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Stereoselective Metabolism of the Monoterpene Carvone by Rat and Human Liver Microsomes

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Abstract

The large amounts of carvone enantiomers consumed as food additives and in dental formulations justifies the evaluation of their biotransformation pathway. The in-vitro metabolism of *R*-(−)- and *S*-(+)-carvone was studied in rat and human liver microsomes using chiral gas chromatography.

Stereoselective biotransformation was observed when each enantiomer was incubated separately with liver microsomes. 4*R*, 6*S*-(−)-Carveol was NADPH-dependently formed from *R*-(−)-carvone, whereas 4*S*, 6*S*-(+)-carveol was produced from *S*-(+)-carvone. Metabolite formation followed Michaelis–Menten kinetics exhibiting a significant lower apparent K_m (Michaelis-Menten Constant) for 4*R*, 6*S*-(−)-carveol compared with 4*S*, 6*S*-(+)-carveol in rat and human liver microsomes ($28{\cdot}4 \pm 10{\cdot}6\,\mu$M and $69{\cdot}4 \pm 10{\cdot}3\,\mu$M vs $33{\cdot}6 \pm 8{\cdot}5\,\mu$M and $98{\cdot}3 \pm 22{\cdot}4\,\mu$M). The maximal formation rate (V_{max}) determined in the same microsomal preparations yielded $30{\cdot}2 \pm 5{\cdot}0$ and $32{\cdot}3 \pm 3{\cdot}9$ pmol (mg protein)$^{-1}$ min^{-1} in rat liver and $55{\cdot}3 \pm 5{\cdot}7$ and $65{\cdot}2 \pm 4{\cdot}3$ pmol (mg protein)$^{-1}$ min^{-1} in human liver microsomes. Phase II conjugation of the carveol isomers by rat and human liver microsomes in the presence of UDPGA (uridine S′-diphosphogluaronic acid) only revealed glucuronidation of 4*R*, 6*S*-(−)-carveol. V_{max} for glucuronide formation was more than 4-fold higher in the rat liver compared with human liver preparations ($185{\cdot}9 \pm 34{\cdot}5$ and $42{\cdot}6 \pm 7{\cdot}1$ pmol (mg protein)$^{-1}$ min^{-1}, respectively). K_m values, however, showed no species-related difference ($13{\cdot}9 \pm 4{\cdot}1\,\mu$M and $10{\cdot}2 \pm 2{\cdot}2\,\mu$M).

This study demonstrated stereoselectivity in phase-I and phase-II metabolism for *R*-(−)- and *S*-(+)-carvone and might be predictive for carvone biotransformation in man.

The *R*-(−)- and *S*-(+)-enantiomers of the monoterpene ketone carvone are found in various plants. While *S*-(+)-carvone is the main constituent of the essential oil of caraway, the oil of spearmint leaves contains about 50% of *R*-(−)-carvone besides other terpenes. Both enantiomers differ in odour and taste; *S*-(+)-carvone has an aromatic flavour while *R*-(−)-carvone has a characteristic minty flavour. They also have different uses in the food, fragrance and pharmaceutical industry (Ziegler 1982; Wichtl 1997). *S*-(+)-Carvone is widely used as a taste enhancer in the food industry and, in smaller amounts, as a carminative in many pharmaceutical formulations, whereas *R*-(−)-carvone is frequently added to toothpastes and chewing gum. Though the acute toxicity of both enantiomers is of low order, with reported LD50 values of 1640 mg kg^{-1} in rats and 766 mg kg^{-1} in guinea-pigs (Opdyke 1973, 1978), the large amounts consumed as food additives and in dental formulations should justify the evaluation of their biotransformation pathway.

So far, only one metabolite has been detected in urine samples after oral administration of *rac*-carvone to rabbits (Ishida et al 1989). Structural elucidation of this biotransformation product revealed a hydroxylation of the isopropenyl group. However, neither reduction of the carbonyl group to the corresponding alcohol, as reported for the terpenoids pulegone and 2,5-bornanedione (Leibmann & Ortiz 1973; Moorthy et al 1989), nor subsequent

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glucuronidation of the formed hydroxyl group has been demonstrated, as has been found for menthol and other hydroxylated terpenes (Yamaguchi et al 1994; Green et al 1995; Green & Tephly 1996).

The aim of the present study was to investigate the implication of stereochemistry on metabolite formation of *R*-(−)- and *S*-(+)-carvone in rat and human liver microsomes using a sensitive chiral gas chromatography method. Furthermore these biotransformation products should be identified by enzymatic hydrolysis, by synthesized standards and by mass spectroscopy.

Material and Methods

Materials

R-(−)- and *S*-(+)-carvone (*p*-mentha-6,8-dien-2-one; optical purity >99%), (−)-carveol (*p*-mentha-6, 8-dien-2-ol; optical purity 97%; Z/E: 50:50), 2,2-dimethoxy-propane and dimethylsulphoxide (DMSO) were obtained from Aldrich, Munich, Germany. *β*-Glucuronidase type B-3 from bovine liver, digitonin and uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma, Munich, Germany. *rac*-Piperitone (1-methyl-4-isopropyl-1-cylohexen-3-one; *R*/*S*: 85/15) was obtained from Roth, Karlsruhe, Germany and methanol (Lichrosolv) was from Merck, Darmstadt, Germany. All other chemicals and solvents were of analytical grade and were used without further purification.

Synthesis of carveol isomers

Stereoselective reductions of *R*-(−)- and *S*-(+)-carvone (1·6 mmol) were carried out at room temperature with $NaBH_4$ (3·2 mmol) in an aqueous solution of sucrose (1 mM) according to the literature (Hervé du Penhoat et al 1991; Adams & Lerner 1992; Denis et al 1996). After stirring for 12 h the final product was extracted with diethylether and the organic layer was evaporated under reduced pressure. The presence of sucrose dramatically enhanced axial entrance of the hydride since more than 75% of 4*R*, 6*S*-(−)- and 4*S*, 6*S*-(+)-carveol, but less than 25% of the 4*R*, 6*R*-(−) and 4*S*, 6*R*-(+) stereoisomers, was formed as determined by chiral gas chromatography (GC). The residue was dissolved in dichloromethane and further purified by preparative GC.

Purification of carveol isomers by preparative GC

For separation of the isomeric mixtures 4*R*, 6*R*-(−)-/4*R*, 6*S*(−)- and 4*S*, 6*S*-(+)-/4*S*, 6*R*-(+)-carveol (T_R: 95 min and 103 min, respectively), a Perkin Elmer Auto System XL Gas Chromatograph (Perkin Elmer, Norwalk, USA) was used. The GC column was a 12 ft×4 mm (i.d.) glass packed column (Chromasorb W-AW 80/100, 10·0% OV-1; Perkin Elmer, Norwalk, USA). The nitrogen carrier-gas flow rate was 30 mL min^{-1}. The injection port and the detector were set at 260°C and the oven temperature was held isothermally at 100°C. Samples (10 μL; 3 mg) were introduced onto the column by split injection (split ratio = 1 : 3) and the corresponding peaks were collected on dry ice (purity of the single isomers >95%, checked by chiral GC).

Additional characterization of the purified carveol isomers was carried out by GC/MS (gas chromatography/mass spectrometry) using a Shimadzu QP-1000EX system (Shimadzu, Kyoto, Japan). The GC column (SE 54) was a 50 m×0·25 mm (i.d.) capillary of 0·25 mm film thickness (Machery & Nagel, Germany) operated with a temperature program of 60°C for 2 min, and then increased at 3°C min^{-1} to 250°C. The helium carrier-gas flow rate was 2 mL min^{-1}. Samples were introduced onto the column by split injection (split ratio = 1 : 2, opened 30 s after the injection; injection port temperature 250°C). The column effluent was directly introduced into the ion source, which was held at 180°C. Electron-impact spectra were recorded at 50–600 amu $(2\,sec)^{-1}$, with an ionization energy of 70 eV and with a vacuum pressure of $8{\cdot}10^{-6}$ torr. Chemical ionization spectra were recorded with ammonia as reactant gas (prepressure of 1 bar, vacuum pressure of $8{\cdot}10^{-6}$ torr, ionization energy 200 eV).

Preparation of microsomes

Male Sprague–Dawley rats (180–250 g, raised at the Institut für Versuchstierzucht und -haltung, University of Vienna, Himberg, Austria) were used as liver donors. The human liver samples HL8 (female, 57 years), HL11 (male, 57 years) and HL12 (male, 55 years) were obtained from patients undergoing partial hepatectomy with their written informed consent (II. University Clinics of Surgery, Innsbruck, Austria). Liver samples were homogenized in 0·1 M phosphate buffer (pH 7·4) and microsomes subsequently prepared using standard procedures in our laboratory (Jäger et al 1996). Microsomes were stored as pellets (overlayed with glycerine/PBS) at −70°C until use. Protein concentration (using bovine serum albumin as the standard), total cytochrome P450 content (rat: 0·92 ± 0·21 ng $(mg\,protein)^{-1}$; human: 0·61 ± 0·17 ng $(mg\,protein)^{-1}$) and Uridine 5′-diphos-

phoglucuronosyl-transferase (UDPGT) activity (rat: 17·38 ± 5·78 mU (mg protein)$^{-1}$; human: 11·03 ± 4·01 mU (mg protein)$^{-1}$) were determined according to the methods of Lowry et al (1951), Omura & Sato (1964) and Jäger et al (1997), respectively.

In-vitro phase-I metabolism of R-(−)- and S-(+)-carvone

Carvone metabolites were formed in-vitro with rat and human liver microsomes. The incubation mixture contained 1 mg microsomal protein, 1 mM NADPH and an NADPH-regenerating system (5 mM $MgCl_2$, 5 mM isocitrate and 0·5 U mL^{-1} isocitrate dehydrogenase) in 0·1 M sodium phosphate buffer, pH 7·4 (Jäger et al 1996). After a 5-min pre-incubation at 37°C, the reaction was started by the addition of 10–200 μM *R*-(−)- and *S*-(+)-carvone (final volume 250 μL) in DMSO (to yield an incubation DMSO concentration of not greater than 0·7% v/v) and allowed to proceed at 37°C for 30 min with constant shaking. The reaction was stopped by addition of 750 μL of ice cold methanol, and piperitone (100 ng mL^{-1}) was added as internal standard. After centrifugation at 5000 *g* for 5 min at 4°C, 1 μL of the supernatant was injected onto the GC column. Control experiments were performed in the absence of NADPH.

In-vitro phase-II metabolism of 4R, 6S-(−)- and 4S, 6S-(+)-carveol

Microsomal glucuronidation of carvone metabolites was studied by incubating 4*R*, 6*R*-(−)- and 4*S*, 6*S*-(+)-carveol (5–100 μM dissolved in DMSO), respectively, with 1 mg microsomal protein in 0·1 M sodium phosphate buffer, pH 7·4, 10 mM $MgCl_2$, and digitonin (1 mg (mg protein)$^{-1}$) in a total volume of 250 μL. The reaction was started by the addition of 2 mM UDPGA and allowed to proceed for 60 min at 37°C. Incubations were stopped by the addition of 750 μL of ice-cold methanol and piperitone (100 ng mL^{-1}), followed by centrifugation (5000 × *g* for 5 min at 4°C). The supernatant (1 μL) was injected onto the GC column. Control experiments in the absence of UDPGA were run in parallel.

Incubation of microsomal samples with β-glucuronidase

For detection of glucuronides, 250 μL of microsomal samples were incubated with 50 μL of β-glucuronidase (50 000 U) in the presence of piperitone (5 μL of a stock solution of 10 μg mL^{-1}) at 37°C in teflon-capped glass tubes. After 2 h the samples were centrifuged at 10 000 *g* for 5 min and the supernatant extracted and analysed for carvone and its metabolites as described previously. In control samples β-glucuronidase was replaced by buffer.

Chiral-GC analysis

For chiral separation of the enantiomers of carvone and its metabolites a Carlo-Erba HRGC 5160 Mega Series instrument with a flame ionization detector and a 40 m × 0·25 mm Chiraldex β-cyclodextrin trifluoroacetyl column connected with a 5 m × 0·25 mm retention gap guard column (both Astec, Whippany, NJ) were used. The carrier gas was helium, with a flow rate of 0·5 mL min^{-1}. Samples were injected onto the column by split injection (split ratio = 1 : 10; injection port temperature, 200°C). The oven temperature was held isothermally at 90°C. The limit of quantification for *R*-(−)- and *S*-(+)-carvone in liver microsomal samples was 20 ± 3·23 ng mL^{-1} and 20 ± 2·87 ng mL^{-1}, respectively, by spiking drug-free liver microsomes with the enantiomers to give final concentrations of 10, 15, 20, 25 and 30 ng mL^{-1} (n = 5). The injection volume was 1·5 μL. The analysed peaks were well separated from the peaks of the internal standard, piperitone, with no overlapping matrix peaks.

Data analysis

Estimates of apparent K_m and V_{max} were obtained by fitting the data to the Michaelis–Menten equation, using the nonlinear curve-fitting package Winzyme (PC version 2·10, Biosoft, Cambridge, UK). Assay conditions were such that metabolite formation and parent consumption were linear with respect to time of incubation and protein concentration. Results are expressed as the mean ± s.d. The significance of differences ($P < 0·05$) was evaluated by the Student's *t*-test. The enzymatic efficacy, that is defined as the ratio V_{max}/K_m, quantifies the capacity of metabolism and corresponds to the intrinsic clearance.

Results and Discussion

Microsomal preparations from three individual rat and human livers were incubated with *R*-(−)- and *S*-(+)-carvone and subsequently analysed by chiral GC. Under the analytical conditions selected, *R*-(−)-carvone and *S*-(+)-carvone, the two isomers of the internal standard, *R*- and *S*-piperitone (*R*/*S*: 85/15), and the two metabolites were resolved

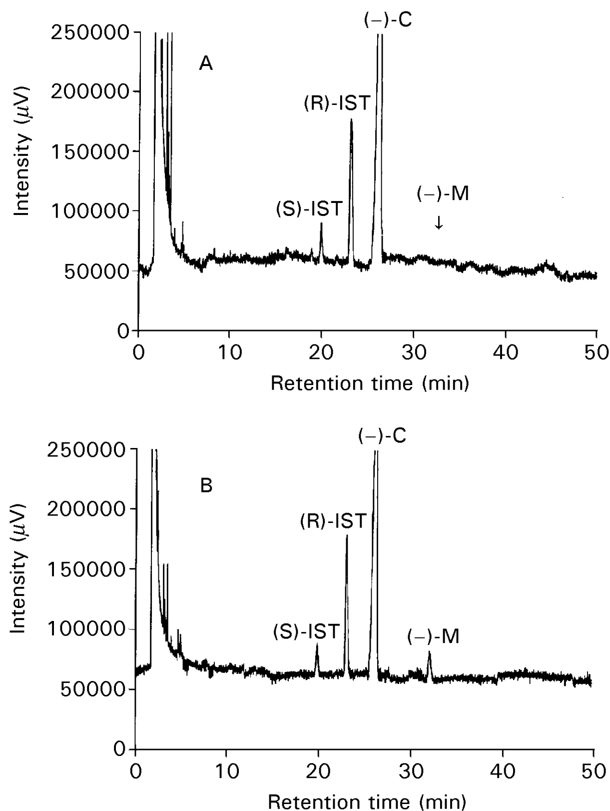

Figure 1. GC chromatogram of medium extracts from the microsomal incubation of *R*-(−)-carvone. Human liver microsomes containing 1·0 mg of microsomal protein were incubated with 200 μM of *R*-(−)-carvone for 30 min at 37°C without (A), and in the presence of (B), NADPH. (−)-C: *R*-(−)-carvone; (R)-IST: *R*-internal standard; (S)-IST: *S*-internal standard; (−)-M: metabolite 4*R*, 6*S*-(−)-carveol.

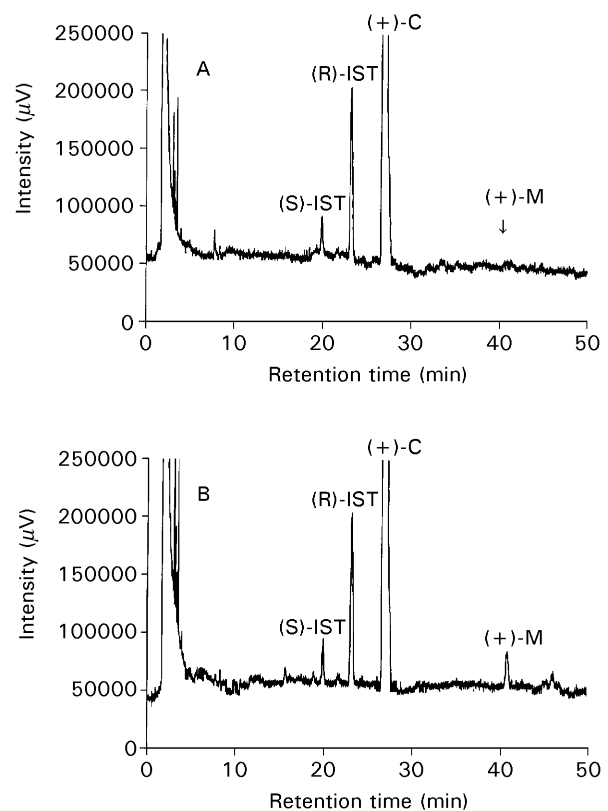

Figure 2. GC chromatogram of medium extracts from the microsomal incubation of *S*-(+)-carvone. Human liver microsomes containing 1·0 mg of microsomal protein were incubated with 200 μM of *S*-(+)-carvone for 30 min at 37°C without (A), and in the presence of (B), NADPH. (+)-C: *S*-(+)-carvone; (R)-IST: *R*-internal standard; (S)-IST: *S*-internal standard; (+)-M: metabolite 4*S*, 6*S*-(+)-carveol.

adequately and there was no interference from endogenous medium constituents. Typical GC chromatograms from the experiments using *R*-(−)-carvone are shown in Figure 1 for the organic phase after solid-phase extraction from an incubation with human liver microsomes without the addition of NADPH (Figure 1A), and from an incubation containing NADPH (Figure 1B). Figure 1B identified one *R*-(−)-carvone-derived peak with a GC retention time of 32·27 min. This peak was not observed in the incubation extract shown in Figure 1A. Parallel incubation with *S*-(+)-carvone (Figure 2) again revealed the formation of only one new peak in the chromatogram with a retention time of 40·95 min (Figure 2B). These new GC peaks were absent when heat-inactivated microsomes were used, or when the substrates or NADPH-generating system were omitted, indicating the involvement of the cytochrome P450 system. Qualitatively similar metabolic profiles were observed in rat microsomal preparations from three individual livers.

GC/MS studies were used in order to identify the chemical structure of these two carvone metabolites. The peaks derived from *R*-(−)-carvone exhibited M^+-ions at m/z 152 amu and main fragment ions at m/z 109, 95, 91 and 84 amu, respectively, and was different from the *S*-(+)-carvone-derived biotransformation product which showed no M^+-ions at m/z 152 amu but did have more prominent fragment ions at m/z 134 and 119 amu. A mass-spectral library search (Adams 1989) confirmed that *R*-(−)-carvone is exclusively metabolized to (−)-carveol, whereas *S*-(+)-carvone is biotransformed to (+)-carveol. By synthesizing or isolating all four possible carveol isomers, we were able to further identify the chiral structure of the carveol metabolites formed. (−)-Carveol, therefore, attributes to 4*R*, 6*S*-(−)-carveol and (+)-carveol to 4*S*, 6*S*-(+)-carveol. The formation of 4*R*, 6*R*-(−)-carveol and 4*S*, 6*R*-(+)-carveol after incubation with either (−)-carvone or (+)-carvone, respectively, was below the detection limit.

Formation of the two carveol isomers were linear with time up to 40 min and with respect to microsomal protein concentrations of 0·4–1·2 mg mL^{-1}

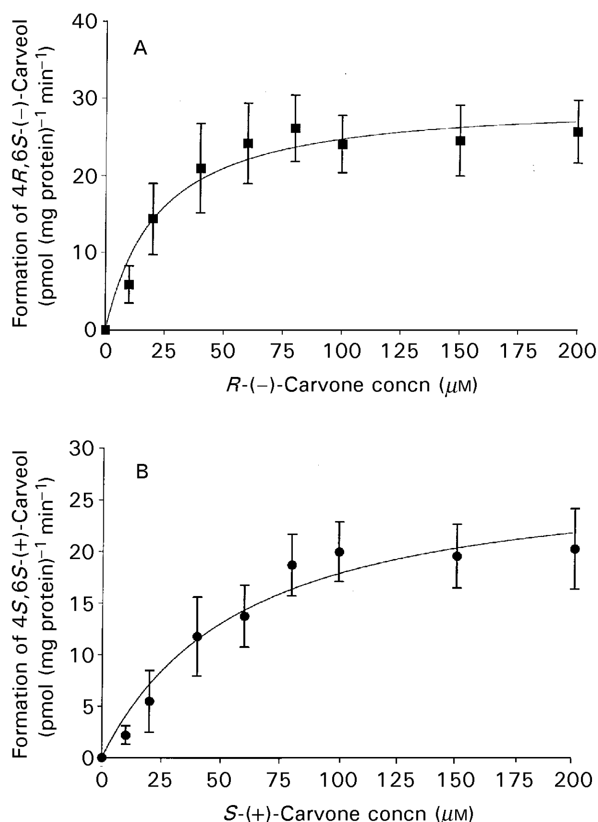

Figure 3. Formation of 4*R*, 6*S*-(−)-carveol (A) and 4*S*, 6*S*-(+)-carveol (B) in rat liver microsomes. A Michaelis–Menten curve was generated by incubating *R*-(−)- and *S*-(+)-carvone at varying concentrations in rat liver microsomes in the presence of NADPH. Data represent the means ± s.d. of three determinations.

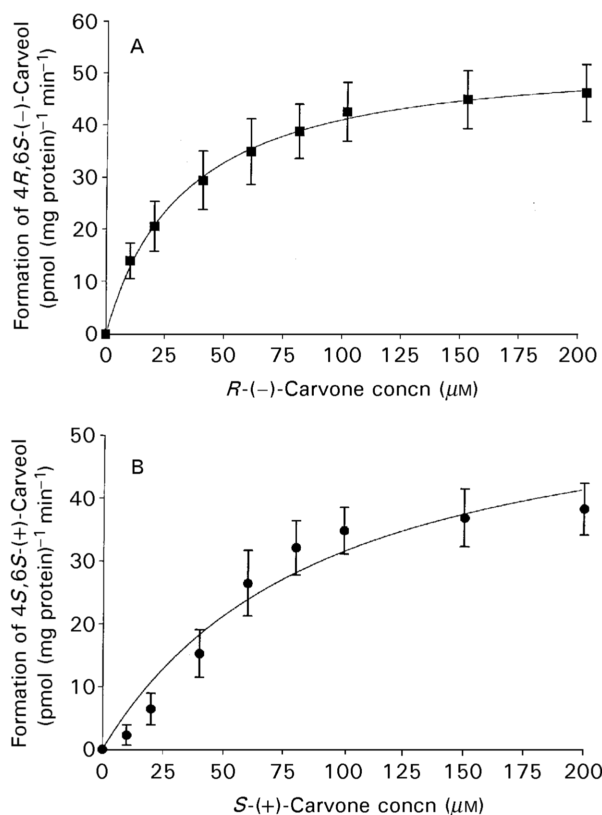

Figure 4. Formation of (−)-(4*R*, 6*S*)-carveol (A) and (+)-(4*S*;6*S*)-carveol (B) in human liver microsomes. A Michaelis–Menten curve was generated by incubating (−)-(*R*)- and (+)-(*S*)-carvone at varying concentrations in human liver microsomes in the presence of NADPH. Data represent the means (± s.d.) of three determinations.

(data not shown). Contrary to findings in a previous study (Ishida et al 1989) investigating the metabolism of (+)-carvone in rabbits, neither *R*-(−)- nor *S*-(+)-carvone showed hydroxylation of the isopropenyl group, indicating a different biotransformation depending on species.

The kinetic constants for this reaction were estimated using *R*-(−)- and *S*-(+)-carvone concentrations of up to 200 μM. Kinetic parameters K_m and V_{max} were determined by direct least squares regression of *R*-(−)- and *S*-(+)-carvone concentrations (substrates) vs velocity data using the Michaelis–Menten equation (Figures 3 and 4). Using this approach, significantly lower K_m values for 4*R*, 6*S*-(−)-carveol were found in rat and human liver microsomes (28·4 ± 10·6 μM and 69·4 ± 10·3 μM, respectively) compared with *S*-(+)-carveol (33·6 ± 8·5 μM and 98·3 ± 22·4 μM). V_{max} determined in the same microsomal preparations yielded higher values in human liver than in rat liver microsomes (55·3 ± 5·7 and 65·2 ± 4·3 pmol (mg protein)$^{-1}$ min^{-1} vs 30·2 ± 5·0 and 32·3 ± 3·9 pmol (mg protein)$^{-1}$ min^{-1}). In terms of enzymatic efficacy (V_{max}/K_m), *R*-(−)-carvone is a better substrate than the *S*-(+) enantiomer. The ratio of enzymatic efficacies of *R*-(−)/*S*-(+) ranges from 2·6 to 2·5 in rat and human liver microsomes.

Biochemical evidence for glucuronic acid conjugates was obtained after incubation of 4*R*, 6*S*-(−)-carveol and 4*S*, 6*S*-(+)-carveol with rat and human liver microsomes in the presence of UDPGA. After UDPGA incubation the microsomal samples were further treated with β-glucuronidase (control experiments were run with buffer). GC analysis of these samples only revealed an increase of the 4*R*, 6*S*-(−)-carveol peak by β-glucuronidase compared with non-treated samples, indicating glucuronic acid conjugation of this biotransformation product. The formation rate of 4*R*, 6*S*-(−)-carveol glucuronidation substantially differed in rat and human liver preparations (Figure 5). While the apparent K_m for 4*R*, 6*S*-(−)-carveol glucuronide was similar in both species (rat: 13·9 ± 4·1 μM; human: 10·2 ± 2·2 μM), V_{max} was more than 4-fold higher in the rat liver microsomes (rat: 185·9 ± 34·5 pmol (mg protein)$^{-1}$ min^{-1}; human: 42·6 ± 7·1 pmol (mg protein)$^{-1}$ min^{-1}). The

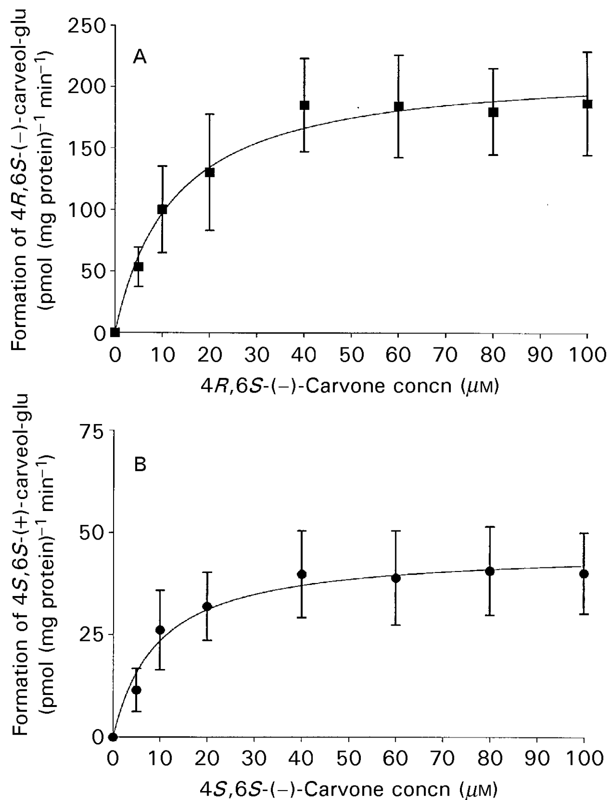

Figure 5. Glucuronidation of 4*R*, 6*S*-(−)-carveol in rat and human liver microsomes. A Michaelis–Menten curve was generated by incubating 4*R*, 6*S*-(−)-carveol at varying concentrations in rat (A) and human (B) liver microsomes in the presence of UDPGA. Data represent the means ± s.d. of three determinations.

V_{max}/K_m ratio (intrinsic clearance), describing the consumption of 4*R*, 6*S*-(−)-carveol by rat and human liver microsomes in the presence of UDPGA, was estimated to be 16·2 ± 2·1 and 4·5 ± 0·2, respectively. The formation of 4*S*, 6*S*-

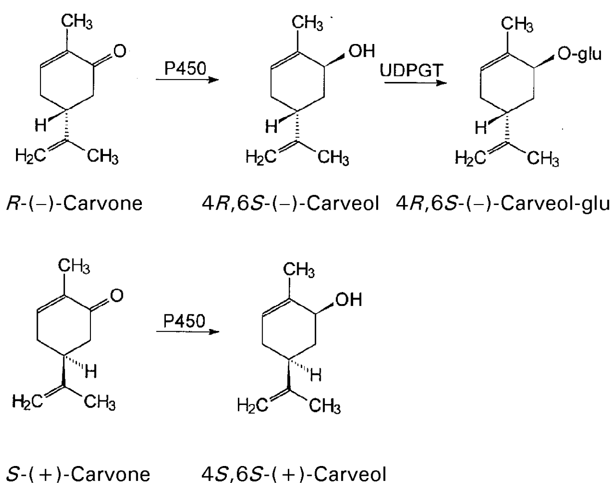

Figure 6. The proposed metabolic pathway of *R*-(−)- and *S*-(+)-carvone in rat and human liver microsomes. P450: cytochrome P450; glu: glucuronic acid; UDPGT: UDP-glucuronosyl transferase.

(+)-, 4*R*, 6*R*-(−)- and 4*S*, 6*R*-(+)-carveol glucuronide was below the detection limit.

The stereoselective metabolism of *R*-(−)- and *S*-(+)-carvone in rat and human liver microsomes is summarized in Figure 6.

In conclusion, our findings show that carvone metabolism is stereoselective and species dependent. As stereoselectivity and stereospecificity of many endogenous biochemical processes, particularly in phase-I and phase II-metabolism have been characterized for many drugs (Williams & Lee 1985; Drayer 1986), it is becoming more apparent that differences in metabolism may also result in altered pharmacokinetics. An ongoing study shall therefore elucidate the stereoselectivity of *R*-(−)-carvone and *S*-(+)-carvone in man.

Acknowledgements

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