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Determination of menthol in plasma and urine of rats and humans by headspace solid phase microextraction and gas chromatography–mass spectrometry

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Abstract

A method for the determination of menthol and menthol glucuronide (M-G) after enzymatic hydrolysis in plasma and urine of rats and humans was developed using headspace solid phase microextraction and gas chromatography–mass spectrometry in the selected ion monitoring mode (HS-SPME/GC–MS). The assay linearity for plasma ranged from 5 to 1000 ng/ml. The limit of quantification (LOQ) in plasma was 5 ng/ml. The intra- and inter-day precision for menthol and M-G were ≤18.1% R.S.D. at the LOQ and ≤4.0% at higher concentrations. Menthol and M-G were determined in rat and human plasma and urine after administration of menthol.

Keyword: Menthol

1. Introduction

Menthol, a monoterpene, is a major component in essential oils of different species of mint (e.g. peppermint oil). It is widely used in food and pharmaceutical industry as flavouring agent and in traditional phytotherapy for the treatment of colds, sport injuries, pruritus, indigestion and migraines. A so far unknown bone resorption inhibiting effect of menthol and some other monoterpenes was observed in rats and in vitro [1]. Therefore, our aim was to develop a bioassay for the study of pharmacokinetic profiles and metabolism of menthol in rats and later on in humans. To date, some metabolites of menthol in rats are known [2,3] but no pharmacokinetic animal data are available. In humans, few studies were performed to elucidate the pharmacokinetic profile of menthol and its main metabolite M-G, but not of other phase-I metabolites. For the quantification of menthol and M-G after enzymatic hydrolysis in plasma and urine different methods including liquid–liquid extraction or headspace sampling followed by analysis with GC and GC–MS were proposed [4–10]. In plasma unconjugated menthol was only detected after a transdermal application [5]. The problem of methods basing on liquid–liquid extraction consists in loosing the lipophilic analytes by volatilisation [9], resulting in loss of recovery, sensitivity and precision. Concerning M-G, the use of high performance liquid chromatography (HPLC) for its direct determination would be an option to avoid such problems. Due to the lack of a chromophore, M-G needs to be derivatised [11,12] or MS has to be used to enhance sensitivity [9]. Headspace solid-phase microextraction (HS-SPME), an alternative sample extraction technique, allows to concentrate volatile and semi-volatile analytes from the headspace above the sample on a coated fiber and to transfer the analytes from the fiber directly into the injector port of a GC without further manipulations. Over the last years, HS-SPME has been established for the qualitative and quantitative determination of essential oils and monoterpenes in plants, foodstuffs, pharmaceuticals and biological matrices [13–20]. To the best of our knowledge, the quantitative determination of menthol in biological fluids using HS-SPME has not been described so far. Here, we present a sensitive, selective, and

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reliable method for the determination of menthol and M-G after enzymatic hydrolysis in rat plasma and urine. Preliminary experiments indicate that this method can as well be used for the determination of menthol and M-G in human plasma and urine without significant modification.

2. Experimental

2.1. Chemicals

(−)-Menthol (>98%) was obtained from Roth (Reinach, Switzerland), (+)-isomenthol (purum >99%) from Fluka Chemie (Buchs, Switzerland), menthol glucuronide (as ammonium salt, M-G) (>99%) and β-glucuronidase (type H-1 from Helix pomatia, 46,700 units/g) from Sigma (Buchs, Switzerland). All other chemicals were of analytical grade purchased from Merck.

2.2. Animals

Wistar Hanlbm rats (RCC Ltd., Füllinsdorf, Switzerland) were reared and kept in standard animal facilities that comply with the Swiss and US National Institutes of Health guidelines for care and use of experimental animals. The experiments performed were approved by the State Committee for the Control of Animal Experimentation.

2.3. Plasma sample preparation

In a pilot study to determine the time profile of menthol and M-G levels, one or two rats were treated for each time point. Blood samples were taken at 5, 15, and 30 min and 1, 2, 4, 8 and 24 h after intragastrical administration of 400 mg/kg b.wt. menthol (in almond oil, 2 ml/kg). 5 ml of blood was collected in centrifuge glass vials containing 625 IU heparin in 125 μl of 0.9% sodium chloride. The blood samples were then immediately centrifuged for 10 min at 800 × *g* and 4 °C. Plasma was transferred into a 4 ml glass vial, closed tightly and stored at −20 °C until analysis.

Human blood samples (5 ml) were taken from a healthy female volunteer at 0, 15, 30, 60, 120 and 360 min after oral administration of 100 mg menthol filled in a gelatine capsule. The heparinised blood specimens were centrifuged for 10 min at 800 × *g*, and the plasma samples stored at −20 °C until analysis.

Five hundred microliters of rat or human plasma, 400 μl of 1.5 M acetate buffer (pH 5.0 at 37 °C), 100 μl of 0.2% sodium chloride with or without 0.26 mg of β-glucuronidase, 50 ng of (+)-isomenthol (internal standard, 10 μl of a 5 μg/ml ethanolic solution) were added to a 4 ml headspace vial and closed tightly. A first sample without β-glucuronidase was analysed immediately after preparation, to determine unconjugated menthol. A second sample with β-glucuronidase was incubated overnight (16 h) at 37 °C to measure the total amount of menthol (unconjugated menthol and menthol released by enzymatic hydrolysis from M-G).

For the determination of the assay linearity blank rat plasma was spiked with 10 μl of menthol standard solutions (0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 17.5, 25.0, 35.0 and 50.0 μg/ml in absolute ethanol), corresponding to menthol plasma concentrations of 5, 10, 20, 50, 100, 200, 350, 500, 700, 1000 ng/ml. Intra- and inter-day precision and accuracy were determined with plasma samples spiked with 10 μl of menthol (0.25, 5 and 50 μg/ml) or M-G standard solutions (0.56, 11.2 and 112 μg/ml, corresponding to 0.25, 5 and 50 μg/ml free menthol) to get menthol plasma concentrations of 5, 100 and 1000 ng/ml.

2.4. Urine sample preparation

Five rats (about 250 g in weight), held in metabolic cages, received 100 mg of menthol mixed with the food. All the rats ingested their food within 1 h after it was placed in the cage. Urine was collected during 24 h in cooled bottles and was then stored at −20 °C until analysis. In the single-subject feasibility study total urine was collected at 2, 4, 6 and 24 h after administration of 100 mg menthol and stored at −20 °C until analysis. Rat and human urine was diluted 1:1000 and 1:10, respectively, with water prior to analysis. Five hundred microliters of diluted urine was then prepared in the same way as described for plasma samples. Samples for the calibration and control of precision and accuracy were prepared with rat urine diluted 1:1000, which was spiked with menthol and M-G standard solutions corresponding to free menthol of 5–1000 μg/ml rat urine.

2.5. HS-SPME extraction

The extractions were performed using a SPME fiber holder for manual sampling and 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB)-coated fibers (Supelco, Buchs, Switzerland). Magnetic stirring at 1315 rpm was applied during the extraction. After being exposed to the sample's headspace for 20 min at 80 °C, the fiber was immediately transferred to the injector port of the GC–MS for desorption (3 min at 250 °C, split valve closed for 1 min). After desorption the fiber was instantly moved to the next sample.

2.6. GC–MS

The analyses were performed on a GC–MS system consisting of a HP 5890A series II GC with electronic pressure control, a HP 5972 mass selective detector (MSD), and a HP 5895A ChemStation software. The GC was equipped with a SPME inlet guide, and a 0.75 mm i.d. liner (Supelco). Chromatographic separation was achieved on a DB-1 capillary column (15 m × 0.25 mm i.d., 0.25 μm film, Agilent) and using helium as carrier gas at a constant flow rate of 1.3 ml/min. The injector port and interface transfer line

were maintained at 250 and 280 °C, respectively. The oven temperature was initially held at 50 °C for 1.3 min, then increased to 90 °C at 10 °C/min, held for 5.5 min, followed by 40 °C/min to 220 °C and held for 2 min. The MS system was used in the single ion monitoring (SIM) mode (*m*/*z* 71, 81, 95, 123, and 138).

3. Results

3.1. Exposure time

To determine the time necessary for the analytes to attend equilibrium between sample, headspace, and fiber, rat plasma samples were spiked with 55 ng/ml menthol and 49 ng/ml isomenthol (internal standard). The fiber was exposed to the sample's headspace at 80 °C for 2, 5, 10, 15, 20, 30 and 45 min, before being desorbed in the injector port of the GC–MS and analysed as described before. The equilibrium was achieved for menthol and isomenthol within 15 min (Fig. 1).

3.2. Selectivity

The mass spectra of menthol and isomenthol showed the same abundant ions, although with slightly different intensity. The molecular peak at *m*/*z* 156 was not detectable for both molecules. The ion at *m*/*z* 123, being the most specific fragment with reasonable intensity, was chosen for the quantitative determination of menthol and isomenthol and *m*/*z* 71, 81, 95 and 138 were used for identification.

All blank plasma and urine samples taken from several rats, and even blank samples using 1 ml of pure water showed an interfering peak at 8.7 min, which was identified as menthol. This unavoidable contamination, apparently resulting from traces of menthol in the air, forced us to set the LOQ in plasma at 5 ng/ml, enabling an acceptable assay precision and accuracy.

There was no interference in any of the blank plasma and urine samples at the retention time of isomenthol (9.3 min). Fig. 2 shows a characteristic chromatogram of a blank rat plasma (A) and a representative sample from a rat plasma 4 h after intragastrical administration of 400 mg/kg b.wt. menthol (B).

3.3. Linearity

Triplicates of spiked blank rat plasma and urine samples were performed for each of the 10 concentration levels. Calculating for both matrices two separate calibration curves, for plasma from 5 to 200, and 200 to 1000 ng/ml and for urine from 5 to 200, and 200 to 1000 μg/ml, good correlation coefficients ($r = 0.9994$–0.9999) were achieved. The linearity data are summarised in Table 1.

3.4. Precision and accuracy

The intra-day precision and accuracy were established at a low, medium, and high concentration level based on the measurement of six samples. For each concentration level blank rat plasma and urine samples were spiked with menthol or an equivalent of M-G. The intra-day precision (%

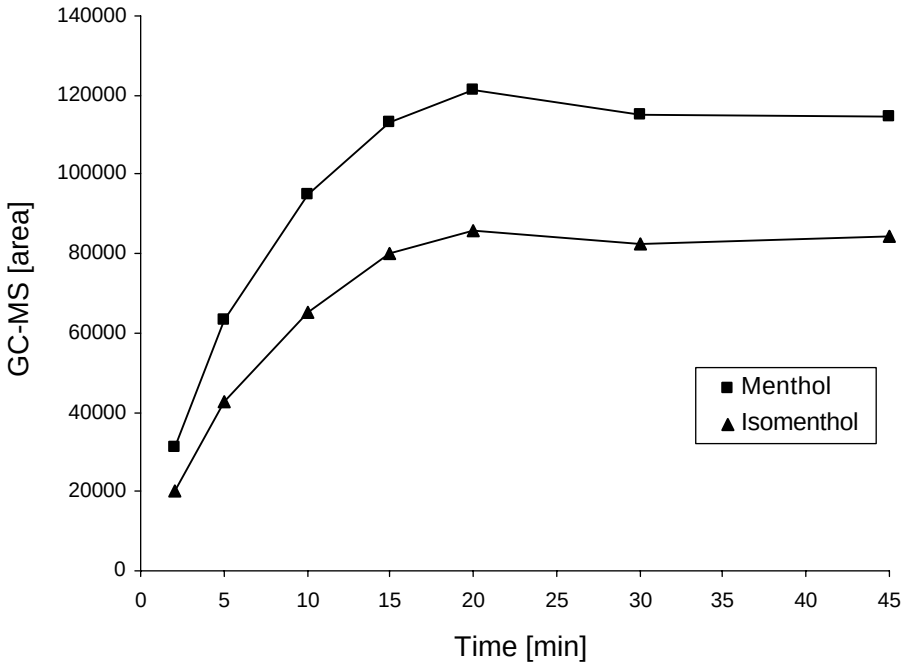

Fig. 1. Extraction time vs. GC–MS signal of menthol (55 ng/ml) and isomenthol (internal standard, 49 ng/ml) in rat plasma at 80 °C using a PDMS/DVB fiber ($n = 2$).

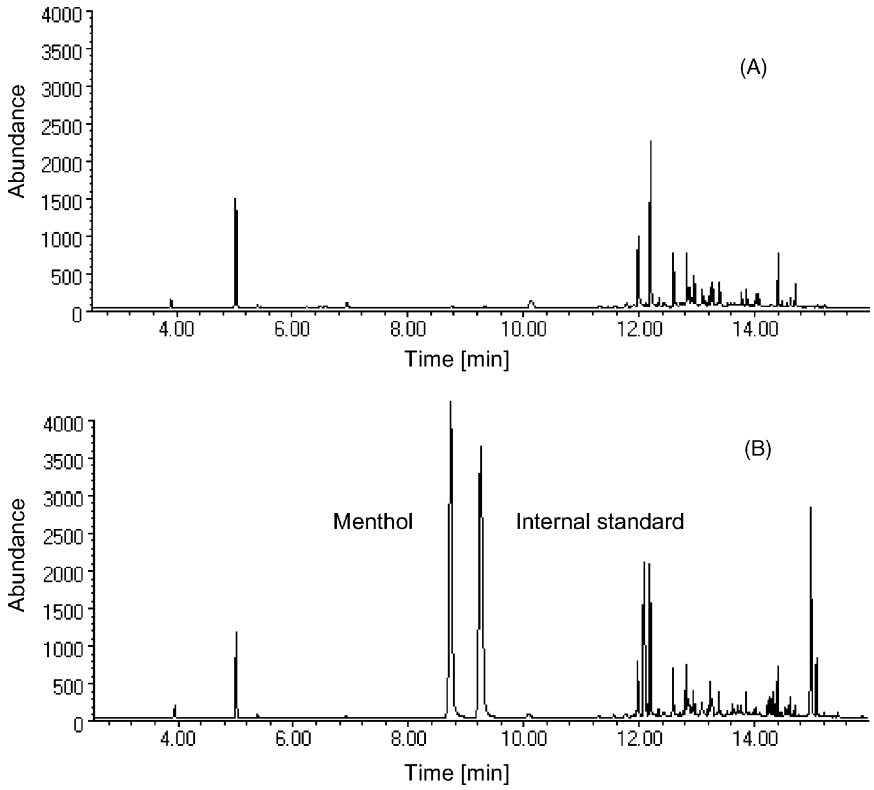

Fig. 2. HS-SPME/GC–MS selected ion chromatograms (*m/z* 123) of blank rat plasma (A) and a rat plasma 4 h after intragastrical administration of 400 mg/kg b.wt. menthol (B).

Table 1
Linearity data for the determination of menthol in rat plasma and urine obtained by performing triplicates for each of 10 concentration levels (5, 10, 20, 50, 100, 200, 350, 500, 700 and 1000 ng/ml and μg/ml in plasma and urine, respectively)

	Concentration range	Slope (mean ± S.D.)	Intercept (mean ± S.D.)	*r* (mean ± S.D.)
Plasma (ng/ml)	5–200	0.0118 ± 0.0002	0.0159 ± 0.0037	0.9999 ± 0.0001
	200–1000	0.0097 ± 0.0001	0.5357 ± 0.0440	0.9994 ± 0.0003
Urine (μg/ml)	5–200	0.0118 ± 0.0001	0.0173 ± 0.0024	0.9998 ± 0.0001
	200–1000	0.0099 ± 0.0001	0.4522 ± 0.0582	0.9998 ± 0.0002

R.S.D.) in plasma and urine for menthol and M-G at the low concentration level was ≤14.7%, at medium and high concentration levels ≤3.5%. The assay accuracy (±% of concentration added) ranged from −12.1 to 4.3% (Table 2).

The inter-day precision and accuracy were determined at the same three concentration levels over a time period of 1 month ($n = 8$). The inter-day precision at the low concentration level was ≤18.1%, at medium and high concentra-

Table 2
Intra- and inter-day precision and accuracy (±% of concentration added) of unconjugated menthol and M-G in rat plasma and urine

Compound	Concentration added to plasma (ng/ml) and urine (μg/ml)	Plasma				Urine			
		Intra-day ($n = 6$)		Inter-day ($n = 8$)		Intra-day ($n = 6$)		Inter-day ($n = 8$)	
		Precision (% R.S.D.)	Accuracy (%)	Precision (% R.S.D.)	Accuracy (%)	Precision (% R.S.D.)	Accuracy (%)	Precision (% R.S.D.)	Accuracy (%)
Unconjugated menthol	5	3.2	4.3	14.9	1.5	12.7	−12.1	10.2	−11.9
	100	0.8	−1.2	4.0	−2.2	0.8	−0.1	0.9	0.1
	1000	3.5	−5.9	2.1	−3.0	1.1	0.6	1.3	−1.1
Menthol glucuronide*	5	14.7	−4.6	18.1	−2.9	6.4	2.5	17.9	−16.1
	100	1.4	−3.8	2.8	−5.4	1.2	−8.4	2.4	−6.2
	1000	1.5	−11.9	1.5	−10.8	1.9	−7.6	1.4	−7.7

*Expressed as menthol after enzymatic hydrolysis.

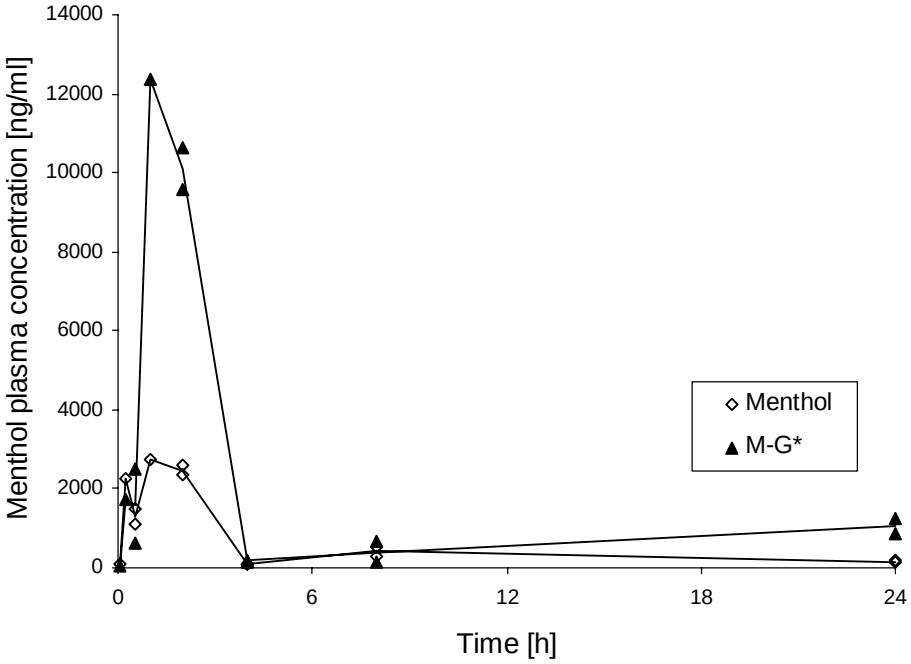

Fig. 3. Plasma levels of unconjugated menthol and M-G (*expressed as menthol after enzymatic hydrolysis) in rats after intragastrical administration of 400 mg/kg b.wt. menthol ($n = 1$ or 2).

Table 3
Urinary excretion of M-G in rats (each about 250 g in weight) during 24 h after administration of 100 mg menthol

Rat	Concentration of M-G* (μg/ml)	Urine volume (ml)	Amount of M-G* excreted (μg)	Percentage of dose excreted as M-G*
1	636.4	14.0	8910	8.9
2	154.8	13.6	2105	2.1
3	307.9	20.6	6344	6.3
4	538.1	8.8	4736	4.7
5	52.0	48.1	2503	2.5

*Expressed as menthol after enzymatic hydrolysis.

tion levels ≤4.0%. The assay accuracy ranged from −16.1 to 1.5% (Table 2).

3.5. Rat plasma and urine profiles of menthol and M-G

A time profile of plasma levels of menthol and M-G in rats after intragastrical administration of 400 mg/kg b.wt. menthol is shown in Fig. 3. In some urine samples traces of free menthol, however below the LOQ, were detected. The concentration of M-G (expressed as menthol after enzymatic hydrolysis) in 24 h urine from rats ($n = 5$, about 250 g in weight) treated with 100 mg of menthol ranged from 52 to 636 μg/ml, which corresponds to 4.9 ± 2.9% of the administered dose (Table 3).

3.6. Determination of menthol and M-G in human plasma and urine

In the single-subject feasibility study very similar results for linearity, precision and accuracy were obtained as for rat plasma and urine. At baseline no free menthol and M-G were detectable in the volunteer's plasma and urine. After ingestion of 100 mg of menthol traces of free menthol, however below the LOQ, were detected in plasma (at 30, 60 and 120 min after administration) and in the 4 h urine. The time profile of the M-G plasma levels is shown in Fig. 4. In the 24 h urine, 53.2% of the menthol dose was recovered as M-G.

4. Discussion

HS-SPME is an equilibrium technique. During extraction the analyte migrates among the three phases, the matrix (e.g. plasma), the headspace above the sample, and the fiber coating, until equilibrium is reached. Therefore, analytes are not completely extracted from the matrix. To improve recovery, parameters affecting the equilibrium should be optimised (fiber type, pH, salt, temperature, etc). Optimising the stirring mode of the sample and the temperature during the

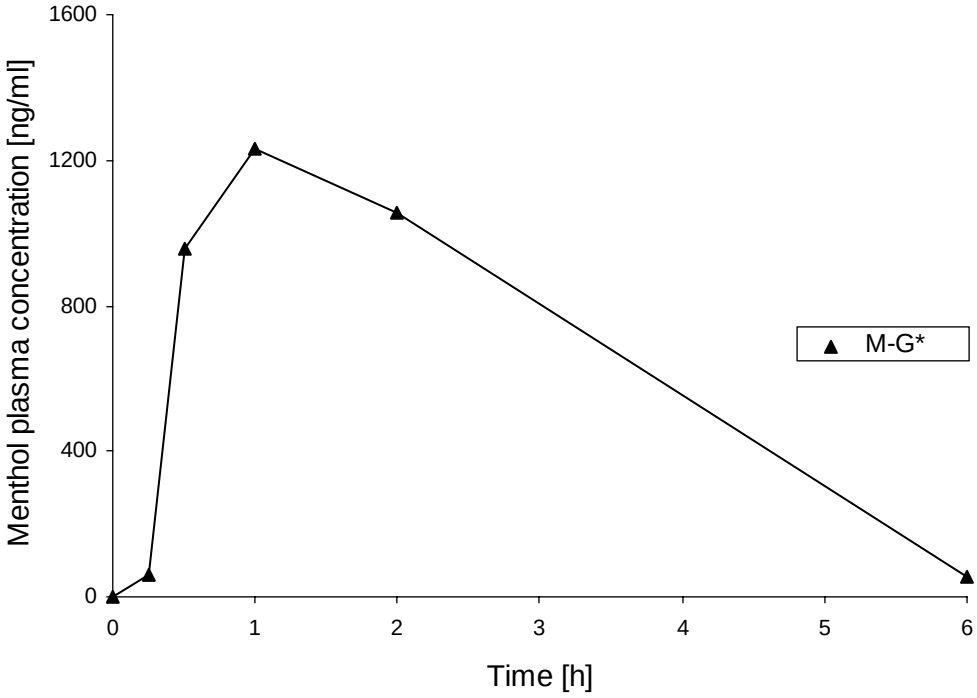

Fig. 4. Plasma levels of M-G (*expressed as menthol after enzymatic hydrolysis) in a human volunteer after ingestion of 100 mg menthol.

extraction can help to reduce the time to reach equilibrium [21]. Three fiber types with different coatings, i.e. 100 μm PDMS, 65 μm PDMS/DVB and 75 μm carboxen/PDMS, were tested. PDMS/DVB coating showed the highest distribution constant for menthol, resulting in the best recovery when using an extraction temperature of 80 °C (data not shown). Adding different salts (sodium chloride, ammonium sulphate, and potassium carbonate) to the samples did not improve the recovery. Intensive magnetic stirring at a constant velocity reduced the extraction time significantly compared to not stirring. With optimised parameters the equilibrium was achieved within 15 min (Fig. 1).

The evaluation of a structural related internal standard, ideally a deuterated isomer of the analyte, is mandatory for a reproducible quantitative analysis based on SPME. Regarding the lack of a commercially available deuterated menthol and the very similar behaviour during SPME and GC–MS (Figs. 1 and 2), isomenthol proved to be an adequate internal standard.

Advantages of SPME compared to liquid–liquid or solid phase extraction (SPE) are (i) the sample preparation is performed in a much shorter time, (ii) the extraction takes place in a mostly closed system and (iii) no concentration step by evaporation of solvents is required. Therefore, the risk of loosing volatile analytes is significantly reduced. In addition, SPME is cost-effective, as each fiber can be used for more than 200 extractions without showing a loss of efficiency.

The linearity of the method was demonstrated in the range of 5–1000 ng/ml and μg/ml menthol in rat plasma and urine, respectively (Table 1). However, it was necessary to calculate two calibration curves, for plasma from 5 to 200, and 200 to 1000 ng/ml and for urine from 5 to 200, and 200 to 1000 μg/ml, to assure accuracy within the limits of 20% at the LOQ and 10% at higher concentrations. Intra- and inter-day precision for the determination of menthol and M-G in plasma and urine was ≤18.1% at the lower LOQ and ≤4.0% at higher concentration levels (Table 2).

According to the accuracy data (Table 2) the hydrolysis of M-G was not complete. However, a higher β-glucuronidase concentration did not increase the hydrolysis rate.

The LOQ in plasma was set at 5 ng/ml allowing a reliable determination of endogenous menthol despite the persisting presence of exogenous menthol (contamination in the laboratory air). As the baseline noise of blank plasma was very small, we estimate that menthol levels below 0.5 ng/ml could be measured in rat plasma in absence of any menthol laboratory contamination.

In humans, no unconjugated menthol was detected in plasma and urine after oral application of menthol [8,9]. Therefore, we expected in rats levels of unconjugated menthol in the low ng/ml range, requiring the development of a highly sensitive method. The upper LOQ of 1000 ng/ml was given by the GC column capacity resulting in loss of peak symmetry (fronting). Analysing the samples for the time profile of menthol and M-G plasma levels in rats, samples taken between 15 min and 2 h had to be diluted for the determination of free and conjugated menthol to fit within the calibration range.

The M-G concentration in 24 h urine samples of rats was in the μg/ml range. As the method developed for plasma

analysis should be used, it was necessary to dilute urine samples 1:1000 prior to analysis not to exceed the upper LOQ. In consequence, the lower LOQ of 5 μg/ml was relatively high. To find out if dilution had an effect on the SPME-equilibrium and subsequently on the reliability of the method, undiluted, 1:10 and 1:100 diluted blank rat urine was analysed, spiked with 5.6 ng of M-G (corresponding to urine levels of 5, 50 and 500 ng/ml of free menthol). With undiluted urine the accuracy was not acceptable, but urine diluted 1:10 and 1:100 showed precision and accuracy data similar to urine diluted 1:1000. Therefore, it would be possible to work with less diluted rat urine in order to lower the LOQ as it was required for the analysis of M-G in human urine. Yamaguchi et al. [3] did not detect unconjugated menthol in urine, faeces and bile of rats treated with 500 mg/kg b.wt. menthol. Therefore, it was very surprising to find a remarkable amount of unconjugated menthol in urine of rats given an equivalent dose of menthol. However, further experiments demonstrated that M-G was not stable in urine, maybe because of the presence of β-glucuronidases originating from intestinal bacteria of the rats. To minimise the hydrolysis of M-G during the collection of the urine, sodium azide as disinfectant was then added to the collecting bottles and the bottles were cooled. In these urine samples, the amount of unconjugated menthol was below the LOQ. With an urinary elimination of $4.9 \pm 2.9\%$ of the dose (Table 3), the rate in rats was rather low compared to other species [22]. This result indicates that other metabolites are predominately formed in the rat. Further studies are ongoing to elucidate the phase-I metabolism of menthol in the rat.

A feasibility study showed that this method is not limited for determination of menthol and M-G in rat plasma and urine but can be used for analysis of human samples, too. Results of the human plasma and urine study with one volunteer corresponded to data reported before by Gelal et al. [8].

In conclusion, the present method using HS-SPME/GC–MS is suited for the determination of menthol and M-G. It is simple, reliable, selective, sensitive, and applicable on biological matrices of different origin.

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