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Celecoxib determination in different layers of skin by a newly developed and validated HPLC-UV method

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ABSTRACT: A simple, rapid and sensitive analytical procedure for the measurement of celecoxib (CXB) levels in skin samples after *in vitro* penetration studies was developed and validated. *In vitro* permeability studies in porcine skin were performed for quantification of CXB at different layers of skin, the stratum corneum (SC) and epidermis plus dermis (EP + D) as well as in the acceptor solution (AS) to assess CXB permeation through skin. CXB was quantified by HPLC using a C_{18} column and UV detection at 251 nm. The mobile phase was methanol–water 72:28 (v/v) and the flow-rate was 0.8 mL/min. The CXB retention time was 5 min. The assay was linear for CBX in the concentration range of 0.1–3.0 μg/mL in the AS (drug permeated through skin) and 5.0–50.0 μg/mL for drug retained in SC and [EP + D] *in vitro*. The linear correlation coefficients for the different calibration curves were equal or greater than 0.99. Intra- and inter-assay variabilities were below 8.0%. Extraction of CXB from skin samples showed recoveries higher than 95.0% after 15 min of ultrasonic sound and centrifugation at 2500 rpm for 3 min. The method was considered appropriate for the assay of CXB in skin samples, after *in vitro* cutaneous penetration studies. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Celecoxib; vertical diffusion cell; tape stripping; skin; HPLC; validation method

Introduction

Celecoxib (CXB) is a nonsteroidal anti-inflammatory drug approved for the treatment of rheumatoid arthritis, osteoarthritis acute pain and primary dysmenorrhea (Fisher *et al*., 1999; Fort, 1999; Schiffman *et al*., 2008). Its use in the treatment of cervical and breast cancer has been highlighted in the literature (Kawamori *et al*., 1998; Harris *et al*., 2000, 2006). Recently, CXB has also been explored as a potential chemopreventive agent in different types of cancer, including UVB light-induced skin cancer, by retarding tumor latency (Fisher *et al*., 1999; Kismet *et al*., 2004). Inhibition of COX-2 enzyme, present in increased amounts in carcinogenic processes, is the probable mechanism of chemoprevention by CXB, a fact that may direct its use in anticancer therapy (Kismet *et al*., 2004).

Currently CXB is marketed in the conventional form of capsules (oral administration) for treatment of various inflammatory conditions due to its effectiveness and safety. However, its side effects in liver and cardiovascular conditions cannot be disregarded in long-term oral treatments. Thus, a safer alternative for the treatment of skin inflammation (including skin cancer induced by UVB radiation) or inflammatory processes arising from rheumatoid arthritis would be topical or transdermal administration of CXB. Drug administration as cutaneous applications directly to the site of injury may minimize or eliminate systemic side effects.

An analytical methodology for rapid and reliable quantification of CXB applied to studies of skin penetration *in* vitro would add support to this alternative. Currently, the scientific literature does not report research addressing precision, accuracy, linearity and recovery of CXB extraction from different layers of the skin, making it an innovative research field. The aim of the present study was to develop and validate an analytical HPLC method with UV detection to determine CXB extracted from different skin layers as a very useful tool for rapid quantification in skin permeation and retention studies *in vitro*.

Material and methods

Chemicals

Celecoxib, 99.4% pure, was purchased from Exim Pharm International (India). Caffeine was from Sigma Chemical Co (St Louis, MO, USA). Tween 20® and methanol of HPLC grade were from Merck (Darmstadt, Germany) and water used in all experiments was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Phosphate buffer pH 7.4 (±0.2) 0.01 M solution was used to prepare the acceptor phase (PBS added 2.0% Tween 20®).

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Abbreviations used: AS, acceptor solution; CXB, celecoxib; EP + D, epidermis plus dermis; SC, stratum corneum.

Apparatus and chromatographic conditions

The HPLC equipment used for CXB analysis was a Shimadzu system (Kyoto, Japan), equipped with 2 LC-10AD pumps and a UV detector SPD-10A/10AV operating at 251 nm. The mobile phase was methanol–water (72:28, v/v) previously degassed with helium gas. The chromatographic column was an RP-C_{18} (10 cm length, 5 µm, 4 mm idLiChrospher; Merck, Darmstadt, Germany), and the flow rate was 0.8 mL/min. The running time was 5 min and the injection volume 20 µL. Caffeine was chosen as internal standard (IS) to correct for any losses during CXB extraction.

Calibration curves and selectivity of the method

A stock solution of celecoxib (1 mg/mL) was prepared by dissolving the appropriate amount of drug in methanol and diluted to prepare standard solutions of concentration range between 0.1 and 3.0 µg/mL in acceptor solution (AS; phosphate buffer added 2.0% Tween 20®) for permeation studies and 5.0–50 µg/mL in methanol–water (72:28) as extractor solvent for *in vitro* retention studies.

The selectivity of the method was confirmed by individual analysis of interfering samples. Solutions containing internal standard, acceptor phase, blank and samples from different skin layers – stratum corneum (SC) or epidermis and dermis (EP + D) – from porcine ear skin extracts were analyzed.

Determination of celecoxib in skin layers

***In vitro* skin penetration studies.** Different skin layers were obtained for *in vitro* skin penetration studies. Fresh porcine ears were obtained from the local slaughterhouse Fripon (Pontal, SP, Brazil) and immediately cleaned with cold running water. After cleaning, skin samples were dissected from the dorsal region of porcine ear and attached to a horizontal support for dermatomization. This process promoted a parallel cut to the skin surface, thereby providing a uniform thickness of tissue, around 500 µm (Dermaton, Nouvag, Switzerland). Skin samples were kept at −18 to −20°C for a maximum period of 30 days. The apparatus used to perform the *in vitro* skin penetration studies consisted of vertical diffusion cells with a receiver compartment of 100 mL and a surface area of 3.8 cm^2. The skin samples were clamped between the two chambers of vertical diffusion cells, with the SC facing the donor compartment and the dermis facing the acceptor phase (0.01 M phosphate buffer pH 7.4 ± 0.2, containing 2.0% v/v Tween 20®. The solubility of CXB in this medium was 630 µg/mL. Lipophilic drugs quantified in *in vitro* penetration studies should have adequate solubility in the AS. In our study, the addition of 2.0% of surfactant Tween 20® to phosphate buffer produced a proper AS for the solubilization of CXB permeated through the skin.

The donor phase consisted of CXB (2.0% w/w) in propylene-glycol. An aliquot of 0.4 g of this formulation was applied on the skin samples, in the donor compartment. In order to keep the skin surface at 32°C, the acceptor phase was maintained at 37°C and stirred at 50 rpm. After 24 h, a 1.0 mL sample was taken from the acceptor phase to determine the total amount of CXB permeated through the skin. The CXB retained in SC and [EP + D] layers was also quantified. This experiment was carried out in triplicate.

Development and validation of the extracting process of CXB retained in skin layers. After *in vitro* skin penetration experiments (as described above), the donor phase was removed and the skin washed with deionized water in order to eliminate donor solution remaining at the surface. The skin was carefully dried with cotton wool and the tape-stripping technique was used to separate the stratum corneum (SC) from the remaining layers ([EP + D]). The SC layer was obtained by tape-stripping with 15 adhesive cellophane tapes (3M, Scotch Book Tape no. 845; 3M, St Paul, MN, USA), the first tape being rejected. The remaining tissue containing the [EP + D] (without stratum corneum) was cut into small pieces. Both stripping tapes [SC] and tape-stripped skin [EP + D] were put in glass tubes and reserved for later extraction.

An optimized CXB extraction process from skin samples (SC and [EP + D]) was developed evaluating different volumes of extractor solvent (3–5 mL), ultrasound bath time (15–30 min), different centrifugation times of incubated samples (3–5 min) and influence of Ultra Turrax tissue cutters.

For the development of the CXB extraction procedure, 50 µL of CXB standard solution (in methanol) was added to glass tubes containing 5 mL of the solvent extractor and samples of SC or 3 mL of the solvent extractor plus EP + D, corresponding to 10 and 16.6 µg/mL of CXB, respectively.

The SC or [EP + D] samples containing the standard solutions were left for 30 min to allow CXB penetration on biological material, followed by a complete evaporation of methanol using air flow. CXB extraction was carried out using the mobile phase as extraction solvent (methanol–water, 72:28 v/v) in different volumes (3 or 5 mL). After addition of extraction solvent, 50 µL of methanolic internal standard caffeine at 1 mg/mL was added to each tube. All samples were shaken for 1 min and maintained in ultrasound bath (15 or 30 min) followed by 2500 rpm centrifugation for 3 or 5 min. The use of Ultra Turrax cutter tissue for 3 min was evaluated in order to verify its influence on extraction of retained CXB. Finally, samples were filtered through a 0.45 µm pore size membrane (Sartorius, Goettingen, Germany) and assayed by the HPLC method described previously.

Linearity of CXB determinations in the acceptor phase (0.01 M phosphate buffer pH 7.4 added 2.0% Tween 20) used for *in vitro* permeation studies was evaluated by consecutive dilutions of CXB stock in acceptor phase to give standards of 3.0, 2.0, 1.5, 0.5 and 0.1 µg/mL and plotting the detector response (peak area) vs nominal concentration of CXB present in samples. These data were used to validate the selectivity, linearity and recovery for penetration studies of CXB extractions from different skin layers. Linear equations and correlation coefficients (r^2) were obtained from calibration curves constructed by plotting ratios between areas of CXB and internal standard (HPLC) and corresponding concentrations of CXB.

To asses CXB linearity in different skin layers of porcine ear skin such as SC and [EP + D], sufficient amounts of drug in methanolic solution were added to glass tubes containing adhesive tapes with SC or tape-stripped skin [EP + D], corresponding to 50.0, 30.0, 20.0, 15.0, 10.0, 8.0 and 5.0 µg/mL of CXB.

Spiked samples were kept for 30 min to allow drug penetration. Samples were then extracted as described previously. Results were compared with the CXB calibration standard in methanolic solution without extraction.

Accuracy and precision evaluations for acceptor phase as well as samples of SC and [EP + D] were performed on both intra-day and inter-day measurements. Intra-day variability of the assay method was determined by repeated analysis of three concentrations of CXB on the same day, such as 3.0, 1.5 and

0.5 μg/mL for acceptor phase and 50.0, 10.0 and 5.0 μg/mL for skin layers. Similarly, inter-day variability was determined by repeated analysis of the same samples in three different days. Precision was expressed as the relative standard deviation (RSD) of replicate measurements.

Accuracy was calculated as the standardized correlation between the measured value and true value. Accuracy was expressed as the relative error of measurement (%).

The lower limit of quantification (LLOQ) was defined as the lowest concentration of CXB methanolic solution which can be determined with both precision (relative standard deviation, RSD) and accuracy levels above 90%. The lower limit of detection (LOD) was calculated by analyzing CXB in known and decreasing concentrations to the lowest detectable level, but still 2–3 times higher than baseline noise (Snyder *et al*., 1997).

Recovery and stability of celecoxib from skin samples: SC and [EP + D]

CXB recovery from skin samples was determined by the ratio of the amount of drug extracted from three spiked samples to the amount of drug added. Three replicates from both adhesive tapes containing SC or tape-stripped skin [EP + D] were spiked with sufficient quantities of CXB in methanolic solution corresponding to 5, 10 and 50 μg/mL. The selected extraction procedure consisted of 5 or 3 mL methanol–water 72:28 (v/v) as extraction solvent for SC and [EP + D] samples, respectively. The internal standard caffeine (50 μL) was added to all samples and shaken for 1 min. Samples were maintained in an ultrasound bath during 15 min in order to extract the CXB retained, followed by centrifugation for 3 min at 2500 rpm, filtration through a 0.45 μm pore size membrane filter (Sartorius, Goettingen, Germany). An aliquot of 20 μL of the samples was assayed by HPLC.

The stability of CXB was verified in different storage conditions. Samples were kept during 12 h at room temperature or 24 h at 4°C. Results were compared with those obtained from freshly prepared samples.

Results

Linearity, selectivity, precision and accuracy of the method to quantify CXB in different media

Chromatographic conditions were validated according to Dhabu and Akamanchi (2002) and completed with criteria reported by Baboota *et al*. (2007). The internal standard was chosen to mimic the behavior of the sample compound in extraction steps. According to Lioyd *et al*. (1997), a proper internal standard: (i) has similar retention (*k*) to the analyte; (ii) mimics the analyte in any sample preparation steps; (iii) does not have to be chemically similar to analyte; (iv) must be commercially available in high purity; (v) must be stable and unreactive with sample or mobile phase; and (vi) should have a similar detector response to analyte at the concentrations used. Caffeine was used as internal standard because an extraction step was involved. Samples containing blank biological matrices and blank AS (Fig. 1a–c respectively) were analyzed by HPLC under the same chromatographic conditions and did not show peaks at the retention times of either CXB nor caffeine, demonstrating the selectivity of analytical methodology. The chromatographic elution times of caffeine

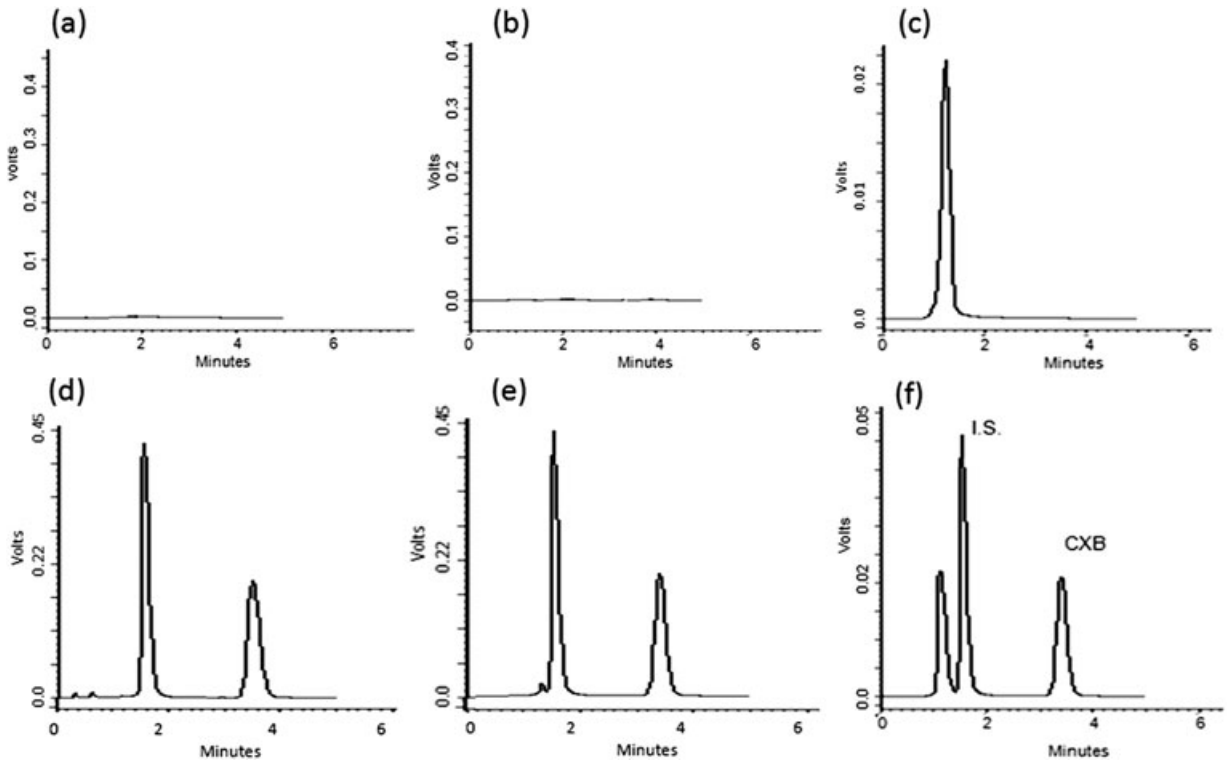

Figure 1. HPLC profiles from different media: (a) blank stratum corneum matrix; (b) blank [EP + D] matrix; (c) blank acceptor phase; (d) 20 μg/mL CXB standard in SC matrix; (e) 20 μg/mL CXB standard in matrix [EP + D] without SC; and (f) 2 μg/mL CXB standard in the acceptor phase. Separation in an RP-C_{18} column, methanol–water (72:28 v /v) as mobile phase, flow rate 0.8 mL/min, detector operating at 251 nm.

and CXB in biological matrices – SC and [EP + D] – or AS (Fig. 1d–f) were 1.5 and 3.7 min respectively.

Linearity of CXB extraction from different penetration experiments and skin layers, SC and [EP + D], were determined by preparing different drug concentrations and also a fixed concentration of internal standard according to criteria reported by Snyder *et al.* (1997). Quantification was determined by the ratio between areas of CXB and internal standard in chromatograms. Figure 2 shows the linearity of CXB at a concentration range of 0.1–3.0 μg/mL in phosphate buffer containing 2% of Tween 20®. Figure 3 shows the linearity of CXB in concentration range of 5.0–50.0 μg/mL from biological matrices (SC and EP + D) in extractor solvent methanol–water (72:28). The linear correlation coefficient (*r*) in these curves was equal or greater than 0.99.

The precision and accuracy of CXB intra and inter assays in different matrices are shown in Table 1. It shows variation coefficients of less than 8% and accuracy above 98.4%. The lower limit of quantification (LLOQ) and detection (LOD) for the analytical methodology was determined as 0.1 μg/mL.

Development and validation of the extraction procedure for CXB retained in skin samples

The extraction of CXB from samples of SC and [EP + D] was validated by selecting the most effective parameters: ideal volume of extraction solvent, ultrasound bath and centrifugation time periods. Table 2 shows values higher than 95 and 98% for CXB extraction from samples of SC and [EP + D], respectively, with 5 and 3 mL of mobile phase as solvent volume, ultrasound for 15 min followed by centrifugation at 2500 rpm for 3 min. The use of tissue cutter Ultra Turrax did not improve CXB extraction values.

Recovery and stability of celecoxib extracted from the SC and [EP + D]

The CXB recovery (%) from different skin layers (SC and [EP + D]) was determined after extraction at three different drug concentrations (5.0, 10.0 and 50.0 μg/mL) by comparing the amounts of drug extracted with the amounts of drug initially added. The recovery rates of CXB in different skin layers (Table 3) was in the range of 97.0–105.0% for SC and 80.0–102.0% for [EP + D] using extraction conditions previously validated. This range is considered satisfactory for the recovery of drugs extracted from samples of SC and [EP + D] (De Paula *et al.*, 2008).

The stability of the CXB extracted from different layers of the skin was assessed within 12 or 24 h after sample preparation and is shown in Table 4. The results show comparisons for samples analyzed in concentrations of 5.0, 10.0 and 50.0 μg/mL. Compared with freshly prepared samples, the CXB concentrations vary between 86.4 and 109.1%.

Method application

The method described and validated was applied for the quantification of CXB in both skin samples (SC and [EP + D]) and samples from the AS collected from diffusion cells after *in vitro* skin permeation studies (Fig. 4).

Results after 24 h of *in vitro* skin permeation and retention of CXB (from a donor phase containing 2.0% drug in propyleneglycol) showed average values of 1.09 (± 0.65) and 8.55 (± 2.90) μg/cm^2 of CXB retained in SC and [EP + D], respectively, while 138.44 (± 55.0) μg/cm^2 of CXB permeated through the skin.

Discussion

Several methodologies have recently explored the determination of CXB in biological and nonbiological samples. The literature reports a number of methods for CXB quantification using spectrophotometry, fluorometry or electrophoretic and chromatographic methods (Rao *et al.*, 2005). Most methods developed and validated for HPLC usually use acetonitrile in the composition of the mobile phase and show quantification of CXB in plasma or serum (Abdel-Hamid *et al.*, 2001; Lutz *et al.*, 2001; Saha *et al.* 2002; Schoenberger *et al.*, 2002; Stormer *et al.*,

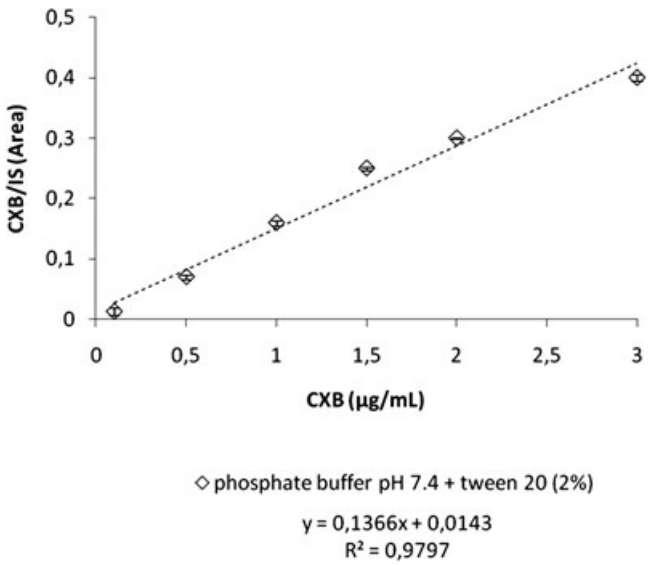

Figure 2. Calibration curve of CXB constructed by plotting ratios of areas of internal standard and drug (HPLC) against CXB concentrations (0.1–3.0 μg/mL). Test solution was dissolved in 0.01 M phosphate buffer 7.4 ± 0.02 containing 2% Tween 20® (acceptor phase). The values of the straight line equation and correlation coefficient are calculated.

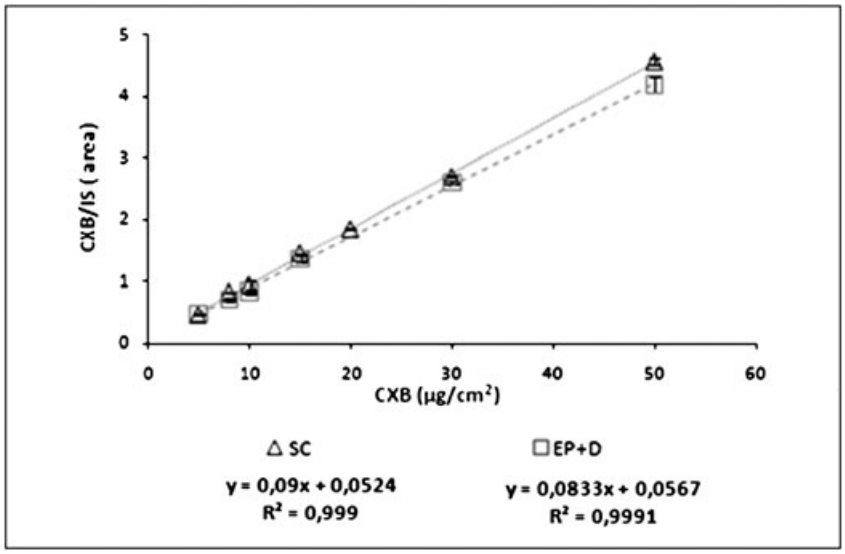

Figure 3. Calibration curves of CXB after extraction from SC and [EP + D] samples constructed by plotting ratios of areas of internal standard and drug (HPLC) against CXB concentrations (5.0–50.0 µg/mL). The values of the straight line equation and correlation coefficient are calculated.

Table 1. Precision and accuracy of intra and inter days assays of CXB quantification by HPLC in different media, obtained by relating the peak areas of caffeine internal standard and CXB

	Intra and Inter days assay			
CXB concentrations	Intra-day Mean (± SEM) $n = 3$	Inter-day Mean (± SEM) $n = 9$	Intra-/Inter-day precision (%)	Intra-/inter-day acuracy (%)
		Acceptor phase		
3000 ng/mL	3319.20 (±83.76)	3158.09 (±309.293)	2.52/9.79	110.64/105.26
1500 ng/mL	1400.00 (±84.285)	1445.6 (±39.359)	6.02/2.72	93.33/96.37
100 ng/mL	103.3 (±2.654)	100.92 (±2.557)	2.56/2.53	103.33/100.92
50 µg/mL	50.02 (±0.098)	45.725 (±1.491)	0.19/3.260	100.04/91.45
10 µg/mL	10.006 (±0.005)	9.264 (±0.736)	0.05/7.94	100.06/92.64
		SC		
5 µg/mL	5.005 (±0.005)	4.889 (±0.398)	0.11/8.146	100.11/97.77
		[EP + D]		
50 µg/mL	49.325 (±1.534)	49.418 (±0.093)	3.10/0.18	98.65/98.83
10 µg/mL	9.871 (±0.385)	10.256 (±0.600)	3.94/5.85	98.70/102.56
5 µg/mL	4.940 (±0.118)	4.475 (±0.193)	2.381/4.328	98.80/89.50

2003; Chow *et al*., 2004; Hassan *et al*., 2004; Zhang *et al*. 2006) in bulk drugs (Jayasagar *et al*., 2002; Abdel-Hamid *et al*., 2001; Satyanarayana *et al*., 2004) and pharmaceutical dosage forms (Schoenberger *et al*., 2002).

Some methods use methanol and water as the mobile phase (Pramod and Krisnacharya, 2002; Krishnaiah *et al*., 2003) for analysis of CXB in pharmaceutical dosage forms (Jalalizadeh *et al*., 2004; Dhabu and Akamanchi, 2002), but none of them are aimed at the quantification of CXB extracted from skin layers. Garcia *et al*. (2006) presented a standardized method for extraction of other anti-inflammatory drug ketoprofen in skin samples also using methanol as a solvent extractor.

To date, there are no methods in the literature about extraction of CXB or other nonsteroidal anti-inflammatory COX-2 selective heterocyclics (rofecoxib, valdecoxib, etorcoxibe, cimicoxibe) in skin samples or their recovery from skin for comparison with our results. The extraction and recovery of lumiracoxib, an anti-inflammatory in the same therapeutic class (but with nonheterocyclic structure) was described in a recent article (Moreira *et al*., 2010) using acetonitrile as solvent extractor in porcine skin samples.

A choice of mobile phase consisting of methanol and water is more advantageous compared with acetonitrile in terms of economy and commercial availability. In addition, the system methanol–water 72:28 used in this study for the CXB was appropriate for extracting the drug with high percentage recovery in biological samples.

Baboota *et al*. (2007) reported a CXB determination by HPLC in microemulsions using reversed-phase chromatography on a C_{18} column with methanol–water 75:25 (%, v/v), as mobile

Table 2. Validation of the extraction method of caffeine and CXB using the mobile phase as extractor, by varying parameters like volumes of solvent extraction, periods of ultrasonication, centrifugation and the influence of using the Ultra Turrax tissue cutter

Stratum corneum (SC)				
Volumes of solvent extraction (mL)	5	5	3	3
Ultrasound bath period (min)	30	15	30	15
Centrifugation period (min)	5	3	5	3
CXB recovery (%)	95.70	95.81	91.33	90.78
Epidermis plus dermis, without SC [EP + D]				
Volumes of solvent extraction (mL)	5	5	3	3
Ultrasound bath period (min)	30	15	30	15
Centrifugation period (min)	5	3	5	3
Turrax tissue cutter used (min)	3	—	3	—
CXB recovery (%)	97.25	94.74	96.40	98.46

Table 3. CXB recovery from different matrices (SC and EP + D) after extraction procedure. The results are presented as percentages of recovery obtained by relating the peak areas between caffeine internal standard and CXB

	Recovery (%)		
CXB concentrations in μg/mL	50	10	5
Extraction from SC	105.2	89.15	96.69
Extraction from [EP + D]	96.5	79.17	102.04
Neat solution	100	100	100

phase and UV detection at 250 nm. The limit of detection was 0.086 μg/mL.

The present study describes, for the first time, an HPLC method for CXB determination in skin samples, using a reverse-phase C_{18} column, mobile phase methanol–water (72:28 v/v) and a drug retention time of 3.7 min. The method showed selectivity (Fig. 1), linearity (Figs 2 and 3), precision and accuracy for the drug in different media (Table 1).

In brief, Fig. 2 shows linearity of the calibration curves of CXB in phosphate buffer (0.01 M, pH 7.4) in the presence of 2.0% Tween 20®. Figure 3 also shows linearity, precision and accuracy of CXB quantification after extraction from different layers of the skin (SC and [EP + D]) using mobile phase methanol–water (72:28 v/v) as the extraction solvent.

The skin extraction procedure was carried out using porcine ear skin. It is considered an excellent skin model, due to histological characteristics similar to human skin in terms of epidermal thickness and composition, pelage density, epidermal lipid biochemistry and general morphology (Bhatia and Singh, 1996); in other words, it is a practical alternative to human skin. An excellent correlation between permeation data using porcine ear skin *in vitro* and human skin *in vivo* has been demonstrated by various investigators (Diembeck *et al*., 1999; Busch *et al*., 1996; Beck *et al*., 1993).

The extraction procedure from skin (total skin or a specific layer) is easy, fast and inexpensive, and most methods use solvents or solutions associated with ultrasound or homogenization of tissues to break the cell membrane and release the drug (Pierre *et al*., 2006; Moreira *et al*., 2010). The most commonly used solvents for extraction of lipophilic substances from the skin are methanol, ethanol (Moreira *et al*., 2010) chloroform, dichloromethane (Steluti *et al*., 2005; Lopes *et al*., 2006), etc. In this study, the best alternative for the extraction of CXB from samples of skin (SC and [EP + D]) was the same mixture used as mobile phase for its quantification by HPLC.

In developing the extraction methodology for CXB from different layers of skin, the following optimum conditions were selected: volumes of 5 and 3 mL of solvent (mobile phase) for extraction of drug in SC and [EP + D], respectively; incubation in an ultrasonic bath (15 min) and centrifugation at 2500 rpm (3 min) in both cases (Table 2). The CXB recovery after extraction from SC and [EP + D] was considered optimal for low, medium and high drug concentrations (Table 3).

A simple, rapid and sensitive method is a valuable tool for skin penetration studies, enabling the evaluation of CXB in samples from *in vitro* penetration studies (permeation through skin) and in different layers of skin (drug retention in the cutaneous tissue). Figure 4 shows the chromatograms of real samples of CXB after 24 h of *in vitro* permeation experiment. In our *in vitro* penetration studies, the highest amounts of CXB were found in deeper layers of skin and in permeation samples. This may be related to lipophilic characteristics of CXB (log $P = 4.21$) and its high permeability through membranes (Biopharmaceutical Class II). Having a pK_a around 11, CXB is in a nonionized form at physiological medium which promotes penetration and permeation through membranes.

In brief, the results showed that CXB can be quantified from samples of AS (corresponding to permeated drug) or extracted from the SC and [EP + D] by tape stripping technique followed by HPLC quantification in a reproducible method.

The stability of the skin samples containing CXB was also evaluated, showing little variation in the concentrations of the drug at room temperature and under refrigeration and after exposure times (Table 4). In both cases, stability was maintained for a reasonable time. However, higher concentrations were observed for CXB concentrations in samples

Table 4. CXB stability in different matrices (SC and EP + D) determined after the extraction process using the mobile phase as solvent. Samples were analyzed at 12 and 24 h after preparation and the results presented in µg/mL of CXB as ratios between CXB and caffeine internal standard compared with samples freshly prepared

Time period/temperature (°C)	CXB (µg/mL)[a] (± SD)		
	50	10	5
12 h at 25°C	SC = 53.72 (±3.99) [EP + D] = 58.41 (±5.10)	SC = 10.36 (±0.1) [EP + D] = 11.71 (±0.90)	SC = 5.45 (±0.65) [EP + D] = 5.13 (±0.02)
24 h at 4°C	SC = 44.16 (±1.06) [EP + D] = 48.15 (±3.67)	SC = 8.64 (±0.10) [EP + D] = 9.70 (±0.5)	SC = 4.96 (±0.04) [EP + D] = 4.71 (±0.01)

[a]Data represent the media of three determinations.

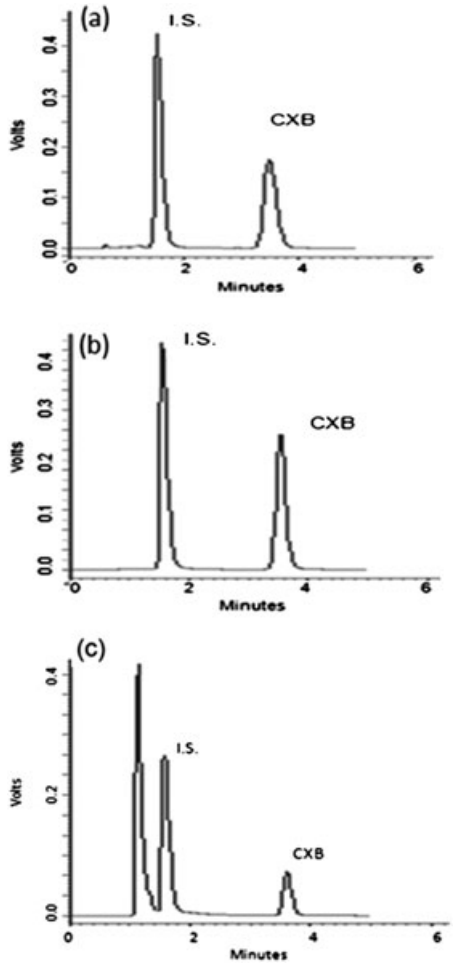

Figure 4. HPLC profiles of CXB and caffeine (IS) in different media (a) stratum corneum matrix; (b) [EP + D] matrix; and (c) acceptor phase after 24 h of *in vitro* permeation studies.

that remained at room temperature (± 25°C), suggesting a possible evaporation of the solvent under these conditions, which was not observed with samples kept in the refrigerator at 4°C.

Conclusion

The present method was successfully applied for the quantification of CXB for *in vitro* cutaneous penetration studies. The results obtained demonstrated that CXB can be quantitatively extracted from porcine skin samples (SC and [EP + D]) as well as samples from AS of vertical diffusion cells, after *in vitro* percutaneous studies, and can be determined by HPLC in a simple, linear, precise, accurate and reproducible assay. This method can be adopted to assess *in vitro* permeation and retention of CXB in the development of topical or transdermal delivery systems.

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