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Development and validation of a high-performance liquid chromatographic method for the determination of cyproterone acetate in human skin☆

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Abstract

In the framework of a preliminary study on the transdermal penetration of cyproterone acetate (CPA), a simple and rapid procedure involving an extraction step coupled to a HPLC-UV determination has been developed for the separation and quantification of CPA in the two main skin layers-epidermis and dermis-after local application. The separation of epidermis and dermis layers was carefully carried out by means of a sharp spatula after skin immersion in heated water at 65 °C. The two skin layers were then treated separately according to the same process: (1) sample homogenization by vibration after freezing with liquid nitrogen in a Mikro-Dismembrator®; (2) CPA extraction with methanol after addition of the internal standard (betamethasone dipropionate); (3) centrifugation; (4) evaporation of a supernatant aliquot; (5) dissolution of the dry residue in methanol and addition of water; (6) centrifugation; (7) injection of a supernatant aliquot into the HPLC system. The separation was achieved on octadecylsilica stationary phase using a mobile phase consisting in a mixture of acetonitrile and water (40:60 (v/v)). The method was then validated using a new approach based on accuracy profiles over a CPA concentration range from 33 to 667 ng/ml for each skin layer. Finally, the method was successfully applied to the determination of CPA to several skin samples after topical application of different gel formulations containing CPA.

Keywords: Cyproterone acetate; HPLC; Human skin; Epidermis; Dermis; Quantitative determination; Validation

1. Introduction

CPA is a particular steroid with antiandrogenic and progestogenic activity used orally by women for the treatment of hirsutism, severe acne and androgenetic alopecia, and by men, for the treatment of hypersexuality and prostate cancer. Its topical activity is debated in the literature with some divergences [1–5]. Different reasons could explain these discrepancies, among which the protocol of the study, the applied quantity and the formulation. A critical point to obtain a topical activity is the absorption of CPA in skin. In order to evaluate CPA penetration in skin, one usual method is to determine CPA concentration recovered in skin after ex vivo application of several formulations onto human skin samples. In the present study, the challenge consists in extracting and determining the whole amount of CPA accumulated in skin.

The objective of this work is to develop a simple analytical procedure to quantify CPA in epidermis and dermis and to evaluate if CPA will be quantifiable following in vitro diffusion studies through human skin. Touitou et al. [6] made a review of the available methods for the quantitative determination of drug localized in the skin. Among the exposed methods, skin extraction followed by HPLC analysis represents an easy and inexpensive method to quantify the drug in epidermis and dermis in comparison with other methods, such as autoradiography or ATR-FTIR, which require

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adequate equipment. HPLC analysis has been therefore selected in the present study in order to quantify CPA in the skin layers. However, before HPLC analysis, the drug has to be quantitatively extracted from the skin tissue. The analysis of CPA in bulk drugs and pharmaceutical products is well described in the litterature [7,8] and it is also mentioned in the European Pharmacopeia [9]. The determination of CPA in biological fluids, such as plasma, serum and urine, has also been reported [10–13] showing in particular the advantages of the HPLC to quantify CPA in plasma and urine in comparison with radioimmunoassay (RIA) [10,11]. A fully automated method to quantify CPA in plasma, using on-line solid-phase extraction prior to the determination by reversed-phase liquid chromatography has also been described [13]. However, as far as we know, no quantitative method for the determination of CPA in skin has been reported in the literature. Therefore, a new method for the quantification of CPA in the skin layers by liquid chromatography was developed for a preliminary study on the transdermal penetration of CPA after its topical application. In the present method, epidermis and dermis layers were first carefully separated before their respective homogenization in a Mikro-Dismembrator® in order to avoid loss of skin tissue and CPA hydrolysis in alkaline solutions. The sample pre-treatment was completed by a methanolic extraction of CPA before injection into the HPLC system. The latter was optimised in order to obtain suitable conditions with respect to selectivity and retention of CPA, the corresponding alcoholic derivative, cyproterone (CP-OH), and the internal standard (IS), betamethasone dipropionate (BMDP). The method was then validated by considering different parameters, such as selectivity, response function, trueness, precision, accuracy and linearity. The most appropriate regression model for the response function was selected during the pre-validation step [14–16]. Finally, the method was successfully used to quantify several human skin samples after topical application of three different gel formulations containing CPA.

2. Material and methods

2.1. Chemicals and reagents

CPA was obtained from Sicor (Lerma, Mexico). Betamethasone dipropionate (BMDP) was supplied by Medeva Pharma (Braine-l'Alleud, Belgium). A sample of CP-OH was synthesized from basic hydrolysis of CPA and its analytical grade was assessed using classical criteria such as elemental analysis, IR spectrum and melting point. The human skin samples used for penetration tests were abdominal skin of Caucasian women obtained immediately after operation of the patients from the Service of Plastical Surgery (University Medical Centre, Liège, Belgium). Methanol, acetonitrile and water were of HPLC grade from Merck (Darmstadt, Germany). In the optimization of the extraction step, different solvents of decreasing polarity were tested: methanol, acetonitrile, dichloromethane and hexane. All the reagents were of analytical grade (Merck). The water used in all experiments was purified on Milli-Q system (Millipore, Bedford, MA, USA).

2.2. HPLC apparatus

CPA content of all human skin samples was determined using an HPLC system from Merck-Hitachi constituted of a Lachrom-7100 high pressure pump, a Lachrom-7200 autosampler and a Lachrom-7455 DAD detector. The injected volume was fixed to 100 μl. A Manu-Cart system which contained an analytical column (125 or 250 mm × 4 mm i.d.) and a short guard column (4 mm × 4 mm i.d.), both filled with octadecylsilica from Merck, was thermostated at 30 °C. The mobile phase consisted of a mixture of acetonitrile and water (40:60 (v/v)). Before use, the mobile phase was degassed for 15 min in an ultrasonic bath. The flow-rate was 1.5 ml/min and UV detection was carried out at 282 nm.

All data obtained were processed and stored using the D7000 Merck-Hitachi chromatography data station software. The e-noval® software (Arlenda, Belgium) was used to determine the accuracy profiles and generate all validation results.

2.3. Skin penetration test

The apparatus to perform the diffusion test through the skin consisted of vertical Franz cells (Hanson Research, Chatsworth, USA) with a receiver compartment of 7.5 ml and a diameter of 15 mm (surface 1.767 cm^2). The receiver solution was composed of a phosphate saline physiological solution containing hydroxypropyl-β-cyclodextrin to ensure CPA solubility. Directly after sampling of the skin in plastical surgery, the fresh tissue was washed twice in physiological solution in order to eliminate blood, disinfectants and other impurities present at the skin surface. After removing the fat tissue, the full skin was mounted on Franz cells. The system was maintained at a controlled temperature of 32 °C, corresponding to the temperature of the human skin surface. Then 200 mg of gels containing 0.5% CPA were applied on the skin surface. After 24 h, the excess of gel was removed and the skin was washed twice with alcohol and twice with desionized water in order to eliminate CPA remaining at the skin surface. The tissue was then carefully dried with a cotton wool.

2.4. Extraction of the skin

At the end of the penetration test, the skin was separated at the epidermis–dermis junction by immersing the skin for 30 s in water heated at 65 °C. The epidermis layer was gently split off with the help of a sharp spatula. After separation,

epidermis and dermis samples were stored at −20 °C until extraction.

2.4.1. Extraction of epidermis samples

The deep-frozen epidermis samples were homogenized by vibration in a mechanical device named Mikro-Dismembrator® S (B. Braun Biotech International, Melsungen, Germany). This technique consisted in shaking the skin tissue placed into a Teflon flask (volume 5 ml) containing a steel ball (10 mm diameter) at 3000 rpm for 1 min. The Teflon container, the percussion ball and the skin samples were previously frozen in liquid nitrogen at −198 °C in order to facilitate fine pulverisation. The pulverised tissue was transferred with a spatula into a polypropylene tube. The Teflon container and the steel ball were rinsed two times with the extraction solvent in order to recover CPA quantitatively. 1.0 ml of a solution containing 5.0 μg/ml of BMDP, the internal standard, was then added to the samples. The final volume of solution was adjusted to 6.0 ml. After vortexing for 2 min, the samples were centrifuged at 10,000 rpm for 20 min. Then 4.0 ml of supernatant were evaporated to dryness under air flux at ambient temperature. The residues were stored at −20 °C until HPLC analysis. Just before injection, the dry residue was dissolved in 1.0 ml of methanol. Then 1.0 ml of water was added to the solvent to precipitate the apolar skin components extracted. After shaking for 3 min, the solutions were centrifuged at 20,000 rpm for 20 min to eliminate these less hydrophilic components. Finally, an aliquot of the clear supernatant was introduced into the HPLC system.

2.4.2. Extraction of dermis samples

The method used for CPA extraction from dermis samples was very similar to the one previously described for the epidermis samples. Nevertheless, since dermis is thicker, it had to be cut in small pieces before freezing in liquid nitrogen and grinding with the Mikro-Dismembrator® in order to obtain fine particles. In the same way, since the mass of dermis powder was higher, the volume of solvent necessary to rinse the Teflon container and the ball as well as to disperse the pulverised tissue had to be increased. Like for epidermis samples, 1.0 ml of a 5.0 μg/ml BMDP solution was added. The final volume of the extraction solution was thus set to 9.0 ml. After centrifugation, an aliquot of supernatant (6.0 ml) was evaporated to dryness. The following steps were the same as for epidermis extraction.

2.5. Standard solutions

Two stock solutions of CPA were daily prepared by dissolving the appropriate amount in methanol in order to obtain a final concentration of about 1.0 mg/ml. These solutions were then diluted with the same solvent to obtain two series of intermediate solutions at final concentrations of 0.1, 0.5 and 2.0 μg/ml. The first series was used to obtain either the external calibration curve (calibration standards) and the second one to spike the skin samples (validation standards). A stock solution of BMDP (IS) was also prepared in methanol at a 5.0 μg/ml concentration.

2.6. Calibration standards

An external calibration curve was constructed by injecting in triplicate ($n = 3$) three different concentrations (33, 167 and 667 ng/ml) of CPA ($m = 3$). Calibration standards were prepared by addition of 1.0 ml volume of the IS solution to 1.0 ml of solution containing 0.1, 0.5, 2.0 μg/ml of CPA, respectively. After adjusting of the volume to 3.0 ml with methanol, a 2.0 ml aliquot was evaporated to dryness under air flux at ambient temperature. The dry residue was dissolved in 1.0 ml of methanol to which 1.0 ml of water was then added. This operation was repeated for three different days ($k = 3$) in order to select the most appropriate regression model for the response function in the pre-validation phase and to determine the intra- and inter-day precision, trueness, accuracy and linearity of the methods in the validation phase. Finally, this calibration curve was also used in routine analysis.

2.7. Validation standards

Three independent series of validation standards were prepared by spiking free CPA epidermis and dermis extracts obtained as described above. 1.0 ml volume of solution containing 0.1, 0.5, 2.0 μg/ml of CPA and 1.0 ml volume of the IS solution (5.0 μg/ml) were added to epidermis or dermis powder. The following preparation steps were identical with those used for the extraction of skin samples. The residues were stored at −20 °C until the HPLC analysis. Before injection, the dry residue was dissolved in 1.0 ml of methanol and 1.0 ml of water. After shaking for 3 min, the mixture was centrifuged at 20,000 rpm for 20 min and 100 μl of the clear supernatant were introduced into the HPLC system. The final concentrations of the validation standards were 33, 167 and 667 ng/ml for each skin layer and each validation standard was analysed three times for three different days.

3. Results and discussion

3.1. Development of the skin extraction method

Among the different available methods to homogenize skin, grinding the deep-frozen skin tissues with a Mikro-Dismembrator® is an appropriate method to obtain very fine particles in comparison with other methods [17]. Laugier et al. [18] extracted acitretin from skin with a mixture of diethylether and ethylacetate (50:50 (v/v)) after homogenization with an Ultra-Turrax®. However, this kind of homogenization requires the washing of the apparatus and its dismounting after each sample. Moreover, for small sample volumes, this method led to a loss of tissue, which was not properly homogenized [17]. Besides, Panus et al.

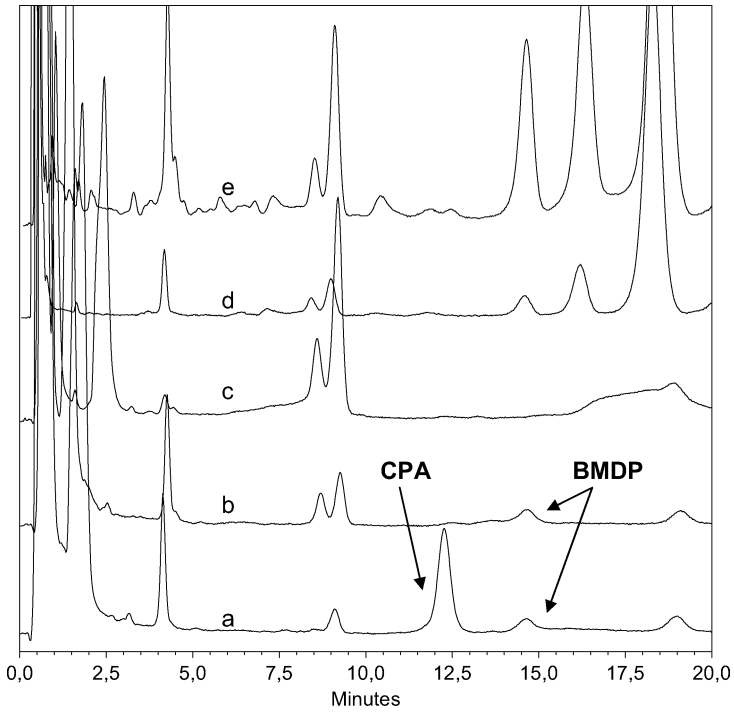

Fig. 1. Chromatograms of dermis extracts. (a) Dermis extracted with methanol and spiked with CPA (333 ng/ml) and BMDP (667 ng/ml). (b) Dermis extracted with methanol and spiked with BMDP (667 ng/ml). (c) Dermis extracted with acetonitrile. (d) Dermis extracted with hexane. (e) Dermis extracted with dichloromethane. Analytical column: Lichrospher RP-18 (125 mm × 4.0 mm, i.d.). HPLC mobile phase: acetonitrile/water (40:60 (v/v)).

[19] used the combination of $NaHCO_3$ and a tissue homogenizer to extract ketoprofen from skin. Nevertheless, in the present work, $NaHCO_3$ would damage CPA, due to a sensitivity to hydrolysis in alkaline solutions. For these reasons, another extraction method using a Mikro-Dismembrator® was investigated. Different authors described the advantages of this apparatus to homogenize various types of tissue, such as lungs [17], stratum corneum [20], colon mucosa [21] and to extract drugs [17], lipids [20], enzymes [21] or even, DNA and RNA [22]. This method avoids loss of skin tissue and allows obtaining very fine particles of skin, which promotes the extraction of compounds by solvents. Moreover, the drug stability is not affected.

After homogenisation of the skin samples, several solvents were tested in order to allow recovering CPA without extracting too many compounds from the skin tissue. Fig. 1 illustrates chromatograms obtained from skin extracted with several solvents and analysed under the same chromatographic conditions. As reference, a chromatogram of a dermis extracted with methanol and spiked with CPA and BMPB was shown (Fig. 1a). The retention times of the two compounds were 12.5 and 15 min, respectively. Fig. 1 shows that the number and the type of extracted components depend on the polarity of the solvent. Dichloromethane and hexane extract many apolar substances, which lead to interferences around the peaks corresponding to CPA and BMDP. On the contrary, extracting skin with acetonitrile and methanol reduces the number of interferences. Finally, methanol was selected as extraction solvent.

3.2. Development of the HPLC method

The HPLC method was optimised with respect to the separation of CPA, BMDP and CP-OH, the selectivity towards interferences from endogenous skin components and detectability. Firstly, different proportions of acetonitrile were tested with a 125 mm column packed with octadecylsilica. Irrespective of the proportion of acetonitrile, a complete separation of CPA and BMDP was obtained. However, interferences were observed at the retention time of BMDP peak for a few tested skin samples. Therefore, a 250 mm column packed with the same material was chosen in order to improve the separation efficiency. Since the number and the area of interfering peaks were different according to the extracted skin sample, the applicability of the method to skin samples from different patients was also checked during the development phase of the method.

Another difficulty consisted in the presence of very large peaks that were eluted only after several injections, probably due to very apolar compounds extracted from skin and strongly retained on the stationary phase. Therefore, a column washing with a mixture of acetonitrile and water (90:10 (v/v)) was performed after each run in order to eliminate all these hydrophobic substances.

Finally, the most suitable operating conditions are as follows: isocratic elution for 40 min with the HPLC mobile phase composed of acetonitrile and water (40:60 (v/v)), then washing of the column for 5 min with a mixture of acetonitrile and water (90:10 (v/v)) and re-equilibration of the

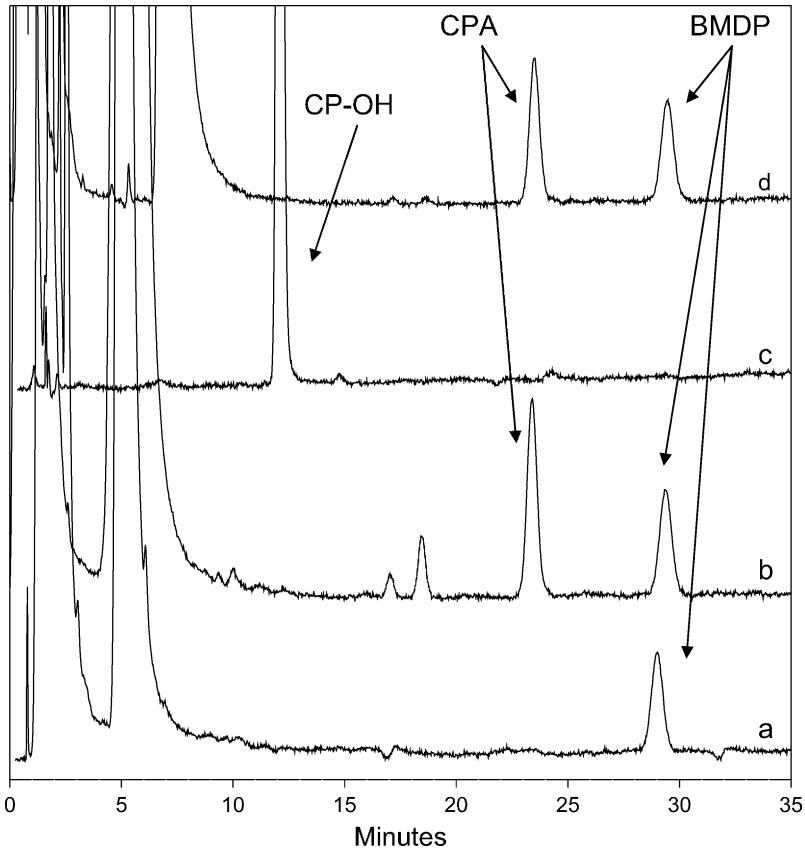

Fig. 2. Chromatograms of epidermis extracted with methanol and of a CP-OH solution. (a) Sample spiked with BMDP (1667 ng/ml). (b) Sample spiked with CPA (33 ng/ml) and BMDP (1667 ng/ml). (c) Solution of CP-OH (500 ng/ml). (d) Sample obtained from diffusion tests through the skin. Analytical column: Lichrospher RP-18 (250 mm × 4.0 mm, i.d.); HPLC mobile phase: acetonitrile/water (40:60 (v/v)).

system with the initial mixture for 10 min. Fig. 2 illustrates chromatograms obtained after analysis of different samples under these conditions. As can be seen in Fig. 2a–c, no interference from extracted skin components was observed at the retention time of the peaks corresponding to CPA and CP-OH.

Epidermis and dermis samples were then spiked with a known amount of CPA at three concentration levels (33, 167 and 667 ng/ml). For the two skin layers, the analyte recovery was calculated for three replicates ($n = 3$) at the three concentration levels of CPA ($m = 3$) and for nine replicates ($n = 9$) at one concentration level of BMDP (Table 1). The mean amounts of recovered CPA and BMDP were between 95% and 106%, which demonstrated a good extraction of the two analytes from skin samples. Moreover, the peak areas obtained for spiked skin samples were statistically compared with the peak areas of calibration standards (repeated measures ANOVA) and there was no significant difference between either CPA areas or BMDP areas of spiked epidermis, dermis and calibration standards ($p \gg 0.05$).

Table 1
Recovery of CPA and BMDP in epidermis and dermis

Concentration (ng/ml)	Epidermis recovery (mean ± S.D.) (%)	Dermis recovery (mean ± S.D.) (%)
CPA		
33 ($n = 3$)	100.7 ± 4.3	106.1 ± 9.0
167 ($n = 3$)	103.7 ± 4.7	97.1 ± 3.1
667 ($n = 3$)	98.1 ± 3.2	105.9 ± 3.2
BMDP		
1667 ($n = 9$)	95.1 ± 4.9	97.8 ± 5.2

3.3. Validation of the HPLC methods

The validation strategy of the described procedures for the quantitative determination of CPA in epidermis and dermis layers involved two steps: a pre-validation phase and a formal validation step. Furthermore, on the basis of preliminary experiments performed in the development phase, a calibration curve without matrix was directly selected in order to avoid the time-consuming preparation of the calibration standards in the skin matrix at different concentration levels. Indeed, only a weak matrix effect was observed with the epidermis and dermis samples.

3.3.1. Pre-validation phase

Before the formal validation phase, an important step consists in the assessment of the relationship between the response and concentration to avoid serious difficulties in

the estimation of other validation criteria. In order to select the most appropriate response function, the approach based on two-sided 95% tolerance intervals for total measurement error-including both bias and precision-of validation samples has been used [14–16]. Such an approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current procedure that compares point estimates of observed bias and precision with the target acceptance criteria, i.e. the mean value for the bias should be within 15% of the actual value and the precision determined at each concentration level should not exceed 15% (CV) except for the lower limit of quantification (LLOQ, with a bias and a CV of 20%) according to the Washington conference [23] or the FDA document [24]. Considering these guidelines, the acceptance limits of the methods under investigation in the pre-validation phase were settled to 30%. Indeed, these limits represent the total error of the methods, i.e. the sum of the systematic and random errors. As illustrated in Figs. 3 and 4, once the validation experiments have been performed, the response function can

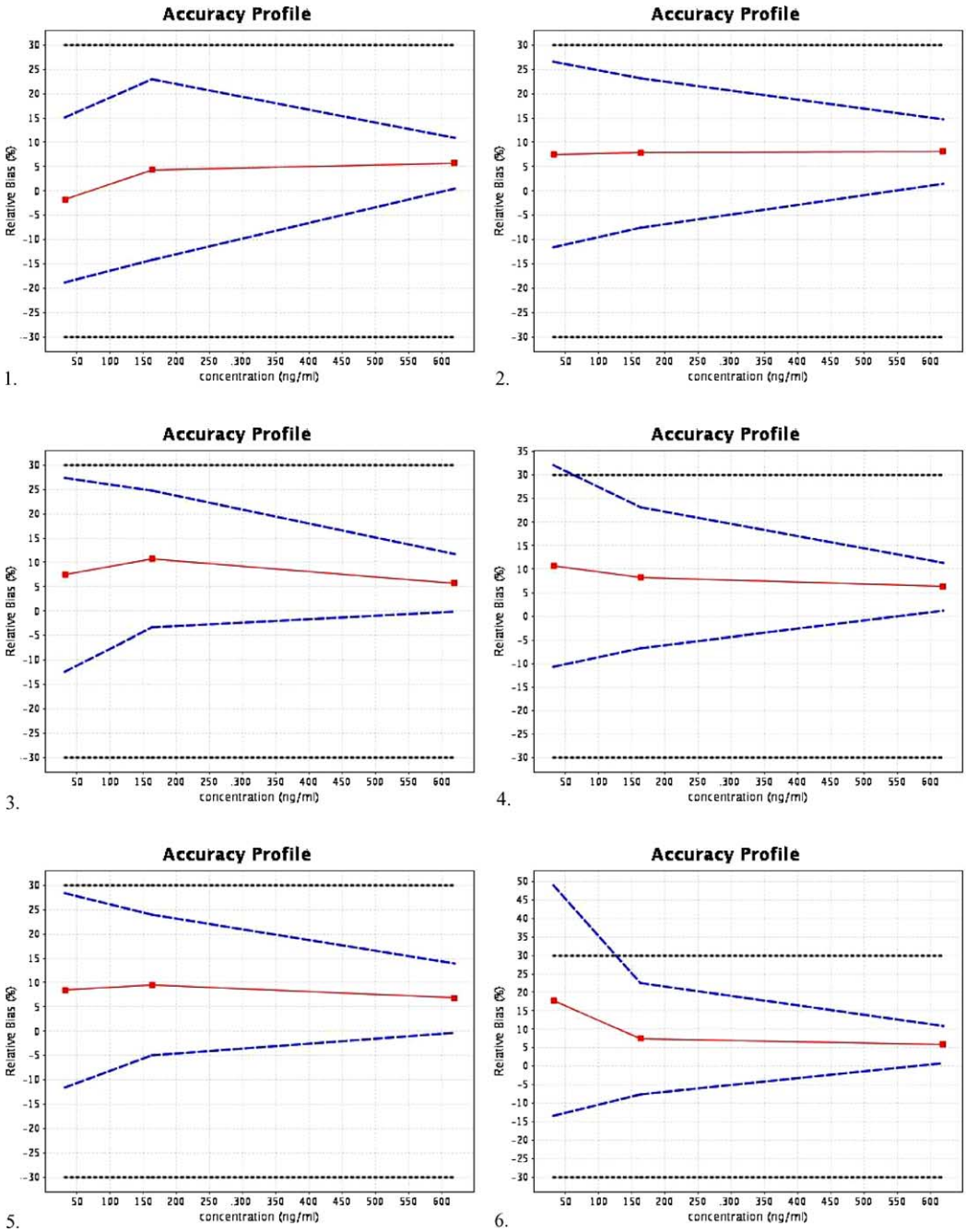

Fig. 3. Accuracy profiles of CPA in dermis using (1) linear regression through 0 fitted with the highest concentration level only, (2) weighted linear regression model, (3) quadratic regression, (4) linear regression model after square root transformation, (5) linear regression model after logarithm transformation, (6) linear regression model.

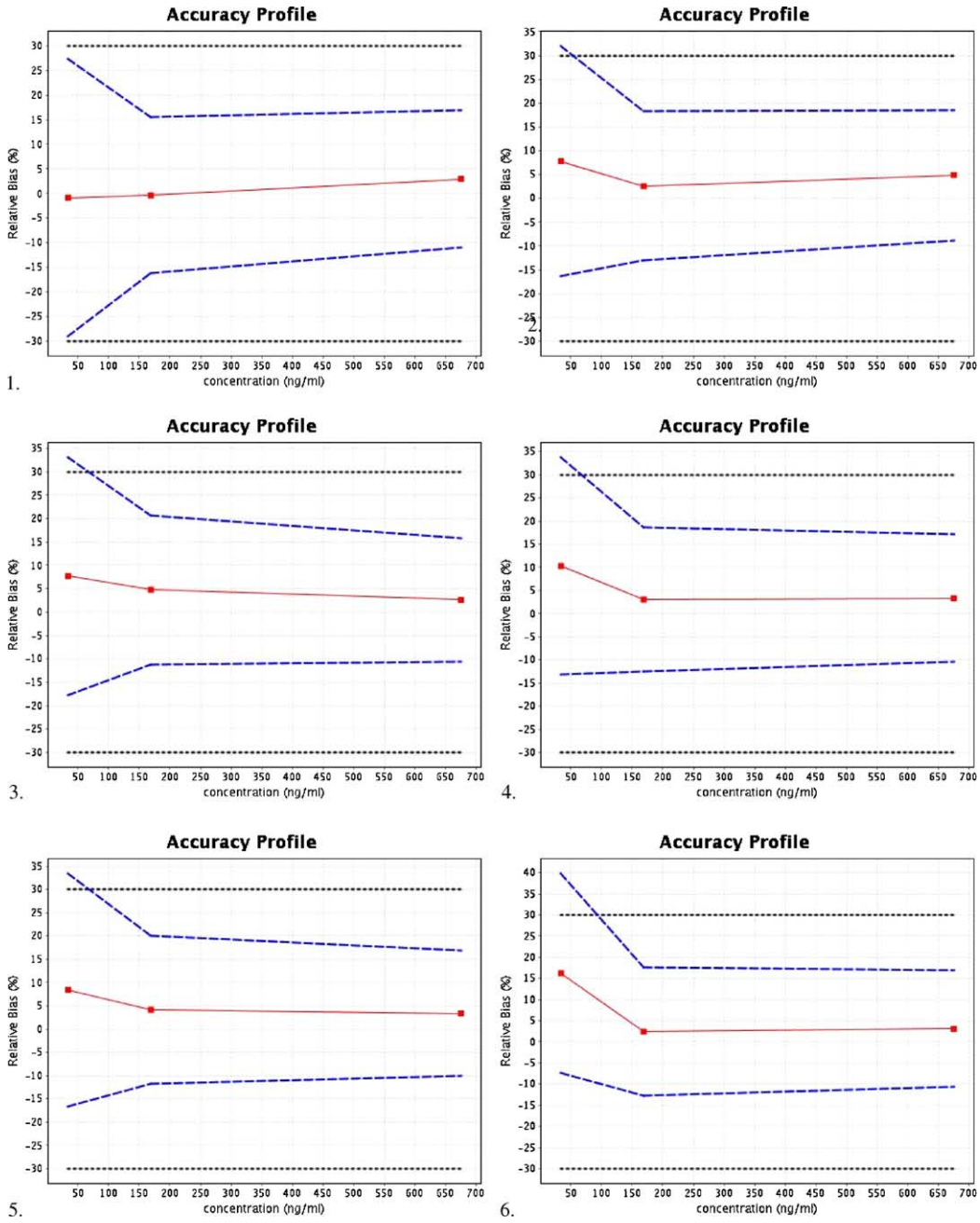

Fig. 4. Accuracy profiles of CPA in epidermis using (1) linear regression through 0 fitted with the highest concentration level only, (2) weighted linear regression model, (3) quadratic regression, (4) linear regression model after square root transformation, (5) linear regression model after logarithm transformation, (6) linear regression model.

be determined by applying different regression models and, from both analytical responses and regression line obtained, selecting the most suitable accuracy profile for the intended use of the analytical methods [14,25]. From the accuracy profiles obtained in the dermis matrix (Fig. 3), except for the accuracy profiles corresponding to the linear regression model and the linear regression model after square root transformation, all the other profiles were within the acceptance limits. On the basis of the accuracy profiles demonstrating the capability of the analytical method [14]-i.e. its ability to quantify with a known accuracy and a risk fixed according to the requirements-a linear regression through 0 fitted with only the highest concentration level could be used as regression model even if a weak bias was observed with two of the three concentration levels. The situation was rather different with the accuracy profiles obtained in the epidermis matrix (Fig. 4). Indeed, regression analysis could only be performed using the linear regression model through 0 fitted with the highest concentration level in order to cover the entire dosing range considered. Consequently, this last regression model was selected for both methods. In addition, it represents the simplest model adequately describing the

Table 2
Validation of the method for the determination of CPA in dermis

Response function ($k = 3$, $m = 3$, $n = 3$)	Slope		
	Day 1 (10.06×10^{-3})	Day 2 (9.04×10^{-3})	Day 3 (9.70×10^{-3})
Trueness ($k = 3$; $n = 3$)	Absolute bias (ng/ml)	Relative bias (%)	
33 ng/ml	−0.6	−1.8	
167 ng/ml	7.2	4.4	
667 ng /ml	35.7	5.8	
Precision ($k = 3$; $n = 3$)	Repeatability (R.S.D., %)	Intermediate precision (R.S.D., %)	
33 ng/ml	6.9	8.0	
167 ng/ml	7.1	8.6	
667 ng /ml	2.6	2.6	
Accuracy ($k = 3$; $n = 3$)	β-Expectation confidence limits in ng/ml (%)		
33 ng/ml	26.6 to 37.7 (−18.7 to 15.1)		
167 ng/ml	140.5 to 201.5 (−14.2 to 23.0)		
667 ng /ml	622.4 to 686.7 (−6.6 to 11.0)		
Linearity (k = 3; m = 3; N = 18)			
Range (ng/ml)	33 to 667		
Slope	1.062		
Intercept	−2.788		
r^2	0.998		
LOD (ng/ml)	10.1		
LOQ (ng/ml)	33.3		
Dilution effect	Factor (μg/ml)	Recovery ± S.D. (%)	
$n = 3$	3 (2.0)	101.6 ± 2.1	
	6 (4.0)	102.0 ± 3.3	

concentration-response relationship and gives better results at the lower concentration levels.

3.3.2. Validation phase

3.3.2.1. Selectivity. Method selectivity was evaluated by treating blank skin samples from six different sources according to the optimized methods in order to demonstrate the absence of interference of endogenous compounds. As shown in Fig. 2, no interference was observed at the retention time of the peaks corresponding to CPA, CP-OH and BMDP, which demonstrates the good selectivity of the methods.

3.3.2.2. Response function. The response function of an analytical procedure is, within the range selected, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample [14–16,23,24]. The validation results for the response function of the two methods are presented in Tables 2 and 3, respectively. As previously mentioned, a linear regression model through 0 fitted with the highest concentration level was used for both methods for the determination of CPA in epidermis and dermis samples.

3.3.2.3. Trueness. Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [14–16,23–26]. As can be seen from the results in Tables 3 and 4, trueness was expressed in terms of absolute bias (ng/ml) or relative bias (%) and was assessed by means of validation standards in the two different matrices at three concentration levels ranging from 33 to 667 ng/ml ($k = 3$, $n = 3$). Mean values are close to the theoretical concentrations, illustrating the rather good trueness of the proposed methods.

3.3.2.4. Precision. The precision of the bioanalytical method was estimated by measuring repeatability and intermediate precision at the same concentration levels as those mentioned above. The variance of repeatability and time dependent intermediate precision as well as the corresponding relative standard deviation (R.S.D.) values were calculated from the estimated concentrations [14–16]. Considering the regulatory requirements [23,24], the precision of both methods for the determination of CPA was acceptable since the R.S.D. values did not exceed the value of 15%, irrespective of the concentration level.

3.3.2.5. Accuracy. The accuracy takes into account the total error, i.e. systematic and random errors, related to the test result [14–16,23–26]. The upper and lower β-expectation tolerance limits for both methods, expressed in ng/ml (or %), are presented in Tables 3 and 4 as a function of the introduced concentrations. As can be seen from these results, the proposed methods were accurate, since the different tolerance limits of the bias did not exceed the acceptance limits of total error for all concentration levels tested including the lowest one (33 ng/ml).

Table 3
Validation of the method for the determination of CPA in epidermis

Response function ($k = 3$, $m = 3$, $n = 3$)	Slope		
	Day 1 (10.06×10^{-3})	Day 2 (9.04×10^{-3})	Day 3 (10.30×10^{-3})
Trueness ($k = 3$; $n = 3$)	Absolute bias (ng/ml)	Relative bias (%)	
33 ng/ml	−0.3	−0.9	
167 ng/ml	−0.6	−0.4	
667 ng /ml	20.1	3.0	
Precision ($k = 3$; $n = 3$)	Repeatibility (R.S.D., %)	Intermediate precision (R.S.D., %)	
33 ng/ml	5.2	11.9	
167 ng/ml	8.4	8.5	
667 ng/ml	5.2	6.8	
Accuracy ($k = 3$; $n = 3$)	β-Expectation confidence limits in ng/ml (%)		
33 ng/ml	24.0 to 43.0 (−29.0 to 27.3)		
167 ng/ml	141.5 to 195.0 (−16.2 to 15.5)		
667 ng/ml	601.5 to 790.0 (−11.0 to 16.9)		
Linearity ($k = 3$; $m = 3$; $N = 18$)			
Range (ng/ml)	33 to 667		
Slope	1.034		
Intercept	−3.649		
r^2	0.992		
LOD (ng/ml)	10.2		
LOQ (ng/ml)	33.3		
Dilution effect	Factor (μg/ml)	Recovery ± S.D. (%)	
$n = 3$	3 (2.0)	99.4 ± 3.2	
	6 (4.0)	103.1 ± 4.1	

3.3.2.6. Linearity. The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (amount) of the analyte in the sample [14–16]. Consequently, for all series, a regression line was fitted on the estimated or back-calculated concentrations as a function of the introduced concentrations by applying the linear regression model based on the least squares method. The regression equations for the two methods are presented in Tables 3 and 4, respectively.

3.3.2.7. Limit of quantitation. The LLOQ of an analytical procedure is the lowest amount of the targeted substance in the sample which can be quantitatively determined under the experimental conditions prescribed with a well defined accuracy [14], i.e. taking into account the systematic and random errors [23,24]. Since the accuracy profiles of the two methods for the determination of CPA in skin layers were comprised within the acceptance limits, the LLOQ was fixed at 33.3 ng/ml, i.e. the smallest concentration level investigated. Indeed, precision and trueness were demonstrated at this concentration level (Tables 3 and 4).

3.3.2.8. Effect of the dilution. Since the amount of CPA in the epidermis and dermis layers was not known a priori, the influence of the dilution procedure, which is intended to be used in routine for samples with a concentration higher than

Table 4
CPA concentrations in epidermis and dermis after Franz diffusion cells

		Epidermis		Dermis	
		Extracted (μg/sample)[a]	Tissue (ng/cm^2)[b]	Extracted (μg/sample)[a]	Tissue (ng/cm^2)[b]
Formulation 1	A	0.480 ± 0.163	272 ± 92	1.958 ± 0.221	1108 ± 125
	B	0.285 ± 0.207	161 ± 116	0.472 ± 0.293	266 ± 168
Formulation 2	A	0.184 ± 0.013	104 ± 7	1.229 ± 0.873	695 ± 493
	B	0.129 ± 0.037	71 ± 20	0.183 ± 0.018	105 ± 12
Formulation 3	A	3.731 ± 0.689	2111 ± 390	6.847 ± 1.824	3875 ± 1032
	B	9.605 ± 3.544	5436 ± 2005	5.009 ± 1.707	2835 ± 968

The diffusion of three different formulations through two skin samples from different individual sources, A and B, was tested. The values are the mean of two replicates ± standard deviation and are calculated taking into account the dilution.

[a] Amount of extracted CPA per sample.

[b] CPA concentration in tissue.

the upper limit of the range, has to be checked [15,16]. In the present study, two dilution factors (three and six) were studied before routine analysis and no significant effect was observed (Tables 3 and 4).

3.4. Method follow-up during routine analysis

Once the assay method has been established for routine use, its performance should be regularly monitored to ensure that it continues to work properly. In order to assess the method performances during routine analysis, quality control samples at different concentration levels have to be analysed. The most widely used procedure for the continuing evaluation of assay performance involves the construction of QC charts. In the present study, the acceptance limits have been fixed at ±15% of the observed bias according to the Washington conference [23] and the FDA document [24]. Three concentration levels for each method were monitored. The QC charts presented in Fig. 5 demonstrate that the analytical procedures were under control during routine analysis. Indeed, at least 67% of the QC samples were within 15% of their nominal values and definitely less than 33% of the QC samples without replicates at the same concentration levels were outside the ±15% of the nominal value [24].

3.5. Application of the method to skin samples from diffusion tests

The analytical method was applied to quantify CPA in epidermis and dermis after application of CPA gels on human skin in diffusion Franz cells. The analysis of skin samples obtained from different patients gave good results and no interference was observed at the retention time of the peaks

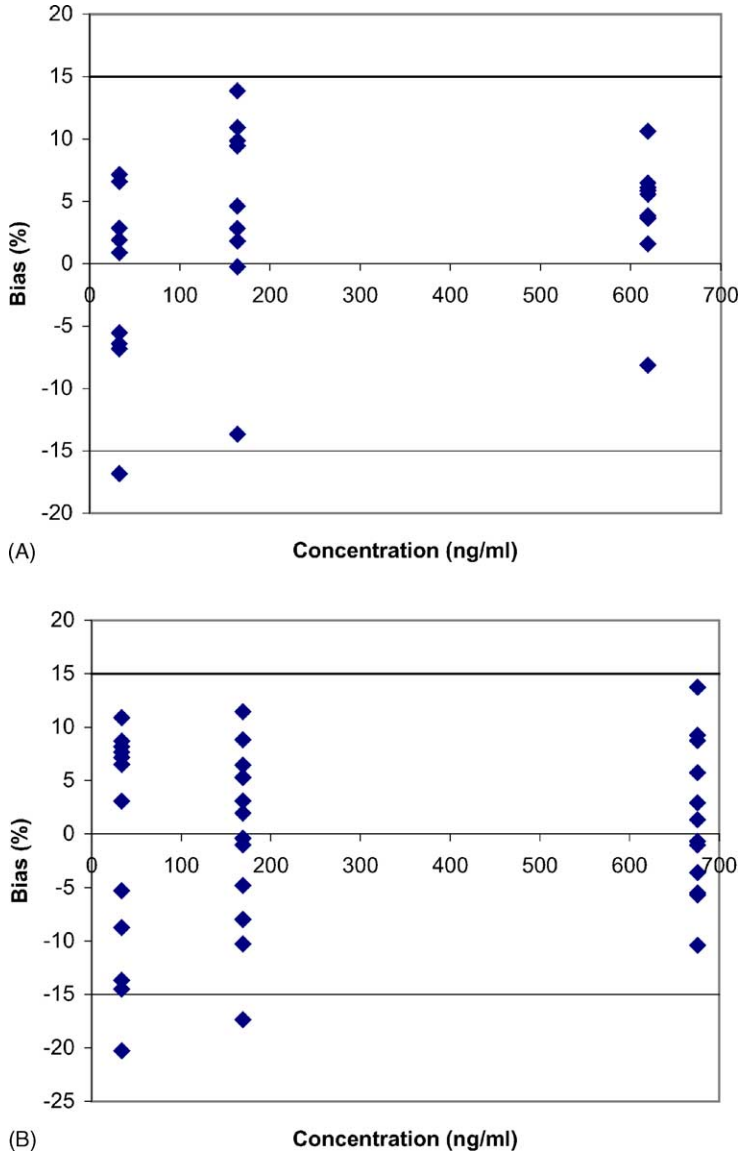

Fig. 5. QC charts of CPA in dermis (A) and epidermis (B).

corresponding to CPA and BMDP. Moreover, as shown in Fig. 2d illustrating a chromatogram of an epidermis sample, no peak corresponding to CP-OH was observed, which seems to mean that the hydrolysis of CPA did not occur in skin under the conditions of the diffusion test. Table 4 shows the results achieved after application of three different formulation gels on skin of two different patients. As can be seen in this table, important inter- and intra-individuals variations were observed. Indeed, the standard deviation values and the differences in the concentrations of CPA from different skin sources were high. However, such variations are usually observed for biological samples. For high CPA concentrations, the use of a higher amount of internal standard and the application of an appropriate dilution was needed in order to obtain a suitable ratio between the peak areas for CPA and BMDP taking into account the calibration range considered. The results in Table 4 also show a higher penetration of CPA in the skin with the formulation 3, which is a hydro-alcoholic gel in opposition to formulations 1 and 2 that are aqueous gels.

4. Conclusions

A simple HPLC method was developed to quantify CPA in epidermis and dermis samples. The extraction method of skin was appropriate since no aggressive product, as sodium hydroxide, susceptible to damage CPA was used. In addition, methanol selected as extraction solvent presents relatively less chronic toxicity than other solvents, such as acetonitrile, dichloromethane or chloroforme. Moreover, the pulverizing of the skin with Dismembrator® after freezing in liquid nitrogen allows obtaining very fine pulverised tissue in comparison to other methods, such as homogenization with an Ultra Turrax®. This method was then validated over a range from 33 to 667 ng/ml after extraction of epidermis and dermis samples.

The successful application of this method to skin samples from diffusion tests on Franz cells allows evaluating the percutaneous penetration of CPA from different formulations, by determining the concentrations of this drug in two skin layers, epidermis and dermis.

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