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Identification of thymol phase I metabolites in human urine by headspace sorptive extraction combined with thermal desorption and gas chromatography mass spectrometry

Bernhard Thalhamer[a,*], Wolfgang Buchberger[a], Mario Waser[b]

[a] Institute of Analytical Chemistry, Johannes-Kepler-University, Altenbergerstrasse 69, A-4040 Linz, Austria
[b] Institute of Organic Chemistry, Johannes-Kepler-University, Linz, Austria

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ABSTRACT

Development of a novel highly sensitive headspace sorptive extraction (HSSE) method in combination with thermal desorption gas chromatography coupled to a mass spectrometer (TD–GC/MS) allowed the identification of thymol and several phase I metabolites in human urine. Combined with an enzymatic hydrolysis of glucuronated or sulphated phase II metabolites of thymol and of the respective phase I metabolites prior to analysis, even trace quantities of hitherto not detected thymol phase I metabolites could be identified in urine samples of test persons after oral administration of 50 mg thymol. It was proven, that human metabolism leads to a hydroxylation of the aromatic ring as well as of the iso-propyl side chain. Hydroxylation of the iso-propyl group results in the formation of the rather unstable p-cymene-3,8-diol and the corresponding dehydration product p-cymene-3-ol-8-ene which could be clearly detected in human urine samples. Furthermore, the aromatic hydroxylation products p-cymene-2,5-diol, its oxidation product p-cymene-2,5-dione and p-cymene-2,3-diol were also unambiguously identified by comparison with synthesized reference compounds.
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1. Introduction

Although a large variety of terpenoid oil components are regularly taken up by the human organisms in significant quantities, only limited details about the metabolism of these compounds are currently known. As an example of special interest, thymol (**1**) is an important additive in cosmetic products (e.g. mouthwash, bath essences, etc.), several traditionally used medicines, and also a main ingredient in different spices and herbs (e.g. thyme, oregano, savory, etc.) which results in the fact that these products are daily consumed in considerable amounts.

Surprisingly enough, only a few studies focusing on the qualitative and/or quantitative analysis of thymol (**1**) in human urine have been reported in the past [1,2]. Furthermore, even less details about the metabolism of **1** are known so far [3–5]. Meesters et al. reported the use of a combined enzymatic hydrolysis, derivatization and solid phase extraction procedure followed by high performance liquid chromatography with postcolumn fluorescence detection (HPLC–FLD) for the identification of thymol and other hydroxylated monoterpenes spiked to human urine [1]. However, in the course of that study, no thymol was orally administered to the probands and therefore no thymol or any metabolites were detected in real urine samples. In a different report, Mills and Walker used solid phase microextraction (SPME) coupled to GC/MS for the analytic profiling of volatile urine components [2]. It was found that healthy test persons who got a normal mixed diet, excreted considerable amounts of thymol besides other organic volatiles in their urine.

On the other hand, early studies focusing on thymol metabolites in humans clearly showed that orally administered **1** is renally secreted as its phase II metabolites thymol glucuronide and thymol sulphate [3]. In addition that report also described the presence of the phase I metabolite p-cymene-2,5-diol (**2**) in human urine, originating from a hydroxylation of the aromatic system of **1**.

The formation of the phase II metabolites thymol glucuronide and thymol sulphate was also proven by LC–MS/MS studies of the urine of probands after oral administration of a herbal medicinal product containing a defined amount of **1** [4]. Furthermore, the thymol content could be quantified by means of GC–FID. However, that study did not report the detection of any phase I metabolites.

The scarcity of detailed information about the biotransformation of thymol can to some extent definitely be attributed to the lack of adequate analytical methods. On the one hand, suitable methods for this purpose have to be very sensitive to determine even trace quantities of metabolites but in addition they also have to be

* Corresponding author. Tel.: +43 732 2468 8732; fax: +43 732 2468 8679.
E-mail address: bernhard.thalhamer@jku.at (B. Thalhamer).

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applicable in analysis of samples with a rather complex matrix of variable composition.

Therefore, in the present work the efforts were first put on the development of a highly sensitive robust method based on headspace sorptive extraction (HSSE) in combination with thermal desorption gas chromatography coupled to a mass spectrometer (TD–GC/MS) for the identification of thymol and – if present – hydroxylated phase I metabolites in human urine. As possible phase II metabolites are not suited for GC analysis, it was decided to carry out an enzymatic hydrolysis reaction prior to the sorptive extraction process to ensure a full hydrolysis of all the glucurinated and sulphated analytes to the corresponding hydroxylated phase I metabolites which should be well suited for TD–GC/MS analysis. In a next step, investigations concerning the identification of hydroxylated phase I thymol metabolites in urine samples of test persons after oral administration of 50 mg **1** were undertaken.

Looking at the possible differently monohydroxylated thymol metabolites, one can rationalize either the formation of derivatives carrying an additional hydroxyl group on the aromatic system (three possible substitution patterns) or compounds being hydroxylated on either the methyl or the iso-propyl side chain (again three possible derivatives). Surprisingly enough, only limited MS-data of these compounds can be found in conventional MS-libraries. Furthermore, the expected spectra and fragmentation patterns of these isomers are supposed to be rather similar, making a clear identification just by GC/MS analysis impossible. In addition, most of these compounds are commercially not available. We therefore decided to synthesize some of the most reasonable phase I metabolites to obtain authentic reference samples for an unambiguous identification of these metabolites in human urine.

Table 1
Structures of tested reference samples.

Compound	Structure
Thymol (**1**)	OH; $C_{10}H_{14}O$ MW: 150.2
p-Cymene-2,5-diol (**2**)	HO, OH; $C_{10}H_{14}O_2$ MW: 166.2
p-Cymene-2,5-dione (**3**)	O, O; $C_{10}H_{12}O_2$ MW: 164.2
p-Cymene-2,3-diol (**4**)	OH, OH; $C_{10}H_{14}O_2$ MW: 166.2
p-Cymene-3,8-diol (**5**)	OH, OH; $C_{10}H_{14}O_2$ MW: 166.2
p-Cymene-3-ol-8-ene (**6**)	OH; $C_{10}H_{12}O$ MW: 148.2

2. Experimental

2.1. Chemicals

2.1.1. Reference compounds

Table 1 shows a list of all the compounds used for these investigations.

Thymol (**1**) was obtained from Roth (Karlsruhe, Germany). The other reference samples were obtained by syntheses according to published methods. The synthesis of p-cymene-2,5-diol (**2**) and the corresponding p-cymene-2,5-dione (**3**) was carried out by oxidation of **1** with meta-chloroperoxybenzoic acid (mCPBA) in $CHCl_3$ as described in the literature [6]. Isolation of **2** and **3** from the crude product mixture could be achieved by normal phase column chromatography. ^{1}H NMR of the synthesized 2,5-diol **2** was in full accordance with the literature [7] as well as in the case of **3** [8]. In addition, the 2,5-regioselective functionalization was also proved by chemical correlation because a $NaBH_4$-mediated reduction of synthesized **3** yielded **2** exclusively. Furthermore, this mCPBA oxidation of **1** also yielded traces of p-cymene-2,3-diol (**4**) (^{1}H NMR in full accordance with the literature [9]). Functionalization of the iso-propyl side chain was achieved according to a recent report [10] by a Grignard reaction between 2-hydroxy-4-methylacetophenone and methylmagnesium bromide yielding the p-cymene-3,8-diol (**5**) exclusively. As described in this report, prolonged thermal exposure of **5** ($T > 160\,^\circ$C) results in the elimination of H_2O giving significant amounts of p-cymene-3-ol-8-ene (**6**). Although most of these compounds were purified by column chromatography, their purities were less than 100%. Therefore, they were suited for qualitative comparison but could not be used as analytical standards for quantitative analysis.

2.1.2. Other chemicals

β-Glucuronidase (with sulphatase activity) type HP-2 from Helix Pomatia was obtained from Sigma Aldrich (St. Louis, MO, USA). Acetic acid, sodium acetate and ethanol were purchased from Merck (Darmstadt, Germany). In all experiments 18 MΩ Milli-Q purified water (Millipore, Bedford, MA, USA) was used. All materials (typically analytical grade or better) were used as purchased without any further purification.

2.2. Instrumentation

2.2.1. GC/MS conditions

Monitoring of reaction progress in the syntheses of the reference samples was carried out by using a Focus GC coupled to a DSQII MS purchased from Thermo (Waltham, MA, USA). The injection (injection volume 1 μl) was performed in the split mode 1:10 at the injection temperature of 200 °C. Separation was carried out on a Rxi-5ms fused silica column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) from Restek (Bad Homburg, Germany) at constant flow. The oven program started at 65 °C (held for 1 min), and the temperature was increased in the first step to 190 °C at 20 °C min^{-1} and in the second step to 280 °C (held for 5 min) at 40 °C min^{-1}. Helium (4.6) was used as carrier gas with a column flow rate of 1 ml min^{-1}.

The mass spectrometer was operated in the scan mode with the scan range *m*/*z*: 50–250. The ion source temperature was 230 °C.

2.2.2. TD–GC/MS conditions

The instrumentation consisted of a 6890N gas chromatograph coupled to a 5973 inert XL MSD mass spectrometer from Agilent (Waldbronn, Germany). The thermodesorption device used as inlet system during this work was a Gerstel TDS 2 system (Gerstel, Mülheim, Germany) equipped with a Gerstel TDS A autosampler and a Gerstel Cooled Injection System (CIS) 4.

The program for thermodesorption started with an initial temperature of 20 °C which was increased to 230 °C at a rate of 60 °C min^{-1} and held for 5 min. The desorbed compounds were cryofocused at −100 °C. After the desorption the temperature of the CIS increased from −100 °C to 280 °C (held for 5 min) at 12 °C s^{-1}. Separation was carried out on a HP-5ms fused silica column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) from Agilent in constant flow. The oven program started at 65 °C (held for 1 min), and the temperature was increased in the first step to 190 °C at 20 °C min^{-1} and in the second step to 280 °C (held for 5 min) at 40 °C min^{-1}. Helium (4.6) was used as carrier gas with a column flow rate of 1 ml min^{-1}. The mass spectrometer was operated in the scan mode (scan range *m*/*z*: 50–250).

2.3. HSSE conditions

For the headspace sorptive extraction, twister stir bars from Gerstel of 10 mm length coated with a 0.5 mm layer of polydimethylsiloxane (PDMS) were employed for all measurements. Before use, the coated stir bars were placed into a cleaning solution containing methanol and dichloromethane (50:50, v/v) for 1 h. Afterwards the stir bars were put into thermodesorption glass tubes and were conditioned for one hour at 300 °C under nitrogen flow in a Gerstel tube conditioner TC 2. After the cleaning procedure the twister stir bars were placed in a commercially available glass insert (Gerstel, Mülheim, Germany) for a 20 ml twister headspace vial (Gerstel, Mülheim, Germany) containing 2 ml sample solution. After a defined extraction time (20 min/60 °C) in a heated block (using an agitator of a multi purpose sampler (Gerstel, Mülheim, Germany) allowing the incubation of the head space vial) and cooling to room temperature (4 min) the twisters were dried with lint-free cloth, placed into the thermodesorption tubes and analysed by TD–GC/MS.

2.4. Sample preparation

2.4.1. Preparation of urine samples for HSSE–TD–GC/MS analysis

850 μl of urine were transferred into a 20 ml crimp cap headspace vial, followed by the addition of 100 μl of a sodium acetate buffer (pH 5; 0.01 M), 50 μl β-glucuronidase and 1000 μl H_2O (total volume 2000 μl). The sealed vial was then placed into a thermostated water bath (37 °C) for 60 min. The vial was cooled to room temperature, the conditioned twister added into a glass insert which was placed into the twister headspace vial which was then crimped and heated to 60 °C for 20 min. After cooling to room temperature (4 min) the twister was placed into the thermodesorption glass tube and analysed by TD–GC/MS.

2.4.2. Preparation of aqueous thymol standard solutions

Thymol (0.1 g) was dissolved in 1 l 18 MΩ H_2O giving the standard stock solution (100 μg/ml) which was then further diluted giving solutions of different concentrations (10/25/50/100/250/500/1000/2500/5000/10,000 ng/ml) which were used in the HSSE–TD–GC/MS quantitation experiments.

1000 μl of these standard solutions were transferred in a 20 ml crimp cap headspace vial, followed by addition of 1000 μl H_2O. Then the twister was placed in the glass insert and put into the twister headspace vial which was closed and heated to 60 °C for 20 min. After cooling to room temperature (4 min) the twister was placed into the thermodesorption glass tube and analysed by TD–GC/MS. This procedure was then used to examine the influence of HSSE extraction time and extraction temperature.

2.5. Study design

2.5.1. Test persons

The test group included six healthy persons (three female and three male). The average age was 33.8 years (s = 8.1) with an average body mass index of 25.6 (s = 4.1). All probands explicitly declared their agreement of taking part in these studies and providing urine samples.

Each proband was administered an oral dose of 50 mg thymol (dissolved in 1 ml ethanol and 200 ml H_2O), which equals approximately 2–3 cups of thyme tea.

2.5.2. Blank urine samples

Blank urine samples were obtained after a period of 48 h during which all the probands just lived on rice or potatoes and mineral water, avoiding all contact to herbs, oils, dietary supplements or cosmetic products which might include terpenoid components. The blank urine samples were collected in PVC-flasks and stored at −20 °C.

2.5.3. Urine samples after administration of thymol

Directly after collection of the blank urine samples, each test person was administered 50 mg thymol and the diet of rice, potato, and mineral water was prolonged for further 24 h. During these 24 h all the urine of the probands was collected in PVC-flasks and stored at −20 °C.

3. Results and discussion

3.1. Method development

3.1.1. HSSE – Influence of extraction temperature

To investigate the influence of the extraction temperature on the extraction yield, aqueous thymol standard samples with a concentration of 5 ng/ml were kept at RT, 40 °C, 60 °C, and 80 °C for 20 min each. It was found that the measured peak area shows a nearly linear dependence of the extraction temperature, doubling from 40 to 60 °C and again from 60 to 80 °C. With respect to the measurement of the real samples it was chosen to carry out the extraction at 60 °C, as this temperature should also allow the measurement of potentially protein-rich samples in future investigations (avoiding denaturation effects like coagulation).

3.1.2. HSSE – influence of extraction time

To investigate the influence of the extraction time on the extraction yield, aqueous thymol standard samples with a concentration of 5 ng/ml were kept at 60 °C for 10, 20, 30, 60, 120, 180, 360, and 1080 min. As depicted in Fig. 1, a linear dependence during the first 180 min was observed, whereas longer extraction times resulted in a non-linear flattened increase. For all further experiments an extraction time of 20 min was implemented which allowed to reduce the overall sample manipulation time to an acceptable minimum and at the same time resulted in a yield that was sufficient for practical applications.

3.1.3. Enzymatic hydrolysis – reaction time

Putting the main focus on the development of an enzymatic hydrolysis which allows to quantitatively hydrolyse all phase II metabolites to the corresponding phase I metabolites, the optimum

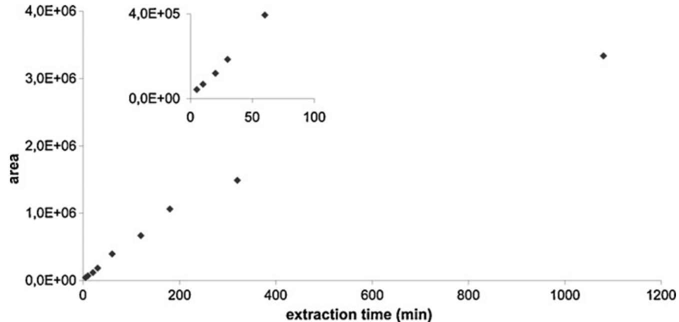

Fig. 1. Influence of HSSE extraction time on extraction yield using aqueous samples of **1** (5 ng/ml).

reaction time had to be elucidated. We therefore used a real urine sample after thymol administration and varied the reaction time in the presence of the enzyme. During the first 30 min, the amount of detected thymol continuously increased. After 30 min the detected amount of **1** remained constant. From this experiment it is clear that the hydrolysis is indeed a rather fast process. However as it cannot be guaranteed that hydrolysis of the other phase II metabolites to the targeted phase I metabolites will talk place with the same reaction rate, it was decided to carry out this sample pretreatment for 60 min in all further real urine samples to ensure a maximum conversion of all the other glucuronidated and sulphate derivatives.

3.2. Identification of thymol phase I metabolites

First, TD–GC/MS analyses of all the synthesized reference samples shown in Table 1 were carried out in order to obtain retention times and mass spectra (Fig. 2).

Subsequent analyses of the blank urine samples showed that in no sample any thymol or any of the possible metabolites were detectable. It can therefore be guaranteed that all observed terpenoid derivatives originated from thymol metabolism.

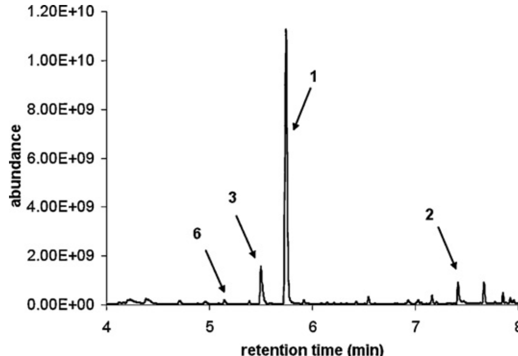

Fig. 3. HSSE–TD–GC/MS analysis (TIC; 50–250 m/z) of human urine 180 min after thymol administration (20 min extraction time).

Typical chromatograms as depicted in Fig. 3 were obtained when analysing urine samples after oral administration of thymol (**1**). As shown in this chromatogram, the major component among the terpenoid structures detected herein is still the parent compound **1** itself. But, out of the synthesized potential phase I metabolites shown in Table 1, also the 2,5-diol **2**, the 2,5-dione **3**, and the elimination product **6** could be detected in this real sample.

3.2.1. Identification of p-cymene-2,5-diol (2) and p-cymene-2,5-dione (3)

The clear identification of p-cymene-2,5-diol (**2**) was achieved by TD–GC/MS analysis of a solution of the synthesized reference compound and comparison of retention time and mass spectrum (Fig. 2, compound **2**). Accordingly **2** could be detected in all six real samples.

p-Cymene-2,5-dione (**3**) was also unambiguously detected in the urine of all test persons by comparison of retention time and mass spectrum (Fig. 2, compound **3**) with the synthesized reference sample.

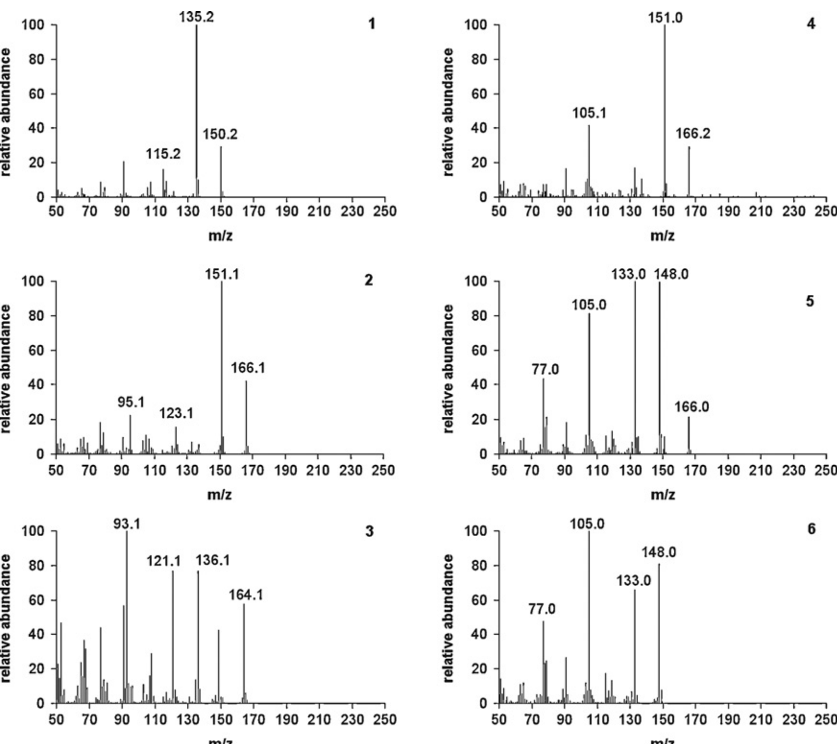

Fig. 2. MS spectra of compounds **1**–**6**.

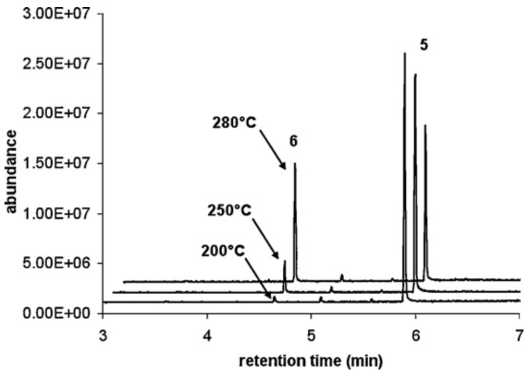

Fig. 4. Influence of injection temperature on the H_2O-elimination of 5 in GC/MS experiments (TIC; 50–250 *m*/*z*).

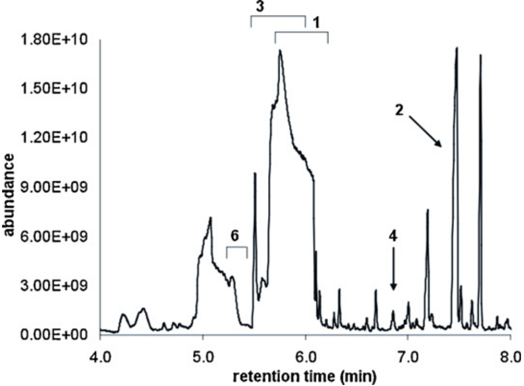

Fig. 5. HSSE–TD–GC/MS analysis (TIC; 50–250 *m*/*z*) of human urine 120 min after thymol application (960 min extraction time).

However, hereby some caution is necessary. Dione **3** is the oxidation product of the diol **2**. During the synthesis of **2** a rather high sensitivity of this compound towards oxygen (especially in the presence of light) was observed, resulting in a considerable oxidation of **2** to **3** under aerobic conditions. This effect was also noticed when a small sample of **3** was reduced to **2** with $NaBH_4$. Carrying out this reaction in an ethanol solution, full conversion of **3** to **2** was obtained within 30 min (proven by NMR and GC/MS). However, when exposed to air and light, within minutes the colourless solution turns partly yellow again, due to re-oxidation to **3** (clearly detected by GC/MS again). To elucidate whether the ratio of **2**:**3** in the urine sample is really as measured with the TD–GC/MS method or whether **2** is partially oxidized in the course of the sample treatment process additional experiments were undertaken. Therefore, a urine sample was first extracted with ethyl acetate and the organic phase directly measured by GC/MS (liquid injection). As expected the sensitivity was much lower in this case but nevertheless, the dione **3** and the diol **2** were clearly detected. However, whilst under the HSSE–TD–GC/MS conditions the dione **3** was the predominant one (see Fig. 3), in this experiment, the diol **2** was definitely the main species of this redox couple, but the dione **3** was also present in considerable amounts. In contrast, just analysing a urine sample with headspace (HS)-GC/MS (thereby avoiding the thermal desorption step of the twister stir bar), the amount of diol **2** decreased and mainly the dione **3** was observed. Accordingly, the measured ratio between **2** and **3** definitely depends on the analysis conditions. Therefore, it is obvious that human metabolism of **1** results in the formation of **2** first and it seems reasonable that a partial oxidation to **3** under the conditions in the human body might occur.

*3.2.2. Identification of p-cymene-3,8-diol (**5**) and p-cymene-3-ol-8-ene (**6**)*

In contrast to the 2,5-functionalized phase I metabolites **2** and **3** we were not able to directly detect the 3,8-diol **5** in any of the measured samples. However, the corresponding elimination product **6** was clearly observed in all cases (by comparison to a synthesized reference compound). As **5** is known to be rather prone to H_2O-elimination under several conditions (e.g. acid catalysed dehydration, thermal dehydration) [10] we were interested whether the synthesized **5** tends to eliminate H_2O under the analysis conditions. As shown in Fig. 4, liquid injection GC/MS measurements of pure diol **5** were carried out at different injection temperatures. Whilst injection at 200 °C resulted in a chromatogram showing only traces of **6**, higher injection temperatures clearly sped up this elimination process. Accordingly, only this very short exposure to high temperatures is already sufficient to result in almost 50% of the dehydrated **6** at 280 °C. This effect was even more pronounced when **5** was analysed under the developed TD–GC/MS conditions. Hereby, a full conversion to **6** was observed and not even traces of **5** were detected anymore. Thus, it cannot be decided, whether **6** was detected in the real samples due to an elimination process of **5** during the sample pretreatment – analysis process, or whether **6** is already present in the collected urine samples themselves.

One might also argue that **6** is not formed from the 8-hydroxylated **5** (OH group in the benzylic position) but from the corresponding 9-hydroxylated isomer (carrying the OH group on the methyl group of the iso-propyl side chain). However, from a chemical point of view, the presence of the 8-substituted **5** leading to **6** seems to be more reasonable than the second alternative mentioned above due to two reasons. First, the benzylic position of an iso-propyl group is normally much more reactive towards oxidation than the methyl group [11]. Secondly, the 8-hydroxylated **5** will easily give a stable benzylic carbocation upon H_2O-cleavage which is the driving force in a lot of elimination reactions, explaining the low stability of **5**. On the other hand, the 9-substituted isomer would be much less prone to H_2O-elimination as this would mean the formation of an unstable primary carbocation and should therefore be more stable under the analysis conditions.

To get further information, a urine sample was extracted with ethyl acetate and the organic phase then analysed by GC-MS with liquid injection at 200 °C. Thereby, the possible impact of the thermal desorption step on the formation of **6** was avoided. In this experiment small amounts of **6** were unambiguously detected, but no diol **5** could be observed.

Accordingly, although we cannot clearly prove the formation of **5** in the human metabolism of **1** by its direct detection in the real samples, the elimination product **6** was unambiguously identified. Although we cannot definitely exclude the possibility of formation of **6** from the corresponding regioisomeric 9-hydroxylated derivative we reason that it most probably originates from **5** by H_2O-occurring in the organism.

*3.2.3. Identification of p-cymene-2,3-diol (**4**)*

p-Cymene-2,3-diol (**4**) could not be detected in the initial experiments (20 min extraction time, 60 °C). However, as in the case of the other phase I metabolites it was observed that some are only present in rather small amounts (see Fig. 3) it was decided to carry out one experiment with a longer extraction time of 960 min (60 °C) to enhance the sensitivity and detect even very low concentrations of metabolites. As shown in Fig. 5, this allowed us to prove also the formation of trace quantities of the 2,3-diol **4** in human metabolism of **1** (comparison of its abundance with **2** illustrates why **4** could not be detected in the initial experiment). We also used this experiment to look for further possible phase I metabolites by checking the MS-spectra of the other peaks. However, no other relevant derivative could be identified hereby. It should be pointed out that the longer

extraction time of 960 min leads to severe overloading of other analytes like **1**, **3**, and **6**, which cannot be quantified reliably under these conditions but require the short extraction time of 20 min as described above.

3.3. Comparison between thymol metabolites in humans and animals

Among the six theoretically possible monohydroxylated thymol phase I metabolites, two could be directly detected in these studies (the 2,5-diol **2** and the 2,3-diol **4**), whereas the formation of the 3,8-diol **5** can be rationalized by the presence of the corresponding elimination product **6**. It was clearly shown that hydroxylation of the aromatic system is the dominant process, resulting in the preferred formation of the 2,5-disubstituted products **2** and **3**, whereas the ortho-substituted **4** is only formed in trace amounts.

Comparing these results with the findings obtained in a systematic study of the thymol metabolism of rats by Austgulen et al. [12] some differences become obvious. In that study, the authors carried out a derivatization of phase I metabolites in the urine of rats followed by a GC/MS analysis (without comparison to synthesized reference samples). In analogy to the results obtained in this study, they observed a significant formation of p-cymene-2,5-diol (**2**) [12]. However, they did not detect the 2,3-diol **4** and the 3,8-diol **5** or its elimination product. On the other hand, they were able to detect the p-cymene-3,9-diol (OH-group on the methyl of the iso-propyl group) as the main metabolite together with the p-cymene-3,7-diol (OH-group on the methyl group). Both compounds were not detected in our experiments. Furthermore, the study of Austgulen et al. also detected some subsequent oxidation products of these two metabolites.

4. Conclusion

A novel highly sensitive and reproducible HSSE–TD–GC/MS method for the determination of thymol and its hydroxylated phase I metabolites in human urine was developed. This method allowed the identification of several hitherto not detected metabolites after oral administration of small quantities of thymol to test persons.

It was clearly shown that human metabolism leads to a preferred hydroxylation of the aromatic system of thymol resulting in the formation of p-cymene-2,5-diol and its corresponding oxidized form (p-cymene-2,5-dione) as the main products. It was also possible to detected small quantities of the p-cymene-2,3-diol upon prolonged extraction times. In addition also a functionalization of the iso-propyl side chain was observed. Although we just detected the elimination product p-cymene-3-ol-8-ene, it can be rationalized that this compound might originate from the p-cymene-3,8-diol upon H_2O-cleavage. Furthermore, it was shown that some of the derivatives which were found in rat metabolism could not be detected in human samples and vice versa. Further investigations concerning the detection of subsequent oxidation products are part of future studies.

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