Journal of Chromatography, 563 (1991) 407–413
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5646

Short Communication

High-performance liquid chromatographic determination of citropten and bergapten in suction blister fluid after solar product application in humans[a]

S. MAKKI*

Department of Analytical Chemistry, Faculty of Medicine and Pharmacy, 2 Place St. Jacques, 25030 Besancon (France)

and

P. TREFFEL, P. HUMBERT and P. AGACHE

Department of Dermatology, Hôpital St. Jacques, 2 Place St. Jacques, 25030 Besancon (France)

(First received April 3rd, 1990; revised manuscript received September 29th, 1990)

ABSTRACT

Citropten (5,7-dimethoxycoumarin) and bergapten (5-methoxypsoralen) are present in bergamot oil which is used as a tanning cosmetic product. The aim of this study was to quantify, using high-performance liquid chromatography, the amount of citropten and bergapten in the skin after suntan products application (an emulsion and an oil formulation). A suction blister technique performed of the anterior aspect of the forearm permitted the collection of these two accumulated molecules. Fluorometric and ultraviolet detections were used for citropten and bergapten determinations, respectively.

INTRODUCTION

Tanning cosmetic products are increasingly used to stimulate human skin pigmentation. One of the substances most frequently used to produce skin-tanning is the essential oil of bergamot [1]. It contains different compounds, including citropten [5,7-dimethoxycoumarin (5,7-DMC)] and bergapten [5-methoxypsoralen (5-MOP)] [2]. Some authors have stressed that this oil together with solar exposition, stimulates melanogenesis, therefore protecting the skin against erythema and even cutaneous cancers. This is the concept of photochemoprotection [3]. On the other hand, citropten and bergapten are known to be photomutagenic and phototoxic [4,5]: citropten is less toxic than bergapten. To our knowledge, citrop-

[a] This paper was presented at *5èmes Journées Nationales de Chimie Analytique, Paris, 4–8 Décembre 1989.*

0378-4347/91/$03.50

ten and bergapten skin absorption and quantification in biological fluids has never been reported in the literature. The present work was carried out to assess the amount of these two non-radiolabelled molecules in the dermo-epidermal junction after application of two galenic forms (emulsion and oil). The interstitial fluid was collected using a suction blister technique [6], followed by high-performance liquid chromatographic (HPLC) determination of the compounds in the blister fluid.

EXPERIMENTAL

Reagents and chemicals

5-Methoxypsoralen, 5,7-dimethoxycoumarin, 8-methoxypsoralen (8-MOP) and 4,5',8-trimethylpsoralen (trioxsalen or TMP) were supplied by the Sigma (St. Louis, MO, U.S.A.). Analytical-reagent grade petroleum ether and other reagents and solvents were obtained from Prolabo (Paris, France) and Carlo Erba (Milan, Italy), respectively.

The composition of the water/oil (W/O) emulsion was: water 60%, petroleum jelly 7.5%, isopropyl myristat 6%, fatty acid ester 10%, sun screens 5%, glycerin 3%, propyleneglycol 1%, groundnut oil 3%, essential oil of bergamot, vitamin E, vitamin A and preservatives in amounts to produce 100%.

The composition of the oil was: groundnut 68%, isopropyl myristat 25%, sun screens 5%, essential oil of bergamot, vitamin E, vitamin A and preservatives in amounts to produce 100%.

Apparatus

A Merck-Hitachi HPLC apparatus (655-A11) (Nogent-sur-Marne, France) with a UV detector (655-A22), a fluorometric detector (F-1000) and an integrator (D-2000) was used. The precolumn and column were of the type RP8 (Merck 125-4 Lichrosphère® 100, 5 μm). UV spectra were recorded using a photodiode array detector Merck-Hitachi (L-3000) and a Sanyo (D-6000) DAD manager computer. The compounds were chromatographed at room temperature. The chromatographic eluents were degassed in an ultrasonic bath. The injected amount was standardized at 20 μl and the flow-rate was set at 1 ml/min. The chromatographic conditions were as follows: for citropten determination, modified HPLC method of Bettero and Benassi [7], fluorometric detection (wavelength excitation 330 nm and wavelength emission 430 nm), eluent of methanol–water (70:30); for bergapten determination, modified HPLC method of Stolk [8], UV detection (258 nm), eluent of methanol–water (60:40). Chromatograms of the two compounds are shown in Figs. 1 and 2. All weighings were performed with a Mettler AE 260 balance.

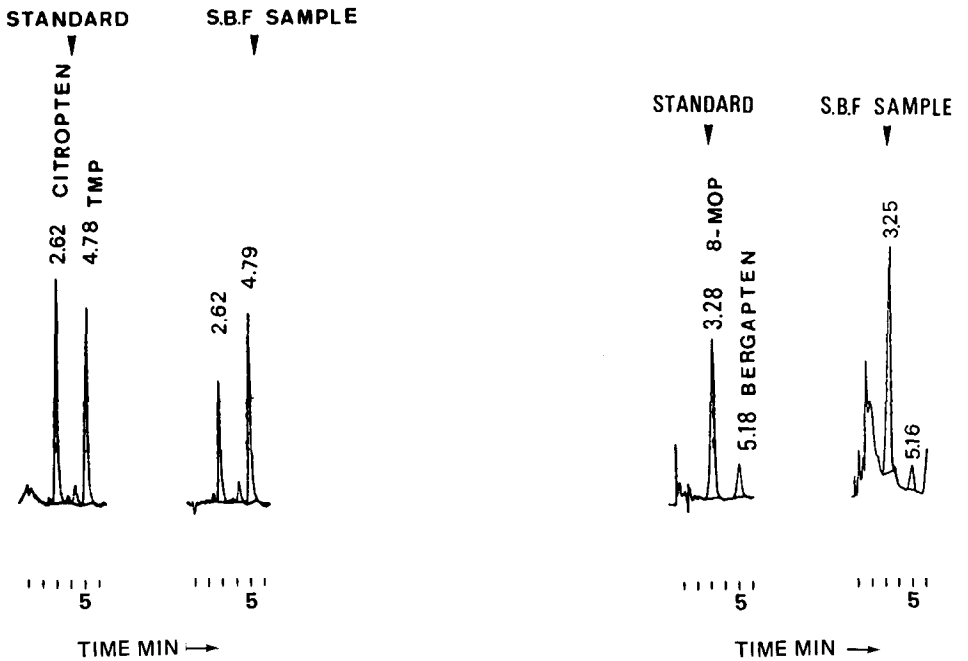

Fig. 1. Chromatograms of (right) an SBF sample and (left) a standard containing to 20 ng/ml citropten. TMP was used as an internal standard. The small middle peak was due to an artifactual substance contained in the internal standard. The chromatographic conditions were: modified Bettero and Benassi HPLC method [7]; fluorometric detection (excitation at 330 nm and emission at 430 nm); eluent, methanol–water (70:30).

Fig. 2. Chromatograms of (right) an SBF sample and (left) a standard containing 80 ng/ml bergapten. 8-MOP was used as an internal standard. The chromatographic conditions were: modified Stolk HPLC method [8]; UV detection at 258 nm; eluent, methanol–water (60:40).

Standard preparation

The standards were prepared by diluting drug-free human serum 1:3 with saline water (9 g/l), giving a concentration of proteins close to that usually found in suction blister fluid (SBF) [6], to obtain concentrations 5, 10, 20 and 40 ng/ml for citropten and 40, 80, 160 and 320 ng/ml for bergapten. The two compounds were simultaneously added to 1-ml diluted human serum aliquots. Furthermore, reference solutions were daily prepared at 20 and 160 ng/ml, respectively, for citropten and bergapten.

Suction blister fluid collection

This experiment was carried out on eleven apparently healthy volunteers, seven women and four men, aged 19–52 years (27 ± 8 years).

Before application of the suction blister device, the cosmetic products were deposited on the volar aspect of subject's forearms. The oil and emulsion were

randomly allocated either on the left or the right forearm. The deposited amounts correspond to the standard cosmetic applications (3.2 ± 0.5 mg/cm^2 for the emulsion and 1.4 ± 0.5 mg/cm^2 for the oil). The suction blister system was then applied on the treated areas with a depression of 0.47 bar. Three groups of blisters (n = 7) were made on each forearm. As soon as possible after blister formation (average time, 100 ± 20 min) the interstitial fluid of the first group was sampled. Similarly the second and the third groups were sampled at 160 and 220 min, respectively.

Extraction procedure

A 0.6 ± 0.2 ml volume of SBF collected for seven blisters was mixed with a 5-ml aliquot of petroleum ether containing 140 ng/ml TMP and 200 ng/ml 8-MOP, subsequently used as internal standards for HPLC quantification of citropten and bergapten, respectively. The solution was stirred for 10 min in a hermetic container and then centrifugated for 7 min at 1700 g before separation and filtration on a 0.45-μm filter. Extraction recovery was 98%. After the extraction procedure the samples were ready for citropten determination. For bergapten, the organic phase (petroleum ether) was evaporated to dryness in a water-bath at 35°C under a nitrogen stream. The residue was redissolved in 50 μl of absolute ethanol, shaken on a vortex and chromatographed. The internal standard concentrations were obtained by dissolving 7 mg of TMP and 10 mg of 8-MOP in 500 ml of petroleum ether, and using an ultrasonic bath to facilitate the dissolution. The final working solution was obtained by diluting the first solution 100-fold. These solutions were stored at 4°C.

Statistical analysis

All the results were expressed as mean ± S.D. Linear regression analysis was performed using the least-squares method on the overall experimental data. Analysis of variance was carried out on the experimental data.

RESULTS

Sensitivity and analytical recovery

Citropten and bergapten peaks were characterized by obtaining the UV spectra of the different eluted peaks completed by the standard addition method.

The linear regression of the calibration curve of citropten was as follows: $y = 0.084 + 0.045x$, $r = 0.9955$ ($p < 0.001$), where x is the citropten concentration (ng/ml) and y the peak-area ratio (citropten/TMP). The regression linearity was verified by variance analysis. The mean and the inter-assay coefficient of variation (n = 10) were, respectively, 20.89 and 9.5% for a 20 ng/ml reference solution. The theoretical detection limit was less than 1 ng/ml at a signal-to-noise ratio of 3: experimentally we assessed 8 ng/ml SBF.

The linear regression of the calibration curve of bergapten was as follows: y =

$5.597 \cdot 10^{-3} + 1.368 \cdot 10^{-3} x$, $r = 0.9939$ ($p < 0.001$), where x is the bergapten concentrations (ng/ml) and y the peak-area ratio (bergapten/8-MOP). The mean and the inter-assay coefficient of variation ($n = 10$) were, respectively, 166.1 and 6.5% for a 160 ng/ml reference solution. The theoretical detection limit was 20 ng/ml at a signal-to-noise ratio of 3: experimentally we assessed 19 ng/ml SBF.

Suction blister technique

The SBF concentrations show a non-significant increase in citropten and bergapten *versus* time following both application routes (Table I). Furthermore, the results show that citropten SBF concentrations were higher when the emulsion was applied ($p < 0.05$). No results were less than the detection limit for citropten, but 18 out of the 66 determinations of bergapten were below its threshold value. They were recorded as negative.

DISCUSSION

To our knowledge, only three studies have been published concerning percutaneous absorption using a suction blister technique. Agren [9] thought that the stratum corneum barrier function following blister formation was damaged. Using a suction blister technique, Huuskonen *et al.* [10] studied 8-MOP penetration during a PUVA (psoralen + ultraviolet A) bath therapy: 8-MOP could be detected in SBF following a bath at 0.4 ppm of 8-MOP. Averbeck *et al.* [11] detected concentrations up to 1.2 μg/ml in SBF from patients treated with 60 μl of an ethanolic solution of 5-MOP at 50 ppm. Such a penetrated amount of a test substance would represent nearly half the deposited mass, which seems surprising

TABLE I

CITROPTEN AND BERGAPTEN CONCENTRATIONS IN SBF

Application in a W/O emulsion (3.2 ± 0.5 mg/cm²) or an oily formulation (1.4 ± 0.5 mg/cm²). Values in ng/ml.

Application	Concentration applied (ppm)	Concentration found (ng/ml)			
		100 min	160 min	220 min	Mean ± S.E.M.
Bergapten emulsion	29	37.9 ± 29	51,.0 ± 48	62.9 ± 48	50.6 ± 24
Bergapten oil	32	18.1 ± 32	28.0 ± 17	22.4 ± 22	22.8 ± 14
Citropten emulsion	19.5	31.1 ± 16	35.5 ± 25	44.9 ± 21	37.2 ± 12
Citropten oil	37	22.7 ± 13	27.2 ± 17	26.8 ± 18	25.6 ± 9

and questionable. Suction blister may be an interesting and useful technique to compare percutaneous absorption of non-radiolabelled compounds *in vivo* in humans.

Our results show that citropten absorption was enhanced when the vehicle was an emulsion. This was not confirmed for bergapten, which is not in agreement with previous works dealing with 8-MOP [12], TMP [13] or 5-MOP skin absorption [14]. The high S.D. mean values of bergapten SBF concentrations (Table I) probably hinder the differentiation of both galenic forms. High S.D. values could be explained by inter-individual variations or/and the lack of reproducibility of the suction blister technique. Performing repeated blisters on the same skin area is very invasive. Consequently the reproducibility cannot be studied as a possible cause of high S.D.

The coefficients of variation (C.V.) of the reproducibility of citropten and bergapten HPLC determination (9.5 and 6.5%, respectively) were high compared with the theoretical level (below 5%). The cause could have been the low concentration of the reference solutions used. The detection limit of citropten determination could be decreased to *ca.* 50 pg/ml by addition of a condensing step. PUVA therapy routinely requires plasma or SBF 5-MOP or 8-MOP determinations, using the HPLC technique with UV or fluorometric detectors [15,16]. In bergamot oil, the presence of citropten, a highly fluorescent compound with a retention time nearly the same as that of bergapten hinders the fluorometric detection of the latter. Consequently we used fluorometric detection for citropten and UV detection for bergapten. Indeed, we did not manage to assess simultaneously bergapten and citropten, as reported by Bettero and Benassi [7]. They used a Perkin-Elmer LS-4 fluorometric detector. This is a very powerful but expensive detector. Unlike Bettero and Benassi [7], who assessed the compounds in cosmetic products with the aid of tetrahydrofuran, we used petroleum ether as extraction solvent, because we performed our determinations in biological fluids. Moreover, methanol–water was preferred to acetonitrile–water as the mobile phase because of its toxicity. Bettero and Benassi's detection limits were 0.5 and 5 ng/ml for citropten and bergapten, respectively.

CONCLUSION

The method presented here is not entirely new, but no analytical procedure has been described in the literature for citropten determination in human biological fluid. The high fluorescence of citropten permits simple routine HPLC analysis with an accurate determination and a very low detection limit. Furthermore, we have demonstrated that the suction blister technique can be used to compare percutaneous absorption of several non-radiolabelled compounds *in vivo* in humans.

REFERENCES

1 S. T. Zaynoun, B. E. Johnson and W. Frain-Bell, *Contact Dermatitis*, 3 (1977) 225.
2 M. Herpol-Borremans, M. O. Masse and R. Grimee, *J. Pharm. Belg.*, 40 (1985) 147.
3 T. B. Fitzpatrick, in T. B. Fitzpatrick, P. Forlot, M. A. Patack and F. Urbach (Editors), *Psoralens: Past, Present and Future of Photochemoprotection and other Biological Activities*, John Libbey Eurotext, Paris, 1989, pp. 5–10.
4 M. J. Ashwood-Smit, G. A. Poulton and M. Liu, *Experientia*, 39 (1983) 262.
5 D. Averbeck and E. Moustacchi, *Photochem. Photobiol.*, 31 (1980) 475.
6 U. Kiistala, *J. Invest. Dermatol.*, 50 (1968) 129.
7 A. Bettero and C. A. Benassi, *J. Chromatogr.*, 280 (1983) 167.
8 L. M. L. Stolk, *Pharm. Weekblad Sci. Ed.*, 2 (1980) 29.
9 M. S. Agren, *Dermatologica*, 180 (1990) 36.
10 H. Huuskonen, L. Koulu and G. Wilen, *Photodermatology*, 1 (1984) 137.
11 D. Averbeck, S. Averbeck, J. Blais, A. Moysan, G. Huppe, B. Morliere, P. Prognon, P.Vigny and L. Dubertret, in H. I. Maibach and N. J. Lowe (Editors), *Models in Dermatology*, Vol. 4, Karger, Basel, 1989, pp. 5–11.
12 J. Gazith, W. Schalla, E. Bauer and H. Schaefer, *J. Invest. Dermatol.*, 71 (1978) 126.
13 N. Vaatainen, *Acta Derm. Venereol. (Stockholm)*, 60 (1980) 327.
14 D. A. W. Bucks and H. I. Maibach, in T. B. Fitzpatrick, P. Forlot and F. Urbach (Editors) *Psoralens: Past, Present and Future of Photochemoprotection and other Biological Activities*, John Libbey Eurotext, Paris, 1989, pp. 111–116.
15 L. M. L. Stolk, W. Westerhof, R. H. Cormane and P. A. van Zwieten, *Br. J. Dermatol.*, 105 (1981) 415.
16 P. Prognon, G. Simon and G. Mahuzier, *J. Chromatogr.*, 272 (1983) 193.