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Percutaneous absorption of the montoterpene carvone: implication of stereoselective metabolism on blood levels

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Abstract

The purpose of this study was to determine whether an enantioselective difference in the metabolism of topically applied *R*-(−)- and *S*-(+)-carvone could be observed in man. In a previous investigation we found that *R*-(−)- and *S*-(+)-carvone are stereoselectively biotransformed by human liver microsomes to 4*R*,6*S*-(−)- and 4*S*,6*S*-(+)-carveol, respectively, and 4*R*,6*S*-(−)-carveol is further glucuronidated. We therefore investigated the metabolism and pharmacokinetics of *R*-(−)- and *S*-(+)-carvone in four healthy subjects using chiral gas chromatography as the analytical method. Following separate topical applications at a dose of 300 mg, *R*-(−)- and *S*-(+)-carvone were rapidly absorbed, resulting in significantly higher C_{max} levels for *S*-(+)-carvone (88.0 vs 23.9 ng mL^{-1}) and longer distribution half-lives ($t_{2\alpha}^{1}$) (19.4 vs 7.8 min), resulting in 3.4-fold higher areas under the blood concentration–time curves (5420 vs 1611 ng min mL^{-1}). The biotransformation products for both enantiomers in plasma were below detection limit. Analysis of control- and β-glucuronidase pretreated urine samples, however, revealed a stereoselective metabolism of *R*-(−)-carvone to 4*R*,6*S*-(−)-carveol and 4*R*,6*S*-(−)-carveol glucuronide. No metabolites could be found in urine samples after *S*-(+)-carvone application. These data indicate that stereoselectivity in phase-I and phase-II metabolism has significant effects on *R*-(−)- and *S*-(+)-carvone pharmacokinetics. This might serve to explain the increased blood levels of *S*-(+)-carvone.

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Introduction

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 (*Carum carvi*), the oil of spearmint leaves (*Mentha spicata* var. *crispa*) contains about 50% of *R*-(−)-carvone besides other terpenes. Both enantiomers are not only different in odour and taste, but they also have different use in the food, fragrance and pharmaceutical industries (Ziegler 1982; Wichtl 1997). Because of its minty odour and taste, large amounts of *R*-(−)-carvone are frequently added to toothpastes, mouth washes and chewing gums. *S*-(+)-Carvone possesses the typical caraway aroma and is therefore mainly used as a taste enhancer in the food and fragrance industries. Due to its spasmolytic effect, *S*-(+)-carvone is also used as a stomachic and carminative in many pharmaceutical formulations (Wichtl 1997). Furthermore, in combination with other essential oils, *S*-(+)- and *R*-(−)-carvone are applied in aromatherapy massage treatments for nervous tension and several skin disorders. Though the acute toxicity of both enantiomers is low, with reported LD50 (median lethal dose) values of 1640 mg kg^{-1} in rats and 766 mg kg^{-1}

in guinea-pigs (Opdyke 1973, 1978), the high concentrations in many products for massage treatments and the large amounts consumed as food additives and in dental formulations justifies the determination of their blood levels after absorption.

We recently demonstrated that *R*-(−)-carvone rapidly penetrates human skin, resulting in measureable blood levels (Fuchs et al 1997). Using rat and human liver microsomes we could also show a stereoselective biotransformation when each entantiomer was incubated separately with liver microsomes (Jäger et al 2000). 4*R*,6*S*-(−)-Carveol was NADPH-dependently formed from *R*-(−)-carvone, whereas 4*S*,6*S*-(+)-carveol was produced from *S*-(+)-carvone. In the presence of uridine 5′-diphosphoglucuronic acid (UDPGA), 4*R*, 6*S*-(−)-carveol and not 4*S*,6*S*-(+)-carveol was further conjugated with glucuronic acid. Glucuronidation of the formed hydroxyl group is in accordance with literature as it has been found for menthol and other hydroxylated terpenes (Yamaguchi et al 1994; Green et al 1995; Green & Tephly 1996).

The aim of this study was to investigate the implication of stereochemistry on the metabolism and pharmacokinetics of *R*-(−)- and *S*-(+)-carvone in healthy subjects after topical absorption from peanut oil using a sensitive chiral gas chromatography (GC) method to analyse blood and urine samples.

Materials 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-dimethoxypropane and dimethyl sulfoxide (DMSO) were obtained from Aldrich, Munich, Germany; β-glucuronidase type B-3 from bovine liver was 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 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 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 were formed as determined by chiral GC. The residue was dissolved in dichloromethane and further purified by preparative GC as described previously (Jäger et al 2000).

Topical application

A target amount of 1.5 g of peanut oil containing 300 mg of *R*-(−) and *S*-(+)-carvone, respectively, were spread on a defined skin area of the lower abdomen (approximately 376 cm^2) of four healthy subjects (body weight 54 ± 5.5 kg, age 20 ± 3.0 years, 1 non smoker, 3 smokers). The subjects provided written informed consent and the protocol was approved by the local ethics committee. To avoid possible uptake of the fragrance compound by inhalation the subjects wore a breathing mask (size 6, King Systems Corp., Noblesville, IN). Blood samples (8 mL) were drawn from the left cubital vein at 0, 5, 10, 15, 20, 25, 30, 40, 55, 70, 85 and 100 min after application of *R*-(−)- and *S*-(+)-carvone-containing peanut oil, respectively, and collected in chilled evacuated tubes (Vacutainer 10 mL, Becton Dickinson, Heidelberg, Germany). Plasma was obtained by centrifugation of blood for 10 min at approximately 2000 *g* at 4°C. Urine samples were collected in teflon-capped bottles and pooled during the following time intervals: 0–4, 4–8, 8–12 and 12–24 h after dosing. During the collection interval, the urine was stored at approximately 4°C. At the end of each collection period, the volume of urine was recorded, and the samples were frozen immediately on dry ice and stored at −20°C until analysis (volumes of urine for *R*-(−)- and *S*-(+)-carvone: 185 ± 60 mL and 162 ± 25 mL at 0–4 h; 179 ± 30 mL and 192 ± 64 mL at 4–8 h; 177 ± 30 mL and 184 ± 57 mL at 8–12 h; 245 ± 30 mL and 177 ± 59 mL at 12–24 h, respectively). Starting a week before the massage, all food and cosmetics containing *R*-(−) and *S*-(+)-carvone were avoided.

Sample extraction

After adding piperitone as the internal standard (5 μL of a stock solution of 10 $\mu g\ mL^{-1}$) to 2 mL of plasma and 2.5 mL of urine, samples were passed through C18 columns (International Technology Ltd, Mid Glamorgan, UK), equilibrated with 3 mL of methanol and water, respectively. The columns were washed with 2 mL

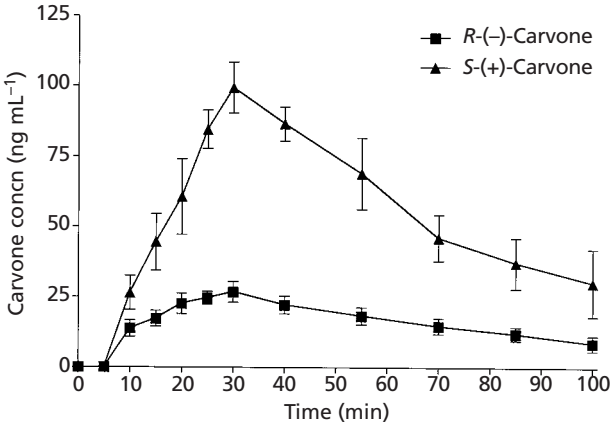

Figure 1 Plasma concentrations of *R*-(−)- and *S*-(+)-carvone in healthy subjects, administered as a single topical application of 300 mg. Data are mean ± s.d., n = 4.

Table 1 Mean pharmacokinetic parameters of *R*-(−)-carvone and *S*-(+)-carvone after dermal administration at a single dose of 300 mg to healthy subjects.

Parameter	*R*-(−)-Carvone	*S*-(+)-Carvone	Stereoselective index[a]
C_{max} (ng mL^{-1})	23.9 ± 2.3	88.0 ± 2.6	0.27**
T_{max} (min)	27.8 ± 3.1	32.4 ± 2.3	0.86
k_a (h^{-1})	2.3 ± 0.53	2.2 ± 0.15	1.07
$t^1_{2\alpha}$ (min)	7.8 ± 2.3	19.4 ± 0.72	0.40**
$t^1_{2\beta}$ (min)	33.5 ± 6.1	37.5 ± 12.4	0.89
AUC (ng min mL^{-1})	1611 ± 28.0	5420 ± 145	0.30**

Each value represents the mean ± s.d. of four percutaneous administrations. [a]Ratio of parameters between both enantiomers; $^{**}P < 0.01$ compared with control. C_{max}, peak plasma concentration; T_{max}, time to reach the peak plasma concentration; k_a, absorption rate constant; $t^1_{2\alpha}$, and $t^1_{2\beta}$, distribution and elimination half-lives, respectively; AUC, area under the blood concentration–time curve from 0 to 100 min.

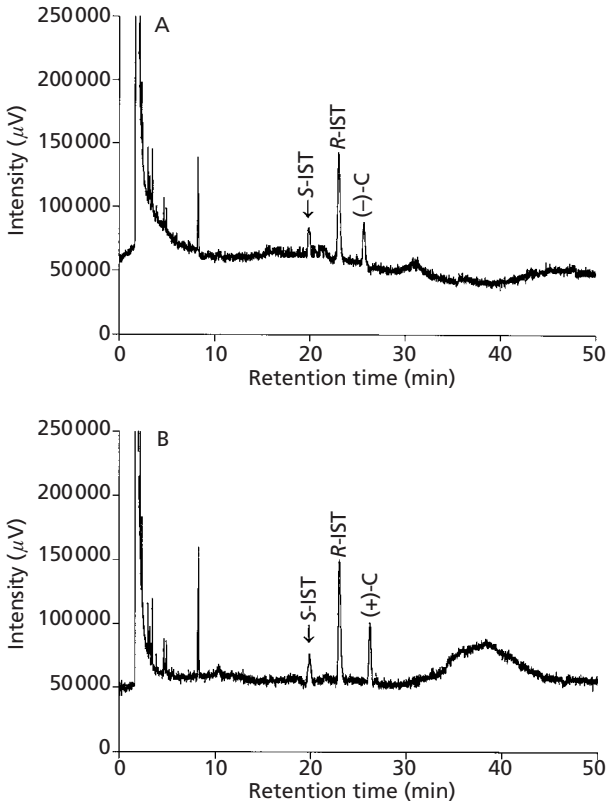

Figure 2 Representative chiral GC chromatogram of a β-glucuronidase pretreated plasma sample after the topical application of *R*-(−)-carvone (A) or *S*-(+)-carvone (B) to a healthy male subject. (−)-C, *R*-(−)carvone; (+)-C, *S*-(+)-carvone; *R*-IST, *R*-(−)-piperitone; *S*-IST, *S*-(+)-piperitone.

of water and *R*-(−)- and *S*-(+)-carvone and their metabolites were eluted with 0.3 mL of methanol (recovery > 95%) and immediately analysed by chiral GC analysis. For detection of glucuronides, selected plasma (2 mL) and urine samples (2.5 mL) were incubated with 50 μL of β-glucuronidase (50000 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 10000 *g* for 5 min and the supernatant extracted as described above. In control samples, β-glucuronidase was replaced by buffer. To test a possible stereo-conversion of both enantiomers in urine samples under storage conditions, drug-free urine samples were spiked with *R*-(−)- and *S*-(+)-carvone, respectively, to give final concentrations of 10 ng mL^{-1} (n = 5). The extent of racemisation for both enantiomers after 24 h at 4°C and −20°C was below detection limit.

Chiral GC analysis

For chiral separation of carvone and its metabolites, a Carlo-Erba HRGC 5160 Mega Series instrument with a flame ionization detector and a 40 mm × 0.25 mm Chiraldex β-cyclodextrin trifluoroacetyl column connected with a 5 mm × 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) and an injection port temperature of 200°C. The oven temperature was held isothermally at 90°C. The limit of quantification for *R*-(−) and *S*-(+)-carvone in plasma and urine samples was 20 ± 3.23 ng mL^{-1} and 20 ± 2.87 ng mL^{-1}, respectively, by spiking

drug-free plasma and urine 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 (retention times for *S*-(+)-piperitone, *R*-(−)-piperitone, *R*-(−)-carvone, *S*-(+)-carvone, 4*R*,6*S*-(−)-carveol, 4*S*,6*R*-(+)-carveol, 4*S*,6*S*-(+)-carveol and 4*R*, 6*R*-(−)-carveol were approximately: 19.9, 23.1, 25.7, 26.3, 31.9, 33.5, 41.0 and 42.6 min, respectively).

Data analysis

The concentration–time curves of *R*-(−)- and *S*-(+)-carvone in plasma samples were adjusted to the data sets via nonlinear iterative least-square regression analysis. Curve modelling was performed using a two-compartment open pharmacokinetic model with the program WinNonlin (vers. 1.5, Scientific Consulting, USA), where AUC represents the area under the concentration–time curve from 0 to 100 min using the linear trapezoidal rule. Results are expressed as the mean ± s.d. The significance of differences ($P < 0.01$) was evaluated by the Wilcoxon test.

Results and Discussion

The plasma concentrations of *R*-(−)- and *S*-(+)-carvone following separate topical application at a dose of 300 mg are shown in Figure 1. Both enantiomers rapidly penetrated the skin and measurable blood levels could already be detected 10 min after dosing. At all sampling time points the *S*-(+)-carvone concentrations were at least 2 times higher than those for the *R*-(−) enantiomer (C_{max}, 88.0 and 23.9 ng mL^{-1}, respectively), resulting

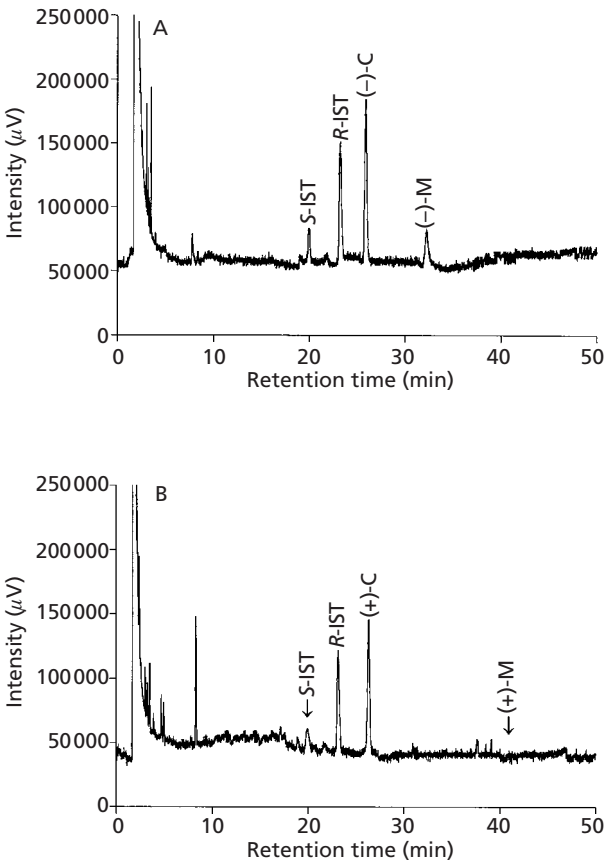

Figure 3 Representative chiral GC chromatogram of a β-glucuronidase pretreated urine sample after the topical application of *R*-(−)-carvone (A) or *S*-(+)-carvone (B) to a healthy male subject. (−)-C, *R*-(−)carvone; (+)-C, *S*-(+)-carvone; *R*-IST, *R*-(−)-piperitone; *S*-IST, *S*-(+)-piperitone; (−)-M, 4*R*,6*S*-(−)-carveol; (+)-M, 4*S*, 6*S*-(+)-carveol.

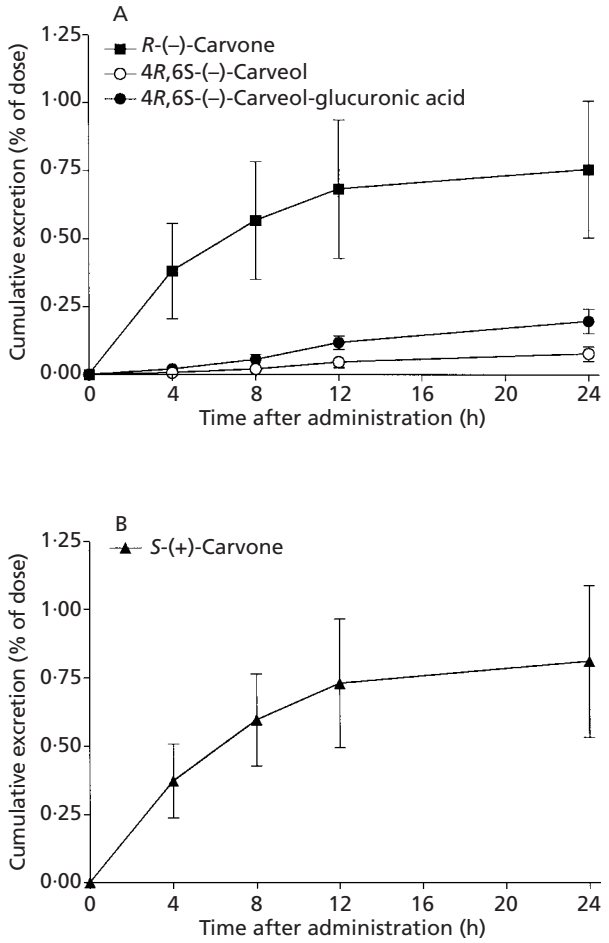

Figure 4 Cumulative urinary excretion after the topical application of 300 mg of *R*-(−)- and *S*-(+)-carvone to healthy subjects. Data are mean ± s.d., n = 4.

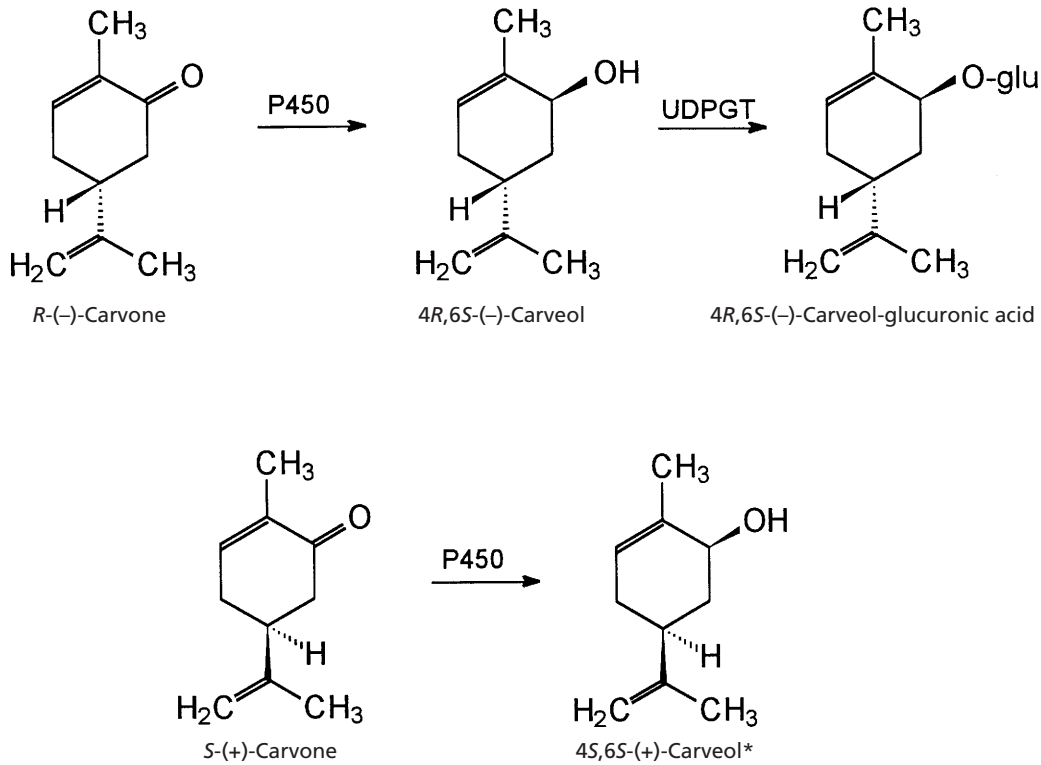

Figure 5 The proposed metabolic pathway of *R*-(−)- and *S*-(+)-carvone in healthy subjects, administered as a single topical application of 300 mg. P450, cytochrome P450 system; UDPGT, UDP-glucuronosyl transferase. *Only found in human liver microsomes.

in a 3.4-fold higher $AUC_{0-100\,min}$(5420 and 1611 ng min mL^{-1}) (Table 1). The elimination of *R*-(−)- and *S*-(+)-carvone from the plasma was best described by a two-compartment open model exhibiting significantly different distribution half-lives ($t_{\frac{1}{2}\alpha}$) of 7.8 and 19.4 min, respectively, but similar elimination half lives ($t_{\frac{1}{2}\beta}$; 33.5 and 37.5 min, respectively). No statistical differences for the values of the absorption rate constant, k_a, and for the time to reach the peak concentration, T_{max}, between the two enantiomers were found (2.3 h^{-1} and 2.2 h^{-1}, 27.8 min and 32.4 min, respectively).

We previously demonstrated in an in-vitro model that *R*-(−)- and *S*-(+)-carvone are stereoselectively biotransformed by human liver microsomes to 4*R*,6*S*-(−)- and 4*S*, 6*S*-(+)-carveol, respectively, whereas only 4*R*,6*S*-(−)-carveol is further glucuronidated. To investigate possible glucuronidation of carveol in man, plasma samples were further treated with β-glucuronidase. Representative chromatograms of plasma samples are shown in Figures 2A and 2B. The extent of racemization was below the detection limit and *R*-(−)- and *S*-(+)-carvone and the two isomers of the internal standard, *R*- and *S*-piperitone (*R*/*S*: 85/15) were baseline-resolved from other endogenous medium constituents. Surprisingly, pre-incubation of plasma samples with β-glucuronidase did not reveal the formation of either 4*R*,6*S*-(−)- or 4*S*,6*S*-(+)-carveol.

However, analysing urine samples after *R*-(−)- but not after *S*-(+)-carvone application revealed an additional peak in the GC chromatogram with a retention time of approximately 31.9 min (Figures 3A and B). This peak exhibited M^+-ions at m/z 152 amu and main fragment ions at m/z 109, 95, 91 and 84 amu, respectively, which is consistent with (−)-carveol using a mass spectral library search (Adams 1989). The retention time for (−)-carveol was the same as the retention time of an authentic standard of 4*R*,6*S*-(−)-carveol. The formation of 4*S*,6*S*-(+)-, 4*R*,6*R*-(−)- and 4*S*,6*R*-(+)-carveol, respectively, was below the detection limit. Parallel incubations of these urine samples with β-glucuronidase revealed a pronounced increase of 4*R*,6*S*-(−)-carveol, indicating that this carvone metabolite is a substrate for UDT-glucuronosyl transferases (UGTs).

The cumulative excretion of native *R*-(−)- and *S*-(+)-carvone eliminated into the urine during 24 h after exposure was low (1.2 and 1.3 %, respectively) whereby the highest concentrations were found in the first 4 h

(Figure 4). The percentage of 4*R*,6*S*-(−)-carveol- and 4*R*,6*S*-(−)-carveol glucuronide on total *R*-(−)-carvone urinary elimination was high and was calculated to be 7.6 and 19.1 %, respectively. As previous experiments in rats showed carvone concentration in the liver was up to 7 times higher than in blood (data not shown), 4*R*,6*S*-(−)-carveol glucuronide excretion into bile and faeces may markedly exceed its excretion into urine and may account for the decreased plasma levels of *R*-(−)-carvone.

The lower excretion of 4*R*,6*S*-(−)-carveol into the urine compared with 4*R*,6*S*-(−)-carveol glucuronide correlates well with in-vitro data using human liver microsomes, where we found the K_m for 4*R*,6*S*-(−)-carveol glucuronide formation to be about 7-times higher than that for 4*R*,6*S*-(−)-carveol. We may also explain the non-detectable amounts of 4*S*,6*S*-(+)-carveol in human plasma and urine after *S*-(+)-carvone administration, as the K_m for 4*S*,6*S*-(+)-carveol formation was significantly higher than that for 4*R*,6*S*-(−)-carveol, indicating lower affinity to the enzyme(s). 4*S*,6*S*-(+)-Carveol glucuronide was not found either in plasma or urine.

Figure 5 gives an overview of the proposed stereoselective metabolism of *R*-(−)- and *S*-(+)-carvone in healthy subjects.

The major metabolic pathway for *R*-(−)-carvone is reduction to the keto group to the secondary alcohol. The hydroxyl metabolite undergoes further conjugation with glucuronic acid. As we have found previously, 4*R*,6*S*-(−)-carveol was NADPH-dependently formed in human liver microsomes, indicating that the cytochrome P450 system, namely NADPH-cytochrome P450 reductase or NADH-cytochrome b5 reductase, is responsible for the reduction of *R*-(−)-carvone to the corresponding alcohol. An ongoing study shall therefore characterize the role of the P450 system on 4*R*,6*S*-(−)- and 4*S*,6*S*-(+)-carveol formation and shall also identify the UGT-isoenzymes involved in 4*R*,6*S*-(−)-carveol glucuronidation.

Plasma level differences of enantiomers based on enzymatic bioconversion is in accordance with the literature, as stereoselectivity of phase-I and phase-II metabolizing enzymes for many compounds is linked to altered pharmacokinetics (Williams & Lee 1985; Drayer 1986). In addition to enzymatic bioconversion, stereoselectivity in transdermal penetration may also influence plasma levels as recently shown for propranolol (Suedee et al 1999). Whether this may also apply to *R*-(−)- and *S*-(+)-carvone has yet to be determined.

In conclusion, *R*-(−)- and *S*-(+)-carvone rapidly penetrated the skin of healthy subjects leading to significantly different blood levels. Only *R*-(−)-carvone was metabolized to 4*R*,6*S*-(−)-carveol, which was further glucuronidated and eliminated into the urine. If it is assumed that 4*R*,6*S*-(−)-carveol glucuronide, like many other conjugated metabolites, is preferably eliminated into bile, the lower plasma levels of *R*-(−)-carvone compared with *S*-(+)-carvone are mainly due to stereoselective biotransformation.

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