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Distribution of the UV filter 3-benzylidene camphor in rat following topical application

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Abstract

A straightforward analytical method for determination of 3-benzylidene camphor (3-BC) in rat adipose tissue, brain, liver, muscle, plasma and testis following topical application was developed and validated. Three exposure levels (60, 180 and 540 mg kg^{-1} day^{-1}) were tested for 65 days in male Sprague–Dawley rats (24 days postnatal). Sample preparation involving homogenization and *n*-heptane or methanol extraction of the tissue was applied before injection into the LC–ESI–MS–MS system. The response was linear from 2 to 100 μg l^{-1} for the qualifier and the quantifier MRM transitions (R^2 (quantifier) > 0.994). Detection limit of the method corresponded to 0.005 μg g^{-1} tissue and 12.5 μg l^{-1} plasma, respectively. Recovery was determined for all tissues (adipose tissue: 40%; all other tissues: 80–100%) at three individual levels. 3-(4-Methyl benzylidene camphor) (4-MBC) was used throughout the study as internal standard. 3-Benzylidene camphor was detected in all tissues at all exposure levels at concentrations between 0.05 μg g^{-1} (liver) and 36 μg g^{-1} (adipose tissue) and in plasma at 16–89 μg l^{-1}. The method allowed for the quantification of 3-benzylidene camphor in all tested tissues following topical application. Furthermore, it was shown that 3-benzylidene camphor can be found in various tissues in the rat following topical application. These findings may suggest that following use of 3-benzylidene camphor containing sunscreen, similar disposition and distribution may occur in humans.

Keywords: Sunscreen; Estrogenic effect; In vivo; Muscle; Plasma; Testis; Adipose tissue; Brain; Liver; Mass spectrometry

1. Introduction

The lipophilic UV filter 3-benzylidene camphor (3-BC) (Fig. 1) is used as a chemical sun protective agent and in cosmetic products at a maximum use concentration of 2% in the European Union [1]. Estrogenic effect of 3-BC has previously been reported in vivo in a fish assay [2], in an uterotrophic assay in immature rats as well as in MCF-7 cell proliferation in vitro cell assay [3,4]. In vivo developmental toxicity in rats has also been demonstrated for 3-BC [3].

Due to the limited molecular size and the lipophilic nature of 3-BC (MW: 240.15, log K_{ow}: 5.37 [5]), skin penetration can be anticipated. The very closely related compound 3-(4-methylbenzylidene camphor) (4-MBC, MW: 254.17, log K_{ow}: 5.92 [5]) (Fig. 1) was indeed found to be systemically absorbed after a whole-body topical application of human volunteers [6].

To the authors knowledge, no studies on the chemical analysis of 3-BC in non-cosmetic matrices have previously been published. One study utilized 3-BC as an internal standard in the GC–MS analysis of 4-MBC [3], but the method was not described, nor referred to in the article. HPLC and GC coupled to different detectors including photo diode arrays and mass spectrometers have previously been applied to detect and quantify other lipophilic UV filters in non-cosmetic matrices like natural waters [7–9], sewage sludge [10], fish [7], swimming pools [11], human urine [12,13], human breast milk [14] and human skin [13].

Previous studies of 4-MBC in fish, rat adipose tissue and human breast milk [3,7,14] utilized various sample preparation techniques. Fish were homogenized with anhydrous

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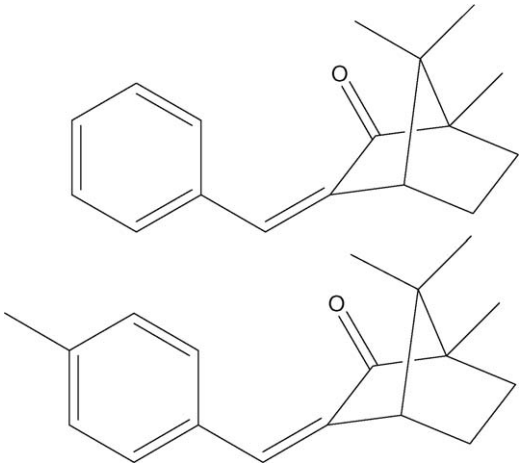

Fig. 1. 3-Benzylidene camphor (3-BC) and 3-(4-methyl-benzylidene camphor) (4-MBC), respectively.

sodium sulphate and sand and subsequently extracted with petrol ether/ethyl acetate (2:1) in a soxhlet apparatus. The extract was further cleaned up using gel permeation chromatography (GPC) and silica gel before injection into a GC–MS system. Homogenization with anhydrous sodium sulphate was also applied for sample preparation of rat adipose tissue. The mixture was eluted with n-hexane and further cleaned up on silica gel before GC–MS analysis. Sample preparation of human breast milk involved homogenization with silica and clean up on a column comprised of anhydrous sodium sulphate and silica. The mixture was eluted with *n*-hexane/ethyl acetate (85:15). Before injection into the GC–MS system, the organic extract was further cleaned up using GPC.

The purpose of the current study was to develop a straightforward analytical method for the quantification of 3-BC in rat adipose tissue, brain, liver, muscle, plasma and testis following topical application of the compound. Sample preparation involving homogenization, removal of water, crude extraction and clean up by centrifugation was applied before LC–ESI–MS–MS analysis. To show the application of the method, tissue from 3-BC exposed rats was prepared and analysed. The toxicological findings of the exposure study will be dealt with elsewhere (Kristensen et al., in preparation).

2. Materials and methods

2.1. Chemicals

3-Benzylidene camphor (3-BC, Unisol S-22, CAS no.: 15087-24-8) was a gift from Lambert Kristensen Aps (Esbjerg, Denmark). 4-Methyl benzylidene camphor (4-MBC, Eusolex® 6300, CAS no.: 36861-47-9) was obtained from VWR International Eurolab S.L. (Malaga, Spain). All other chemicals used were of analytical grade.

2.2. Animals

A total of 32 Sprague–Dawley male rats (Taconic-SD, Taconic Europe, Lille Skensved, Denmark) were divided randomly into four groups with eight animals in each. The animals were kept in cages (Makrolon type IV, Tecniplast, Buguggiate, Va, Italy) at $22 \pm 3\,^{\circ}C$ in groups of four with a 12 h light/12 h dark cycle. Access to food (Altromin 1324, Brogaarden, Gentofte, Denmark) and water was ad libitum. Furthermore, paper strips (Enviro-Dri, PharmaServ, Framingham, MA, USA) and chew blocks were available in the group cages. Animals were 24 days postnatal at the onset of the exposure weighing 63 ± 3 g.

2.3. Solutions

Stock solutions of 3-BC and 4-MBC were prepared in methanol at a concentration of $1000\,mg\,l^{-1}$ and kept at $-18\,^{\circ}C$ until use. A $50\,\mu g\,l^{-1}$ sample of both compounds was stabile in 75% methanol kept in a glass vial at $20\,^{\circ}C$ for more than 30 days (data not shown).

2.4. Study design

Three groups of animals ($n = 8$) were topically exposed to 3-BC at 60, 180 and $540\,mg\,kg^{-1}\,day^{-1}$, respectively for 65 successive days, followed by euthanasia (CO_2 chamber) and sampling (all samples were kept at $-80\,^{\circ}C$ until analyses). A fourth group was topically exposed to the vehicle alone (propylene glycol/isopropanol (80/20, w/w)) (control).

For application, 3-BC was dissolved in the vehicle at A = 2.4% and B = 7.1% (w/v) corresponding to $2.50\,\mu l\,A\,g^{-1}\,day^{-1}$, $2.53\,\mu l\,B\,g^{-1}\,day^{-1}$ and $7.59\,\mu l\,B\,g^{-1}\,day^{-1}$ for 60, 180 and 540 mg 3-BC kg^{-1} day^{-1}, respectively. After exposure (once daily on the shaved back), the animals were fitted with collars to avoid oral uptake of the applied test substance and isolated for up to 6 h in single cages (Makrolon type III).

2.5. Sample preparation

2.5.1. Adipose tissue

0.5 g adipose tissue and 2.0 g anhydrous sodium sulphate was homogenized in a mortar. The mixture was transferred to a 5 ml Merck LiChrolut® glass column (Merck KGaA, Darmstadt, Germany) stopped with quartz wool (Mikro Kemi AB, Uppsala, Sweden). The column was placed on an IST VacMaster (Mikrolab Aarhus A/S, Højbjerg, Denmark). The internal standard (4-MBC) was added and the mixture was eluted using 10 ml of methanol with the use of vacuum. Subsequently, the solvent was evaporated at $60\,^{\circ}C$ under a gentle nitrogen stream and the analytes were reconstituted in 500 μl 75% (v/v) methanol. Following centrifugation for 5 min at $8000 \times g$ in a Sigma 1-13 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), the supernatant was carefully aspirated using a 23 gauge needle. Following appropriate dilution, 10 μl was injected into the LC–ESI–MS–MS system.

2.5.2. Brain, liver, muscle and testis

0.5 g tissue and 2.0 g anhydrous sodium sulphate (2.5 g for the liver samples) was homogenised in a mortar. The mixture was transferred to a 5 ml Merck LiChrolut® glass column and placed on an IST VacMaster. The internal standard (4-MBC)

was added and the mixture was eluted using 10 ml of *n*-heptane with the use of vacuum. Subsequently, the solvent was evaporated at 45 °C under a gentle nitrogen stream and the analytes were reconstituted in 500 μl 75% (v/v) methanol. Following centrifugation for 5 min at 8000 × *g* in a Sigma 1–13 centrifuge, the supernatant was carefully aspirated using a 23 gauge needle. Following appropriate dilution, 10 μl of the supernatant was injected into the LC–ESI–MS–MS system.

2.5.3. Plasma

Immediately after sacrifice of the animal, blood was sampled from the heart using a syringe and a heparinized needle. The blood was centrifuged for 10 min at 2880 × *g* at 4 °C in a heparinized test tube. Plasma was aspirated and kept at −80 °C in 0.5 ml Eppendorff tubes until further preparation and analyses. Before analyses, the plasma tubes were allowed to thaw at room temperature and 200 μl was transferred to a new Eppendorff tube. Five hundred microliters of *n*-heptane including the internal standard (4-MBC in *n*-heptane) was added to the plasma and vortexed for 20 s. Following extraction, the organic phase was carefully aspirated using a 23 gauge needle. The solvent was evaporated at 45 °C under a gentle nitrogen stream and the analytes were reconstituted in 500 μl 75% (v/v) methanol. Following appropriate dilution, 10 μl was injected into the LC–ESI–MS–MS system.

2.6. LC–ESI–MS–MS

LC–ESI–MS–MS analyses were performed on an Agilent 1100 series HPLC (Agilent Technologies, Inc., Palo Alto, CA, USA) coupled to a PE Sciex 2000 triple quadropole mass spectrometer equipped with a turbo ion spray. Data were collected using Analyst® 1.4 software (MDS Sciex, Concord, Ont., Canada).

The analytical column was a Phenomenex® Synergi™ 4 μ Max-RP 80A, 150 × 2.0 mm (Phenomenex, Torrance, CA, USA) with a Phenomenex® Securityguard™ Max-RP 4 × 2.0 mm guard cartridge. Flow rate was 0.2 ml min^{-1} and column temperature was controlled at 45 °C. A methanol gradient was used for elution of the two compounds within 7 min. Initially, isocratic elution with 83.3% methanol was used for the first min. Hereafter, the methanol concentration was linearly increased to 86.9% in 3 min, further increased linearly to 92.3% in 0.5 min, then brought back to 83.3% in 1 min and kept at this concentration for the remaining 1.5 min. The eluents were buffered with 0.1% (v/v) formic acid. The effluent from the HPLC was directed to waste for the first 3 min of the analysis time. MS–MS detection was performed in the positive ion mode using multiple reaction monitoring (MRM). $[M+H]^+$ ions were used for monitoring in Q1. In Q3, the camphor moiety was used for both compounds as qualifying *m/z* value and the benzyl and the 4-methyl benzyl moieties as quantifying *m/z* values for 3-BC and 4-MBC, respectively (see Fig. 2 for 3-BC). According to the European Communities Council Directive 96/23/ECU [15], four identification points are required when analysing official samples using LC–MS–MS. In the current study, four identification points were obtained by using one precursor and two daughter ions for 3-BC in the analyses. The MRM transition resulting in the most intense signal was used for quantification and the less intense signal for qualification, respectively.

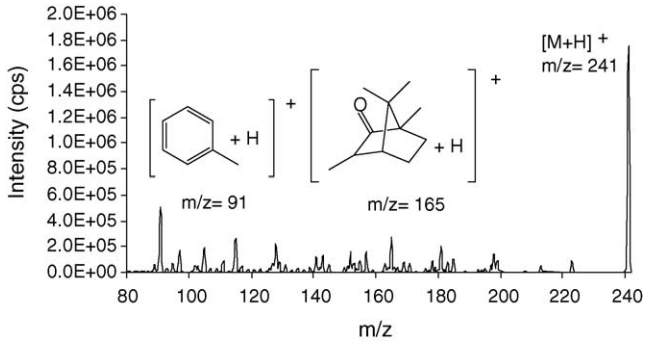

Fig. 2. 3-BC fragments used for the MS–MS detection.

MRM transition and compound dependent parameters (declustering potential (DP), focusing potential (FP), entrance potential (EP) and cell exit potential (CXP), all in V) were optimised using the automated infusion analysis program: DP: 16.5; FP: 390; EP: 6 and CXP: 2. Collision energies were 54, 60, 46 and 66 V, respectively, corresponding to the MRM transitions 241.2–91.1 (3-BC quantifier), 241.2–165.1 (3-BC qualifier), 255.2–105.1 (4-MBC quantifier) and 255.2–165.1 (4-MBC qualifier), respectively. Gas parameters (curtain gas (CUR) in l min^{-1}, collision gas (CAD) in l min^{-1}, temperature, (TEM) in °C, needle voltage (IS) in V, gas 1 (GS1) and gas 2 (GS2) in l min^{-1}) were optimised using the automated flow injection analysis program. Nitrogen was used for all gas purposes. Gas parameters were: CUR: 35; CAD: 9; TEM: 500; IS: 5500; GS1: 10; GS2: 40, ihe: on.

2.7. Validation

Two calibration curves corresponding to the quantifier and the qualifier MRM transitions of 3-BC, respectively, were established using concentrations of 2, 5, 10, 20, 40, 60, 80 and 100 μg l^{-1} in 75% (v/v) methanol. All calibration standards were analysed in replicates of six. The calibration curves were linear with R^2-values above 0.994. Furthermore, the 95% confidence intervals of the intercepts with the *y*-axis included zero for both curves. 4-MBC at a concentration of 50 μg l^{-1} was included as quality control in all sample sets to ensure a constant response of the mass spectrometer during the analyses (inter day variation: 3.3%, $n = 60$ injections; intra day variation: 4.4%, $n = 14$ days). The equation corresponding to the calibration curve of the 3-BC quantifier MRM transition was: $y = 982x - 556$. The limit of quantification (LOQ) was defined as a signal to noise ratio (S/N) of 10 and estimated using the build-in function in Analyst® 1.4. LOQ was estimated to 5 μg l^{-1} (0.005 μg g^{-1} sample or 12.5 μg l^{-1} plasma). A Savitzky-Golay smoothed (10 points) chromatogram of a 5 μg l^{-1} 3-BC calibration standard with quantifier and qualifier MRM transitions is given in Fig. 3.

Recovery was determined at three levels in all tissues (adipose tissue, brain, liver, muscle, plasma and testis). Results are given in Table 1.

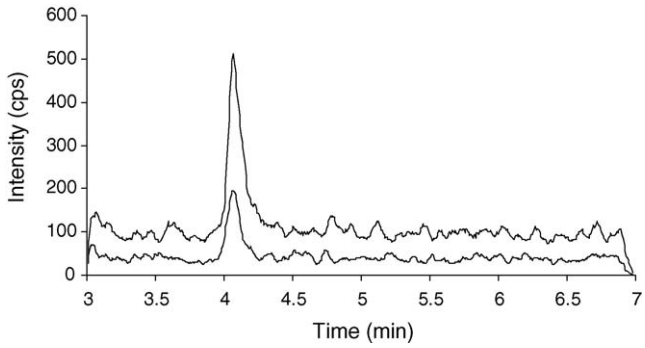

Fig. 3. 3-BC, 5 μg l^{-1} calibration sample. Peeks correspond to quantifier and qualifier MRM transitions, respectively.

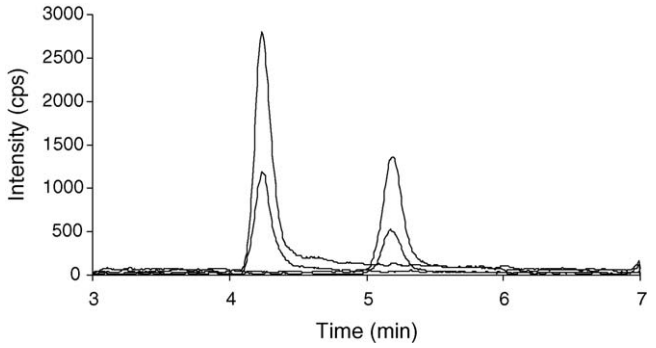

Fig. 4. Brain extract, exposure level 1 (60 mg 3-BC kg^{-1} day^{-1}) diluted five times. 3-BC quantifier and qualifier MRM transitions, respectively, elute at retention time 4.3 min. 4-MBC quantifier and qualifier MRM transitions, respectively, elute at retention time 5.2 min.

Table 1
Recovery (%) of 3-BC at three levels

Tissue	Level 1	Level 2	Level 3
Adipose tissue	39.8 ± 12.7	39.2 ± 13.4	41.8 ± 13.3
Brain	106.7 ± 7.6	95.2 ± 7.9	78.0 ± 7.8
Liver	101.2 ± 12.6	90.8 ± 9.8	96.8 ± 15.2
Muscle	98.6 ± 8.0	93.8 ± 10.1	84.2 ± 9.9
Plasma	96.4 ± 5.1	86.6 ± 15.4	89.3 ± 8.3
Testis	93.7 ± 14.1	96.1 ± 7.9	87.4 ± 10.9

Blank tissue samples from eight animals were pooled and recovery at each level was determined in triplicate. Levels 1, 2 and 3, respectively (in μg g^{-1} or μg l^{-1} (plasma)): adipose tissue: 20, 200, 1000; brain, liver and testis: 0.02, 0.2, 2; muscle: 0.1, 1, 10; plasma: 20, 200, 2000.

3. Results and discussion

Based on the lipophilic nature of 3-BC (calculated log K_{ow} value of 5.37 [5]), it was expected that the compound would be found in highest concentrations in adipose tissue. This was indeed true with concentrations in the tissue reaching 36 μg g^{-1} after exposure to 180 mg 3-BC kg^{-1} day^{-1} applied topically for 65 days (Table 2). The concentration of 3-BC in adipose tissue did not increase with increasing topical exposure (31 μg g^{-1} corresponding to 540 mg 3-BC kg^{-1} day^{-1}). This is most likely explained by saturation of the tissue reaching a dynamic equilibrium with the systemic circulation. Another possible explanation includes an upper limit of topical permeation of 3-BC. If this was the case though, similar saturation patterns would have been expected with the other tissues analysed. This was not observed.

The high concentration in plasma (89 μg l^{-1} after exposure to 540 mg 3-BC kg^{-1} day^{-1} applied topically for 65 days; Table 2) indicates that the compound is systemically available.

As expected, the concentration of 3-BC in muscle was much lower than in adipose tissue (Table 2).

3-BC was also found in brain (Fig. 4) and in testis at all exposure levels. The rat blood–brain barrier (BBB) is formed around postnatal days 14–24 [16–18]. Thus, the BBB should be fully developed in the animals of the current study. The limited molecular size, the lack of functional groups and the high log K_{ow} value of 3-BC may explain the ability of the compound to bypass the BBB and thus the findings of the chemical in brain tissue.

The limited amounts of 3-BC found in liver samples could possibly be explained by metabolism. Metabolic processes may have altered the molecular structure of 3-BC resulting in 3-BC metabolites, which are not recognised by the *m/z* detection in the mass spectrometer.

The recovery of 3-BC from adipose tissue was expected to be limited due to the choice of extraction solvent (methanol), which was not able to extract all of the analyte from the matrix. On the other hand, only a limited amount of interfering lipids was extracted using methanol. During method development, *n*-heptane was tested for extraction of adipose tissue (data not shown). *n*-Heptane completely dissolved the adipose tissue, which complicated the sample preparation compared to the methanol extraction. Recovery values resulting from *n*-heptane extraction were almost identical compared to those obtained with methanol. However, LOD was at the same time increased by a factor of ten due to dilution of the sample. Dilution was necessary in order to remove interfering lipids before injection into the LC–ESI–MS–MS system. Thus, methanol was chosen as the extraction solvent for adipose tissue. The recovery could

Table 2
Concentration (μg g^{-1}) of 3-BC in rat tissues

Tissue	Control (n = 3)	60 mg kg^{-1} day^{-1}	180 mg kg^{-1} day^{-1}	540 mg kg^{-1} day^{-1}
Adipose tissue	<LOQ	18.6 ± 3.6	36.4 ± 8.8	30.7 ± 3.2
Brain	<LOQ	0.13 ± 0.03	0.35 ± 0.07	1.2 ± 0.6
Liver	<LOQ	0.05 ± 0.02	0.20 ± 0.05	0.44 ± 0.09
Muscle	<LOQ	0.18 ± 0.06	1.0 ± 0.6	1.3 ± 0.7
Plasma (μg l^{-1})	<LOQ	15.5 ± 3.6	51.2 ± 13.7	88.9 ± 14.0
Testis	<LOQ	0.13 ± 0.03	0.34 ± 0.12	0.62 ± 0.33

n = 8.

definitely be increased using *n*-heptane as extraction solvent followed by clean up using GPC or similar. However, the vast amounts of 3-BC present in our samples (some methanol extracts were diluted 1000 times before analyses) made the time consuming clean up step redundant. For future studies, *n*-heptane extraction of adipose tissue and clean up by GPC could be considered if higher recovery values are needed.

The lowest exposure level (60 mg 3-BC kg^{-1} day^{-1}) is comparable to the amount of 3-BC applied by three whole-body applications of sunscreen (2 mg sunscreen cm^{-2}, 2% 3-BC, w/w). As summarized by the European Commission [19], rat skin is typically two to ten times more permeable than human skin but the permeability also occasionally resembles that of human skin. A mixture of propylene glycol/isopropanol (80/20, w/w) was used to dissolve 3-BC before application. This mixture facilitates the penetration into the skin of 3-BC compared to conventional semi-liquid topical dosage forms. Thus, the systemically absorbed amount of 3-BC resulting from the lowest exposure level in the current study is expected to be higher than the corresponding amount in humans.

Nevertheless, at the lowest exposure level, 3-BC was found in all analysed tissues at concentrations above LOD indicating that 3-BC might also be found in human tissue after prolonged exposure to commercial 3-BC containing sunscreen.

4. Conclusion

A straightforward and sensitive method involving LC–ESI–MS–MS for quantification of the lipophilic, xenoestrogenic UV filter 3-BC in rat adipose tissue, brain, liver, muscle, plasma and testis was developed and validated.

The method was applied to tissue samples from rats being topically exposed to 3-BC for 65 days. 3-BC was found in all tissues in concentrations ranging from 0.05 $\mu g\, g^{-1}$ (liver) to 36 $\mu g\, g^{-1}$ (adipose tissue) and 89 $\mu g\, l^{-1}$ in plasma.

For providing data related to the human risk assessment of UV filters in sunscreens, this method could be a useful tool.

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