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Validation of a microdialysis–gas chromatographic–mass spectrometric method to assess 8-methoxypsoralen in psoriatic patient dermis

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Abstract

8-Methoxypsoralen (8-MOP) is currently used in PUVA therapy (psoralen+UVA) to treat dermatological diseases such as psoriasis, vitiligo and atopic dermatitis. The aim of this work was to validate a method for collecting 8-MOP from patient dermis by a non invasive technique, microdialysis, and then to assess this molecule by gas chromatography–mass spectrometry (GC–MS). 5-Methoxypsoralen (5-MOP) was used as an internal standard. The calibration curve demonstrated a linear relationship between the peak areas of 8-MOP and 5-MOP over a wide range of 8-MOP concentrations (0.9–100 ng/ml). Within- and between-run precisions were measured, using four different 8-MOP concentrations, which varied from 98.0 to 102.0% and from 98.5 to 101.8%, respectively. The limits of detection and quantification were 0.29 and 0.52 ng/ml, respectively. The method was validated and then applied to determine the pharmacokinetic of 8-MOP in ten psoriatic patient dermis, after oral intake of this drug. The results demonstrated that the association of microdialysis with the GC–MS method was an efficient procedure to collect and assess 8-MOP in human dermis, in vivo.

Keywords: Microdialysis; Psoriatic patient dermis; 8-Methoxypsoralen

1. Introduction

Some psoralens (P), particularly 8-methoxypsoralen (8-MOP), are currently employed in dermatology (orally or topically), associated with ultraviolet A (UVA) irradiation to treat skin diseases such as psoriasis, vitiligo and atopic dermatitis. The combination of the previous elements (P+UVA) is known as PUVA therapy [1]. The efficacy of PUVA therapy has been confirmed to be related to the 8-MOP level in the plasma, which was inversely proportional to the amount of UVA needed for delivery [2,3]. 8-MOP interacts with nuclear deoxy-

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ribonucleic acid (DNA) in the presence of UVA, forming photoproducts which cause the inhibition of cellular DNA synthesis [4]. It is believed that this mechanism can explain the beneficial effect of PUVA in proliferative disorders like psoriasis [4].

The literature indicates that 8-MOP plasma concentration is an appropriate marker of this psoralen level in the skin [5]. However, it is in the skin that the interaction of psoralens with UVA has a therapeutic effect [6]. Therefore, the kinetic investigation of 8-MOP had sometimes been carried out in suction blister fluid (SBF) which reflects the psoralen concentration in the skin tissue more precisely than in the serum level [7–10]. However, due to the invasive character of in vivo methods, such as suction blister fluids and biopsies, there are few reports on 8-MOP assessment in psoriatic patient dermis [7]. Mary et al. demonstrated that microdialysis was a powerful tool for collecting 8-MOP from ex vivo human dermis [11]. The dermis insertion of microdialysis probes is slightly painful due to the topical application of a local anaesthesia (Elma®). Consequently, the discomfort is nearly similar to what is perceived during ordinary blood sampling [12]. Therefore, in vivo sampling of 8-MOP dermis by microdialysis can be considered to be acceptable for the patients.

The principle of the microdialysis technique relies on the solute diffusion across a semipermeable membrane which prohibits the passage of macromolecules and proteins [13]. Only the nonprotein bound analyte fraction is sampled. For this reason, drug transfer across the membrane is incomplete [14]. Therefore, it is necessary to determine the 8-MOP relative recovery [11], which gives the possibility of calculating the total amount of 8-MOP in the dermis.

Plasma or suction blister fluid psoralen levels are routinely assessed by high-performance liquid chromatography (HPLC) after extraction with organic solvents [15]. The psoralen organic phase extraction is time consuming as it involves several steps. There are few reports of gas chromatography–mass spectrometry (GC–MS) application in psoralen analysis [16,17]. According to Cracco et al. [16], GC–MS is one of the most sensitive technique to assess 8-MOP levels in plasma. In addition, no GC–MS method has been used, until now, to determine 8-MOP concentrations in microdialysis samples collected from human dermis.

In this work, a microdialysis system was used to collect 8-MOP from patient dermis. The GC–MS procedure was used to assess 8-MOP in the microdialysis samples. Before determining the drug level in patient dermis, linearity, repeatability, reproducibility and detection limit studies were carried out to validate the method.

2. Materials and methods

2.1. Chemicals

All chemicals, 8-MOP (purity 99%) and 5-MOP (purity 99%), were purchased from Sigma–Aldrich (St Quentin Fallavier, France). Ringer buffer solution (pH 6, Cl^-: 111 m*M*; K^+: 5.3 m*M*; Ca^{2+}: 1.8 m*M*; Na^+: 130 m*M*, lactate: 27.6 m*M*) was obtained from Maco Pharma (Paris, France). Emla® (Astra, France) is composed of lidocaıne (2.5 g/100 g of cream), prilocaıne (2.5 g/100 g of cream).

2.2. Microdialysis system

The microdialysis system consisted of a CMA/100® syringe pump (Phymep, Paris, France) and a CMA/140 microfraction collector. The microdialysis probes (CMA/100) had a polycarbonate membrane (length 10 mm) with a 20 000 molecular mass cut off. The probes were sterilized with ethylene oxide.

2.3. Gas chromatography–mass spectrometry

The GC–MS apparatus used was a Hewlett-Packard 5890 gas chromatograph and a 5971A mass spectrometer. The RTX-5MS gas chromatography column (30 m×0.25 mm I.D., 0.25 μm film thickness) was obtained from Supelco (Paris, France).

2.4. Standard sample preparation

8-MOP solution was prepared with ethanol to obtain 8-MOP solution at 9 mg/ml. Then, 20 μl of 8-MOP ethanolic solution were diluted in 19.8 ml of Ringer solution to obtain a solution at 9 μg/ml. 5-MOP solution was prepared by diluting 5.8 mg of 5-MOP in 1 ml of ethanol. A 1-μl volume of this 5-MOP ethanolic solution was diluted in 100 ml of Ringer solution. The concentration of the 5-MOP

solution was 5.8 ng/ml. These 8-MOP (9 μg/ml) and 5-MOP (58 ng/ml) stock solutions were stored in a darkroom at −4 °C. Standard 8-MOP solutions were prepared by diluting the stock 8-MOP solution (9 μg/ml) in Ringer buffer solution to obtain 8-MOP concentrations of 0.9, 1.25, 2.5, 5, 10, 20, 40, 60, 80 and 100 ng/ml.

2.5. In vitro microdialysis samples preparation

Probes were placed in different vials containing the different 8-MOP concentrations and were perfused with Ringer solution at a flow-rate of 3 μl/min. The microdialysis samples were collected every 30 min over a period of 5 h. This flow-rate was found to be an optimal condition for obtaining the highest substance extraction from human dermis [11]. From the 90 μl collected by microdialysis over the 30 min, 50 μl of each 8-MOP solution were added to 50 μl of 5-MOP (58 ng/ml). The two psoralen solutions were mixed together and frozen for 15 min at −80 °C and then lyophilized to eliminate the water. For 15 samples, the lyophilization took about 60 min. The resultant residue was dissolved in 100 μl of ethanol and 4 μl were analyzed by GC–MS.

2.6. GC–MS conditions

The following temperature conditions were applied to the column: MS interface temperature at 290 °C; column oven at 150 °C, 150–170 °C at the rate of 15 °C/min, 5 min at 170 °C and 170–300 °C (15 °C/min). The injector temperature was 280 °C. A 4-μl volume of the sample was injected in splitless mode (30 s of purge-off time). Ionization was performed by electron impact (at 70 eV). The electron multiplier was set at 2200 V. Selected ion monitoring (SIM) was used to assess 8-MOP and 5-MOP—the internal standard (I.S.). To perform mass fragmentography, the ions m/z 145 and 173 for 8-MOP and 5-MOP, respectively, were recorded.

2.7. Validation of the microdialysis–gas chromatographic–mass spectrometric method

To validate the method, it was necessary to determine the following parameters: (1) selectivity, (2) linearity, (3) recovery, (4) limit of detection and quantification, (5) precision and accuracy and (6) stability.

2.7.1. Selectivity

The 8-MOP solution (9 μg/ml) was diluted in the Ringer buffer solution to obtain a 8-MOP concentration of 50 ng/ml. Probes were placed in a vial containing this solution and were perfused at a flow-rate of 3 μl/min for 5 h. Samples (50 μl) of the collected 8-MOP and 5-MOP solutions (58 ng/ml) were mixed. The samples were analysed by GC–MS.

Selectivity was validated by analysing six microdialysis samples obtained from psoriatic patient dermis, after 8-MOP oral intake. This was necessary to verify that no endogenous substances interfered with the 8-MOP and 5-MOP peaks. The collected solutions were analysed according to the procedure described above.

2.7.2. Linearity

Probes were placed in different vials containing ten different 8-MOP concentrations ranging from 0.9 to 100 ng/ml and were perfused with Ringer buffer solution. The collected solutions were analysed according to the procedure described above. Peak area ratios of 8-MOP and the I.S. (5-MOP) were used to plot the calibration curve in relation to the increased 8-MOP concentration. A least square regression analysis was carried out. The linearity of this method was checked by preparing five calibration curves in triplicate at ten different concentrations.

2.7.3. Recovery

2.7.3.1. Recovery of in vitro 8-methoxypsoralen prepared solution without the microdialysis technique. The recovery of the prepared 8-MOP solution (resultant residue dissolved in 100 μl of ethanol) consisted of determining the 8-MOP values of low (5 ng/ml), medium (20 and 50 ng/ml) and high (100 ng/ml) concentrations in relation to the standard 8-MOP ethanolic solutions which represented a recovery of 100%.

2.7.3.2. Recovery of in vitro 8-methoxypsoralen prepared solution with microdialysis technique. Microdialysis probes were placed in vials containing

four different 8-MOP concentrations (5, 20, 50 and 100 ng/ml). These probes were perfused with sterile Ringer solution at a flow-rate of 3 μl/min for 5 h. A 50-μl volume of 5-MOP was added to the collected samples. The samples were lyophilized and analysed by GC–MS.

2.7.4. Limits of 8-methoxypsoralen detection and quantification

Twelve injections of a blank sample containing Ringer and 5-MOP (I.S., 58 ng/ml) were analysed by the GC–MS method. Noise was measured at the 8-MOP retention time (6.21 min). The limit of detection was determined by considering the lowest 8-MOP concentration which gave a signal-to-noise ratio greater than 3 [18].

The limit of quantification was defined as the concentration at which the ratio between the area of 8-MOP and 5-MOP peaks which gave a relative standard deviation (RSD) lower than 20% [18].

2.7.5. Precision and accuracy

The precision of the assay is expressed as the RSD of the concentration values obtained for low (5 ng/ml), medium (20; 50 ng/ml) and high (100 ng/ml) control samples [19]. In this work, the precision was calculated after the analysis of five replicates on the same day (intra-assay precision) and after repeated analysis over 5 days (inter-assay precision).

2.7.6. Stability

The stability of 8-MOP in standard solutions was determined by analysing standard solutions containing 5, 20, 50 and 100 ng/ml ($n=5$). These samples were stored at −4 °C for 7, 14 and 21 days before analysis.

The stability of 8-MOP in microdialysis samples collected from human dermis was assessed by determining the 8-MOP concentration after 7, 14 and 21 days storage at −4 °C.

2.8. In vivo application

2.8.1. Patients

Ten psoriatic patients (eight men and two women, average age 41±16 years, mean±standard deviation) were enrolled in the present study. Oral 8-MOP tablets were administered to psoriatic patients (0.6 mg/kg of body mass). Microdialysis probes were inserted into the psoriatic dermis area localized on the volar forearm. None of the patients had cutaneous cancer, hepatic disorders or renal disease. The study was approved by the local ethics committee. Informed consent was obtained from all subjects.

2.8.2. 8-Methoxypsoralen sampling by microdialysis from psoriatic patient dermis

After cutaneous application of an anaesthesia cream (Emla), the probes were inserted into the patient dermis. Probes were perfused with Ringer solution at an optimal flow-rate of 3 μl/min. 8-Methoxypsoralen dermis samples were collected by microdialysis every 30 min over a period of 5 h.

2.8.3. Preparation of microdialysis samples

A 50-μl volume of microdialysis samples collected from psoriatic patients and 50 μl of 5-MOP stock solution (58 ng/ml) were frozen for 15 min at −80 °C and then lyophilized. A 4-μl volume of the residue, which was dissolved in 100 μl of ethanol, was injected into the GC–MS system.

3. Results

3.1. Method development

The following oven temperatures were used: from 160 to 250 °C (20 °C/min), 1 min at 250 °C, 20 °C/min to 290 °C [16]. To optimize the method, the oven temperature was modified in the following way: a temperature gradient from 150 to 170 °C at the rate of 15 °C/min, 5 min at 170 °C and 170–300 °C (15 °C/min). The separation of the two peaks of 8-MOP and 5-MOP, using these new GC–MS conditions, was satisfactory. The second oven temperature ramp was used to eliminate the microdialysate products which were not of interest in this work.

3.2. In vitro relative recovery of 8-MOP

Microdialysis permits only a fraction of 8-MOP to be collected. Therefore, it was necessary to determine the relative recovery of 8-MOP. The method consists in continuously perfusing a fluid, i.e. Ringer

solution, which creates a concentration gradient along the dialysis membrane. The compounds diffuse through the membrane from the interstitial fluid to the perfusate or from the perfusate to the interstitial fluid. The outlet perfusate is collected in microtubes. To determine the relative recovery (RR), the following formula is used:

$$\mathrm{RR} = \frac{C_{\mathrm{in}} - C_{\mathrm{out}}}{C_{\mathrm{in}}}$$

which gives the ratio between the concentration in the dialysate (C_{out}) and the concentration in the medium surrounding the probe (C_{in}) [14].

The recovery of 8-MOP from the concentrations of 5, 20, 50 and 100 ng/ml, were 31.8±0.5, 31±1, 30.8±0.8 and 31.2±1.2%, respectively ($n=3$). These recoveries were not statistically different ($P>0.05$, Mann–Whitney test).

3.3. Selectivity

Chromatograms obtained by GC–MS of an 8-MOP standard solution and microdialysis samples are represented in Figs. 1 and 2. The retention times for 8-MOP and 5-MOP were 6.21 and 6.36 min, respectively. The resolution of the peaks is equal to 1. Determination of 8-MOP was not affected by endogenous compounds (Figs. 1 and 2).

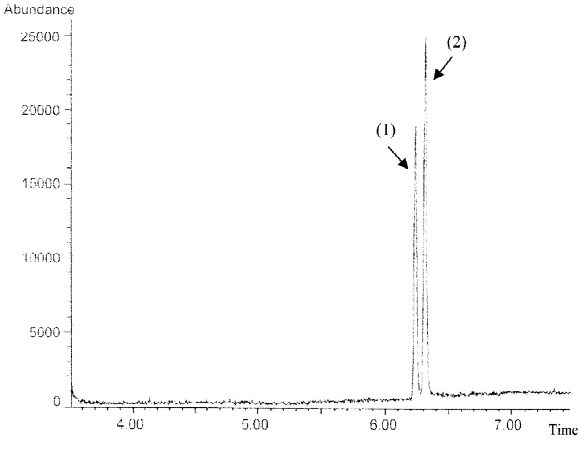

Fig. 1. Chromatogram of 8-methoxypsoralen in standard solution: 8-MOP (1); 5-MOP (2).

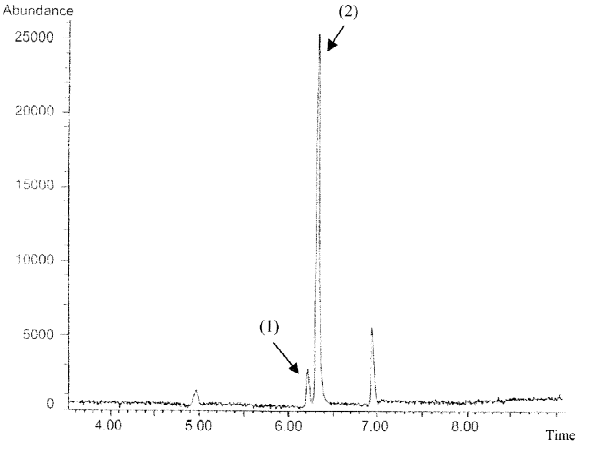

Fig. 2. Chromatogram of 8-methoxypsoralen in a microdialysis sample from psoriatic patients: 8-MOP (1); 5-MOP (2).

3.4. Calibration

The 8-MOP calibration curve ranging from 0.9 to 100 ng/ml revealed that the standard curve was linear with the correlation coefficient R^2: 0.999±0.001 and slope: 0.057±0.001. The curve intercepted y axis at 0.013±0.002. The data demonstrated that the assay response was proportional to

Table 1
Linearity of 8-methoxypsoralen assay responses to concentrations

Standard 8-MOP concentration (ng/ml)	Response	Standard deviation	Response/concentration
0.9	0.054	0.003	0.060
1.25	0.080	0.008	0.064
2.5	0.160	0.010	0.062
5	0.315	0.005	0.063
10	0.561	0.006	0.056
20	1.247	0.015	0.062
40	2.263	0.012	0.057
60	3.277	0.015	0.055
80	4.553	0.075	0.057
100	5.753	0.119	0.058
Mean			0.059
Standard deviation			0.003
RSD (%)			5.394

Slope = 0.057; standard deviation of slope = 0.001; intercept = 0.013; standard deviation of intercept = 0.002; $R^2 = 0.999$; standard deviation of $R^2 = 0.001$. 8-MOP data were compiled from an average of five different standard curves, each performed by triplicate injection.

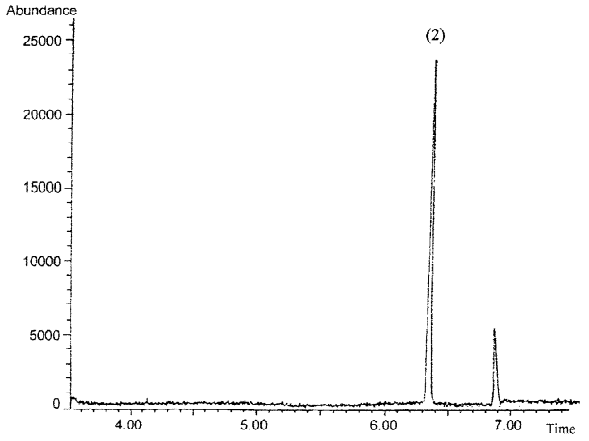

Fig. 3. Chromatogram of a microdialysis blank: microdialysis sample without 8-MOP (1); with 5-MOP (2).

concentrations across the range of standards (Table 1).

3.5. Recovery of in vitro 8-methoxypsoralen prepared solution without microdialysis technique

In order to establish the 8-MOP loss due to the solution preparations, the percentage recovery of the method used was determined [20]. The absolute recovery ranged from 95.9 to 104.9%.

3.6. Limits of detection and quantification

Blank samples (Fig. 3) were used to determine the limits of detection and quantification. The 8-MOP detection limit was equal to 0.29 ng/ml and the limit of quantification was equal to 0.52 ng/ml.

3.7. Precision and accuracy

The intra- and inter-assay precision values (% standard deviation) at the four 8-MOP concentrations ranged from 98.0 to 102.0% and from 98.5 to 101.8%, respectively (Table 2). Accuracy was expressed as the bias which was calculated by using the formula suggested by Causon [19]. The accuracy bias was $1.7 \pm 0.5\%$ ($n = 5$) for intra-assay experiments and $1.1 \pm 0.6\%$ ($n = 5$) for inter-assay experiments.

3.8. Stability

According to Dadgar et al. [21], solutions are considered to be stable if the change in sample concentration is less than 10%. 8-MOP concentrations in all in vitro microdialysis samples reduced by 8% after 1 month in storage.

As the microdialysis samples collected in vitro were stable at the different levels, the stability of 8-MOP in microdialysates from psoriatic patient dermis was determined. The results demonstrated

Table 2
Intra- and inter-assay accuracy and precision results of dermal 8-methoxypsoralen concentrations, using microdialysis associated with gas chromatography–mass spectrometry

8-MOP concentrations (ng/ml)		RSD (%)	Recovery (%)	Error (%)
Nominal	Assessed ± SD ($n = 5$)			
Intra-assay ($n = 5$)				
5	5.1 ± 0.5	9.6	102.0 ± 10.0	2.0
20	20.4 ± 1.0	4.9	102.0 ± 5.0	1.9
50	50.5 ± 1.9	3.7	101.0 ± 3.8	0.9
100	98.0 ± 4.4	4.5	98.0 ± 4.4	−2.0
Inter-assay ($n = 5$)				
5	5.1 ± 0.1	2.7	101.8 ± 2.0	−1.8
20	19.9 ± 0.6	3.2	99.5 ± 2.9	−0.5
50	50.3 ± 1.8	3.6	100.6 ± 2.8	0.6
100	98.5 ± 7.5	7.6	98.5 ± 7.5	−1.5

that 8-MOP was stable over 1 month (decrease of 9% of 8-MOP concentrations).

3.9. Application

The validated method was applied to determine the concentrations of 8-MOP in ten human psoriatic dermis following oral administration of this drug at a dose of 0.6 mg/kg of body mass. Microdialysis samples were collected every 30 min over a period of 5 h and assessed by GC–MS. Typical curves of 8-MOP pharmacokinetic, from psoriatic dermis patients, are shown in Fig. 4.

4. Discussion

The GC–MS technique has already been demonstrated to be sensitive and specific to determine 8-MOP in plasma and in suction blister fluid [17]. The limit of detection obtained in our work (0.29 ng/ml), was nearly equal to the results of Cracco et

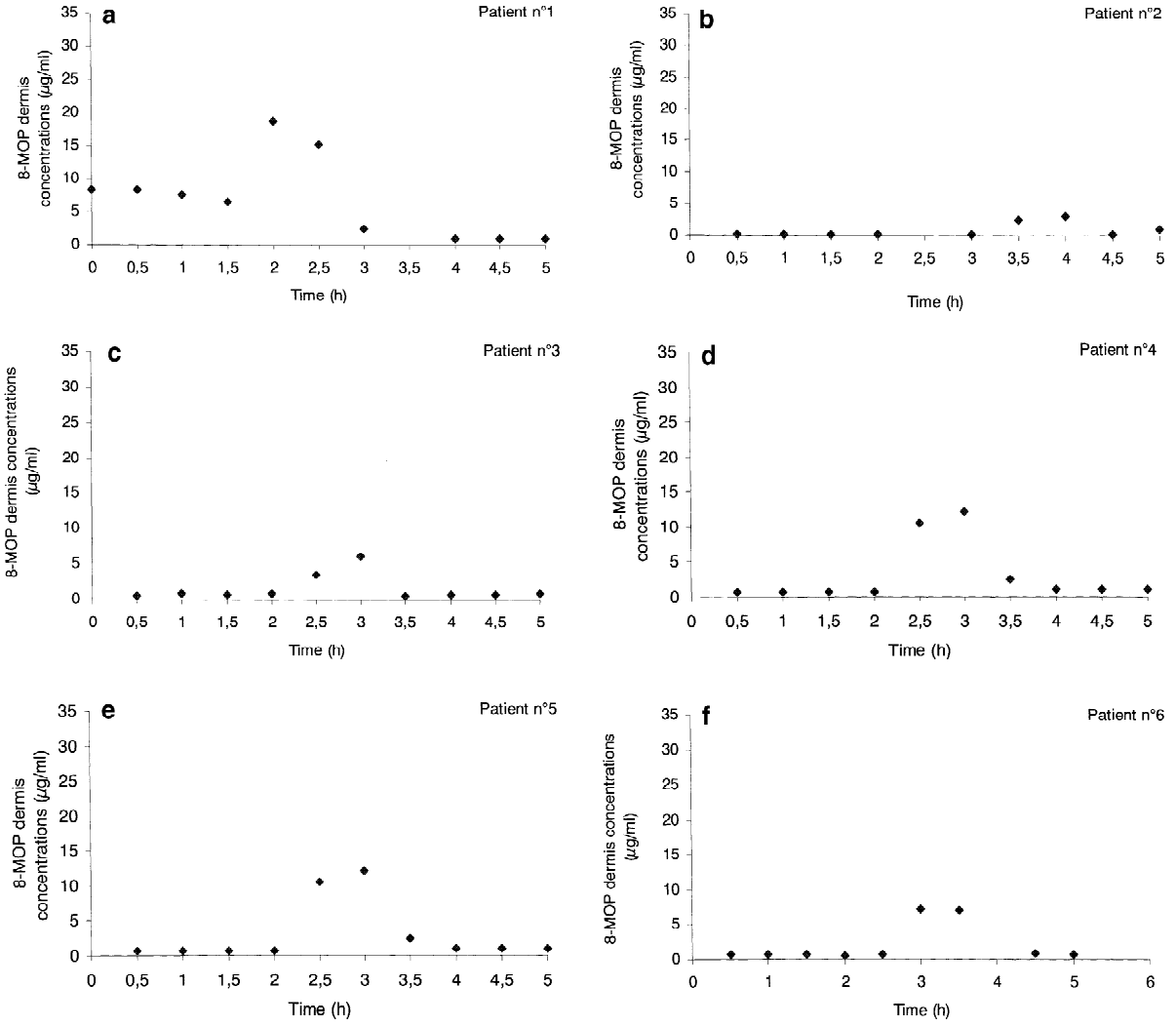

Fig. 4. 8-Methoxypsoralen (8-MOP) pharmacokinetics in psoriatic patient dermis (mean±SEM, $n=10$).

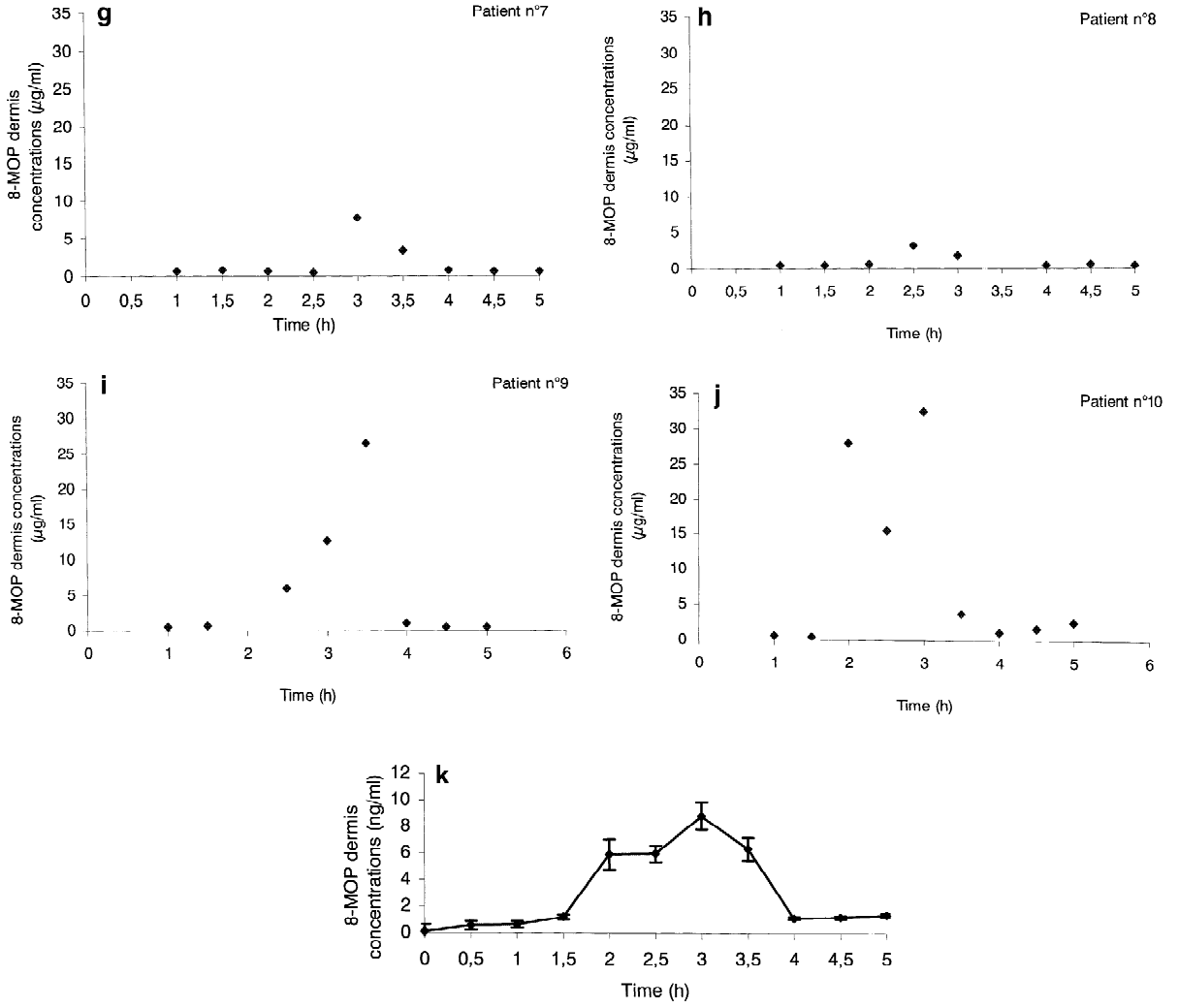

Fig. 4. (*continued*)

al. (0.4 ng/ml) [16] and Huuskonen et al. (3 ng/ml) [17] (Table 3). Our method detected less than 1 ng/ml of psoralen in psoriatic patient dermis. Using this technique, only 50 μl of volume sample were needed, whereas Cracco et al. and Huuskonen et al. used minimally 400 μl (Table 3). As microdialysis is suitable for collecting relatively pure solution without protein, the association of microdialysis and GC–MS avoids several extraction steps and as a consequence the sample preparation time is considerably reduced.

In this work, the 8-MOP concentrations in psoriatic patient dermis were collected by a non invasive method, microdialysis and then assessed by a sensitive method, GC–MS. A precise GC–MS procedure was developed and validated for 8-MOP assessment in microdialysis samples collected, in vivo, from psoriatic patients dermis. The advantages of the GC–MS method over other techniques [22] are sensibility, short analysis time and the possibility of working with a very small sample volume.

The combination of microdialysis and GC–MS seems to be an efficient method of collecting and determining 8-MOP dermis concentrations. The aim

Table 3
Comparison of 8-MOP experimental conditions and results determined by GC–MS

Criteria	Huuskonen et al. 1984 [17]	Cracco et al. 1992 [16]	Current work 2002
Internal standard	5-MOP	TMP	5-MOP
Concentration range studied (ng/ml)	10–1000	1–500	0.9–100
Matrix	Plasma and suction blister fluid	Plasma	Microdialysis dermis samples
Volume required for analysis	0.5–1 ml (plasma) 10–400 μl (suction blister fluid)	1 ml (plasma)	50 μl (dermis microdialysates)
Precision (RSD, %)	<2.0	<2.0	<2.4
8-MOP extraction	Yes	Yes	No
Limit of detection (ng/ml)	3.0	0.4	0.29

5-MOP, 5-methoxypsoralen; 8-MOP, 8-methoxypsoralen; TMP, trimethylpsoralen.

of this work was fulfilled: validation of a method for assessing 8-MOP in patient dermis by GC–MS after microdialysis sampling. This method could be used to determine other psoralen skin concentrations (5-MOP and TMP) and to correlate their dermis levels with their therapeutic efficiency.

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