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Short communication

LC analysis of benzophenone-3 in pigskin and in saline solution
Application to determination of in vitro skin penetration

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1. Introduction

The use of sunscreens has increased with awareness of the detrimental effects (erythema, skin cancers) of UV light on human skin. In Europe sunscreens are classified as cosmetics, whereas in the USA they are OTC drugs. When a high sun protection factor in sunscreens is desired, benzophenone derivatives, most notably benzophenone-3 or oxybenzone, are usually employed. Oxybenzone is currently used in many cosmetic formulations on the market, such as lipcares, sunscreen lotions or emulsions, shampoo and hair sprays. The efficacy of a sunscreen formulation depends on low penetration profiles and high photoprotection. These characteristics are important for cosmetic sunscreen safety. Sunscreens are applied repeatedly to exposed skin over a large surface area — approximately 1.8 m^2 for a 70-kg adult [1] There is little but recently published data describing their permeation through the animal, human skins or synthetic membrane and it is important to know how much penetrates. In these studies, preferentially formulations (emulsions: oil in water or water in oil, gel and lotions) were investigated in vivo or in vitro [2–5]. These preparations contained a combination of various ingredients and some of them are established absorption enhancers (ethanol, sodium lauryl sulfate, Tween 20, fatty alcohols) [6,7] and in these cases it is difficult to generalize findings from one to all preparations. The vehicle used is a factor influencing percutaneous absorption: it may enhance or block the movement of the UV-filter

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through the skin. Very recently Brinon et al. [8] have demonstrated that the transcutaneous fluxes of sunscreens was modulated by a liquid crystalline phase. In the literature mineral solvents are preferentially used (petrolatum jelly, paraffin, liquid paraffin) [3,9,10] but oxybenzone is poorly soluble in these products [11–13]. In this work the vehicles used to deliver oxybenzone to the skin surface were three different solvents (non-polar and semi-polar) used in dermatology: coconut oil (natural oil), capric-caprilic triglyceride (neutral oil, with high skin compatibility), and propylene glycol. These solvents were chosen as vehicles in order to achieve sufficiently high solubility.

The examination of the literature revealed some existing chromatographic methods for determination of oxybenzone or others UV-filters but they are applied for the analysis of cosmetic preparations (frequently emulsions). These methods are designed predominantly for product evaluation, and where LC method is applied to determine concentration and separation of sunscreening agents in cosmetic preparations [14–18]. In commercial sunscreen products, the concentration in sunscreen agents is very important. For formulations, a step of cleanup is not always necessary and sample preparations require extraction step and dilution. Other papers report the development of reversed-phase LC assay for quantifying oxybenzone in biological fluid including bovine serum albumin [19]. An interesting novel technique using a solid-phase microextraction combined with a gas chromatography-quadrupole ion trap GC-MS is also adapted for water and human urine [20]. Literature dealing with the determination of benzophenone-3 in tissues provides very few data on tissues, Kadry et al. [21] have developed and validated a LC method for identification of benzophenone-3 in the liver and kidney of rat. This study was performed to investigate the pharmacokinetic of benzophenone-3 after oral administration. This work is focused on the oxybenzone quantification in saline solution and in skin after in vitro dermal application of benzophenone-3 in various solvents. Due to the complexity of the skin matrices, artifacts arising from the interaction or due to environmental factors tend to complicate the analysis. The second major difficulty particularly in tissues is the ability to quantify low levels of the drug. In this field this ability is often viewed in terms of purification and limit of detection, and clean-up is performed using solid-phase extraction cartridges. The aim of the present work was to develop an easy and adapted LC analytical method and validation procedure for the determination of oxybenzone levels in pigskin ear and saline solution. This method was applied to determine the amounts of oxybenzone from 'in vitro' transdermal experiments.

2. Experimental

2.1. Reagents and chemicals

Oxybenzone or benzophenone-3 (2-hydroxy-4-methoxybenzophenone), was used without further purification (stated purity 99.5%, Sigma, St. Louis, MO). Standard stock solutions: the compound was weighed (accuracy of 20 mg) and dissolved in 100 ml of methyl alcohol and ethyl alcohol (99.9%, Carlo Erba RPE-ACS, FarmaItalia, Milano, Italy). Three standard stock solutions were prepared. Different working solutions of oxybenzone were prepared in the range 0.16–4 mg l^{-1} in methyl alcohol (R.P. Normapur, Prolabo, Fontenay s/bois, France) and stored at -4°C in the dark. Standard stock and work solution were also prepared in hexane (R.P. Normapur, Prolabo, Fontenay s/bois, France) for recovery studies. Saline solution was prepared with 9‰ (W/W) NaCl (Merck, Darmstadt, Germany). LC mobile phase was methyl alcohol (99.9%, Carlo Erba RS HPLC, FarmaItalia, Milano, Italy) and freshly redistilled water. Extractions were performed with hexane and ethyl acetate (Normapur, Prolabo, Fontenay s/bois, France), and dried with anhydrous sodium sulfate (Normapur, Prolabo, Fontenay s/bois, France) stored at 90°C. Silica gel disposable extraction cartridges (6 ml, 500 mg, SDS, Valdonne, France) were used for chromatography. The chosen solvents are capric-caprilic triglycerides (Miglyol 812, Condea), coconut oil (Laboratory CPF, Melun) and propylene glycol (Prolabo).

2.2. Apparatus

A Uvikon 922 spectrophotometer (Kontron Instrument) was used to record the absorption spectrum of oxybenzone in methanol. Ultra Turrax (Ika-Tron labortechnik) was used for extraction. The LC system consisted of a Beckmann 344 System with a variable wavelength detector (set at 287 nm). The chromatograph was equipped with a Merck Lichrosph® 60 RP select B (250 mm × 5 μm) column. The mobile phase was isocratic methanol–water (92:8, v/v) with a flow rate of 0.75 ml min^{-1}. The injection volume was always 20 μl. Franz vertical glass diffusion cells were used. The area for diffusion was 0.785 cm^2 and the receptor chamber volume varied from 10.2 to 10.8 ml.

2.3. LC methodology

Quantitative analysis was performed using an external standard, which further simplified sample preparation.

2.3.1. Statistical tests and validation procedure

Linearity of detector response was checked using increasing amounts of oxybenzone (with two different initial standard solutions). Peak areas and retention times were measured and a calibration curve plotted. Linear regression analysis and test result evaluation was made using the appropriate statistical methods with Statgraphics software [22].

Detection limit (DL). Several approaches for determining the detection limit are possible. Those based on the standard deviation (S.D.) of the blank sample were used [23]. Measurement of the magnitude of background noise was made by analyzing three blank samples injected in triplicate and calculating the S.D. of the responses $DL = \bar{X} + 3S_{blank}$

The quantification limit (QL) may be expressed as: $QL = \bar{X} + 10S_{blank}$

2.3.2. Analysis of spiked sample — recovery

To determine method efficiency, untreated samples were fortified with known amounts of analytical standards dissolved in methyl alcohol for saline solutions and in hexane for pigskin. Three different concentration levels were used, covering the range to be determined. Percentage recovery was calculated for each [24]. Each analysis was performed in duplicate or triplicate and injected in triplicate.

2.4. Saline solution, preparation, extraction procedures and analysis

Twenty millilitres of saline solution was spiked (0.2–1 mg l^{-1}) with standard solution in methyl alcohol. The oxybenzone was extracted successively with 20–15–10 ml hexane. The organic phases were combined, filtered through anhydrous sodium sulfate, then evaporated to dryness and taken into in methyl alcohol (20 ml). The final step was LC analysis under the conditions described above. Three concentrations were analyzed in duplicate.

2.5. Pigskin ear, preparation, extraction procedures and analysis

Pigskin and particularly pig ear resembles human skin morphologically and functionally, and percutaneous permeation rates appear to be similar [25]. Furthermore this model is useful for its availability and ease of preparation, in contrast to using human skin obtained from surgery or cadavers, which is normally not frequently available and which is quite often already damaged. Pig ears were obtained from a slaughterhouse from freshly-killed animals (within 4 h of death). After cold water cleaning and shaving, full thickness skin (about 1 0.1 mm) was removed with a scalpel from the cartilage of the outer region. Only intact skin discs with 3-cm inner diameter were kept and sealed in plastic bags [26] then stored at −20°C until use. For in vitro tests, the maximum storage time under these conditions to ensure maintenance of skin integrity was shown to be 1.5 months.

A piece of pigskin (approximately 0.2 g) was placed in a 10-ml bender pot. Spiked samples (10–45 mg kg^{-1}) were usually left for 2 h before analysis to ensure even distribution of oxybenzone after solvent evaporation [27]. Spiking levels were

based on previous works. The first step was extraction with 10, 7, 5 ml of methanol for 3, 3, 3 min at 15 000 rpm with the Ultra-Turax. The organic phases were combined, filtered, and evaporated to dryness in a rotary evaporator, with water bath at 30°C. The second step was a clean-up. A silica column was washed with 5-ml hexane-ethyl acetate (70:30, v/v) and 5-ml hexane, which were rejected. Eluate 1 (E1): the residue in the flask was rinsed successively with 3 and 3 ml hexane and placed on the column. Eluate 2 (E2): the column was then eluted with 5 ml hexane-ethyl acetate (90:10, v/v). This elute (which contains the oxybenzone) was evaporated just to dryness under air. Exactly 2 or 5 ml of methyl alcohol were added. This elute could be analyzed directly. For repeatability studies, each experiment was performed in triplicate.

2.6. Diffusion studies

Percutaneous absorption could be assessed in vitro using a diffusion cell skin absorption model. The 'static diffusion Franz-type cells' diffusion cell consists of donor and receptor chambers between which the skin is positioned [28]. It is nowadays desirable to replace human skin with animal skin, and pig epidermal membrane is a relevant model for in vitro percutaneous absorption studies [29–32]. The receptor fluid must not adversely affect the barrier properties of the skin or the physicochemical properties of oxybenzone. Physiological saline solution was used for this work.

Skin penetration was measured using static diffusion Franz vertical cells. A portion of pigskin (3 cm^2) was placed as a barrier between the two halves of a horizontal skin permeation system. The dermis side of the skin was in contact with the receiver compartment and stratum corneum with the donor compartment. The area available for diffusion was 0.785 cm^2 which correspond to $0.217\ g \pm 0.056$ of skin and the receptor chamber volume varied from 10.2 to 10.8 ml. The receptor chamber was maintained at 37.0 ± 0.5°C throughout the experiment. The receptor chamber content was continuously agitated by a small bar magnet. Oxybenzone in these conditions is readily soluble in the receptor fluid (4.7 ± 0.2 μg ml^{-1} ($n = 6$)).

The diffusion cell was prepared to equilibrate for 1 h before application of the oxybenzone formulation. The quantity of oxybenzone applied to the surface was 2.00 mg cm^{-2}, the product was spread uniformly over the whole area using a digital pipette. At the end of each application time the receptor fluid was removed, the skin surface was washed four times with 5 ml ethanol, 5 ml distilled water, 5 ml methanol, 5 ml distilled water. Analyses were performed on the exposed skin area and only on this part.

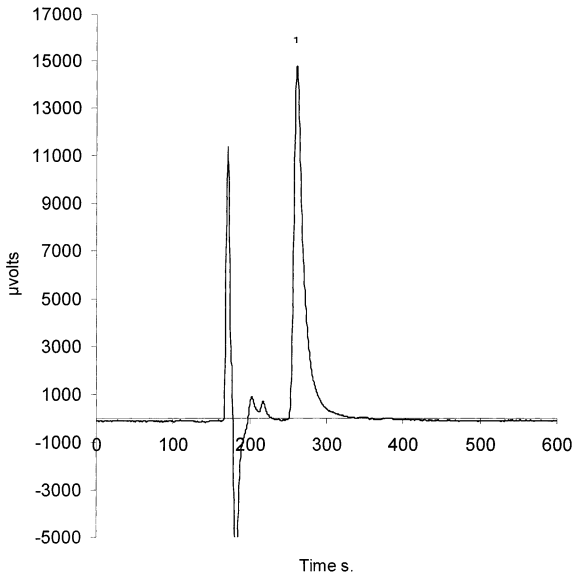
3. Results and discussions

3.1. Absorption spectrum and LC analysis of benzophenone-3

The absorption spectrum was recorded between 210 and 360 nm. The spectrum showed maxima at 242, 287 and 326 nm and minima at 230, 263 and 309 nm. The strongest maximum was chosen for LC analysis, in the case 287 nm.

Before commencing this study, we recorded the retention time of oxybenzone by injecting an oxybenzone standard solution and matrix with oxybenzone. Under the conditions described above, the 95% confidence limits are given by $\bar{X} \pm t_{(\alpha,v)}(s/\sqrt{n})$ [33], 4.29 ± 0.17 with $n = 15$. A chromatogram is shown in Fig. 1.

Next linearity across the range previously specified must be confirmed [34]. The condition of homoscedasticity of variances was checked using the most common method — Cochran's C test. For this procedure, the test statistics were $C = 0.38$, $P = 0.23$, eight levels. The variances between the groups were not found to be significantly different, which is a precondition for the regression test. The best-fit equation was $y = 112354.5x + 862.5$, where $y =$ peak area (dependent variable) and $x =$ mg l^{-1} of oxybenzone (independent variable). The R-squared statistic (R^2) indicated that the model explains 99.7% of the variation in peak area. All regression parameters are listed in Table 1. An analysis of variance (ANOVA, Table 2) on data allowed a



Ref.	Nom	Tp.R	S(µV*s)	H(µV)
1	oxybenzone	4,358	223545	14788

Fig. 1. Chromatogram of oxybenzone (2 mg l^{-1} — work solution in methyl alcohol). Column Merck Lichrosph® 60 RP select B (250 mm × 5 µm), 287 nm.

linear regression between peak area and oxybenzone concentration to be calculated. A statistically significant relationship between peak area and concentration in oxybenzone was found, P-value < 0.001. The P-value for the lack of fit test in the ANOVA table was 0.8, so the model appears to be adequate for the observed data.

The calibration curve for oxybenzone showed a linear response over the range 0.16–4 mg l^{-1}. A linear regression equation applied to the results should have an intercept not significantly different from zero. This was obtained (T-statistic = 0.44, DF = 24, P-value = 0.66), demonstrating that there is no effect on the accuracy of the method.

3.2. Recovery rates and expression of results

Untreated samples were analyzed in triplicate in each case and are reported in Table 3. Analysis of control blanks is very important for accurate qualitative and quantitative results. From these assays, the sensitivity of the method was assessed, and the limit of determination and quantification of oxybenzone in saline solution and in pig ear were calculated. These values are also reported Table 3.

The recovery rates for saline solution and pigskin for the method described above are summarized in Table 4. The method must be validated over the whole concentration range, and the

Table 1
Linearity of detector response to oxybenzone — saline solution and tissue recovery

	Linearity of detector response	Linearity of recoveries (saline solution)	Linearity of recoveries (skin)
Range used (µg ml^{-1})	0.16–4	0.2–1	10.4–45.9
DF (total)	24	13	24
b (slope)	112354.5	0.87	0.83
Standard error S_b	1183.0	0.023	0.03
RSD_b ($S/\bar{X}$)	0.01	0.02	0.03
a (intercept)	862.5	0.015	1.11
Standard error S_a	1957.9	0.014	0.93
RSD_a ($S/\bar{X}$)	2.2	0.9	0.8
R^2	0.99	0.99	0.97
Standard error of estimate, $S_{y,x}$	6884.76	0.03	1.97
Slope, 95% confidence intervals (with appropriate t value)	[+109 906 − +114 801]	[+0.82 − +0.92]	[+0.76 − +0.89]
Intercept, 95% confidence intervals (with appropriate t value)	[−3188.4 − +4913.3]	[−0.016 − +0.046]	[−0.82 − +3.05]

Table 2
Analysis of variance with lack-of-fit

Source	Sum of squares	DF	Mean square	*F*-Ratio	*P*-value
Model	4.27523×10^{11}	1	4.27523×10^{11}	9019.47	0.0000
Residual		24			
Lack-of-fit	1.62466×10^{8}	6	2.70777×10^{7}	0.50	0.8003
Pure error	9.75133×10^{8}	18	5.41741×10^{7}		
Total (corr.)	4.2866×10^{11}	25			

amount found was plotted against the amount added. Several additions of known different concentrations were made. To determine the slope of the regression line (the results obtained are a function of known quantities of oxybenzone added to the matrix). All data are reported in Table 1. The homogeneity of variances was checked. In each case, for recoveries from saline solution and pigskin, the variances between levels 3 and 9 were not found to be significantly different (P-value > 0.05). Saline solution: C-value $= 0.42$, P-value $= 0.90$. Pig ear. C-value $= 0.37$, P-value $= 0.23$. In these recovery experiments, the two matrix slopes were estimated, giving 0.87 and 0.84 for saline solution and skin, respectively. In each case, the intercept was not significantly different from zero. Recoveries were determined by external standard methods and they attained close to 88–98 and 83–92% for saline solution and pig ear, respectively. From these recovery rates, it can be concluded that this procedure can be used to provide reliable quantitative determination of this UV-filter in saline solution and pig ear.

3.3. Diffusion study

With the previous conditions, the quantity of product applied to the surface of skin was 2 mg cm^{-2}, which corresponds to the FDA recommended test dose for measurement of the UV light protection factor. The initial concentrations of oxybenzone in solvents were respectively 5% in coconut oil and in capric-caprilic triglycerides, and 3% in propylene glycol. In the present study it seemed not appropriate to investigate 48 h with a sunscreen. In fact, long applications of sunscreen formulation were not realistic. Independent samples (skin and receptor phase) were analyzed in triplicate at 1, 2, 4 and 8 h. The data obtained following application of oxybenzone were shown in Tables 5 and 6 from pigskin and physiological solution, respectively. These data correspond to the amount of oxybenzone (μg cm^{-2}) accumulated in skin and in receptor fluid 1, 2, 4, and 8 h after application. These results clearly confirm the vehicle effect on penetration of oxybenzone. The greatest concentration of benzophenone-3 in pigskin was obtained with propylene glycol where benzophenone-3 concentration was near the saturated solution. Profiles of absorption for benzophenone-3 (Fig. 2) show that propylene glycol acts as a penetration enhancer. The literature contains opposing work as to whether this solvent increases skin permeability [35], but in this case propylene glycol acts by promoting drug and particularly oxybenzone partitioning into the skin. Although propylene glycol was widely used in dermatological formulations, it must be limited in sunscreen formulations. No differences were

Table 3
Analysis of untreated samples

	Saline solution	Pigskin
	Performed in triplicate with three injections for each replicate	
Mean (μg ml^{-1})	0.0043	0.16
S.E.	0.006	0.08
Detection limit (DL) (μg ml^{-1})	0.03	0.43
Quantification limit (QL) (μg ml^{-1})	0.07	1.05

Table 4
Recovery rates from saline solution and pigskin ear shown as mean ± S.D.

	Saline solution (% recovery) Performed in duplicate with three injections for each replicate			Pigskin (% recovery) Performed in triplicate with three injections for each replicate		
	Sample: 20 ml exactly of NaCl solution			Sample: 0.2 ± 0.03 g of skin[a]		
Fortification (μg ml^{-1})	0.2	0.4	1	10.4–11.1	28.2–28.9	35.5–45.9
Recovery rates (%)	98.2	88.0	88.5	85.4	92.8	83.5
S.D.	15.5	6.3	3.4	6.6	5.3	5.2

[a] Fortification ranges because the skin weight was not exactly the same.

Table 5
Data from saline solution show as mean ± S.D.

Time (h)	Capric-caprylic triglycerides (μg cm^{-2})[b]	Coconut oil (μg cm^{-2})	Propylene glycol (μg cm^{-2})
	S.D.		
0[a]	0.04 ± 0.04	0.01 ± 0.02	0.01 ± 0.01
1	0.10 ± 0.07	0.13 ± 0.04	0.18 ± 0.05
2	0.07 ± 0.08	0.16 ± 0.10	0.20 ± 0.07
4	0.17 ± 0.14	0.17 ± 0.14	0.43 ± 0.09
8	0.42 ± 0.08	0.47 ± 0.13	0.47 ± 0.06

[a] Time 0 correspond to a point where the solvent (without oxybenzone) was added with 4 h of contact.
[b] Area available for diffusion was 0.785 cm^2 which correspond to 10.5 ± 0.3 ml of saline solution.

Table 6
Data from pigskin ear show as mean ± S.D.

Time (h)	Capric-caprylic triglycerides (μg cm^{-2})[b]	Coconut oil (μg cm^{-2})	Propylene glycol (μg cm^{-2})
	S.D.		
0[a]	0.07 ± 0.07	0.00 ± 0.00	0.22 ± 0.03
1	3.38 ± 0.60	4.40 ± 1.22	10.04 ± 1.94
2	4.18 ± 0.21	5.10 ± 0.72	10.51 ± 2.62
4	4.23 ± 0.79	3.73 ± 0.87	12.95 ± 3.36
8	7.56 ± 0.98	6.06 ± 1.27	20.43 ± 2.86

[a] Time 0 correspond to a point where the solvent (without oxybenzone) was added with 4 h of contact.
[b] Area available for diffusion was 0.785 cm^2, which corresponds to 0.217 ± 0.056 g of skin.

found between the two oily phases: capric-caprilic triglycerides and coconut oil. In contrast to the propylene glycol lower values were generated with these two solvents. It seems important to use these solvents for sunscreen formulation intended to be limit percutaneous penetration.

The exposure time must reflect in-use conditions, 8 h were investigated so the slope of the linear portion of the curve could not be estimated and the steady state flux was not given. Drug penetration measurements were done with the glass cell system and this method allows the measurement of even small drug fluxes. Table 5 shows that significant amounts of this UV-filter was

found in saline solution but these low concentrations measured in the receptor fluid suggesting that percutaneous absorption of this UV-filter would be minimal across the skin. In Fig. 3, time courses of fluxes (J) from solvents show that they are no solvent effect between 2 and 8 h. It may be concluded that cutaneously applied oxybenzone is able to penetrate though the skin

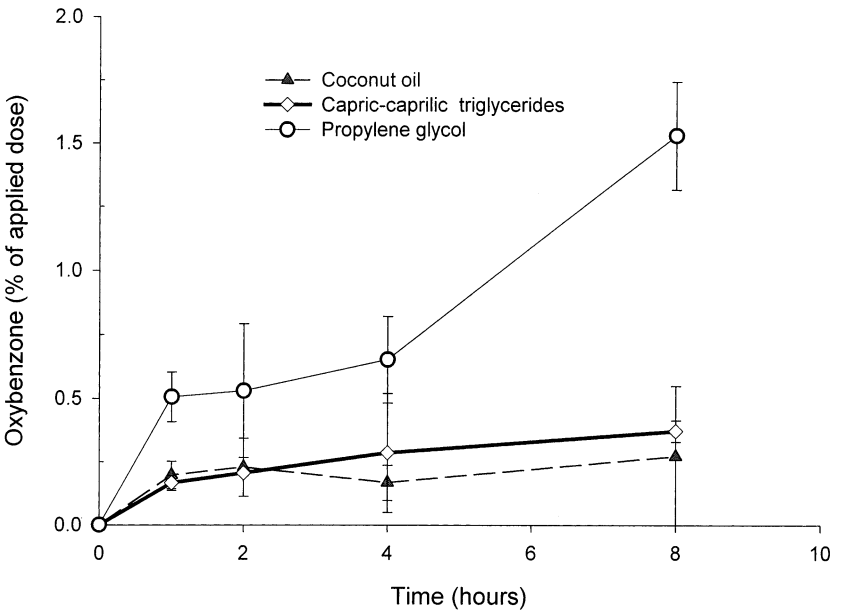


Fig. 2. Skin penetration of oxybenzone from various solvents, expressed as % applied dose (mean ± S.E.).

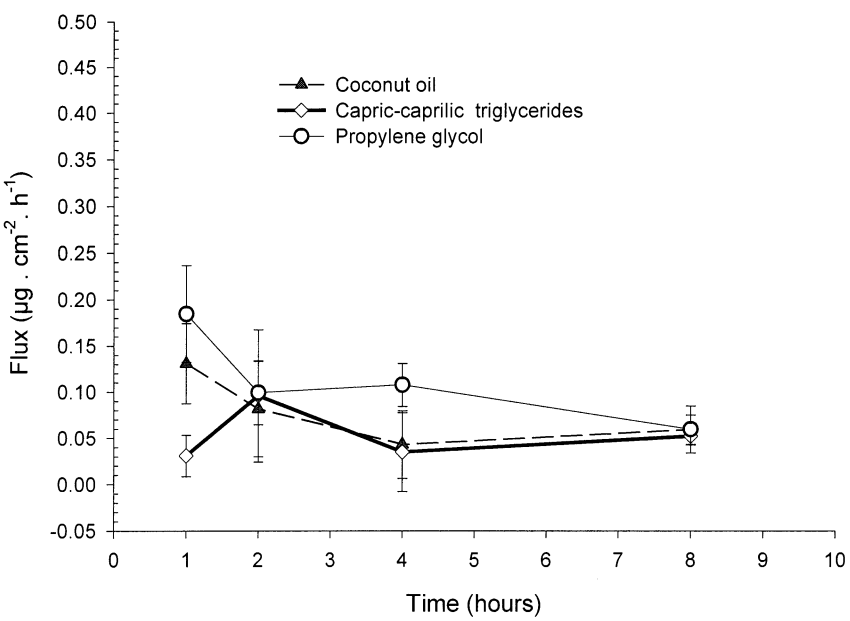


Fig. 3. Mean flux of oxybenzone from various solvents through pigskin membrane.

but in these cases the major part remains in the skin.

4. Conclusions

The purpose was first to develop a method adapted to the accurate and relatively simple determination of the oxybenzone in matrices used in transdermal field. Oxybenzone was selectively and reproducibly determined in each sample with sufficient sensitivity, after SPE-cartridge clean-up and LC chromatography. The recovery of each sample was close to 85–90% and the results of each test indicate that the method is stable. The last step is to evaluate the transdermal penetration of this UV-filter in different vehicles and formulations with a method developed and validated prior to use witch could engender confidence in the results generated. In this study, minimal penetration is achieved by choosing vehicles with higher oxybenzone solubility and a low polarity, the intention in a last step is to formulate optimized emulsions with these oily phases that hindered or reduced transdermal and systemic absorption.

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