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High-performance liquid chromatographic assay for common sunscreening agents in cosmetic products, bovine serum albumin solution and human plasma

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Abstract

This paper reports the development of a reversed-phase high-performance liquid chromatographic assay for quantifying five of the most common sunscreen agents, namely 2-ethylhexyl-*p*-dimethyl aminobenzoate (Escalol 507), 2-ethylhexyl-*p*-methoxycinnamate (Parsol MCX); 4-*tert*.-butyl-4′-methoxydibenzoylmethane (Parsol 1789), 2-hydroxy-4-methoxybenzophenone-3 (oxybenzone) and 2-ethylhexyl-salicylate (octylsalicylate). The assay permits analysis of the sunscreen agents in formulations and in biological fluids, including bovine serum albumin (BSA) solution, a common additive to in vitro skin diffusion cell receptor fluids, as well as human plasma. Separation was achieved using an ODS C_{18} column with a methanol–water (88:12) mobile phase. The analytes were detected by ultraviolet light absorption at a wavelength of 315 nm. The assay was linear with minimum detectable limits, calculated as greater than 3-times the baseline noise level: for oxybenzone and Escalol 507, 0.05 μg/ml; for Parsol 1789 and Parsol MCX, 0.1 μg/ml; for octylsalicylate, 1 μg/ml. Recoveries from both plasma and 2% BSA were within the range 89–107%. The inter- and intra-day coefficients of variation for the five agents were not more than 4% at the upper end of the linear range and not more than 10% at the lower end. Preliminary stability studies of the sunscreen agents in a commercial product and in two diffusion cell receptor fluids were also conducted.

Keywords: 2-Ethylhexyl-*p*-dimethyl aminobenzoate; 2-Ethylhexyl-*p*-methoxycinnamate; 4-*tert*.-Butyl-4′-methoxydibenzoylmethane; Oxybenzone; Octylsalicylate

1. Introduction

The topical application of sunscreens is widely practiced to protect healthy and photosensitive skins from the sun. However, there is little published literature describing the percutaneous absorption and consequent systemic distribution of these chemicals. Studies in this area require an assay to accurately determine the sunscreen agents from various media. The chemicals employed in topical preparations to filter UV radiation are generally classed as either chemical sunscreens or physical blockers [1,2]. Physical sunscreens are described as containing particles that interrupt the path of UV light by scattering or reflection [3] while chemical absorbers undergo photochemical excitation exposure to UV light. The high-energy radiation is absorbed and

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released as less dangerous long-wavelength radiation, and so protects the skin from a potentially damaging dose of ultraviolet light [1]. These topical photoprotectants have been in use for at least sixty years. The early chemical sunscreens were less efficient at absorbing radiation and were easily removed by sweat or swimming. However, modern formulations have taken advantage of chemicals which are capable of absorbing larger amounts of energy in a broad spectrum range of both UVA and UVB and due to their lipid solubility, they are retained in the skin, and resist removal. Today, sunscreens are applied regularly for everyday sunburn protection [4] and are especially useful for people with photosensitivities [5,6]. The modern UV filters are a broad range of compounds that include salicylates, benzophenones, cinnamates, *p*-aminobenzoic acid derivatives, anthranilates, dibenzoylmethanes, some camphor derivatives and a few miscellaneous chemicals. The most common active ingredients used in sunscreen formulations are oxybenzone, parsol MCX and Parsol 1789, other ingredients that feature commonly are Escalol 507 and octylsalicylate (Fig. 1). Sunscreens almost always appear as a combination of two or three chemical absorbers with a physical blocker. This type of formulation is marketed as broad spectrum, as it will filter UVB (290–320 nm) and UVA (320–400 nm) light.

Little is known about skin penetration of sunscreen agents following topical application and consequent systemic distribution. Therefore, in order to facilitate these studies, a suitable assay method is required. A number of publications describe high-performance liquid chromatography (HPLC) assays of sunscreening vehicles [7–12]. These methods are designed predominantly for product evaluation. For example, Ikeda et al. [11] applied a simple HPLC method to determine the concentration of sunscreening agents in cosmetic formulations. The sample pre-treatment was, in relation to previously reported methods, fast and simple while a number of sunscreen agents could be resolved. Other assays deal with trace contaminants in sunscreen products

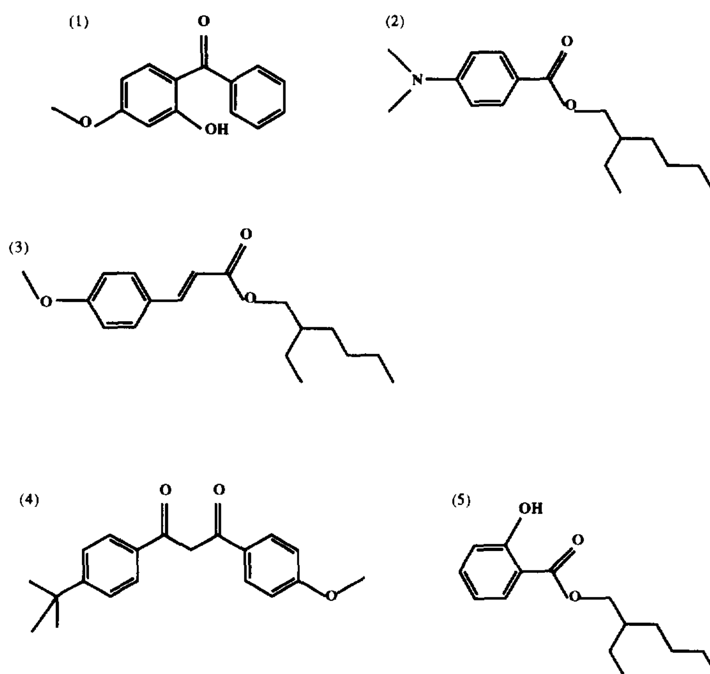

Fig. 1. Structures of five sunscreen agents most common used in formulations. (1) Oxybenzone; (2) Escalol 507; (3) Parsol MCX; (4) Parsol 1789; (5) octylsalicylate.

[8,13]. Few papers have been published which were designed for analysing these chemicals from biological media. Abdel-Nabi et al. [14] described a sensitive HPLC assay for quantifying one of the common sunscreen agents, namely oxybenzone, in blood, tissue and urine.

Percutaneous penetration of substances are frequently determined in vitro by using diffusion cells that consist of a donor phase, containing the drug in question, and a receptor phase which represents the dermal blood supply. These are separated by a membrane that mimics the barrier function of the skin. The membrane can be either excised human or animal skin or a suitable synthetic membrane (silicone or polyethylene). Both bovine serum albumin (BSA) and aqueous ethanol are common choices as receptor fluids in vitro skin diffusion studies when lipophilic compounds are involved [15,16]. BSA has good solubilizing power because of the ability of its non-specific hydrophobic site binding with lipophilic compounds. The binding is reversible and the protein can be easily removed before analysis [15]. Stability of sunscreen agents in receptor phase should be taken into account during the period of penetration study due to the possibility of photochemical reactions when exposed to sunlight. However, little has been reported in the area [17,18]. A preliminary stability study of the sunscreen agents in the two receptor phases was carried out in order to determine whether significant degradation occurs during the penetration study period. The preliminary stability study was also conducted in a sunscreen product using conditions that mimicked human application (ambient sunlight condition with no temperature control) to obtain an initial view of potential degradation after topical application of sunscreen cosmetics on human skin. Vital to these investigations is the accurate quantification of sunscreen agents in biological media to allow both in vitro and in vivo studies of skin penetration of sunscreen agents. The paper provides a reproducible and accurate assay in which five of the most common sunscreen agents can be resolved simultaneously. This is particularly important due to the inclusion of a minimum of three sunscreen agents in their formulation. Using this assay procedure these agents can be quantified from their formulation and biological media.

2. Experimental

2.1. Materials and reagents

The following materials and reagents were used: oxybenzone and BSA (Sigma–Aldrich, Australia), Escalol 507 (Van Dyk, USA) Parsol MCX and Parsol 1789 (Givaudan–Roure, Dee Why, Australia), octylsalicylate (Tokyo Kasei Kogyo, Japan), Coppertone sunblock lotion (Cosmetic Suppliers, Australia), Sun Sense cream (EGO Pharmaceuticals, Australia), Reef coconut spray (Colgate–Palmolive, Australia), HPLC-grade methanol (EM Science, USA), acetonitrile and sodium phosphate dibasic anhydrous (Mallinckrodt, Australia), sodium dihydrogen orthophosphate (BDH Chemicals, Australia). Distilled water was filtered (0.45-μm membrane filter, Alltech Australia) before use.

2.2. HPLC instrumentation

A Model LC-6 A liquid chromatograph (Shimadzu, Japan) equipped with a SIL-9A autoinjector (Shimadzu) and a 20-μl loop, a Nova Pak C_{18} RCM (4 μm, 100×8 mm I.D, Waters-Millipore) analytical column protected by an in-line column prefilter (2 μm, 4 mm I.D., Alltech Australia) with a 0.005-in. I.D. tubing (Alltech Australia) connected from the injector to the column, a Model 486 tunable absorbance detector (Waters-Millipore) and a C-R4A Chromatopac Integrator (Shimadzu) was used.

2.3. HPLC conditions

The mobile phase was methanol–water (88:12, %). This was filtered through a 0.45-μm membrane (Nylon 66, Alltech Australia) and degassed before use at a flow-rate of 1.0 ml/min. The column temperature was ambient, the injection volume was 10 μl and the detector wavelength was 315 nm.

2.4. Assay precision

Stock solutions were prepared by accurately weighing the agents and dissolving in methanol. Three working solutions of the five sunscreens were freshly prepared from their stock solutions by a 1:10 dilution with 85% (v/v) aqueous methanol. Appro-

priate dilution of these working solutions gave concentrations of 0.1 to 5 μg/ml, except for octylsalicylate, where the concentrations were 1.0 to 16 μg/ml. The entire procedure was repeated on three different days to test inter-day variation and repeated six times at low and high concentrations to test intra-day variation. Volumes of 10 μl of the standards were used for the assay.

2.5. Minimum detectable limits and low limit of quantitation

The detectable limits were measured by diluting the sunscreen agents with methanol to give a appropriate range from 0.01 to 20 μg/ml. Aliquots of 10 μl of the samples were injected onto the HPLC column.

2.6. Recovery study in human plasma and 2% BSA in phosphate buffer

The five sunscreen standards were spiked into plasma and the receptor phase (2%, w/v BSA in phosphate buffer, pH 7.2) at low and high concentrations (0.5 and 5.0 μg/ml, except for octylsalicylate, 2.0 and 16.0 μg/ml). The sample solutions were stirred for 30 min following spiking to ensure complete dissolution of the sunscreen agents. After protein precipitation with two sample volumes of acetonitrile (200 μl of acetonitrile to 100 μl of sample), the samples were centrifuged at 10 000 *g* for 10 min before injecting onto the HPLC system. Six replicates were performed at each concentration. Blank plasma and BSA in phosphate buffer were treated identically and injected onto the HPLC to ensure there were no peaks interfering with the sunscreen active substances.

2.7. Sunscreen agents in cosmetics

The sunscreen content in the following commercial sunscreen products was evaluated: Coppertone sunblock lotion, Sun Sense cream and Reef coconut spray. Preparation of samples of the sunscreen products was carried out using a two-step dilution procedure. After weighing an appropriate amount of the products and diluting with 50% methanol (cream and lotion) or 85% methanol (spray), the final dilution was conducted in absolute methanol to ensure no further precipitation occurred during eluting in the mobile phase. After centrifuging at 10 000 *g* for 10 min, the diluted supernatants were then injected onto the HPLC system.

2.8. Preliminary stability study

2.8.1. Coppertone shade SPF 15^{+} moisturising sunblock lotion

Approximately 5-ml aliquots of the lotion were kept in glass vials and placed in full sunlight at ambient temperature (17–34°C during the day). After either exposing them to the sun or protecting from the sun by covering with aluminium foil during a period of 120 h, these samples were assayed at the following time points: 0, 2, 4, 6, 24, 72 and 120 h. Of each of the samples, 100 mg were taken at the time points, then diluted to 100 ml with 50% methanol. The final dilutions were carried out by diluting 0.2 ml of the diluted samples to 10 ml with absolute methanol.

2.8.2. Sunscreen agents in 2% (w/v) BSA in phosphate buffer (pH 7.2) and in 20% (v/v) aqueous ethanol

The stability of the five sunscreen agents in both 2% BSA in phosphate buffer (pH 7.2) and in 20% ethanol (typical receptor phases for skin penetration studies) was investigated during a period of 24 h. Concentrations of sunscreens in the two media were 2.5 μg/ml, except for octylsalicylate, which was 8 μg/ml. The procedure was as described above. The samples were taken at 0, 4, 6 and 24 h. A 0.2-ml aliquot of sample was mixed with 0.4 ml of acetonitrile, then centrifuged at 10 000 *g* for 10 min (2% BSA in phosphate buffer only) to precipitate protein prior to analysis.

3. Results and discussion

3.1. Chromatography and resolution

Fig. 2 and Fig. 3 show chromatograms of the HPLC separation for the five most common sunscreen agents after sample preparation from

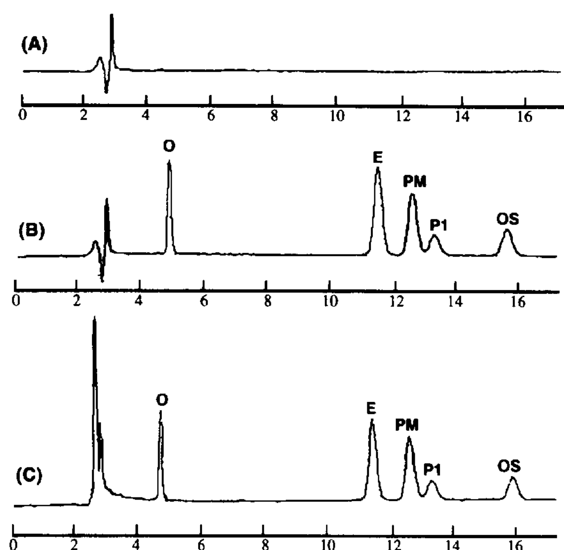

Fig. 2. Chromatograms of a blank of 2% BSA (A), an extract from 2% BSA in phosphate buffer (B) and plasma (C) with the HPLC conditions described in the text. Peaks: O=oxybenzone; E=Escalol 507; PM=Parsol MCX; P1=Parsol 1789 and OS=octylsalicylate.

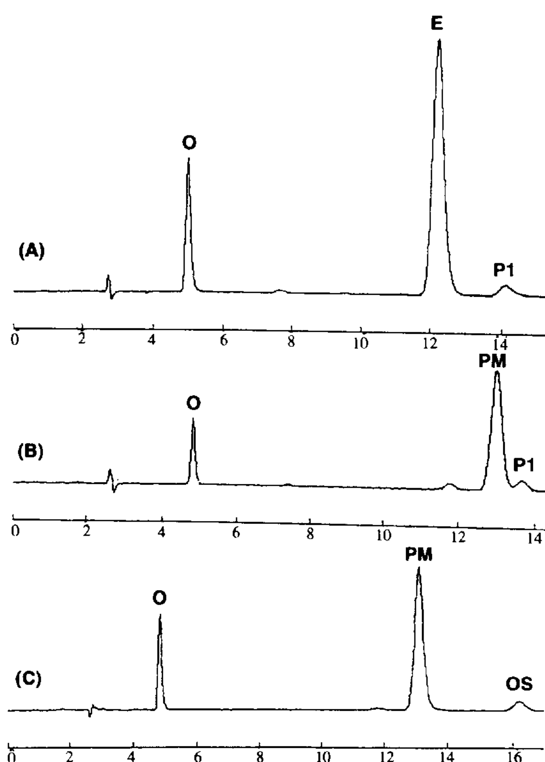

Fig. 3. Chromatograms of sunscreen agents in cosmetics. (A) Coppertone sunblock lotion; (B) Sun Sense cream; (C) Reef coconut spray with the HPLC conditions as described in the text.

different media. Satisfactory resolution could be achieved within a range up to 2 μg/ml when all five sunscreen agents are to be analysed simultaneously using narrow-bore tubing (0.005 inch I.D.) to connect the injector to the column. This system gave better resolution of the five agents than when standard tubing (0.009 in. I.D.) was used due to reduce band broadening. The restricted loading (up to 2 μg/ml) is necessary to maintain good resolution between Parsol MCX and Parsol 1789. The overlap of the two compounds was worse when the loading concentration was over 2 μg/ml. Excellent resolution can be achieved with a range up to 5 μg/ml of each component if one of the Parsol actives is absent. The procedure is relatively rapid with an analytical run time of 16 min at room temperature (around 22°C).

3.2. Linearity

Table 1 reports the results for calibration plot linearity. Excellent linearity was obtained over the range 0.1–5.0 μg/ml for all compounds except octyl salicylate for which the range was 1.0–16.0 μg/ml.

3.3. Assay precision

Calibration graphs were constructed by plotting the peak area versus concentration of standards injected. The best-fit straight lines were determined using the method of least squares. All plots passed through the origin. To obtain a satisfactory UV response for all analytes, the detection wavelength was selected at 315 nm, which is a compromise absorption wavelength for the five sunscreen agents. The intra- and inter-day precisions of the assay are summarized in Table 2. There was no significant difference between day-to-day analysis (slopes evaluation, $p<0.01$). Intra- and inter-day coefficients of variation (C.V.) of the assay for the five sunscreen agents were below 10%.

Table 1
Data for calibration plots[a]

Compound	Regression equation[b]	Correlation coefficient (r^2)	Linear range (μg/ml)
Oxybenzone	$y=24575x+716$	0.9998	0.1–5.0
	$y=23430x+615$	0.9998	
	$y=23930x+603$	0.9997	
Escalol 507	$y=55229x+2171$	0.9998	0.1–5.0
	$y=54288x+2123$	0.9999	
	$y=55619x+1323$	0.9999	
Parsol MCX	$y=42476x+673$	0.9998	0.1–5.0
	$y=41106x+285$	1.0000	
	$y=40381x+513$	0.9999	
Parsol 1789	$y=18447x+230$	0.9998	0.1–5.0
	$y=18745x-333$	0.9996	
	$y=17608x-358$	0.9997	
Octylsalicylate	$y=7370x+364$	0.9996	1.0–16.0
	$y=7005x+15$	0.9999	
	$y=6919x+142$	0.9997	

[a] Each data is the mean of triplicates; three equations represent three individual days.
[b] x=content (μg/ml); y=peak area.

3.4. Minimum detectable limits and low limit quantitation

The minimum detectable limits, calculated as greater than three times the baseline noise level in the assay, were 0.01 μg/ml for oxybenzone, 0.05 μg/ml for Escalol 507, 0.1 μg/ml for Parsol MCX and Parsol 1789, and 0.5 μg/ml for octylsalicylate. The poor sensitivity for octylsalicylate resulted from the compromise wavelength used for the assay. Use of a multiple-wavelength detector would provide improved assay characteristics for octylsalicylate by assaying at its wavelength maximum of 307 nm. The lower limits of quantitation, calculated as greater than ten times the baseline noise level in the assay, were 0.05 μg/ml for oxybenzone, 0.1 μg/ml for Escalol 507, 0.2 μg/ml for Parsol MCX and Parsol 1789, and 1.0 μg/ml for octylsalicylate.

3.5. Recovery study in human plasma and 2% BSA in phosphate buffer

It was necessary to evaluate the recovery of the five sunscreen agents from plasma and 2% (w/v) BSA solution. Table 3 summarizes recovery of the five sunscreen agents from these media. A range of

Table 2
Intra-day and inter-day variations

Compound	Intra-day variation (n=6)		Inter-day variation (n=9)	
	0.1 μg/ml[a]	5.0 μg/ml[a]	0.1 μg/ml[a]	5.0 μg/ml[a]
Oxybenzone	0.091±0.006	4.985±0.202	0.081±0.003	4.995±0.010
C.V. (%)	6.6	4.0	3.2	0.2
Escalol 507	0.108±0.009	4.956±0.076	0.107±0.010	5.002±0.007
C.V. (%)	8.4	1.5	9.4	0.1
Parsol MCX	0.102±0.006	4.998±0.048	0.092±0.008	5.002±0.005
C.V. (%)	5.8	1.0	9.2	0.1
Parsol 1789	0.091±0.009	4.950±0.106	0.107±0.010	5.024±0.009
C.V. (%)	9.7	2.1	10.0	0.3
Octylsalicylate	1.150±0.069	15.77±0.092	1.080±0.036	16.06±0.044
C.V. (%)	6.0	0.6	3.3	0.3

[a] Concentration of octylsalicylate is 1.0 (low) and 16.0 μg/ml (high).
Data are expressed as mean ±S.D.

Table 3
Recovery of the sunscreen agents from both plasma and 2% BSA in phosphate buffer

Compound	Amount added (μg/ml)	Plasma		2% BSA in phosphate buffer	
		Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Oxybenzone	0.5	99.2	3.3	103.1	2.4
	5.0	97.0	3.6	101.6	0.7
Escalol 507	0.5	107.4	3.8	103.7	2.6
	5.0	96.4	3.9	101.4	1.8
Parsol MCX	0.5	104.3	2.8	99.0	3.3
	5.0	95.3	3.9	102.6	0.9
Parsol 1789	0.5	102.9	4.8	97.4	5.0
	5.0	106.4	2.5	93.2	3.2
Octylsalicylate	2.0	96.4	6.4	102.9	3.0
	16.0	88.7	4.3	100.1	2.7

*Each value is the mean of six replicates.

89–107% recovery for the five agents was observed at both low and high concentrations. The coefficients of variance calculated from six replicates were all less than 7%. An interference peak with the same retention time as octylsalicylate was observed in the plasma blank. The interfering peak was shown to be present in plasma from both fresh and stored blood, and hence was not due to the very lipophilic plasticizers commonly found in blood bags. It was also not due to heparin. After subtracting the background absorbance, the percentage recovery of octylsalicylate from plasma was estimated. Degradation peaks were observed in the sample spiked with a high concentration of octylsalicylate, but were negligible in the sample with a low concentration. Further investigation is required to ensure the stability behaviour of octylsalicylate in plasma.

3.6. Applications of the assay

3.6.1. Active content in formulated products

Fig. 3 presents satisfactory resolution of the sunscreen agents in cosmetics when analysed by the present HPLC assay.

3.6.2. Preliminary stability study

It is known that chemical sunscreens undergo electronic excitation when exposed to UV light which may make them susceptible to decomposition [1]. Therefore, it is of interest to know if any degradation occurs after application of the sunscreen product to the skin during a period of sun exposure. Table 4 presents changes in a sunscreen product after 120 h sun exposure at ambient temperature. A degradation product of Escalol 507 (confirmed by exposure of Escalol 507 alone to the sun) increased, while the concentration of Escalol 507 decreased, in contrast to a sample protected from the sun. The unknown product, therefore, is presented as a percentage of the initial amount of Escalol 507. The experiment lasted 120 h at ambient conditions (temperature and sunlight varying with time during the day) in order to mimic degradation under actual conditions. After 72 h Parsol 1789 in the sample exposed to the sun, was decreased by up to 25% of the initial concentration. Degradation products of Parsol 1789 could not be found in the chromatogram, which may suggest overlapping with peaks for other sunscreen agents. The preliminary stability study presents an initial indication of sunscreen behaviour in the formulation during sun exposure. However, it should be pointed out that the assay was neither designed nor optimised for analysing degradation products with a polar nature (e.g., products from hydrolysis of esters such as Escalol 507, which appeared at the solvent front), but may still be useful for the less polar photodegradation products of the agents. The overlapping of degradation product with sunscreen agents can be overcome by studying them individually.

Assessment of sunscreen penetration through epidermal or synthetic membranes in vitro needs to be

Table 4
Stability of sunscreen agents in Coppertone sunblock lotion

Time (h)	Oxybenzone		Escalol 507		Parsol 1789		Degradation product	
	Sun	Dark	Sun	Dark	Sun	Dark	Sun	Dark
0	100.0±1.7	100.0±1.7	100.0±1.5	100.0±1.5	100.0±2.7	100.0±2.7	0.67±0.05	0.67±0.05
2	100.0±1.9	98.3±1.4	101.0±2.4	98.1±2.2	98.4±5.8	95.4±2.8	1.27±0.07	0.56±0.08
4	101.0±0.6	102.8±2.8	99.2±0.8	102.0±5.0	103.5±1.4	104.7±5.6	2.90±0.15	0.94±0.07
6	100.0±1.3	103.8±1.5	91.1±2.1	100.4±2.9	95.5±2.3	97.4±5.0	4.30±0.11	0.90±0.09
24	99.5±0.1	100.0±0.4	89.7±0.7	104.3±0.8	94.1±1.6	104.7±3.5	5.70±0.05	0.80±0.02
72	103.1±0.2	103.2±2.3	58.1±2.2	105.1±5.5	74.5±3.0	106.8±6.4	15.00±0.28	0.93±0.01
120	99.6±0.7	102.4±1.1	41.6±2.8	102.8±1.7	70.4±0.9	109.1±1.7	15.30±0.06	0.91±0.02

*The data is the mean±S.E. of triplicates.

cognisant of the stability of the agents in the receptor phase. Results of limited concentration–time profiles of the five sunscreen agents in two possible receptor phases (2% BSA in phosphate buffer and 20% aqueous ethanol) are shown in Fig. 4 and Fig. 5. In general, the sunscreen agents exposed to ambient

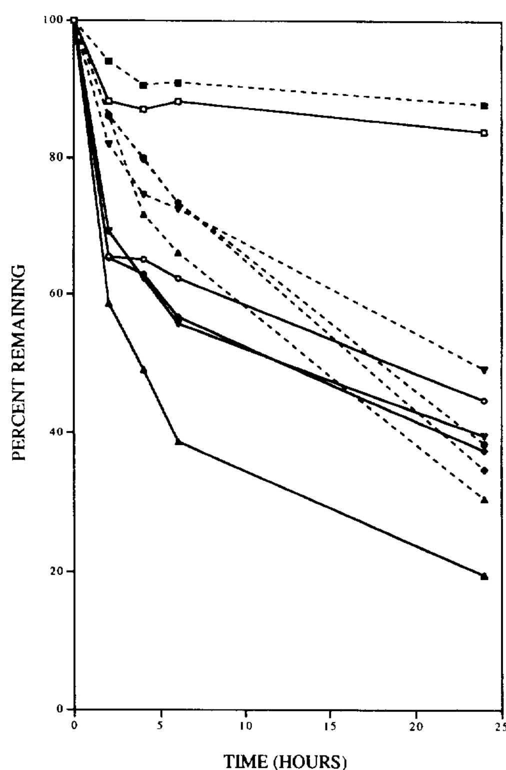

Fig. 4. Stability study of the five sunscreen agents in 20% ethanol during a period of 24 h. The data is the mean of triplicates with the standard error for each point being less than 5%. The solid line (open symbols) represents sunscreen agents in sun exposure conditions and dotted line (closed symbols) represents sunscreen agents in dark conditions. (□) Oxybenzone; (○) Escalol 507; (△) Parsol MCX; (▽) Parsol 1789; (◇) octylsalicylate.

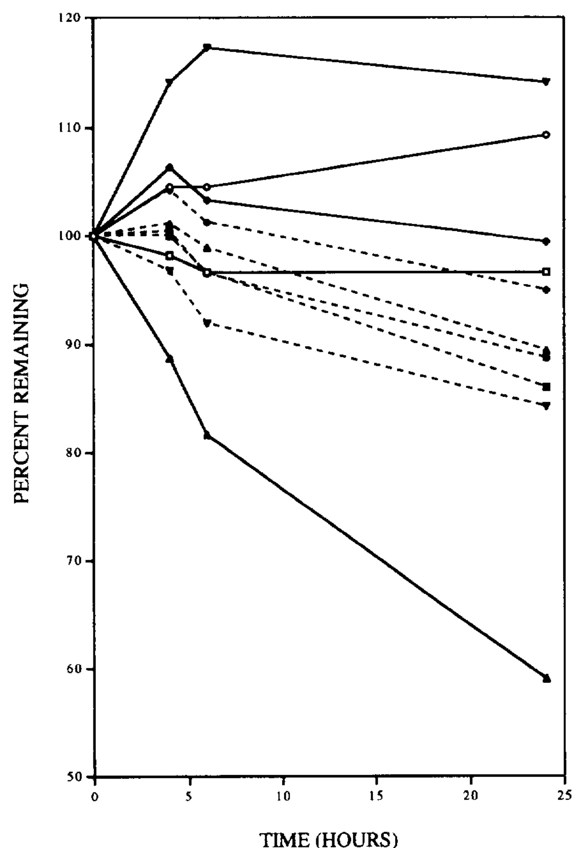

Fig. 5. Stability study of the five sunscreen agents in 2% BSA during a period of 24 h. The data is the mean of triplicates with the standard error for each point being less than 4%. The solid line (open symbols) represents sunscreen agents in sun exposure conditions and dotted line (closed symbols) represents sunscreen agents in dark conditions. (□) Oxybenzone; (○) Escalol 507; (△) Parsol MCX; (▽) Parsol 1789; (◇) octylsalicylate.

light showed increased degradation in 20% aqueous ethanol during the study period, compared to samples protected from light. Marked degradation was also found when sunscreen actives were dissolved in methanol. Both photochemical reactions and solvolytic reactions appear to be involved in the loss of all agents in the aqueous ethanol (20%). In 2% BSA, degradation was generally less than in 20% ethanol. The samples protected from light displayed less degradation than light-exposed samples (<5%, except for Parsol 1789, 8%) at 6 h and not more than a 15% decrease in concentration at 24 h. Irregular results were found in the samples exposed to the light, for example, Parsol MCX had a concentration decrease of up to 40% after 24 h ambient light exposure, while Parsol 1789 and Escalol 507 had apparently increased concentrations at the same time point. The most likely reason for this result is interference in HPLC chromatograms by co-elution of one or more degradation products. Very high albumin binding of the sunscreens is expected, due to their hydrophobic nature, which may in turn influence the rate of degradation.

A potential problem in these stability studies is co-elution of degradation products with other components in the receptor phase, caused by analysing all five agents together. Consequently, degradation of each active must be examined separately and a mass balance determined to account for all degradation products. Further research on the stability of sunscreen agents in receptor phases is currently being undertaken in our laboratory to quantify and identify degradation of sunscreen agents under controlled light and temperature conditions. Based on the knowledge that sunscreen agents are photodegradable, penetration experiments must be shielded from light during the study period.

In conclusion, the HPLC assay developed is simple and rapid. It is suitable for quantification of five of the most commonly used sunscreen agents in different biological media, to allow in vitro and in vivo skin penetration studies. It can also accurately quantify the sunscreen agents in commercial sunscreen products.

Acknowledgments

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