

## LETTER TO THE EDITOR

## RETENTION OF FLUNISOLIDE IN RAT LUNGS AFTER SINGLE INHALATION

A. KANTAR<sup>1</sup>, M. CARLONI<sup>2</sup>, N. KULKARNI<sup>1,3</sup>, D. FEDELI<sup>2</sup> and G. FALCIONI<sup>2</sup>

<sup>1</sup>High Altitude Pediatrics Asthma Centre, Pio XII Institute, Misurina, Belluno, Italy; <sup>2</sup>School of Pharmacy and Health Products, University of Camerino, Italy; <sup>3</sup>Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, United Kingdom

Received March 2, 2011 – Accepted December 18, 2011

**Flunisolide (6 $\alpha$ -fluorine 11 $\beta$ , 16 $\alpha$ , 17 $\alpha$ , 21-tetrahydroxypregna-1, 4-diene-3,20-dione 16,17 acetonide) is a potent inhaled corticosteroid, as demonstrated by its anti-inflammatory activities. Clinical data have widely demonstrated that inhaled flunisolide administered twice daily is effective for the treatment of bronchial asthma. However, pharmacokinetic studies suggest that the high solubility rate of flunisolide in bronchial fluid reduces the residence time of the drug in the lungs to a few minutes. The aim of this study is to determine the concentration of flunisolide in lung tissue of rats after administration by inhalation at varying time periods. Male Wistar rats weighing approximately 300 g were divided into four groups and administered a single dose of 1 mg flunisolide via inhalation. Rats were sacrificed with the exposure to CO<sub>2</sub> either immediately or 3, 6, 12 hours after inhalation. The whole lung was then surgically removed and analysed for flunisolide concentration using gas chromatography. The mean concentration (2 standard deviation) of flunisolide detected in the lung tissue at 0, 3, 6 hours after inhalation were 66.4 (11.9), 48.6 (5.9), 42.7 (8.1) ng/mg of proteins, respectively. No flunisolide was detected after 12 hours in lung tissue. We conclude that flunisolide is retained for long duration (more than 6 hours) in lung tissue. This finding partially explains the mechanisms of action of the drug.**

The mechanism of the anti-inflammatory action of endogenous or exogenous corticosteroids administered either systemically or through the inhaled route is similar. However, the pharmacokinetic and pharmacodynamic properties of the corticosteroid formulations determine the effectiveness, onset and duration of action. These properties are utilised to maximise the clinical benefit with minimal adverse systemic effects (1). Altering the basic structure modifies the properties of corticosteroids, resulting in significant variation in clinical anti-inflammatory potency and adverse effects (2).

A drug that is retained in the lung for longer periods

should have higher therapeutic index. Flunisolide (FLU) is a synthetic corticosteroid with a potent anti-inflammatory activity and is highly soluble in water (140  $\mu$ g/ml) (3). FLU has low lipophilicity therefore dissolves quickly in human bronchial fluid (4). In addition, high solubility rate reduces tissue retention and increases body elimination, reducing the risk of systemic effects (5). FLU's receptor affinity is relatively low; however this can be overcome by increasing the drug dose (6). The bioavailability after oral administration is poor (6.7%) (7), with a rapid and extensive first pass metabolism through the liver after gastrointestinal absorption, resulting in

*Key words: flunisolide, gas chromatography, lung, rat*

Mailing address: A. Kantar, MD, PhD  
High Altitude Paediatric Asthma Centre,  
Via Monte Piana, 4  
32040 Misurina, BL, Italy  
Tel: +39 043539008  
Fax: +39 043539210  
e-mail: kantar@tin.it

an inactive 6 $\beta$ -hydroxylated metabolite. The plasma half-life is estimated to be between 3.9 and 4.6 hours. FLU has a low distribution volume rate at steady state (96 litres) and a succinct terminal half-life after inhalation (1.6 hours) (8). FLU, like Budesonide (BUD), is characterized by a short pulmonary residence time and it is hypothesized that, inside the cell, it might be subjected to esterification due to the presence of a free C-21 hydroxyl group. Clinical data have widely demonstrated that inhaled flunisolide administered twice-daily is effective for treatment of bronchial asthma (9). The intrapulmonary retention time of flunisolide is not known; therefore we aimed to assess the concentration of flunisolide in rat lungs after inhalation using gas chromatography. The dose of FLU investigated corresponds to the current amount administered to children by nebulisation (9).

## MATERIALS AND METHODS

All reagents were characterised by a pure analytical grade and purchased from Sigma (Milan Italy).

### *Animals*

Male Wistar rats from Harlan, Italy (S. Pietro al Natisone, Italy), weighing about 300 g, were used. The rodents were placed in plastic cages (five rats per cage) in a temperature (21 $\pm$ 5°C) and light-controlled room (12:12 h light-dark cycle), with free access to water and food. The experiments were approved by Camerino University Joint Ethics Committee.

### *Flunisolide Inhalation*

The animals were divided into four groups (5 rodents per group) and all rats were administered with a single dose of 1 mg FLU (Lunibron, VALEAS Joint-Stock Company, Milan, Italy) via inhalation using a jet nebuliser (Nebula Markos-Mefar, Bovezzo, Italy). The dose of FLU in the study corresponds to the amount currently administered to children by nebulisation (9). Each group of rats was sacrificed with the exposure to CO<sub>2</sub> after inhalation at varying periods, i.e. either immediately or after 3, 6, and 12 hours. The lungs were surgically removed.

### *Extraction of FLU from the lungs*

Each lung was minced and homogenized in Phosphate Buffered Saline. In this homogenized substance, the protein concentration was determined according to the Lowry method (10). Then, it was put in a separatory funnel and was extracted thrice with 15 ml of chloroform. The chloroform fractions were collected and dried under

a nitrogen flux. The samples were kept at -20°C before being analysed.

### *Determination of flunisolide via gas chromatography*

The dried samples were dissolved in 50  $\mu$ l of chloroform, and 1  $\mu$ l was analysed via gas chromatography using a Shimadzu A17 apparatus fitted with a split-injection system and equipped with a flame-ionization detector and an Agilent HP-5MS column (30m x 0.25 $\mu$ m x 0.1 $\mu$ m thickness). Nitrogen was the carrier gas. The programmed column was kept at 150°C for 1 minute. Soon after injection, the temperature was increased at a rate of 30°C/min. until 275°C, then at 20°C/min. until 310°C and the column was held at this temperature for 10 min. Injector and detector were both at 280°C (11, 12). FLU concentration was determined through a comparison with commercial standard FLU provided by Sigma (Milan Italy).

### *Statistical analysis*

Statistical analysis was performed using SPSS version 14 (SPSS Inc., Chicago, IL). The data (ng FLU/mg proteins) is presented as mean  $\pm$  standard deviation. Comparisons between FLU content analysed immediately after the treatment and those analysed 3, 6, and 12 h afterwards were made via One Way ANOVA followed by Student Newman Keuls test.  $P < 0.05$  was considered statistically significant.

## RESULTS

Rodents treated with 1 mg FLU showed a 0.5% deposit in the lung immediately after inhalation. The mean concentration (2 standard deviation) of flunisolide detected in the lung tissue at 0, 3, 6 hours after inhalation were 66.4 (11.9), 48.6 (5.9), 42.7 (8.1) ng/mg of proteins respectively (Fig. 1). There was a reduction in FLU concentration at 3 and 6 hours ( $p < 0.05$ ) in comparison with that measured immediately after inhalation. The FLU was undetectable after 12 hours, suggesting retention of FLU in the lungs is between 6-12 hours.

## DISCUSSION

This is the first study to demonstrate the prolonged retention of inhaled flunisolide in rat lung tissue. The pharmacokinetic and pharmacodynamic profile of Inhaled Corticosteroids (ICS) is important for its clinical effectiveness and is influenced by many of its physiochemical properties. In particular, retention in

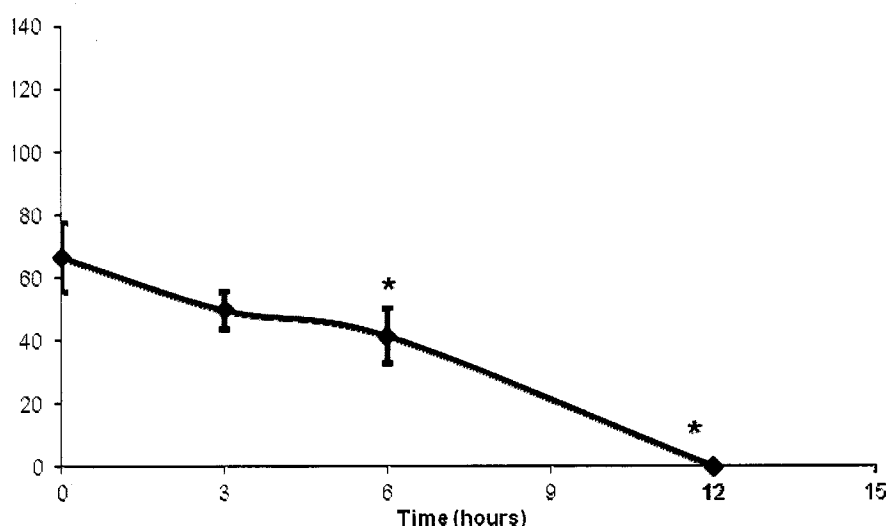
the lung would enhance the therapeutic index, either due to slow dissolution or the ability to form esters in the airways. The pharmacokinetic properties of ICS determine how much of the drug reaches the target cells, as well as the fraction that reaches the systemic circulation, producing side effects. The poor oral absorption and rapid systematic clearance properties of ICS contribute to favourable therapeutic index.

Esterification of sterols, such as cholesterol, with fatty acids in the body is a well-documented phenomenon (13). The properties that make synthetic alkyl esters of steroids useful as pharmacological agents have been widely recognised (14, 15). A recent study reported that acetates of corticoids at C-21 are formed biosynthetically during the incubation of [ $^3\text{H}$ ] cortisol with newborn rat homogenized brain (16). The enzyme responsible was an acyl-Coenzyme-A-transferase. However, the identification of the biosynthetic esters as acetates was probably incorrect. Similar studies in rat mammary glands and other tissues, including brain, using modern chromatographic and analytic techniques have definitely identified the non-polar hydrolysable corticoid metabolites as fatty acid esters and not acetates (17, 18).

Among the naturally occurring steroids, it has

been demonstrated that only corticosterone and 5-dihydrocorticosterone form C-21 esters. Data suggest that this mechanism is relatively stereoselective. Biochemical and kinetic studies have shown that a significant portion of BUD, and to a smaller extent Triamcinolone acetonide (TAA), is esterified intracellularly (1, 19). Corticosteroids that lack a free C-21 hydroxy group, such as Fluticasone propionate (FP) and Beclometasone dipropionate (BDP), do not form free fatty acid esters. It has been suggested that this reversible esterification slows down the release of active drug at the targeted site of activity, providing the observed level of airway specificity (20). As TAA, FLU and Ciclesonide all contain a free C-21 hydroxyl group; it is possible that they might all be esterified as well. This has been confirmed by some recent data regarding Ciclesonide and TAA, but not FLU (8, 21).

Although the mechanism is yet to be defined, several hypotheses regarding this observation are to be considered. It is likely that several unspecific cellular binding sites exist with both different capacities and affinities. In this context, reversible fatty acid esterification is a newly discovered way of intracellular ICS storing. Moreover, it should be borne in mind that in the steady-state treatment a



**Fig. 1.** Concentration of Flunisolide (ng/mg proteins) obtained by gas chromatography analysis of chloroform extract of lung obtained from Wistar rats treated with 1 mg of inhaled flunisolide and sacrificed after 0, 3, 6 and 12 h from the treatment. \*  $p < 0.05$  compared to 0 hour.

real equilibrium is never reached; instead, a series of drug concentration gradients, depending on absolute doses and dosing frequencies are obtained. The free drug will thus continuously flow between different intracellular compartments and between intracellular and extracellular sites. The pulmonary absorption of FLU is faster than the one of BUD, although FLU is an effective drug (9). The rapid pulmonary absorption combined with a significant retention within the airways and lung parenchyma, shown in the mid- and late 1980s (22), remained unexplained for about 10 years. From relatively recent kinetic and biochemical studies, it became clear that a significant portion of BUD, and to a smaller extent TAA, are intracellularly esterified (23). After 6-hour incubation of normal human bronchial epithelial cells with 3H-budesonide, 93% of the total cellular BUD was in the form of fatty acid esters (24). Hydrocortisone appears to follow the same biotransformation pathway, but here hydrolysis is much more prompt and only a small quantity of ester is therefore detected in tissue samples. FP, BDP and probably other corticosteroids lacking the C-21 hydroxyl group do not form fatty acid esters (23). FLU, like BUD, has a short pulmonary deposit time and it is hypothesized that it might as well be subjected to esterification in the cell, due to the presence of a free C-21 hydroxyl group.

In conclusion, our study demonstrates retention of FLU in rat lungs. This may be attributed to non-specific binding processes, esterification, or gradient formation in the cell. Further exploratory studies are now in progress in our laboratories to shed light on the handling of FLU in rat lung.

#### ACKNOWLEDGEMENTS

This research was supported by a grant offered by VALEAS Chemical and Pharmaceutical Industry, Milan, Italy.

Conflict of interest statement: A.K. has received lecture fees for non-promotional CME activities from Merck Sharp & Dohme, Istituto Gentili, Valeas, and Italchimici.

#### REFERENCES

1. Edsbäcker S. Uptake, retention, and biotransformation of corticosteroids in the lung and airways. In *Inhaled Steroids In Asthma. Optimizing Effects in the Airways*. Schleimer RP, O'Byrne PM, Szeffler SJ and Brattsand R (Eds). New York: Marcel Dekker, 2002; 163:213-46.
2. Kelly HW. Establishing a therapeutic index for the inhaled corticosteroids: part I. Pharmacokinetic / pharmacodynamic comparison of the inhaled corticosteroids. *J Allergy Clin Immunol* 1998; 102:S36-S51.
3. Högger P, Rohdewald P. Glucocorticoid receptors and fluticasone propionate. *Rev Contemp Pharmacother* 1998; 9:501-22.
4. Johnson M. Pharmacodynamics and pharmacokinetics of inhaled glucocorticoids. *J Allergy Clin Immunol* 1996; 97:169-76.
5. Derendorf H. Corticosteroid pharmacokinetic/ pharmacodynamic parameters and their relationship to safety and efficacy. *Allergy Asthma Proc* 2005; 26:327-35.
6. Kelly HW. Comparative potency and clinical efficacy of inhaled corticosteroids. *Respir Care Clin N Am* 1999; 5:537-53.
7. Dickens GR, Wermeling DP, Matheny CJ, John W, Abramowitz W, Sista SM, Foster T, Choudhury S. Pharmacokinetics of flunisolide administered via metered dose inhaler with and without a spacer device and following oral administration. *Ann Allergy Asthma Immunol* 2000; 84:528-32.
8. Kelly HW. Pharmaceutical characteristics that influence the clinical efficacy of inhaled corticosteroids. *Ann Allergy Asthma Immunol* 2003; 91:326-34.
9. Kantar, Mroueh S, Fiocchi A. A reappraisal of the clinical efficacy of nebulised flunisolide in pediatric asthma: the Italian experience. *Allergy Asthma Proc* 2007; 28:671-87.
10. Lowry OH, Rosenbrough NJ, Farr AL, Randall RL. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193:265-75.
11. Shimada K, Mitamura K, Higashi T. Gas chromatography and high-performance liquid chromatography of natural steroids. *J Chromatogr A* 2001; 935:141-72.
12. Yap BK, Johnston GA, Kazaluskas R. Routine screening and quantization of urinary corticosteroids

- using bench-top gas chromatography-mass-selective detection. *J Chromatogr* 1992; 573:183-90.
13. Neuhausen T. Das Cholesterin: Vorstellungen über seine Rolle im Körper. Forschungsstelle Robert-Koh-Strasse, Köln, Germany 1997.
  14. Pedersen-Bjergaard K, Tonnesen M. The influence of esterification upon the biological activity of oestradiol in rats and mice. *Acta Endocrinol (Copenh)* 1948; 1:350-61.
  15. Hochberg RB. Biological esterification of steroids. *Endocr Rev* 1998; 19:331-48.
  16. Grosser BI, Axelrod LR. Acetylation of cortisol by neonatal rat brain *in vitro*. *Steroids* 1967; 9:229-34.
  17. Pearlman WH, Lamay EN, Peg LH, Pearlman MR, Hass JR. *In vitro* metabolism of [3H] corticosterone by mammary glands from lactating rats: isolation and identification of 21-acyl[3H]corticosterone. *J Biol Chem* 1985; 260:5296-301.
  18. Pahuja SL, Hochberg RB. A comparison of the fatty acid esters of estradiol and corticosterone synthesized by tissues of the rat. *J Biol Chem* 1989; 264:3216-22.
  19. Miller-Larsson A, Mattsson H, Hjertberg E, Dahlbäck M, Tunek A, Brattsand R. Reversible fatty acid conjugation of budesonide: novel mechanism for prolonged retention of topically applied steroid in airway tissue. *Drug Metab Dispos* 1998; 26:623-30.
  20. Miller-Larsson A, Jansson P, Runström A, Brattsand R. Prolonged airway activity and improved selectivity of budesonide possibly due to esterification. *Am J Respir Crit Care Med* 2000; 162:1455-61.
  21. Kelly HW. Comparison of inhaled corticosteroids: an update. *Ann Pharmacother* 2009; 43:519-27.
  22. Ryrfeldt A, Persson G, Nilsson E. Pulmonary disposition of the potent glucocorticoid budesonide, evaluated in an isolated perfused rat lung model. *Biochem Pharmacol* 1989; 38:17-22.
  23. Tunek A, Sjödin K, Hallström G. Reversible formation of fatty esters of budesonide, an antiasthma glucocorticoid, in human lung and liver microsomes. *Drug Metab Dispos* 1997; 25:1311-7.
  24. Wieslander EI, Delander EL, Järkelid L, Hjerberg E, Tunek A, Brattsand R. Pharmacological importance of the reversible fatty acid conjugation of budesonide studied in a rat cell line *in vitro*. *Am J Respir Cell Mol Biol* 1998; 19:477-84.