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HPLC Quantification of Dexamethasone Palmitate in Bronchoalveolar Lavage Fluid of Rat after Lung Delivery with Large Porous Particles

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Abstract

A high-performance liquid chromatography (HPLC) method has been developed and validated for the determination of dexamethasone palmitate (DXP) in bronchoalveolar fluid lavage samples (BALF). DXP in rat BALFs containing the internal standard (IS), testosterone decanoate (TD), was extracted using a mixture of chloroform and methanol (9:1, v/v). Extracts were then centrifuged, dried and dissolved in acetonitrile. A chromatographic separation based on an isocratic elution was done using acetonitrile and water (85:15, v/v) as a mobile phase at a flow rate of 1.2 mL/min. The graph of the developed method was linear within the tested calibration range of 0.5 - 40 µg/mL. The overall extraction recovery of DXP from BALF samples was 84.3% ± 1.6%. The accuracy (relative error) and precision (coefficient of variation) values were within the pre-defined limits of ≤15% at all concentrations. This methodology has been applied to determine levels of DXP in BALF samples collected from rats treated with DXP large porous particles. The measured concentrations were successfully evaluated using a non-compartment pharmacokinetic model. Since the developed method requires only a microvolume (100 µL) of BALF sample for analysis, it is therefore particularly suitable for the evaluation of drug biodistribution in lung.

Keywords

Dexamethasone Palmitate, Bronchoalveolar Lavage Fluid, HPLC

1. Introduction

Glucocorticoids are the most effective therapy available for patients with asthma

[1] [2]. Glucocorticoids could be administered either orally, parenteral route or much more safely, by inhalation [3]. Inhaled glucocorticoids may have direct inhibitory effects on many of the cells involved in airway inflammation in asthma, including macrophages, T-lymphocytes, eosinophils, and airway epithelial cells. Inhalation allowed to have high local concentrations of glucocorticoids in the airway mucosa which limit the increase of steroid concentrations in the rest of the body [4] [5]. In previous work, we have formulated large porous particles containing dexamethasone palmitate intended to be administered by the pulmonary route for a prolonged action [6] [7]. Indeed, dexamethasone palmitate is a lipophilic molecule which allows high distribution to inflammatory lesions, high uptake by macrophages [8] [9]. Although inhaled formulations of dexamethasone palmitate have been extensively studied, little is known about the lung distribution of dexamethasone palmitate and its rate of conversion to dexamethasone after administration. However, for dexamethasone palmitate, only one assay method in lung fluid was reported in the literature. Indeed, Wijagkanalan *et al*. (2008) described a method for in vitro assay of DXP by HPLC using the hydroxyprogesterone caproate as internal standard [10]. The concentration of DXP in lung tissue was determined using Proteostain Protein Quantification Kit (Dojindo, Kumamoto, Japan) without any description [10] [11]. The objective of this work is therefore to develop a simple and sensitive method for the determination of dexamethasone palmitate in a bronchoalveolar fluid lavage (BALF) and its application to a pulmonary biodistribution study.

2. Materials and Methods

2.1. Chemicals and Reagents

Dexamethasone palmitate (DXP, 99%) was provided by Interchain (France), Testosterone decanoate (TD, 98%) for use as internal standard (IS) (**Figure 1**), was provided by Sigma-Aldrich (France), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC, 99%) by Corden Pharma (Switzerland) and hyaluronic acid, sodium salt 95% (HA) (MW = 1000 kDa) by Acros Organics. HPLC-grade methanol and acetonitrile were purchased from Carlo Erba (Italy). All chemicals used, were of analytical grade. Water was purified using a RIOS/MilliQ system from Millipore (France).

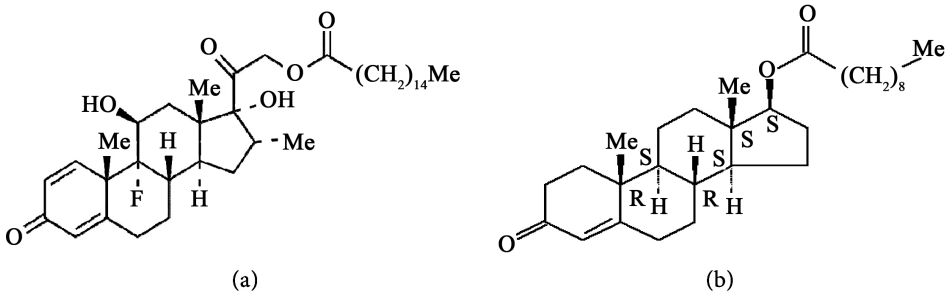

Figure 1. Chemical structures of dexamethasone palmitate (a) and testosterone decanoate (b).

2.2. Porous Particles Preparation

An aqueous solution of HA was prepared dissolving 200 mg of HA into 150 ml of water upon magnetic stirring at room temperature. An ethanolic solution was prepared by dissolving 750 mg of DPPC and 50 mg of DXP into 350 ml of ethanol absolute. Ethanolic and aqueous solutions were then mixed at a ratio of 70/30 (v/v) prior to spray-drying and the mixture maintained under moderate stirring while fed into the spray-dryer. Spray drying was performed using a mini spray-dryer BÜCHI B-290 (France) equipped with a 0.7 mm diameter two-fluid nozzle, which operates in a co-current mode according to conditions detailed in **Table 1** [12].

2.3. Animals

Sprague Dawley male rats with average weight of 300 g were obtained from Harlan Laboratory (Gannat, France). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the EU guidelines for Animal Experiments (86/609/EEC and 2010/63/EU) and Legislation in force in France (Decree No. 2013-118 of February 1, 2013) [13]. They were housed 4 per cage while on study in accordance to EEC guidelines. The light/dark cycle was 12 h/12 h. The temperature in the animal room was ambient room temperature of approximately 25˚C and the ambient humidity was in the range of approximately 35% - 60%. Animals were allowed access to food and water ad libitum throughout the duration of the study [14].

2.4. Bronchoalveolar Administration

Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 $mg \cdot kg^{-1}$) and xylazine (10 $mg \cdot kg^{-1}$) and then intratracheally administered with 3 mg of DXP powder corresponding to 150 µg of DXP using a Microsprayer (Penn Century, Philadelphia, PA) [15] [16]. Powder was delivered through the insufflation device by rapidly pushing a 2 cm^3 bolus of air through

Table 1. Spray drying conditions used to obtain the DXP porous particles.

Parameters	Operational conditions
Feed flow rate (mL/min)	17
Inlet temperature (˚C)	150 ± 2
Outlet temperature (˚C)	55 ± 4
Aspiration setting (%)	100
Air-flow rate (L/h)	414

the device. After administration, rats were supported vertically for 1 min. Bronchoalveolar fluid was collected at t = 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 18 h and 24 h after administration. At least 3 different rats were used for each time point. Bronchoalveolar lavage fluid (BALF) was collected by lavaging lungs three times with 1 mL of PBS per intratracheal lavage [17] [18]. The lungs were massaged and the fluid withdrawn immediately and collected in centrifuge tube. The rats were euthanized by cardiac puncture under deep isoflurane anesthesia before BALF collection. The trachea was cannulated using an 18-gauge needle adaptor for subsequent injection and retrieval of BALF. Then BALF collected containing the lung cells was frozen at −80˚C until further analysis [19].

2.5. Preparation of Calibration Standards and Quality Control Samples

Acetonitrile stock solutions of DXP (100 µg/mL) and TD (10 µg/mL) were prepared and stored at (−20˚C) until use. The DXP stock solution was diluted with acetonitrile to give a series of standard solutions with concentrations ranging from 1 µg/mL to 80 µg/mL. The calibration samples were prepared by diluting 1 volume of each standard solution with 1 volume of BALF. The final concentrations of calibration samples used to draw the calibration-curve ranged from 0.5 to 40 µg/mL (0.5, 2, 5, 10, 20 and 40 µg/mL). Standards for calibration were stored at −20˚C until the use [20] [21].

2.6. Sample Preparation

Extraction method of DXP in BALF was performed by using a mixture chloroform: methanol (9:1 v:v) [22] [23]. In an amber glass tube, 100 µL of BALF, 100 µL of different concentrations of standard solutions of DXP (0.5 - 40 µg/mL) and 100 µL of IS (2.64 µg/mL) were mixed with a vortex during 30 s. Then 3 mL of a mixture chloroform: methanol (9:1, v:v) were added in the tube. Sample was vortexed for 3 min to obtain protein precipitation then was centrifuged for 10 min at 10,000 rpm using a centrifuge (Jouan GR 412, France). The organic phase in the bottom was transferred into a clean amber vial and evaporated to dryness under a stream of nitrogen at 30˚C. The residue was then reconstituted into 100 µL of acetonitrile and vortexed prior to analysis using HPLC conditions described below [24] [25].

2.7. HPLC Method

Determination of DXP concentration in BALF was performed by HPLC. A Waters 2707 autosampler chromatographic system was employed equipped with a Waters™ 1525 binary HPLC pump, a Waters™ 2998 photodiode array detector, and a Waters™ Breeze software. The analysis was performed at 240 nm using a Symmetry Shield™ RP18 column (5 µm, 250 × 4.6 mm; Waters, France). Column temperature was maintained at 40˚C using a column Heater (Model 1500, Waters Corporation). The mobile phase consisted in a mixture of water: acetonitrile (15:85 (v: v)) and was pumped in isocratic mode for 40 min through the column with a

flow rate of 1.2 mL/min. The sample volume injected was of 50 µL [26] [27].

2.8. Validation

The method was validated according to the criteria developed by the International Conference of Harmonization (ICH) [28]. The parameters evaluated to assess the reliability of the results consist of the linearity of the chromatographic response as a function of analyte concentration from 0.5 to 40 mg/mL, the repeatability of the chromatographic analysis of the reference solution at three (3) levels of concentration: 2, 10 and 40 mg/mL (n = 6), the repeatability of chromatographic analysis of a sample of BALF (n = 6), the repeatability of the procedure (n = 6). The limits of detection (signal-to-noise ratio of 3) and quantification (signal-to-noise ratio of 10) determined were assessed from serial dilutions of the standard solution of DXP [29] [30].

2.9. Statistical Treatment

The linearity of the response of DXP was assessed from a scatter plot. The regression line was determined according to the least squares method. An analysis of variance (ANOVA) was performed to test the statistical significance and the overall slope of the regression line. The accuracy was assessed through the coefficient of variation calculated from the ratio of the standard deviation of the mean of each series of measurements. The level of statistical significance was set at 0.05 [31] [32].

2.10. Application

The validated method was applied to a series of DXP in BALF samples collected from rats treated with DXP large porous powder and the measured concentrations were fitted using a non-compartment pharmacokinetic model [33] [34].

3. Results and Discussions

3.1. Method Optimisation

3.1.1. Selection of Solvents and Separation Conditions

Different dilutions of the mixture acetonitrile:water (40:60, 50:50, 75:25, 85:15, 100: 0, v/v) at different temperatures (20˚C, 30˚C and 40˚C) were tested. Better peak shapes were obtained with acetonitrile:water (85:15, v/v) at 40˚C. Using a short column (5 µm, 250 × 4.6 mm) to achieve the separation in the shortest time possible resulted in an optimal separation. Under these conditions, a minimum of 50 separations can be achieved with the same set of separation vials without noticeable drift of migration times due to elution solvents.

3.1.2. Selection of Internal Standard

The use of an IS seems useful to take into account the rate of DXP extraction by the extraction method used. Potential candidates of IS were tested for their structural similarity with DXP as testosterone decanoate, hydroxyprogesterone caproate and dexamethasone acetate. Testosterone decanoate was selected as it

gives a similar profil of extraction with DXP and a retention time (mn) near that of DXP peak.

3.1.3. Selection of Detection Wavelength

A screening of DXP and IS standard sample was carried out and the maximum absorption wavelength of DXP and IS peaks was at 240 nm.

3.2. Validation

Chromatographic conditions used provided sufficient resolution with elution of DXP and IS respectively at 27.1 min and 23.9 min (**Figure 2**). There were no significant (≥10% of LLQ area) interferences at either of these retention times and the selectivity of the method is demonstrated by the representative HPLC chromatograms presented in **Figure 2** [27]. A linearity range has been set to the concentration range of 0.5 to 40 µg/mL of DXP with a coefficient of determination R^2 = 0.999. The line of regression equation was established: $Y = (0.262 \pm 0.007)X - (0.071 \pm 0.022)$, Y the chromatographic response and X, the concentration in µg/mL [30] [35]. The repeatability tests reported that relative standard deviations were less than 0.1% for the reference solutions and at 3.7% to a DXP in BALF sample for the chromatographic analysis and 2.7% for the whole procedure [36]. The overall recovery of DXP from BALF samples for concentrations 2, 10 and 40 µg/mL was 84.3% ± 1.6%. Consistent recovery values at low, medium and high concentrations indicate the extraction process is acceptable across this range. The high recoveries observed indicate analyte stability under the extraction conditions applied and good extraction. The limits of detection and quantification were, respectively, 0.03 and 0.1 µg/mL [37].

3.3. Application of Assay

An example of chromatograms obtained from the extract of a DXP and IS (internal standard) in BALF samples from standard solutions and rats treated with DXP powder is given in **Figure 2**. DXP contents in BALF from rats treated, ranged from 31.5 to 0.1 µg/mL corresponding to DXP levels from 30 min to 24 h after lung administration. The results of DXP obtained in BALF were converted to epithelial lining fluid (ELF) concentrations according to the theoretical volume of ELF reported in the literature (30 µL/kg) and to the volume of PBS used for bronchoalveolar lavage (1 mL). Thus, for a rat of 300 g, the dilution factor determined by calculation was 333 (**Table 2**) [38] [39]. All the observed concentrations of DXP in BALF and ELF lie within the ranges predicted by the PK model for the dosing regimen. This suggests that the assay is performing well and that the current HPLC method can be used successfully for the measurement of DXP levels in BALF samples Results indicate a greater degree of sensitivity is required to adequately detect DXP levels 18h after administration in lung where the dose is low that the LOQ. Further work would include using a tandem mass spectrometer for greater selectivity and potentially increased detection sensitivity [40].

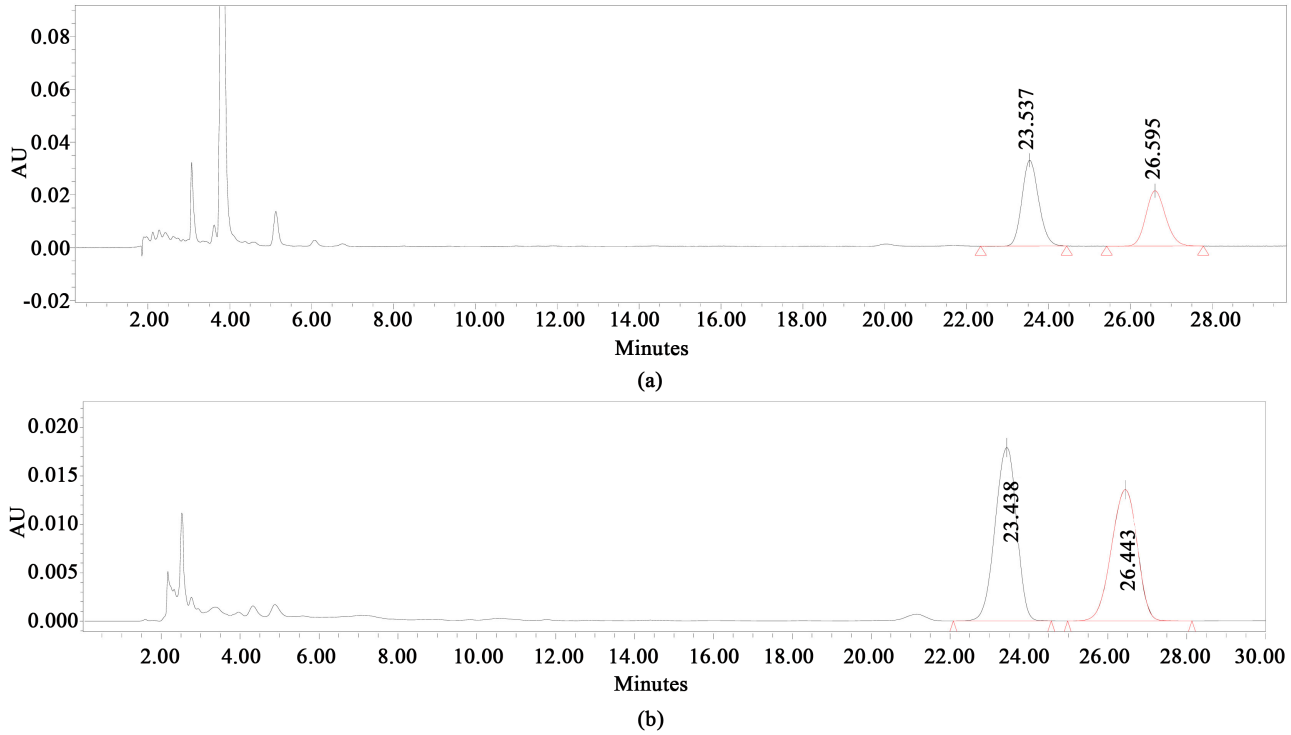

Figure 2. Chromatograms obtained from the extract of a DXP and IS in BALF samples from standard solutions (a) and rats treated sample (b).

Table 2. DXP contents in rats treated BALF and ELF after intratracheal insufflation of 3 mg of DXP micro particles (DXP dose = 150 µg), n = 3.

Temps (h)	0.5	1	2	3	4	6	18	24
BALF (µg/ml)	31.5 ± 4.4	26.7 ± 5.2	20.4 ± 1.9	15.7 ± 1.5	5.1 ± 2.1	0.6 ± 1.0	0.1 ± 0.0	0.1 ± 0.0
ELF (µg/ml)	10,510.3 ± 1471.7	8894.4 ± 1737.9	6798.5 ± 638.3	5233.1 ± 506.3	1689.3 ± 705.5	204.2 ± 333.6	16.7 ± 3.3	12.5 ± 1.7

4. Conclusion

A simple and sensitive HPLC method after chloroform-methanol extraction has been successfully developed for the quantification of DXP in BALF. The validated method has been shown to be accurate and precise with a RE and CV ≤ 15% at all tested concentrations. The validated method has been shown to be accurate and precise with an RE and CV ≤ 15% at all tested concentrations. Requiring only a 100 µL BALF sample, the method is well suited for application to inhaled drugs. The developed DXP method has been successfully applied to BALF samples collected from rats treated with a candidate innovating large porous particle and is being used to support an on-going PK study of inhaled DXP dry powder.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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