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Short Communication

High-performance liquid chromatographic determination of methoxsalen in plasma after liquid–solid extraction

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ABSTRACT

An improved method suitable for the determination of 8-methoxypsoralen in the range 50–1500 ng/ml in the plasma of psoriatic patients undergoing PUVA (psoralens and long-wave ultraviolet light) therapy is proposed. A 5-ml aliquot of plasma containing sodium citrate as anticoagulant was centrifuged, griseofulvin was added as internal standard and the sample was denatured with acetonitrile. The supernatant was applied to C_{18} cartridges and 8-methoxypsoralen was eluted with methanol. The evaporated eluate was reconstituted in the mobile phase for high-performance liquid chromatography (HPLC) and applied to the HPLC column: mobile phase, acetonitrile–0.01 *M* phosphoric acid (34:66); flow-rate, 1 ml/min; temperature, 40°C; column, Spherisorb 5 ODS, 100 mm × 4.6 mm I.D., 5 μm particle size; UV detection at 248 nm; detection limit, 15 ng/ml of plasma.

INTRODUCTION

8-Methoxypsoralen (8-MOP, methoxsalen, xanthotoxin), a fourocoumarinic derivative, is used in phototherapy [so-called psoralens and long-wave ultraviolet light (PUVA) therapy] in the treatment of psoriasis, a hyperproliferative disease of the skin. This therapy is also effective in some other diseases, *e.g.* in cutaneous T-cell lymphoma [1]. Plasma and serum levels of 8-MOP after oral treatment vary greatly between individuals, ranging from 0 to 500 ng/ml [2,3] and from 0 to 380 ng/ml, respectively [4–6]. The effect of PUVA therapy is very poor in patients with plasma levels below 40 ng/ml [7]. Thus, monitoring of 8-MOP plasma levels should be an important part of every patient's treatment.

Different methods of analysis of 8-MOP in

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plasma have been published [8]. Since 8-MOP has a very high UV absorbance, high-performance liquid chromatographic (HPLC) methods with UV detection are mainly recommended, using either normal-phase (NP) columns [6,9–11] or reversed-phase (RP) columns [1,4,8,12–14].

We have used our experience in work with preseparation cartridges to develop an improved method suitable for the determination of 8-MOP in the plasma of psoriatic patients undergoing PUVA therapy.

EXPERIMENTAL

Materials

8-MOP of high purity was synthesized and analysed by the Institute of Pharmaceutical Chemistry of the University of Padua (Padua, Italy). Griseofulvin was the product of Orion (Espoo, Finland). Methanol was UV grade from Lachema (Brno, Czech Republic). Other chemicals — dimethylformamide, phosphoric acid, hydrochloric acid and sodium citrate — were also from Lachema, and were of p.a. grade. Acetonitrile, p.a. grade, from VEB Laborchemie (Apolda, Germany) was redistilled before use. Plasma for calibration was supplied by the transfusion centre of the Teaching Hospital in Hradec Králové, Czech Republic.

Equipment

Plasma samples were centrifuged on the Media 415 centrifuge (Chirana, Piešťany, Czech Republic). A Dorcus vacuum pump (Tessek, Prague, Czech Republic) was used for the elution of 8-MOP from the Silicacart C_{18} preseparation cartridges — volume 1 ml, particle size 60 μm (Tessek). HPLC analysis was performed on a PU 4100/44 liquid chromatograph with a PU 4110 UV–VIS detector and PU 4810 integrator (all from Pye Unicam, Cambridge, UK). A Spherisorb 5 ODS analytical column, 100 mm × 4.6 mm I.D., particle size 5 μm (Pye Unicam), was used.

Blood sample preparation

A 5-ml aliquot of venous blood from psoriatic patients treated with Oxsoralen (Gerot, Austria; one capsule containing 10 mg of 8-MOP) was taken to the centrifugal tube containing 0.5 ml of 3.8% sodium citrate as an anticoagulant. The sample was centrifuged at 500 *g* for 10 min, 2 ml of plasma were transferred to another centrifugal tube, and 2 ml of acetonitrile and 1 ml of the methanolic solution of griseofulvin ($6.25 \cdot 10^3$ ng/ml) as the internal standard (I.S.) were added. After a brief manual shaking (1–2 s) the sample was centrifuged at 500 *g* for 10 min and 4 ml of the supernatant were applied to the preseparation cartridge.

Liquid–solid extraction

Prior to extraction, the preseparation cartridges were activated with 5 ml of methanol. Then 5 ml of water were passed through to remove the rest of the methanol. The samples were applied to the cartridges and washed with 5 ml of water. 8-MOP and the I.S. were eluted using 2 ml of methanol. The cartridges were reconstituted with 4 ml of a 0.5 *M* solution of HCl in methanol followed by 5 ml of water. For the separation the vacuum pump and a pressure of 10 kPa were used.

The eluate was evaporated to dryness under vacuum at 38°C. The residue was reconstituted in 1 ml of the mobile phase and 50 μl of this solution were injected into the column.

Calibration

A 1-ml volume of acetonitrile, 1 ml of the methanolic solution of griseofulvin ($6.25 \cdot 10^3$ ng/ml) and 1 ml of the methanolic solutions of 8-MOP (100, 200, 400, $1.0 \cdot 10^3$, $1.6 \cdot 10^3$, $2.0 \cdot 10^3$, and $3.0 \cdot 10^3$ ng/ml) were added to 1 ml of the mixed plasma of several healthy donors. After a short manual shaking (1–2 s), the samples were centrifuged at 500 *g* for 10 min. The supernatant was treated in the same manner as the samples from the patients.

High-performance liquid chromatography

The mixture of acetonitrile and 0.01 *M* phosphoric acid (34:66, v/v, pH 2.82) was used as the mobile phase, flow-rate 1 ml/min, temperature

40°C. The mobile phase was filtered before use and continuously degassed using helium. The UV detector was set at wavelength 248 nm.

Intra-day assay variation of HPLC determination

Two sets of ten samples containing 250 and 500 ng of 8-MOP per ml of plasma (prepared from the same mixed plasma of healthy donors) were measured, respectively, nine or ten times during one day as described above.

Inter-day assay variation of HPLC determination

From the stocks of two plasma samples containing 250 and 500 ng of 8-MOP per ml of plasma stored in well closed bottles placed in the refrigerator (4°C), 1-ml portions were taken nine times during the period of three weeks and measured as described above together with the calibration solutions.

RESULTS AND DISCUSSION

Several compounds have been used as I.S. for the HPLC determination of 8-MOP, *e.g.* ammidin [6], trimethylpsoralen [4,12], 5-methoxypsoralen [8], griscofulvin [9], and coumarin [13]. Our first attempts were made with coumarin as the I.S. and with the mobile phase acetonitrile–water according to Ashwood-Smith *et al.* [13], who also carried out liquid–solid extraction. However, when applied to plasma samples, an uncontrolled decomposition of coumarin probably occurred. Therefore, we tried griseofulvin as another I.S. It was necessary to optimize the HPLC parameters, especially the composition of the mobile phase, the length and type of the column and the wavelength of UV detection. After these improvements griseofulvin as an I.S. possessed suitable properties. Its peak retention time (t_R), approximately 12 min, was sufficiently distinguishable from the peaks of 8-MOP ($t_R \sim 7.6$ min) and of endogenous compounds ($t_R \sim 1.3$–3.4 min), as shown in from Fig. 1a and b. In addition, both measured compounds (*i.e.* griseofulvin and 8-MOP) had good stability under all the experimental conditions. Also, at the wavelength used, 248 nm (maximum absorbance of 8-MOP), the

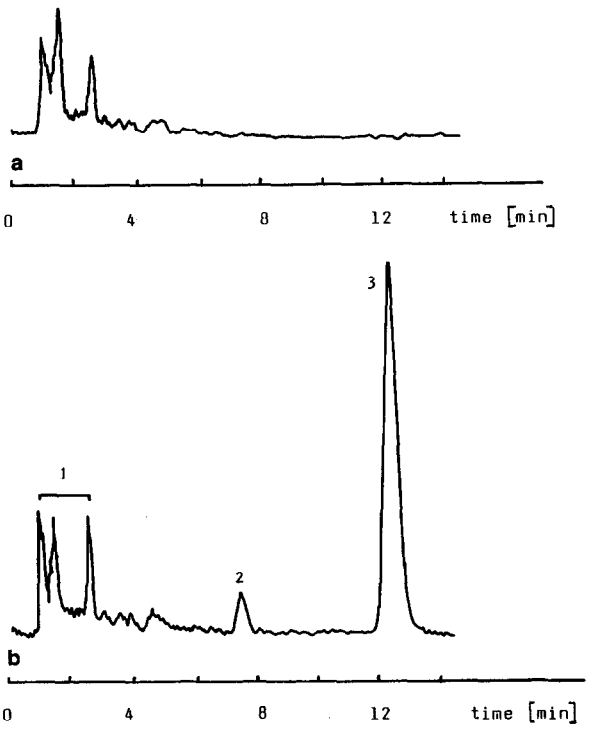

Fig. 1 (a) Chromatogram of blank plasma after liquid–solid extraction. (b) Chromatogram of 8-MOP and griseofulvin after extraction from plasma. Peaks: 1 = endogenous compounds (t_R = 1.3–3.4 min); 2 = 8-MOP (t_R = 7.59 min, concentration = 170 ng/ml of plasma); 3 = griseofulvin (t_R = 12.13 min, concentration = $6.25 \cdot 10^3$ ng/ml). Column: Spherisorb 5 ODS, 100 mm × 4.6 mm I.D., particle size 5 μm. Mobile phase: acetonitrile–0.01 *M* phosphoric acid (34:66, v/v), 1 ml/min, 40°C. UV detection at 248 nm.

sensitivity of UV detection and determination of 8-MOP were found to be sufficient to make griseofulvin a satisfactory I.S. The detection limit of 8-MOP under the experimental conditions described is 15 ng/ml of plasma.

A calibration graph was derived from the results of HPLC measurement of plasma samples containing known amounts of 8-MOP and griseofulvin. The calibration curve was linear in the studied concentration range of 50–1500 ng/ml of plasma ($y = ax$; $a = 0.4329$; S.D. = 0.0162; correlation coefficient $r = 0.9900$). According to our previous experiments the extraction efficiency of the preseparation cartridges for 8-MOP and griseofulvin is in the range 96–100%. HPLC measurements of standard methanolic solutions

TABLE I

PLASMA LEVELS OF 8-MOP IN PATIENTS UNDERGOING PUVA THERAPY 3 h AFTER THE APPLICATION OF OXSORALEN, AS DETERMINED BY THE REVERSED-PHASE HPLC INTERNAL STANDARD METHOD WITH GRISEOFULVIN AFTER PRESEPARATION ON SILICACART C_{18} CARTRIDGES

Patient No.	Dose (mg)	Concentration (ng/ml)
1	60	620
2	90	680
3	30	322
4	20	170

TABLE III

INTER-ASSAY VARIATION OF HPLC DETERMINATION OF 8-MOP IN PLASMA

Theoretical concentration (ng/ml)	n^a	Concentration found (mean ± S.D.) (ng/ml)		v^b (%)
250	9	243.1	22.2	± 18.1
500	9	502.3	36.2	± 14.3

[a] n = number of measurements in a three-week period.
[b] v = variability for the 95% confidence limit.

of 8-MOP and griseofulvin practically did not differ from those of 8-MOP and griseofulvin in plasma after liquid–solid extraction. Potential losses of 8-MOP on the preseparation cartridges were followed by the same losses of griseofulvin, so that the ratio of the AUC (area under the curve) of 8-MOP to the AUC of griseofulvin from which the calibration curve and further results were taken, was the same, dependent on the original concentration of 8-MOP.

Plasma levels of 8-MOP in four psoriatic patients were measured twice (see Table I). The variability of the method for a 95% confidence limit (calculated on the basis of the estimated standard deviation for the number of measurements $\leqslant 7$ [15]) is about 13%, as determined from double measurements of all the real plasma samples. This result agrees with the results of the intra-assay variation tests (see Table II). Slightly poorer results were obtained in the inter-assay variation studies (see Table III).

The measurement of 8-MOP in plasma confirmed the usefulness of the proposed method for routine screening of psoriatic patients. The Silicacart cartridges in connection with the Dorcus vacuum manifold have proved to be useful for the extraction of plasma samples for routine analysis, because up to twelve samples can be extracted simultaneously.

Our method offers an alternative to the methods published or used for the determination of 8-MOP in plasma. Griseofulvin as the internal standard has been used previously [9] but in connection with a complicated and long procedure. However, the use of liquid–solid extraction is much simpler and shorter. Thus, the presented method is simple and can be directly used for monitoring of the 8-MOP plasma levels in psoriatic patients undergoing PUVA therapy.

TABLE II

INTRA-ASSAY VARIATION OF HPLC DETERMINATION OF 8-MOP IN PLASMA

Concentration (ng/ml)	n	S.D. (ng/ml)	v^a (%)
250	9	21.1	± 16.8
500	10	28.2	± 11.2

[a] v = variability for the 95% confidence limit.

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