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Short communication

High-performance liquid chromatographic method for the determination of budesonide in bronchoalveolar lavage of asthmatic patients

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Abstract

A simple, sensitive and selective method for the determination of budesonide in bronchoalveolar lavage (BAL) using high-performance liquid chromatography (HPLC) with UV detection was developed. BAL samples were extracted twice with methylene chloride, the extracts were centrifuged and the organic layer separated and dried under nitrogen. The samples were reconstituted in the mobile phase and 80 μl were injected on to a Spherisorb ODS column with UV absorbance detection at 250 nm. The mobile phase was methanol–aqueous buffer (69:31, v/v). Inter-assay coefficients of variation were measured at 7.81 and 500 ng/ml with ranges of 0.89–7.31%. Average recoveries were 97% or greater. This method was successfully implemented for the analysis of BAL from asthmatics, in order to establish the amount of budesonide available to the lung and to investigate the efficacy of inhaler systems. Patients ($n = 9$) inhaled four puffs of 200 μg of budesonide and BAL was performed 10 min after the last inhalation. Only four BAL out of the nine presented detectable amounts of budesonide. The concentrations in BAL in these four patients were 13.44–84.18 ng/ml, corresponding to total amounts of 0.847–7.997 μg.

1. Introduction

Glucocorticosteroids with high local anti-inflammatory activity but low systemic effects are of interest in the treatment of inflammatory diseases of the respiratory tract. Despite the extensive use of corticosteroids in the treatment of disorders such as asthma and interstitial pneumonitis, the knowledge of its tissue delivery in the lung has been surprisingly limited.

Budesonide (BDS) is a topically active glucocorticosteroid used in inhalation treatment of respiratory disorders [1,2]. BDS has been available for a number of years and has been shown to be both effective and safe in the treatment of asthma [3,4]. BDS may be delivered via a pressurized metered dose inhaler or via a Turbuhaler, a new inhaler system.

Some studies have already reported the plas-

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ma concentrations of budesonide using HPLC with UV detection [5–8], but to our knowledge, no study has been made of on the amount of BDS in the lung after inhalation of drug. We therefore developed an original HPLC method to determine BDS in bronchoalveolar lavage (BAL) specifically and without interference with drug. A study using the technique of technetium marking has shown that the rate of penetration of an aerosol into the lung was 10–20% [9]. However, the actual concentrations of BDS in the alveolar–capillary unit are still unknown. The superior penetration, and thus exposure, of a glucocorticoid to the pathogenic site may result in a greater effect on local inflammatory cells involved in diseases such as asthma and interstitial lung disease. The purpose of this paper is to report a simple method to determine budesonide directly in BAL, to establish the real amount of BDS in the lung and to investigate the efficacy of inhaler systems.

2. Experimental

2.1. Chemicals

Budesonide (BDS) was obtained from Astra Labs. (Montargis, France). Acetic acid (Rectapur) and methanol (HPLC grade) were obtained from Prolabo (Paris, France). Aqueous buffers were prepared using doubly distilled, deionized water obtained from Maco-Pharma Labs. (Tourcoing, France). Human BAL were performed in the Pneumology Department, Calmette Hospital (Lille, France).

2.2. Preparation of standard solutions and BAL samples

A stock standard solution of BDS was prepared in methanol at a concentration of 1 mg/ml in polypropylene tubes and stored at −20°C for 8 weeks. Working BDS solutions ranging between 7.81 and 500 ng/ml were prepared weekly by suitable dilution of the stock standard solution with the mobile phase in a polypropylene tube. Known amounts of BDS were added to aliquots of 1 ml of human BAL. Calibration graphs were obtained by analysing 1 ml of BAL sample spiked with 7.81, 15.62, 31.25, 62.50, 125, 250 and 500 ng of BDS (five samples for each concentration).

2.3. Chromatographic conditions and instrumentation

Chromatographic analyses were performed with an HP 1090 high-performance liquid chromatograph (Hewlett-Packard, Orsay, France) equipped with a variable-volume injector, an automatic sampling system and a Hewlett-Packard Model 79994A diode-array UV detector operating at 250 nm. The output from the detector was connected to a Hewlett-Packard 9000 Model 300 integrator and the data were recorded on a HP ThinkJet printer. Separation was achieved using a 5-μm Spherisorb ODS C_{18} column (250 × 4.6 mm I.D.) (Touzard et Matignon, Vitry sur Seine, France) operating at 20 ± 2°C. During assay development, BDS was eluted isocratically with a mobile phase consisting of methanol–aqueous buffer mixture (69:31, v/v) at a flow-rate of 1.0 ml/min with a system back-pressure averaging about 232 kPa. The buffer was prepared in water containing 0.1% acetic acid at pH 3. The mobile phase was filtered through a 0.45-μm membrane and degassed under a helium stream before use. The run time was 15 min.

2.4. BAL extraction procedure

An aliquot of BAL was centrifuged for 10 min at 1150 *g* 1 ml of the supernatant was extracted with 5 ml of methylene chloride. The mixture was shaken for 10 min using an alternating agitator. The solution was then centrifuged for 10 min at 1150 *g*. The lower organic phase (fraction 1) was transferred into a clean conical tube. The aqueous phase was extracted again with 5 ml of methylene chloride and the mixture was treated as above. The lower organic phase (fraction 2) was combined with fraction 1 and the total organic phase was evaporated to dryness in a water-bath at 50°C under nitrogen. The

residue was dissolved in 100 μl of mobile phase and, after centrifugation, 80 μl of the supernatant were finally injected into the chromatograph.

2.5. Quantification

For BDS quantification, calibration graphs were constructed by unweighted linear regression analysis of the peak area of BDS versus the theoretical BDS concentration. Unknown concentrations of BDS were calculated by inverse prediction for each BAL sample by reference to the calibration graph.

No internal standard was used because we obtained an average recovery of drug near 100% (≥97%) when we employed a double extraction procedure.

3. Results and discussion

3.1. Chromatography

Typical chromatograms obtained from extracted BAL samples are shown in Fig. 1.

Fig. 1A show a representative chromatogram of a processed blank, indicating that no co-extracted endogenous compound from the BAL exist at the retention time of BDS. Fig. 1B is for a standard BAL sample spiked with 500 ng of budesonide. Identification of BDS in BAL samples was achieved by comparison with an authen-

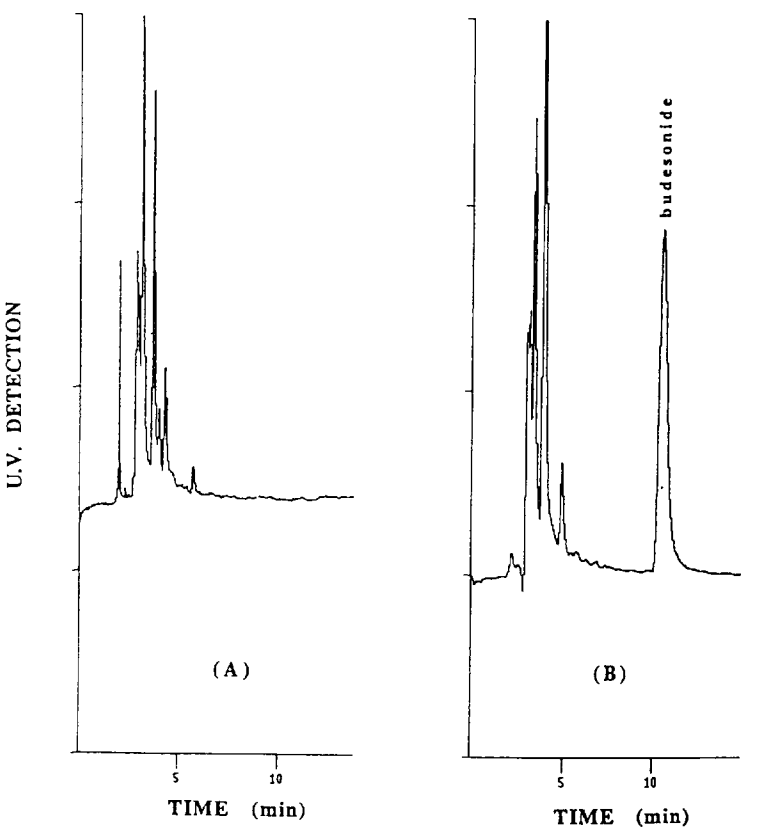

Fig. 1. Chromatograms obtained after extraction from BAL samples: (A) blank BAL sample; (B) blank BAL sample spiked with 500 ng of budesonide. Extraction procedure and chromatographic conditions are described in the text.

tic specimen obtained from Astra Labs. The retention time of BDS was 10.51 min. The capacity factor (k') was 3.50 for BDS, showing a satisfactory separation and a relatively short analysis time.

3.2. Linearity, precision and accuracy

The calibration graphs of the peak area of BDS versus drug concentration were linear over the range 7.81–500 ng/ml. Linearity data were analysed by using the Student's t-test. The mean linear regression equation was $y = 0.879x + 4.012$ (y = peak area of BDS, x = drug concentration in ng/ml). Mean values for the slope and intercept (±standard deviations) were 0.879 ± 0.028 and 4.012 ± 0.084, respectively, obtained from five replicates. The correlation coefficients (r) of the regression lines were better than 0.999. No significant differences were observed between the equation parameters. The calibration graphs were not forced through the origin. The precision of the method was good, as shown by the low values of the intra-assay and inter-assay coefficients of variation.

The inter-assay reproducibility and accuracy were measured using spiked budesonide BAL with different calibration graphs. The inter-assay reproducibility (Table 1) was within 7.3% and the absolute accuracy [which was defined as (measured concentration − theoretical concentration)/theoretical concentration] was 15% or better. Recoveries from BAL were 97% or greater ($n = 5$).

The intra-assay reproducibility and accuracy were determined by simultaneously assaying replicates of BAL spiked with BDS. The intra-assay reproducibility was 3.2% and the accuracy was 2.4%.

The detection limit was the minimum quantifiable concentration of BDS, that is, 5 ng/ml at a signal-to-noise ratio of 2.

3.3. Clinical application

This HPLC method is suitable for determining BDS in BAL from asthmatic patients after drug inhalation. Fig. 2 shows a chromatogram obtained from BAL from an asthmatic patient after budesonide treatment.

Nine patients received BDS (4×200 μg) by inhalation via a Turbuhaler or through a metered dose inhaler, and BAL were performed 10 min after the last inhalation. Only four of the BAL presented detectable amounts of BDS. The BDS concentrations in these four BAL samples were 13.44–84.18 ng/ml, corresponding to total amounts of budesonide of 0.847–7.997 μg.

To our knowledge, this is the first study of the determination of BDS in BAL from asthmatic patients. Previous in vitro and in vivo investigations have demonstrated a clear correlation between glucocorticosteroid cell concentrations and anti-inflammatory response [10].

The local action obtained through inhalation of BDS could therefore be linked to an effective concentration resulting in anti-inflammatory properties on cells involved in asthma. In fact,

Table 1
Reproducibility and accuracy of budesonide determinations

Amount added (ng/ml)	Amount found (mean ± S.D.) (ng/ml)	Inter-assay R.S.D. (%)	Accuracy (%)
7.81 ($n = 7$)	6.71 ± 0.20	2.98	14
15.62 ($n = 7$)	13.22 ± 0.60	4.53	15
31.25 ($n = 5$)	31.87 ± 2.33	7.31	1.79
62.5 ($n = 5$)	64.88 ± 1.76	2.71	3.80
125 ($n = 5$)	124.85 ± 3.23	2.58	0.12
250 ($n = 6$)	254.41 ± 3.41	1.34	1.76
500 ($n = 6$)	500.56 ± 4.47	0.89	0.11

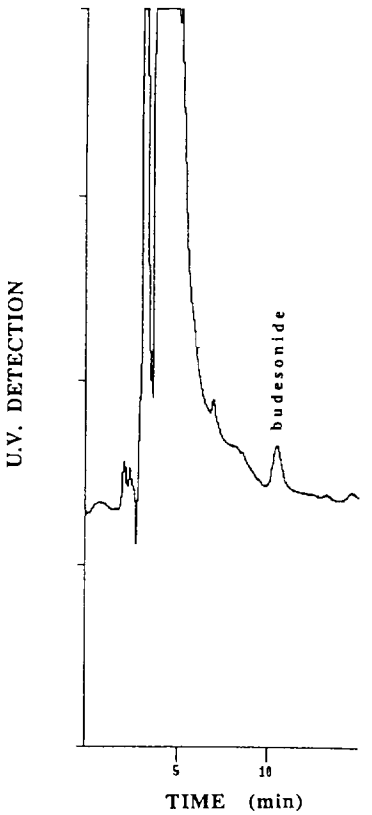

Fig. 2. Chromatogram obtained from BAL from an asthmatic patient who had inhaled 4 × 200 μg of budesonide via a Turbuhaler.

previous studies [10] carried out in vitro on alveolar macrophages showed that a BDS concentration of 40 ng/ml could limit inflammatory processes. Further studies are needed to determine the concentrations in BAL of patients and to compare the clinical responses of inhaled budesonide delivered either via a pressurized metered dose inhaler or via a Turbuhaler.

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