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Determination of 8-methoxypsoralen in human plasma, and microdialysates using liquid chromatography–tandem mass spectrometry

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Abstract

The validation of a LC/MS/MS method for the determination of 8-methoxypsoralen (8-MOP) in human plasma and microdialysates after topical application is described. Plasma samples were extracted by liquid–liquid extraction with diisopropylether using 4,5′,8-trimethylpsoralen (TMP) as internal standard. Chromatographic separation of plasma sample extracts was carried out using a short narrow-bore Nucleosil C18 column (30 mm × 2.0 mm i.d.) with acetonitrile/(2 mM ammonium acetate buffer, 2 mM acetic acid) (80:20, v/v). For mass spectrometric analysis an API 3000 triple quadrupole mass spectrometer was employed. The mass transitions used were m/z 217.2 → 174.0 for 8-MOP and m/z 229.1 → 142.1 for TMP. Microdialysis samples diluted with an equal amount of acetonitrile did not require any extraction and were analyzed directly on a narrow-bore Nucleosil C18 column (70 mm × 2.0 mm i.d.) with acetonitrile/(2 mM ammonium acetate buffer, 2 mM acetic acid) (50:50, v/v) with the mass transition m/z 217.2 → 174.0. The assays were validated over the concentration ranges of 0.5–50 ng/ml for plasma samples and 0.25–50 ng/ml for microdialysates, respectively.
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Keyword: 8-Methoxypsoralen

1. Introduction

In the treatment of dermatoses, the combination of psoralens and UV radiation (PUVA therapy) is a widely used strategy [1]. Oral administration of 8-methoxypsoralen (8-MOP) is easy to accomplish and inexpensive. Since plasma concentrations reach the maximum after 2 h UV radiation is performed at this time [2]. It has been reported, however, that plasma peak concentrations and the time they are reached vary highly between individuals [3–5]. Moreover, oral PUVA therapy is associated with a variety of potential side effects caused by the systemic administration of the drug [6–8]. These side effects may limit the long-term use of oral PUVA therapy for some patients.

Topical PUVA therapy by bath or cream is an alternative treatment which is as effective as oral PUVA therapy [9–12]. The skin concentrations might be even higher and less side effects are seen, because the systemic availability of topical applied 8-MOP is limited [4,13].

Optimal therapeutic response is achieved by UVA radiation at the maximum tissue concentration of 8-MOP. Since an exact knowledge of the tissue concentration-time course of 8-MOP would help to optimize the therapeutic regimens, we used microdialysis to assess skin concentrations of 8-MOP after oral, bath and cream application of the drug [14]. Microdialysis allows a dynamic and direct assessment of drug concentrations in the extracellular water space [15] and has been extensively used in the investigation of dermal

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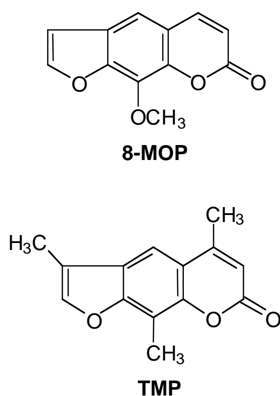

Fig. 1. Chemical structures of 8-methoxypsoralen (8-MOP) and 4,5′,8-trimethylpsoralen (TMP).

drug penetration [16–18], as well as skin physiology and pathology [19,20].

Several methods for the determination of 8-methoxypsoralen are described in the literature. A HPLC assay using UV detection and 4,5′,8-trimethylpsoralen (TMP) as internal standard with a limit of quantification of 10 ng/ml is described [21]. Other assays have used TMP and 5-methoxypsoralen as internal standard with comparable sensitivity [22–24]. Another assay with increased sensitivity (limit of quantification of 1.0 ng/ml) is based on gas chromatography–mass spectrometry with TMP as internal standard [25].

In this paper, the validation of a LC/MS/MS assay to determine 8-MOP with TMP (Fig. 1) as internal standard in human plasma and in microdialysates is described. Since plasma concentrations of 8-MOP were expected to be very low following topical application, samples need to be concentrated by a factor of 10–100 if using a method with UV detection. Microdialysis samples typically have a small volume of about 50 μl. LC/MS/MS is the method of choice to cope with these challenges. It delivers an improved sensitivity as compared to previously published methods and employs only small sample volumes. Additionally, short retention times are possible.

2. Experimental

2.1. Materials

8-Methoxypsoralen (purity 99%) and the internal standard 4,5′,8-trimethylpsoralen (purity 98%) were obtained from Sigma–Aldrich (Steinheim, Germany). Acetonitrile gradient grade was obtained from Merck KgaA (Darmstadt, Germany). The water used had HPLC grade and was obtained from Mallinckrodt Baker (Griesheim, Germany). Potassium dihydrogen phosphate and acetic acid were purchased from Riedel-de-Haen (Seelze, Germany) and ammonium acetate from Fluka (Seelze, Germany). Saline (0.9%) was obtained from Braun (Melsungen, Germany). Control human plasma was a gift from the Blutspendedienst Hessen des Deutschen Roten Kreuzes (Frankfurt am Main, Germany).

2.2. Instrumentation

The LC/MS/MS system consisted of a Jasco LC unit (Gross-Umstadt, Germany) equipped with a DG 1580-53 degasser, a LG-1580-02 ternary gradient unit, a PU-1585 pump and an AS 1550 autosampler. The mass spectrometer consisted of an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) and a turbo ion spray interface. The set was equipped with a Whatman nitrogen generator (Goettingen, Germany), which delivered nitrogen with a purity >97%.

2.3. LC/MS/MS conditions for plasma samples

Chromatographic separation of extracted plasma samples was performed in isocratic mode with a Nucleosil C18 column (30 mm × 2.0 mm i.d., 5 μm particle size and 100 Å pore size, Macherey-Nagel, Dueren, Germany). The mobile phase consisted of acetonitrile/(2 mM ammonium acetate, 2 mM acetic acid) (80:20, v/v). The flow rate was set at 0.2 ml/min. The injection volume was 10 μl and the runtime was set at 3 min. 8-MOP and TMP were eluted at 0.7 ($k' = 0.5$) and 0.9 ($k' = 0.7$) min, respectively. The turbo ion spray interface operated in the positive ion mode at 450 °C and 4800 V and was supplied by an auxiliary gas flow at 5.0 l/min. The nebulizer gas was set at 1.23 l/min (setting 10) and the curtain gas flow was set at 1.08 l/min (setting 9), the collision gas at 3×10^{-5} hPa (setting 4).

Quantification was performed in MRM mode of the protonated precursor ion and the related product ion using an internal standard method with peak area ratios and $1/x$ weighting. The mass transitions used for quantification were m/z 217.2 → 174.0 (collision energy 35 eV, dwell time 400 ms) and m/z 229.1 → 142.1 (collision energy 35 eV, dwell time 400 ms) for 8-MOP and TMP, respectively. The quadrupoles Q1 and Q3 were set at unit resolution. The analytical data output was processed by Analyst software (version 1.1).

2.4. LC/MS/MS conditions for microdialysates

Chromatographic separation of microdialysis samples was performed in isocratic mode with a Nucleosil C18 column (70 mm × 2.0 mm i.d., 5 μm particle size and 100 Å pore size, Macherey-Nagel). The mobile phase consisted of acetonitrile/(2 mM ammonium acetate, 2 mM acetic acid) (50:50, v/v). The flow rate was set at 0.12 ml/min. The injection volume was 10 μl and the runtime was 9 min. 8-MOP was eluted at 4.8 min ($k' = 1.4$).

The turbo ion spray interface operated in the positive ion mode at 450 °C and 4900 V and was supplied by an auxiliary gas flow at 5.5 l/min. The nebulizer gas was set at 1.31 l/min (setting 11), the curtain gas flow was set at 1.25 l/min

(setting 10) and the collision gas at 3.75×10^{-5} hPa. Quantification was performed as described earlier without the internal standard TMP.

2.5. Plasma extraction procedure

The liquid–liquid extraction method previously described [21] was used in a slightly modified way. Plasma samples were vortexed prior to extraction. Twenty microliters TMP (500 ng/ml) were pipetted into a 6 ml polypropylene tube and a sample aliquot of 500 μl plasma and 1 ml phosphate buffer (0.02 M, pH 12) were added and cautiously mixed. After administration of 3 ml diisopropylether the samples were shaken for 10 min and centrifuged for 15 min at 10 °C and 3220 × *g*. An amount equal to 2.5 ml of the upper organic layer were transferred in conical glass tubes. The solvent was evaporated at 50 °C under a gentle stream of nitrogen and the residue reconstituted with 400 μl acetonitrile/(2 mM ammonium acetate, 2 mM acetic acid) (80:20, v/v) and added to micro reaction tubes prior to injection into the analytical system.

2.6. Stability of the solutions

8-MOP and TMP are light sensitive. The stock solutions were stable in acetonitrile stored light protected at 4 °C for at least 6 months. Standards were prepared in brown polypropylene micro reaction vials (Brand, Wertheim, Germany).

Addition of acetonitrile reduced light sensitivity (e.g. in acetonitrile/NaCl 0.9% (50:50, v/v) no degradation of 8-MOP was found after 48 h under daylight conditions) as compared to samples without acetonitrile (e.g. in water degradation was found after 12 h under daylight conditions). Therefore, before sampling the equal volume of acetonitrile of expected volume of microdialysates was added to the sample vials.

2.7. Stock solutions and plasma standards

The stock solutions of 8-MOP and TMP had a concentration of 1 mg/ml. They were prepared by weighing 10 mg of reference material into a volumetric flask and diluting to volume with acetonitrile. From these stock solutions (1 mg/ml in acetonitrile), working standards were prepared freshly by diluting with acetonitrile/water (50:50, v/v) to 100, 10 and 1 μg/ml. From this working standards, calibration standards were prepared freshly by diluting subsequently with drug-free control plasma in the range of 0.1–100 ng/ml (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0, and 100.0 ng/ml).

2.8. Standards for microdialysis samples

Standards for microdialysis samples were prepared from the stock solutions mentioned earlier. Working standards of 100, 10 and 1 μg/ml were prepared freshly in acetonitrile/water (50:50, v/v). Standards were prepared freshly in acetonitrile/NaCl 0.9% (50:50, v/v) by diluting subsequently in the concentration range of 0.1–100 ng/ml (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0, and 100.0 ng/ml).

2.9. Determination of the capacity factor k'

Ions such as Na^+ or Cl^- are not expected to have any retention on reversed phase columns. Saline, however, suppresses the baseline as shown in the chromatograms in Fig. 2. It produces a negative peak. The minimum of this peak was set as the hold-up time t_0. The hold-up time (t_0) for the determination of the capacity factor was determined by injecting NaCl 0.01%. Using a concentration of 0.01% results in less suppression and better accuracy. Six injections with NaCl 0.01% were made to determine t_0.

The capacity factor k' is a measure of the degree to which a component is retained by the column relative to an unretained component. When t_R is the elution time of the retained component and t_0 the hold-up time of an unretained sample, the capacity factor is calculated according to $k' = (t_R - t_0)/t_0$.

2.10. Application of the method

The microdialysis technique has been reviewed elsewhere [18]. In brief, microdialysis catheters were inserted

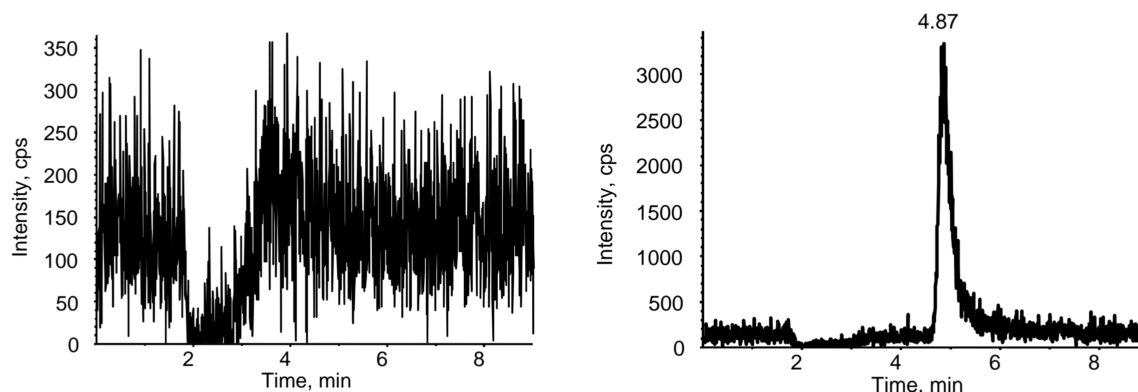

Fig. 2. Chromatograms of a blank dialysate and a microdialysate sample (each diluted with an equal volume acetonitrile).

underneath the skin of the dorsal forearm. The site of skin puncture was tightly covered with a waterproof plaster. In addition, the direct area surrounding the puncture site was excluded from cream or bath. The microdialysis catheters were perfused with sterile water at a flow rate of 2 μl/min using a microdialysis pump (CMA 102, CMA Microdialysis AB, Solna, Sweden). Dialysates were collected in 20 min intervals up to 4 h and in 30 min intervals from 4 to 8 h. The outlet tube of the catheter was clipped at 12 cm to reduce the dead space. Blood samples were drawn at the end of each dialysate sampling interval.

The subjects received three different formulations of 8-methoxypsoralen: (1) 8-methoxypsoralen tablets (10 mg Meladinine tablets; Galderma, Freiburg, Germany; ingredients: methoxsalen, lactose, cornstarch, gelatin, and magnesiumstearate); (2) 8-methoxypsoralen bath (Meladinine solution concentrate in 95% ethanol; Galderma, Freiburg, Germany); and (3) 8-methoxypsoralen cream. The cream (8-methoxypsoralen in Dorithin cream; Asta Medica, Vienna, Austria) was produced as described previously [26].

Five subjects received 1 mg/kg 8-MOP orally.

Five subjects were treated by bath. For bath application (8-MOP concentration 3 mg/l), the right or left arm (forearm and upper arm without wrist and hand) was submerged for 30 min into the 8-methoxypsoralen bath (0.3% 8-methoxypsoralen solution in 9 l water), which was kept at 36–38 °C and covered with aluminum foil.

Five subjects were treated with cream. The 8-methoxypsoralen cream (0.1% 8-MOP) was applied onto the skin of the right or left arm (forearm and upper arm without wrist and hand) for 30 min. The amount of cream per square centimeter approximated the amount used in patients (50–60 mg/cm^2). During the application time, the arm of the patient was wrapped in transparent foil and aluminum foil according to the standard clinical procedure. At the end of the application time, the remaining cream was gently removed with paper cloths without rubbing of the skin [14].

3. Results

3.1. Development of the LC/MS/MS method

The aim of the development was to find a sufficiently sensitive procedure and run times as short as possible because of the high selectivity of the MS/MS technology no proper chromatographic separation was necessary.

Infusion of 500 ng/ml 8-MOP and TMP in acetonitrile/water (70:30, v/v) at 10 μl/min in the positive ion mode resulted in the fragment spectra as shown in Fig. 3. The respective settings of the mass spectrometer were set by the automatic tuning tool of the Analyst software. The two most abundant fragments of each substance were taken for quantification and as qualifier, respectively. The ion spray parameters such as nebulizer gas, curtain gas, ion spray voltage and collision gas as well as the declustering and focussing potential were optimized by flow injection analysis (FIA) for plasma samples using a solution of 10 ng/ml of 8-MOP in acetonitrile/2 mM ammonium acetate buffer with a flow rate of 0.2 ml/min.

Moreover, the composition of the mobile phase was tested for sensitivity. For plasma sample extracts a mobile phase consisting of acetonitrile/(2 mM ammonium acetate, 2 mM acetic acid) (80:20, v/v) resulted in best sensitivity.

Chromatographic separation is not necessary for plasma samples in contrast to microdialysis samples, which contained a reasonable amount of salts. Salts can completely suppress the analyte signal by the formation of clusters. Therefore, a chromatographic separation was accomplished by using a longer column and a mobile phase which consisted of less organic solvent (acetonitrile/(2 mM ammonium acetate buffer, 2 mM acetic acid) (50:50, v/v)). The ion spray settings were optimized for this mobile phase by FIA.

3.2. Matrix effects

Extracted components of the plasma can cause suppression or enhancement of the signal of the analyte. To assess

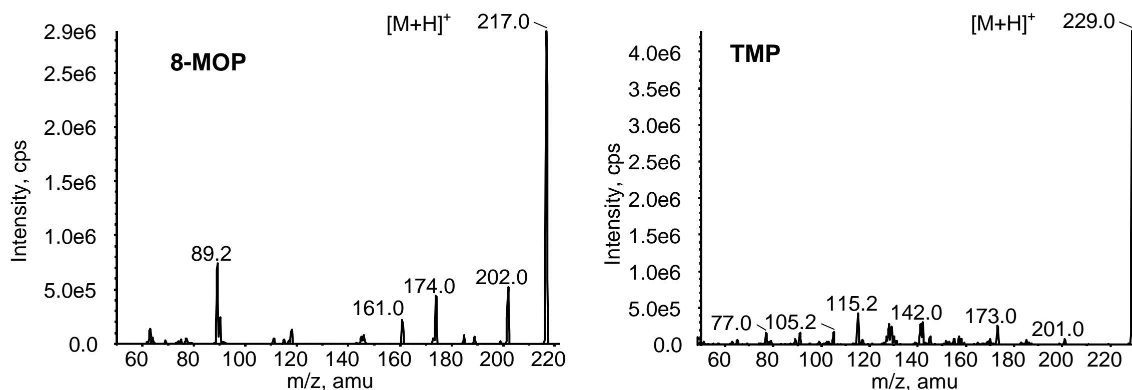

Fig. 3. Product ion scans of 8-MOP and TMP in the positive ion mode.

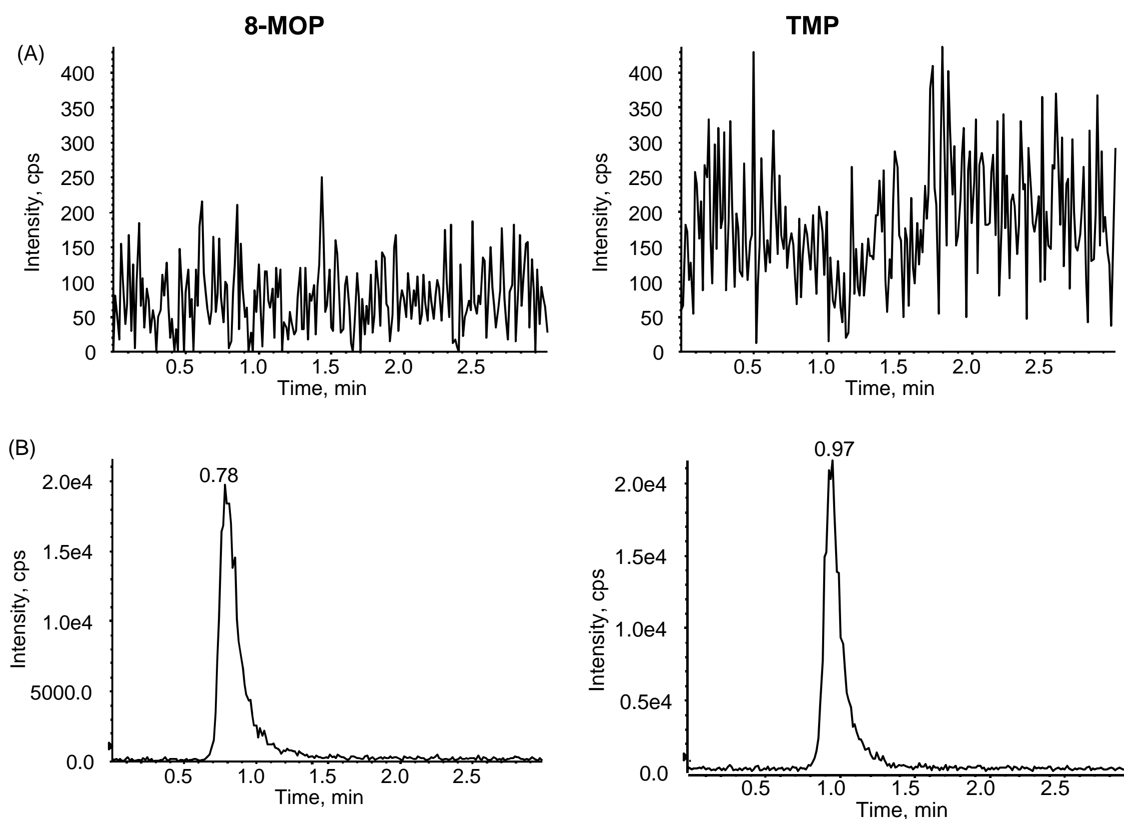

Fig. 4. Chromatograms of 8-MOP and TMP of extracted plasma blanks (A) and of an extracted plasma sample containing 10 ng/ml 8-MOP and 50 ng/ml TMP (B).

these matrix effects, blank plasma samples from six different individuals were extracted. Before evaporating, the organic solvent was spiked with 400 μl of a 10 ng/ml 8-MOP and 50 ng/ml TMP solution in acetonitrile (Fig. 4). The organic solvent was evaporated and reconstituted in 400 μl mobile phase. Reference standards were prepared in the same fashion to make the standards comparable to the samples: 400 μl of a 10 ng/ml 8-MOP and 50 ng/ml TMP solution in acetonitrile were diluted in 2.5 ml diisopropylether, evaporated and reconstituted in mobile phase. The samples and the standards were measured as described. The peak areas of the spiked samples and the standards were compared to determine matrix effects. No significant differences between the plasma samples of the different individuals were detected. A signal suppression by plasma matrix of 35% was detected for 8-MOP and of 45% for TMP.

Microdialysis samples contain salts and small endogenous compounds. NaCl 0.9% is comparable to in vivo microdialysates and was used as a substitute to validate the method. To determine the influence of NaCl 0.9% on the signal intensity of 8-MOP the peak areas of samples in acetonitrile/NaCl 0.9% (50:50, v/v) containing 10 ng/ml of 8-MOP were compared to corresponding standards in mobile phase. The suppression due to NaCl 0.9% was by 73%.

3.3. Assay validation of plasma samples

Seven concentrations were used to assess the inter-day variability in six independent series of spiked plasma samples. The resulting assay precision and accuracy data are presented in Table 1. The intra-day precision was assessed using quality control samples with concentrations representing the range of calibration (1, 25, and 100 ng/ml; $n = 6$). The intra-day precision was determined measuring the coefficient of variation (C.V.) and was found to be 10, 3 and 4% for the respective concentrations. Assay accuracy was found to be within 10% of nominal values in every case.

Table 1
Precision and accuracy for quantification of 8-MOP with TMP as internal standard in human plasma

Nominal concentration (ng/ml)	Mean analyzed concentration ± S.D. (ng/ml; $n = 6$)	Coefficient of variation (%)	Accuracy (%)
0.5	0.51 ± 0.06	11.9	103.0
1.0	0.95 ± 0.05	5.0	95.1
2.5	2.5 ± 0.2	6.8	100.3
5.0	5.0 ± 0.2	4.2	99.3
10.0	10.0 ± 0.6	6.4	100.2
25.0	25.3 ± 1.1	4.5	100.7
50.0	51.2 ± 1.7	3.3	102.5

The limit of quantification was defined as the lowest concentration with a C.V. of ≤15% and an accuracy between 85 and 115% of nominal concentrations. The limit of quantification was 0.5 ng/ml. The limit of detection was 0.25 ng/ml.

The extraction recovery of plasma samples was tested at three different concentrations representing the standard curve (1, 10, and 50 ng/ml; $n = 6$). The recovery of TMP was determined at its working concentration of 50 ng/ml. Recoveries were calculated by comparing the absolute peak areas of the extracted samples with extracted blank plasma samples spiked with the corresponding amount of analyte. Standards were diluted in diisopropylether, evaporated and reconstituted in the same way as extracted samples to make them equal. The mean recovery for 8-MOP was 90% (S.D. = 5%) independent of the concentrations tested and for TMP 85% (S.D. = 3%).

3.4. Assay validation for microdialysis samples

The inter-day variability was assessed using seven concentrations in six independent series of spiked acetonitrile/NaCl 0.9% (50:50, v/v). The resulting assay precision and accuracy data are presented in Table 2. The intra-day precision was assessed using quality control samples with concentrations representing the range of calibration (1, 10, and 50 ng/ml; $n = 6$). The intra-day precision was determined measuring the coefficient of variation and was found to be 10, 5 and 5% for the respective concentrations. Assay accuracy was found to be within 11% of nominal values in every case.

Table 2
Precision and accuracy for quantification of 8-MOP in human microdialysates

Nominal concentration (ng/ml)	Mean analyzed concentration ± S.D. (ng/ml; $n = 6$)	Coefficient of variation (%)	Accuracy (%)
0.25	0.27 ± 0.03	13.3	106.7
0.5	0.49 ± 0.03	7.4	97.8
1.0	1.0 ± 0.1	11.2	97.2
2.5	2.4 ± 0.3	14.2	95.2
5.0	4.7 ± 0.3	7.4	93.4
10.0	9.9 ± 0.5	5.2	98.6
25.0	25.2 ± 0.6	2.3	100.9
50.0	51.2 ± 0.5	0.3	104.0

The limit of quantification was defined as the lowest concentration with a C.V. of ≤15% and an accuracy between 85 and 115% of nominal concentrations. The limit of quantification was 0.25 ng/ml. The limit of detection was 0.1 ng/ml.

3.5. Application of the assay

The resulting concentration versus time profiles of microdialysates and plasma samples are presented in Figs. 5 and 6,

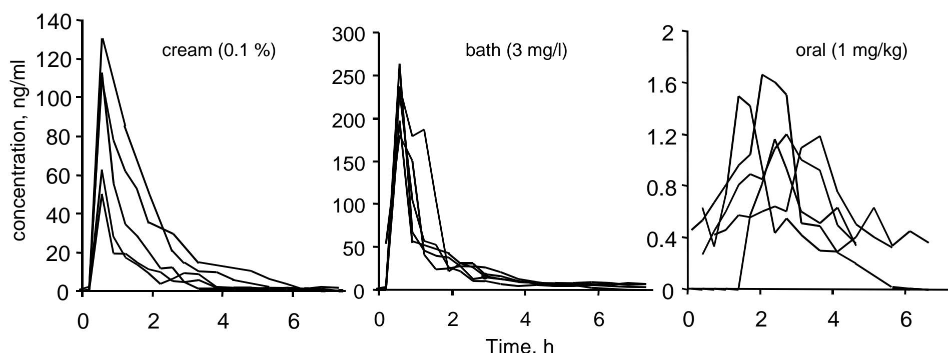

Fig. 5. Concentration–time courses of 8-MOP in microdialysates after cream (0.1%), bath (3 mg/l) and oral administration (1 mg/kg).

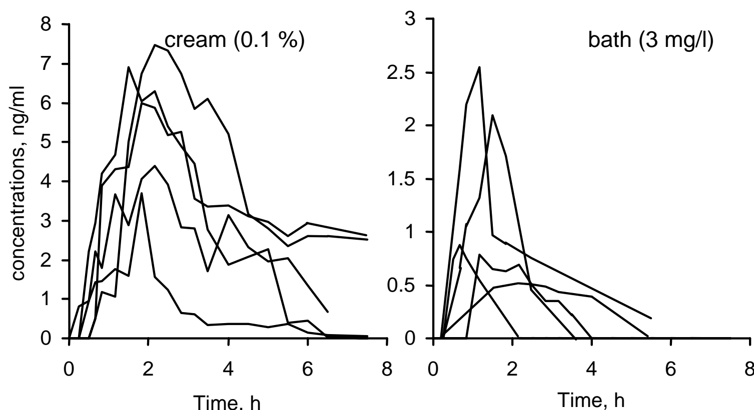

Fig. 6. Concentration–time courses of 8-MOP in plasma samples of five subjects after cream (0.1%) and bath (3 mg/l) administration.

respectively. Concentrations in microdialysates above the calibration range were diluted with acetonitrile/NaCl 0.9% (50:50, v/v) to appropriate levels.

4. Conclusions

To monitor the concentrations of 8-MOP in plasma and microdialysates after topical and systemic application of the drug a sensitive assay using LC/MS/MS with electrospray ionization was developed and validated. This assay allows a faster analysis of plasma samples with an improved sensitivity at a concentration range of 0.5–50 ng/ml. Using this assay it was possible to determine 8-MOP concentrations in plasma after topical application. Using UV detection such sensitivity might be possible by concentrating plasma samples by a factor of 10–100 requiring, however, large volumes of blood. Since endogenous compounds are also concentrated, the resulting chromatograms may be dominated by these peaks. Moreover, the runtime of the analysis was improved. Since 10 μl of sample volume are sufficient for the assay the quantification of 8-MOP was also possible in microdialysates. Using LC/MS/MS in the present paper the sensitivity of the assay for microdialysates was improved to a concentration range of 0.25–50 ng/ml. Compared with gas chromatographic assays the sensitivity is still improved by a factor of 4.

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