed that the level of p-cresol (VIII) in the assay medium increased considerably if the reaction was terminated by the addition of 10% TCA suggesting the ready conversion of VII to VIII in the acidic medium.

Studies carried out in vivo clearly demonstrated that 4-hydroxy-4-methyl-2-cyclohexenone (VII) and p-cresol (VIII) are among the metabolites formed from menthofuran (II). This

is a very significant observation as far as toxicity mediated by R-(+)-pulegone (I)/menthofuran (II) is concerned since p-cresol (VIII) is a known liver and lung toxin (15, 16). However, at this stage it is difficult to assess the contribution of p-cresol (VIII) towards the overall toxicity elicited by R-(+)-pulegone (I)/menthofuran (II) since additional routes for their bioactivation resulting in the formation of other reactive metabolite(s) may be operative.

One can envisage the formation of 4-hydroxy-4-methyl-2-cyclohexenone (VII) and p-cresol (VIII) from menthofuran (II) through the intermediacy of α, β-unsaturated γ-ketoaldehyde (III, Fig.2). Reductive elimination of the keto group, hydration of the exocyclic double bond followed by a retro-aldol type reaction could easily yield 4-methyl-2-cyclohexenone (V). This assumption is rationalised on the basis of the following evidences. We have now demonstrated the ability of PB-induced rat liver microsomes to hydroxylate

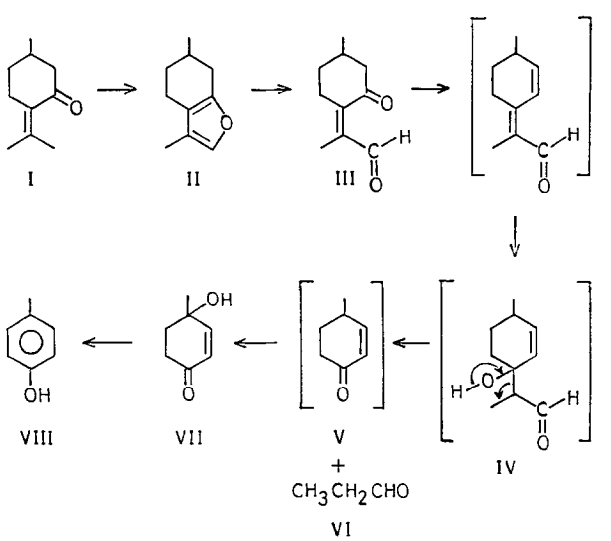

Fig.2. Proposed scheme for the formation of p-Cresol (VIII) from menthofuran (II).

V to 4-hydroxy-4-methyl-2-cyclohexenone (VII) in the presence of NADPH and O_2. This keto alcohol (VII) readily loses water to yield p-cresol (VIII). In fact the compound VII has been isolated earlier from a plant source and its ready conversion to p-cresol (VIII) has been demonstrated (13). The other evidence which supports the proposed route for the formation of compound VII stems from the fact that the GC-MS analyses of the non-acidic metabolites indicated the presence of propanaldehyde (VI, Fig.2) as one of the metabolites formed from menthofuran (II) (unpublished observation).

Earlier it was reported (1) that S-(-)-pulegone is less toxic than R-(+)-pulegone (I). If the hydroxylation of 4-methyl-2-cyclohexenone (V) is stereospecific, then formation of p-cresol (VIII) from S-(-)-pulegone is prevented. This is just s speculation and certainly needs further experimental proof.

The present report has demonstrated that metabolism of menthofuran (II) in rats results in the formation of p-cresol (VIII), a known toxin. It is quite possible that this toxin could have been derived from α , β -unsaturated γ -keto aldehyde (III). However, it is not known to what extent p-cresol (VIII) is involved in the toxicity mediated by R-(+)-pulegone.

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