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Determination of 5-methoxypsoralen in human serum

Lai-Hao Wang *, Mey Tso

Department of Applied Chemistry, China Nan University of Pharmacy and Science, Tainan 71743, Taiwan

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Abstract

A high performance liquid chromatography with fluorometric and ultraviolet detection described to quantify 5-methoxypsoralen (5-MOP) percutaneous absorption in humans after the application of an essential oil, as well as 5-MOP in bergamot oil and cosmetics by fluorometric and voltammetric measurement, respectively. A μBondapack C_{18} analytical column (particle size 5 μm, 3.9 × 300 mm) eluted with acetonitrile–tetrahydrofuran–water (70:15:15, v/v/v) containing 0.07% trifluoroacetic acid. The quantification limits are 0.05 and 0.26 ng for 5-MOP and 5-geranoxypsoralen, respectively. For fluorometric measurement was found linear over the range 0.05–3.00 μg/ml for psoralens. Five volunteers blood samples were collected over a 2-day period were investigated before and after treatment with bergamot oil. Serum levels of 5-MOP were significantly increased from the 4 h after the application of bergamot essential oil. The hourly mean levels were significantly higher after the application of bergamot essential oil compared to baseline values.

Keywords: 5-Methoxypsoralen; Human serum; Bergamot essential oil; Fluorescence detection

1. Introduction

Bergapten (5-methoxypsoralen, 5-MOP) is found in bergamot oil, which is combination with UV radiation, promotes melanogenesis and causes thickening of the stratum corneum. These properties can be used in suntan product to accelerate the tanning (pigmentation) of the skin [1]. When photoactivated with sunlight or long-wavelength (UVA = 320–400 nm) a photoreaction occurs leading to covalent binding of psoralens to DNA and to a lesser extent, to RNA and proteins [2–4]. It has been reported that bergapten (5-MOP) is the only significant phototoxic component of bergamot essential oil. Recently, bergapten was shown to be a frameshift mutagen in *Ames Salmonella typhimurium* plating assay [5–8]. 5-MOP make up approximately 0.11–0.32% by weight of bergamot oil [9]. Some of nonvolatile compounds are photosensitive and have mutagenic activity [10,11]. The literature contains several methods for the determination of 5-MOP [12–21]. A Finnigan ITD ion trap detector coupled to a gas chromatograph was used to identify of some coumarin derivatives in deterpe-

* Corresponding author. Fax: +886-7-8024538
E-mail address: e201466@ms47.hinet.net (L.-H. Wang).

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nated citrus peel oil [12]. Separation of some coumarins were performed on a normal-phase silica column in hexane–ethyl acetate and on a C_{18} reversed-phase column with aqueous acetonitrile or methanol with ultraviolet detector [13–17]. For improvement of the peak identification, the HPLC system was equipped with a photodiode array detector which is not suitable for the determination of psoralens present in trace amounts [18,19]. The determination of 5-MOP with selective fluorescence detection to give more specificity by eliminating any interference of natural coumarins and furocoumarins by refined the method has been reported [20]. Improvement in the fluorometric detection of 5-MOP by using β-cyclodextrin (β-CD) in the mobile phase and a cross-linked β-CD column have developed [21]. However, there is some information on skin photosensitization and some biochemical effect of 5-MOP. To our knowledge, only one study has been published concerning percutaneous absorption after solar product application in humans [19]. Quantitative blood levels with 5-MOP provided pharmacokinetic model by fluorometric measurement might be expected to study. The current investigation was performed to determine the pharmacokinetics of 5-MOP on the percutaneous absorption in the human after the application of bergamot essential oil. The approach adopted by the International Fragrance Association is to recommend a maximum of 15 μg/ml 5-MOP in final products [8]. The LC determination has many advantage, particularly its sensitivity and low detection limit with appropriate solvents systems, but psoralens and coumarin derivatives in essential oil and complicated organic matrix in cosmetics absorbance interferences comparatively large. The differential pulse voltammetry (DPV) offers greater specificity and metal oxides dispersed on electrode surface enhances the sensitivity of their voltammetric measurement. Several randomly selected commercial products such as essential oil, suntan and body lotions were analyzed by liquid chromatographic fluorescence (LCFL) and DPV.

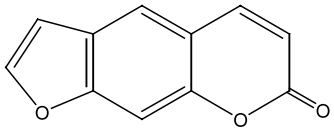
2. Experimental

2.1. Instrumentation

A high-performance liquid chromatography (GL Science Model 576 pump, Japan) with spectrofluorometric RF-10A_{XL} and SPD 10A spectrophotometric detectors (Shimadzu, Japan) were used for the analysis. All electrochemical experiments were performed using an EG&G Princeton Applied Research Model 394 polarographic analyzer (Princeton, NJ, USA) The absorption spectra of human skin were obtained using a Perkin-Elmer System 2000/IRDM FTIR spectrophotometer with a Grase by Specac attenuated total reflection system (ATR).

2.2. Materials

5-Methoxypsoralen (bergapten, 5-MOP) was purchased from Sigma Chemical Company. 5-geranoxypsoralen (bergamotin, 5-GOP) was purchased from Extrasynthese (Genay, France). The bergapten and bergamotin are derivatives of the following basic structure.



Psoraleh

All other chemicals were analytical reagent grade. Sample of essential oil and cosmetics were bought from a number of retail outlets in the south of Taiwan. Formulation, the test article dosage forms were provided in amber vials containing 385 ng 5-MOP in 2 ml jojoba oil and ethanol-tricaprylin (C_8) (5:2, w/w), respectively.

3. Procedure

3.1. Human volunteers

Five normal healthy subjects (two males and three females, mean age, 22 ± 1.0 years) were investigated before (drug-free) and after 2-day

periods of treatment with a formulation of bergamot essential oil. Six circular area of 20 cm^2 each were delineated and the emulsion was randomly allocated either to the left or the right forearm. An application of 12 mg/cm^2 of emulsion represented 385 ng/cm^2 of 5-MOP. The skin was not occluded; therefore, the vehicles were allowed to evaporate as they would during conditions of normal use. All skin surfaces washed at 6 h with ethanol to remove unabsorbed material. Test compound not removed from the skin by washing was considered to be absorbed.

3.2. Fourier transform infrared spectroscopy in vivo

The marked skin area of the forearm was pressed on the thallium chloride (KRS-5) crystal (the window of the Skin Analyser) by its own weight. After each measurement, the window was cleaned with alcohol. All in vivo spectra were obtained under ambient laboratory conditions and recorded at $t=0$ (before emulsion application) and $t=0.5$, 1.0, 1.5, 2.0 and 2.5 h after application. At each time, the values of absorption band integration were noted for O–H (3271 cm^{-1} stretching absorbance), C–H (2915 and 2847 cm^{-1}) and C=O (1732 cm^{-1}) for both treated and non treated arms.

3.3. Sample preparation

All standard solutions were freshly prepared in methanol from 1000 μg/ml stock solutions of psoralens in methanol. Taking into account the 5-MOP content of essential oil and cosmetics, a sample ($\sim$0.2 to 1.0 g) of the latter was weighed accurately in a 15 ml beaker, dissolved in approximately 5 ml methanol by stirring for 5–6 min, and centrifugated. The supernatant was eluted through a 6 ml solid phase extraction (Supelco) glass column (15 mm i.d.) with a Teflon frits at the lower end and containing 3.0 g of silica gel 60 (0.063–0.04 mm; Merck, Darmstadt, Germany) in the bottom. The column was eluted with 12 ml of hexane–ethyl acetate (75:25, v/v) led to the isolation of several fractions. The first fraction that contained neither 5-MOP nor coumarin derivatives, and these were followed by two fractions in which 5,7-dimethyl coumarin only was found. Finally about six fractions containing 5-MOP was eluted. The factions were combined and evaporated under a nitrogen flux to dryness, at room temperature. After clean-up, the dried extracts were solubilized in 2 ml of methanol for HPLC and DPV analysis.

Since the analyte and internal standard in any sample or standard receive the sample treatment, the ratio of their signals will be unaffected by any lack of reproducibility in the procedure. A calibration curve of peak height 5-MOP/5-GOP versus concentration of 5-MOP is linear (0.05–3.00 μg/ml) with a slope of 0.8 ($r=0.9999$). To 2 ml samples of serum were added: 0.4 ng of bergamotin as internal standard; and 4 ml of ethanol. Samples were extracted with 5 ml of chloroform at room temperature on a reciprocating shaker for 15 min. After centrifugation for 30 min ($2100 \times g$) the organic layer was transferred to another centrifuge tube and evaporated at 30 °C under a stream of dry nitrogen. Immediately prior to the chromatographic analysis the dry residue was redissolved by agitating in 200 μl of methanol.

3.4. Determination of 5-MOP by HPLC

The stationary phase was a μBondapack C_{18} (5 μm, 3.9×300 mm) and the mobile phase was a 70:15:15 (v/v/v) mixture of acetonitrile–tetrahydrofuran–water (0.07% trifluoroacetic acid (TFA)). The mobile phase flow rate was 1.0 ml/min and the fluorometric detector was operated at Ex (excitation wavelength) 325 nm and Em (emission wavelength) 470 nm. Injections (20 μl) of sample and standard solutions were performed by means of the injection valve. Chromatograms and peak height were calculated by means of a SISC chromatogram data integrator. Quantitation was based on compound peak height.

3.5. Serum kinetics of 5-MOP

Five millilitre of blood was collected into centrifuge tubes at the following time intervals after 5-MOP emulsion application: 2, 4, 6, 8 and 16 h. 5-MOP serum levels were determined after

extraction from the serum by high-performance LCFL and UV detection.

3.6. Determination of 5-MOP by DPV

A typical composite metal oxide carbon paste preparation procedure was as follows: 1.2 g of graphite powder (Merck) and 0.01–0.03 g of metal oxide were mixed for 5 min, followed by the addition of 0.8 g of liquid paraffin (Merck) and then were mixed in a mortar and pestle. The body of the composite metal oxide carbon paste working electrode was fabricated from a PTFE rod (o.d. 7 mm) with a 3 mm deep hole bored (diameter 3 mm) into one side for the composite metal oxide carbon-paste filling. The composite metal oxide carbon paste was placed in the body of the electrode using a PTFE spatula, and then smoothed off.

The supporting electrolyte was 0.1 M phosphate buffer (pH 2.31) prepared from 1+1 mixture of 0.1 M KH_2PO_4 and 0.1 M H_3PO_4. In order to obtain calibration plots for the 5-MOP, 10 ml of supporting electrolyte was pipetted into a voltammetric cell and de-aerated with nitrogen for 4 min before voltammetric measurement. By micropipette, aliquots of 1000 ppm 5-MOP solution were added. After each addition, voltammograms were obtained. Quantitative analyses were performed in the differential pulse mode. The potential was set at 0.0 to +1.2 V versus Ag/AgCl. The pulse height was 50 mV, and the scan rate 10 mV/s. For sample solution analysis, 0.1 ml of the solution was pipetted into a 10 ml calibrated flask, and diluted to volume with phosphate buffer. The solution was analyzed by DPV using the same condition as that for the calibration plot.

4. Results and discussion

4.1. Optimization of chromatographic conditions

Separation parameters such as the kind and concentration of organic modifier, addition of certain salts and variation of pH were examined to establish the optimum conditions for the separation of psoralens in the essential oil and cosmetics by HPLC using an ODS column. In reverse-phase liquid chromatography, the retention of any solute depends on the proportion of the organic modifier in the aqueous eluant: An organic-enriched composition results in a decreased retention time or capacity factor (k′). To illustrate this point, three different eluants acetonitrile–water (70:30, 50:50, 33:67, 25:75, v/v); acetonitrile–methanol–water (20:60:20, 30:40:30, 40:20:40, 70:10:20, v/v) and acetonitrile–tetrahydrofuran–water (25:25:50, 50:20:30, 75:15:15 containing 0.07% TFA, v/v/v) were prepared. The acetonitrile content in the mobile phase affects on the capacity factors and sensitivity. The retention time decreases and sensitivity increases of 5-MOP as the proportion of acetonitrile in the mobile phase is increased. The use of methanol in the mobile phase is judged inappropriate for determination of 5-MOP under our analytical conditions. Only tetrahydrofuran displayed improved sensitivity characteristics and afforded a good resolution for 5-MOP. The addition of TFA to the mobile phase and variation of the pH of the mobile phase did not affect the capacity factors of the 5-MOP. The optimum composition of the mobile phase was acetonitrile–tetrahydrofuran–water containing 0.07% TFA.

Table 1
Recovery of 5-MOP fortified human serum by LCFL

Serum in μg/ml	5-MOP		
	Added (μg/ml)	Found (μg/ml)	Recovery (%)
Control (Sigma)	1.00	1.01	101.0 (1.0)[a]
Volunteer			
1	0.25	0.20	80.0 (2.4)
2	0.50	0.49	98.0 (4.3)
3	1.00	1.02	102.0 (3.8)

Number of determination ($n = 5$).
[a] Relative standard deviation.

4.2. Linearity, recovery and limit of quantification

The calibration graphs of 5-MOP and 5-GOP show good linearity over the range of 0.05–3.00

μg/ml, the regression equations being $y = 1.6 + 51.0 \times$ (correlation coefficient $r = 0.9998$) and $y = 0.3 + 22.8 \times (r = 0.9992)$, respectively. The limit of quantification are (LOQ) 0.05 and 0.26 ng for 5-MOP and 5-GOP, respectively. The recovery was studied on an essential oil or an emulsion type suntan accelerator, which was shown previously not to contain 5-MOP and 5-GOP. This sample was spiked to levels of 10 μg/ml 5-MOP and 4 μg/ml 5-GOP. Six determinations based on peak height measurements gave a recovery for 5-MOP of 98%, with a relative standard deviation (RSD) of 1.0%, for 5-GOP in a recovery of 104% with a RSD of 0.3%. Table 1 show the LCFL traces obtained for a control and volunteer serum sample spiked with 5-MOP. Recoveries and precision were observed (recoveries ranging from 80.0 ± 2.4 to 102 ± 3.8%).

4.3. Interferences

The composition of bergamot oil consists of a mixture of monoterpene and sequiterpene hydrocarbons, oxygenated derivatives and nonvolatile residue. Terpenes and oxygenated compounds make up 93–96% by weight of the oil, while the nonvolatile residue constituted the remaining 4–7%. The nonvolatile fraction early studies on the composition of psoralens and coumarins [9]. In recent years, there has been much concern about the addition of bergamot oil to sun cosmetics (emulsion) and body cleansing lotion products as accelerator or perfume. The type emulsion and lotion included a lot of surfactant, humectant (glycerin), softening agent emollient (oils), thickening agent (gum, carboxyvinyl polymer), alcohol, small amount perfume and preservatives (methyl paraben, phenoyethanol), purified water and pharmaceutical agents (vitamins). The effect of the ingredients such as anionic surfactant sodium dodecylbenzenesulfonate, non-ionic surfactant Triton X-100, triethanolamine, dimethione, cetyl alcohol, vitamin A palmitate, vitamin E acetate in cosmetic products on the determination of 5-MOP was investigated. These ingredients with fluorometric detection did not appear exceed 20 times of 5-MOP and no interference effects were observed.

Table 2
Analytical results of determination of bergapten (5-MOP) in commercial bergamot essential oil and cosmetics by DPV and LCFL detection

Samples	Concentration (10^{-3}%, w/w)	
	5-MOP	
	DPV (%) $N = 5$[a]	LCFL (%)
Bergamot essential oil		
1	76.000 (5.0)[b]	68.000 (0.5)
2	255.000 (5.0)	250.000 (5.2)
3	28.000 (5.0)	24.000 (3.6)
4	69.000 (2.8)	55.900 (0.8)
5	53.000 (5.0)	54.000 (0.9)
Suntan		
1	–[c]	0.214 (5.8)
2	–[c]	0.199 (0.7)
Body lotion with bergamot oil	–[c]	0.742 (5.0)
Cleansing foam with bergamot oil		
1	–[c]	1.124 (1.5)
2	–[c]	0.925 (3.7)

[a] Number of determination ($n = 5$).
[b] Relative standard deviation.
[c] Not determined.

4.4. Compound stability

Analytes were stored in the dark for 24 h at −20, 28 and 40 °C and the chromatograms were recorded. The peak height remained almost constant over the whole range of temperatures. To determine the effect of light on psoralens stability two bags were prepared, one was protected from the light, the other not; both were maintained at room temperature for 0.5, 2.0, 3.0, 4.0, 6.0 and 24 h. The concentration of psoralens remained almost constant, irrespective of whether or not they are exposed to the light. Psoralens upon irradiation with long-wavelength UV light undergo photodimerization to give cyclobutyl derivatives. [2] Therefore, at UV (366 nm) irradiation the concentration decreased by approximately 3 and 45% for 5-MOP and 5-GOP, respectively, with 1 h.

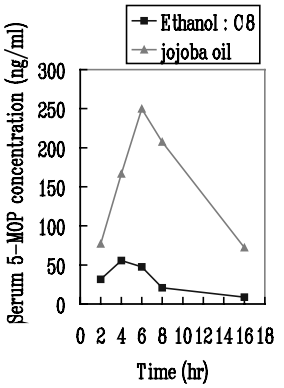


Fig. 1. Time course of percutaneous absorption of 5-MOP after application of a formulation of 125 mg/ml bergamot oil to human skin: (■) application with a solution in ethanol-tricaprylin (C_8) (5:2, w/w); (▲) a oil in jojoba oil.

4.5. Application to bergamot oil and cosmetics

The repeatability of the method was studied on a commercial sample. Five independent measurements gave an average result of 0.171% 5-MOP in bergamot essential oil, and a RSD of 3.9%. In Table 2 the data obtained by LCFL and DPV are compared. In DPV the suntan, body lotion and cleansing foam are not detected probably because they are present in small amount but they are found by LCFL.

5. Application to humans

5.1. 5-MOP percutaneous absorption

The percutaneous absorption of 5-MOP was measured from a oil and ethanol vehicle; the time course of absorption into the human skin is shown in Fig. 1. Peak percutaneous absorption occurred during the first 2.0 h collection interval. 5-MOP was absorbed with maximum concentrations of ca. 250 ng/ml 4–6 h after application of a formation of bergamot oil. At all the time points tested 5-MOP level was significantly higher in the serum of human serum for up to 16 h after of 5-MOP formation compared with time zero The 5-MOP from a tricaprylin in ethanol into skin was much lower than that observed with a jojoba oil as a receptor fluid. 5-MOP is a highly hydrophobic molecule; the higher solubility in the oil facilitates the partition of 5-MOP into skin lipid horny layer (stratum corneum). Fig. 2 is the infrared absorbance spectra of human skin before (a) and after (b and c) contact with bergamot essential oil. The bands at 2915, 2847 and 1732 cm^{-1} are due to C–H and C=O vibrations, respectively. The strong C=O band in the 1700 cm^{-1} range is mainly due to the C=O bonds present in the psoralens. The intensity of the C=O bond in the skin spectra after essential oil contact is much lower in the C–H

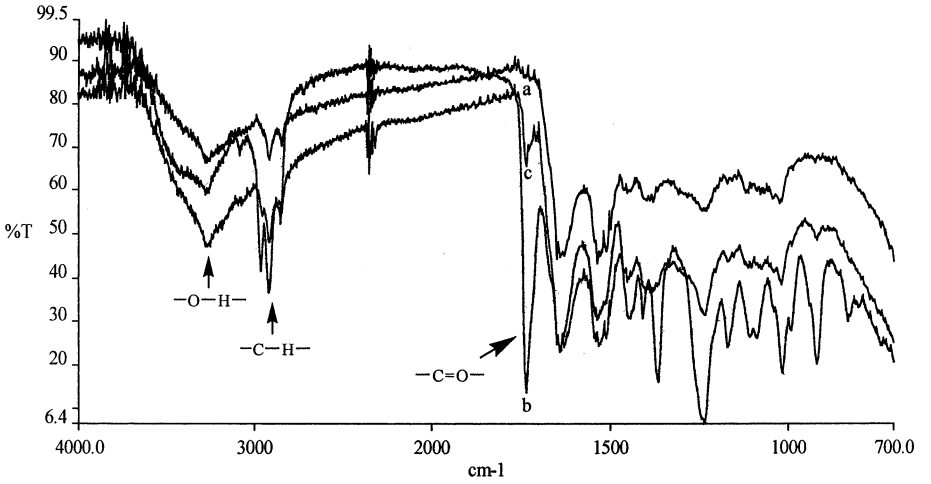


Fig. 2. ATR-FTIR spectra of human skin treated with 10.6 mg/cm^2 bergamot essential oil (BO) from 700 to 4000 cm^{-1}: (a) untreated BO (b) at 30 min for C=O characteristic (c) at 90 min for C=O characteristic.

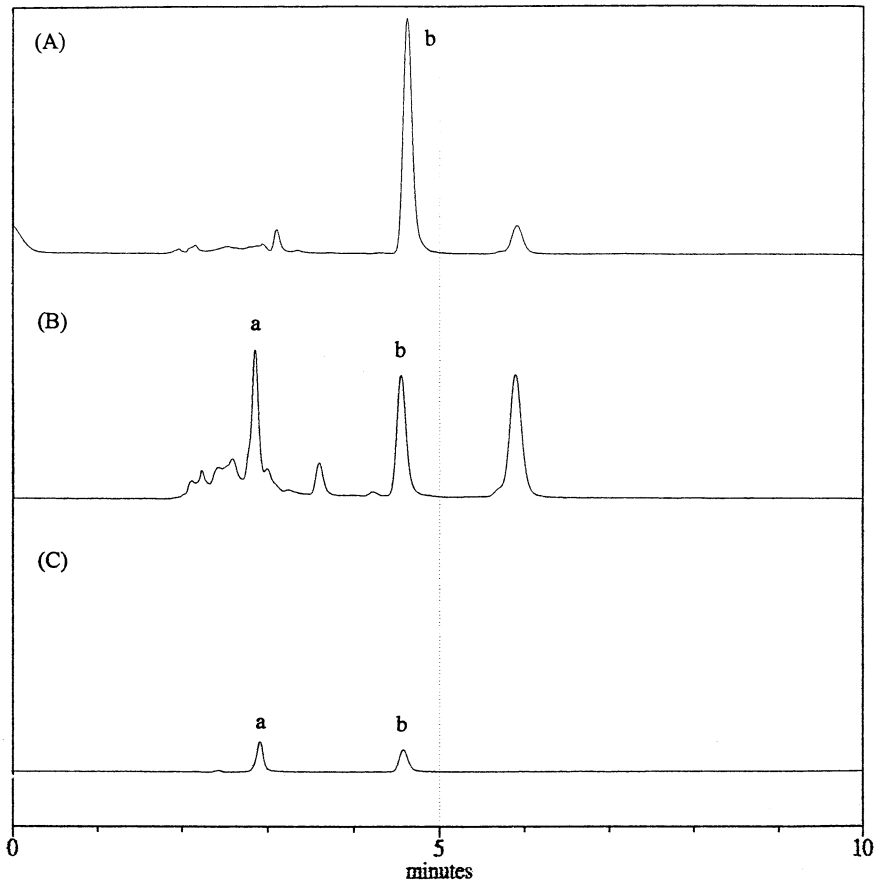


Fig. 3. Chromatograms obtained by LCFL from the (A) before and (B) after application with bergamot essential oil of human serum (C) calibration standard, 200 ng/ml of 5-MOP and internal standard (I.S.) of bergamotin. Peak a is the 5-MOP and peak b is the bergamotin. Stationary phase, μBondapack C_{18} (3.9 mm × 30 cm); mobile phase, acetonitrile–tetrahydrofuran–water (70:15:15, v/v/v), containing 0.07% TFA; flow rate, 1.0 ml/min; fluorescence

bonds. It is, therefore, concluded that the adsorbed psoralen is largely responsible for the C=O bond in the spectra of essential oil treated skin. Two hours after essential oil application, the essential oil components had penetrated the stratum corneum; this could be demonstrated by the lack of characteristic C=O band of the applied essential oil in the skin print.

5.2. Blood analysis of 5-MOP

Fig. 3 shows chromatograms obtain from a blank human serum, blank serum spiked with 5-MOP and a serum sample from a human with received a single application 385 ng/cm^2 of 5-

Table 3
After 4 h treatment of bergamot oil to human skin from healthy adult and control subjects

Serum concentration (ng/ml, $n = 6$)[a]			
Subjects		5-MOP	
Number	Sex	FL (%)	UV (%)
1	F	235.0 (0.3)[b]	207.9 (6.9)
2	F	147.0 (1.3)	143.0 (1.7)
3	M	162.0 (2.0)	241.1 (13.0)
4	M	172.4 (2.2)	191.0 (3.5)
5	M	84.7 (2.6)	141.8 (3.1)
6	Control	–	–

[a] Number of determination.
[b] Relative standard deviation.

MOP. 5-MOP and 5-GOP (internal standard) were eluted as well resolved sharp peaks with retention times of 2.9 and 4.6 min, respectively. The samples were processed in the same manner as described for 5-MOP. Table 3 summarizes the results with these samples by LCFL and LCUV methods. The mean concentration of 5-MOP in the application bergamot oil was 160 ng/ml, this value might be increased after treatment.

6. Conclusions

The most profound advantage of fluorescence detection is sensitivity. Although any sensitivity comparison depends on the type of molecule being detected, if a molecule is amenable to fluorescence detection in general, detection limits will be 2–3 orders of magnitude lower than detection limits normally seen with an absorbance detector. The improvement in the detection limit of 5-MOP in HPLC with a β-CD cross-linked column require a balance between the β-CD and methanol contents of the mobile phase. A procedure is presented here for a rapid and routine analysis of 5-MOP and 5-GOP in various cosmetics and human serum, based on HPLC separation and subsequent fluorometric detection. Each determination requires a total time of less than 5 min. The measurement of 5-MOP of percutaneous absorption confirmed the usefulness of the proposed method for routine screening of phototoxic patients. The use of liquid-liquid extraction is much simpler and shorter. Thus, the presented method is simple and can be directly used for monitoring of the 5-MOP serum levels in phototoxic and psoriatic patients undergoing psoralen and long-wavelength ultraviolet and psoriatic light (PUVA) therapy. The clean-up procedure for crude extracts is very easy and efficient. Further work will be done to examine the 5-MOP contents of various essential oils and cosmetics, together with the phototoxic and photomutagenic activities.

Acknowledgements

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