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ACTIVATION OF BECLOMETHASONE DIPROPIONATE BY HYDROLYSIS TO BECLOMETHASONE-17-MONOPROPIONATE

GUDRUN WÜRTHWEIN AND PETER ROHDEWALD*

Institute of Pharmaceutical Chemistry, University of Münster, Hittorfstr. 58–62, D-4400 Münster, West Germany

ABSTRACT

The relative affinity of beclomethasone (B), beclomethasone-17-monopropionate (17-BMP), beclomethasone-21-monopropionate (21-BMP), and beclomethasone dipropionate (BDP) has been determined. BDP binds to the glucocorticoid receptor with about half the affinity of the potent glucocorticoid dexamethasone (Dexa), B was found to be 0·75 times less active than Dexa. The 21-BMP has no binding affinity whereas the 17-BMP is about 13 times as potent as Dexa. The affinity data indicate that metabolism of BDP to 17-BMP is an important activation step. To evaluate the relationship between local and systemic activity incubation studies with BDP in human lung, simulated gastric and intestinal fluid and plasma were performed. In cytosol from human lung cells BDP is hydrolysed rapidly to the more stable 17-BMP. During gastric passage BDP is stable but is immediately hydrolysed to 17-BMP in intestinal fluid. In human plasma BDP is hydrolysed to 17-BMP and an interesterification of 17-BMP to the inactive 21-BMP was also found.

KEY WORDS Beclomethasone dipropionate Beclomethasone monopropionates Beclomethasone Human lung cytosol Gastric and intestinal fluid Human plasma Relative receptor affinity

INTRODUCTION

After inhalation of a beclomethasone-17,21-dipropionate (BDP)-containing aerosol less than 25 per cent of the steroid enters the respiratory tract, the majority of the dose being swallowed.[8] Pharmacological activity and systemic side-effects may be influenced by several factors, e.g. by absorption from the lung or from the gastrointestinal tract, the metabolic activity of different tissues and biological fluids, as well as by the receptor affinities of BDP and its hydrolysis products.

Hydrolysis of BDP to beclomethasone-17-monopropionate (17-BMP) and beclomethasone (B) was found in rat, dog, and human tissues and has been discussed by some authors in terms of an inactivation of BDP to less active compounds,[4,5,7] whereas Yamamoto[16] suggested B to be the active compound after hydrolysis of BDP by plasma- and tissue-esterases.

* Correspondence to: Professor Dr P. Rohdewald, Institut für Pharmazeutische Chemie, Hittorfstr. 58–62, 4400 Münster, West Germany.

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However, examination of the relative receptor affinity (RRA)[12] indicated that BDP had about the same affinity for the glucocorticoid receptor as B itself. Additionally, the primary hydrolysis product found in human lung and liver, the 17-monopropionatester of B, showed, compared with other glucocorticoids, a much higher affinity. Therefore, metabolism of BDP to 17-BMP must be regarded as an activation step resulting in a much more potent substance with high intrinsic activity.

To assess the potential relationship between local and systemic activity it is therefore not only important to know the stability of BDP itself in biological fluids, but also to monitor the stability of the highly potent 17-BMP, too.

Therefore, we evaluated an HPLC procedure to separate BDP, 17-BMP, beclomethasone-21-monopropionate (21-BMP), and B; we determined the stability of BDP and its primary hydrolysis products *in vitro* in human lung cytosol, simulated gastric and intestinal fluid and in plasma. The RRA of 21-BMP was evaluated and the RRAs of BDP, 17-BMP, and B to glucocorticoid receptors from human lung cytosol were reinvestigated because we had improved our method described earlier[12] by using a more effective esterase inhibitor.

MATERIALS AND METHODS

Determination of the relative receptor affinity

Diisopropylfluorophosphate was obtained from Janssen Chimica (Nettetal, FRG); ^{3}H-dexamethasone and ^{3}H-triamcinolone acetonide were obtained from NEN (Boston, MA, USA). Radiochemical purity was demonstrated by TLC. Scintillation counting was done with Tricarb 300 C from Packard Instruments (Zürich, Switzerland) using Quickszint 212 from Zinsser (Frankfurt/Main, FRG).

The human lung tissue samples were obtained immediately after resection of human lung, frozen, and stored in liquid nitrogen until required for analysis.

Determination of the stability

Beclomethasone-17,21-dipropionate (BDP), beclomethasone-17-monopropionate (17-BMP), beclomethasone-21-monopropionate (21-BMP), and beclomethasone (B) were donated by Glaxo Group, Ware, UK. Acetonitrile was HPLC grade (Baker, Deventer, NL), the other reagents were obtained from Merck, Darmstadt, FRG, in pro analysi quality. TRIS-buffer, pH 7·4 was prepared by dissolving 12·1 g TRIS and 68·46 g sucrose in 800 ml distilled water, adjusting the resulting solution with 0·1 N HCl to a pH of 7·4 and diluting with distilled water to 1000 ml. Phosphate buffer, pH 7·4, 0·2 M, was made by dissolving 1·361 g KH_2PO_4 in 50 ml distilled water, adding 39·1 ml of 0·1 M NaOH and diluting with water to 200 ml. Simulated gastric fluid (USP XXI) was prepared by dissolving 2·0 g of NaCl and 3·2 g of 0·1 M

NaOH and diluting with water to 200 ml; simulated intestinal fluid (USP XXI) by dissolving 6·8 g of K_2HPO_4 in 250 ml of distilled water, mixing, adding 190 ml of 0·2 N NaOH and 400 ml of distilled water; then adding 10·0 g of pancreatin, adjusting the resulting solution with 0·2 N NaOH to a pH of 7·5, and diluting with distilled water to 1000 ml.

Six human lung samples were obtained from patients with lung cancer after lobectomy (Centre for Pneumonology and Thorax-surgery, Großharnsdorf Hospital). Lung samples were stored until homogenization in dry ice/acetone.

Relative receptor affinity

Cytosol preparation. Preparation of cytosol was performed as described by Pörtner *et al.*[11] with the exception that buffer contained in addition 50 mmol l^{-1} NaCl and 10 per cent glycerin. The protein concentration of cytosol was measured using the method of Lowry.[6]

Glucocorticoid receptor test. In incubation experiments using different concentrations of ^{3}H-dexamethasone alone or in the presence of an excess of unlabeled dexamethasone we could demonstrate the presence of a dexamethasone binding protein (for experimental details see Pörtner *et al.*[11]).

Relative receptor affinities (RRA) of BDP, 17-BMP, 21-BMP, and B. RRAs were determined by competition assays as described by Pörtner *et al.*[11] Receptor affinity of the reference substance dexamethasone was defined as 100. RRAs of glucocorticoids are defined:

$$RRA_x = 100 * \frac{C_{Dexa,50}}{C_{x,50}} \quad (1)$$

with:

RRA_x : relative receptor affinity of glucocorticoid,

$C_{Dexa,50}$: concentration of dexamethasone, reducing the specific binding of the tracer to 50 per cent,

$C_{x,50}$: concentration of glucocorticoid, reducing the specific binding of the tracer to 50 per cent.

Ester cleavage and analysis of the starting material. Glucocorticoid by-products in B, 17-BMP, 21-BMP, and BDP were quantified by HPLC. Ester cleavage following 6 h incubation at 20 °C in human lung cytosol was determined by analysing samples ($n = 6$) before and after incubation.

After adding 100µl internal standard solution (cloprednol or amcinonide, 20 µg ml^{-1}) and 3 ml ether samples were extracted by shaking for 10 min. Following centrifugation (g = 3000) the organic phase was evaporated to dryness under nitrogen. Residues were dissolved in 200 µl mobile phase

– methanol:water:acetonitrile:acetic acid/400:200:100:1; 50 µl were injected and separated on a Nucleosil R 10 C 18 column (Macherey-Nagel, Düren, FRG). Steroids were detected at 254 nm with a Shimadzu SPD-6A UV-detector (Shimadzu, Latek, Heidelberg) and integrated with a HP 3390 A integrator (HP, Avondale, USA).

Methods to examine stability

Incubation with cytosol from human lung samples. Each lung sample was homogenized in a five-fold amount of ice cold TRIS-buffer using an Ultraturrax and a Potter-Elvejhem homogenizer. The homogenates were divided in equal parts, one half was centrifuged at 1000 g (cytosol L), the other at 100 000 g (cytosol C) at 4°. Supernatants were stored in liquid nitrogen until incubation. Before incubation, protein concentration was estimated according to Lowry *et al.*[6] Then, samples were diluted with TRIS-buffer to 10 mg protein ml^{-1}. BDP was incubated at 37° at a concentration of 50 µg ml^{-1} by addition of 25 µl ethanolic BDP solution (5 mg ml^{-1}) with 2·5 ml of the supernatants containing 50 µg ml^{-1} penicillin-k and streptomycin.

At suitable intervals 100 µl samples were taken and immediately frozen in dry ice/acetone.

Incubation with human plasma, simulated gastric fluid, and simulated intestinal fluid. Plasma from volunteers was stored in liquid nitrogen for a maximum of 2 weeks; 2 ml plasma, simulated gastric or intestinal fluid or phosphate buffer pH 7·4 were incubated at 37° at a concentration of 50 µg ml^{-1} by adding 20 µl of an ethanolic solution of 5 mg ml^{-1} BDP or 100 µl of 1 mg ml^{-1} 17-BMP or 21-BMP. Aliquots of 50 µl were taken at intervals and frozen in dry ice/acetone. In the case of intestinal fluid 10 mg ml^{-1} phenol was added 1 h after starting the incubation.

HPLC analysis of BDP and its hydrolysis products. Extraction procedure and chromatographic conditions were the same as described above. As internal standard we used prednisone or cloprednol (20 µg ml^{-1}). The limit of detection for all glucocorticoids was 0·5 µg ml^{-1}.

For calibration, solutions of BDP, 17-BMP, 21-BMP, and B in the range from 0·5–100 µg ml^{-1} in cytosol or human plasma were analysed according to the procedure given above. Extracts of glucocorticoids from cytosol did not differ significantly from directly injected standards for the indicated range of concentrations; recovery was between 89 and 116 per cent and the calibration curve was linear. Coefficients of variation (CV) for extractions from cytosol varied between ±1·1 and 3·1 per cent (100 µg ml^{-1} and 50 µg ml^{-1}). Near the limit of detection (2 µg ml^{-1}) CV varied between ±5·7 and 14·3 per cent for the different glucocorticoids. Half-life ($t_{½}$) was calculated from the slope m of the linear parts of the plot ln conc. vs time: $t_{½} = \ln 2/m$.

RESULTS

Relative receptor affinity

Values for RRA are influenced by the following factors: the affinity of the glucocorticoid itself (RRA_p), the affinity of impurities of isomeric or other glucocorticoids (RRA_i) in the starting material, and finally by the affinity of the glucocorticoid produced by hydrolysis or interesterification during incubation in human lung cytosol (RRA_h). Correcting the experimentally found RRA_{exp} the RRA of the pure glucocorticoid RRA_p can be calculated as follows:

$$RRA_p = \frac{RRA_{exp} - i - h}{(100 - A) * (100 - B)} * 10000 \quad (2)$$

with:

$$i = \frac{A}{100} * RRA_i \quad (3)$$

$$h = \frac{(100 - A) * B}{10000} * RRA_h \quad (4)$$

with

A : percental contents of impurity
B : percental hydrolysis during incubation
RRA_i : relative receptor affinity of impurity
RRA_h : relative receptor affinity of hydrolysis product.

Results for impurities present in glucocorticoid samples as well as results for ester hydrolysis following incubation in human lung cytosol are listed in Table 1. The logit-log diagram of the competition curves for B, 17-BMP, 21-BMP, BDP, and the reference substance dexamethasone are shown in Figure 1. In Table 2 we summarize values for RRA described earlier[12] and values evaluated in this study, including corrections according to the equations (2)–(4).

Table 1. Impurity of the glucocorticoids and substances found after incubation in human lung cytosol

Glucocorticoid	Impurity of the glucocorticoid		Substances found after incubation	
	Per cent	Substance	Per cent	Substance
B	–		–	
17-BMP	1·9	21-BMP	15·9	21-BMP
21-BMP	4·3	B	4·3	B
BDP	–		–	

Table 2. RRA values from earlier studies[12] compared with values from this study: experimentally evaluated values (RRA_{exp}) and RRA of the pure steroid (RRA_p)

Glucocorticoid	RRA[12]	RRA_{exp}	RRA_p
B	59	76	76
17-BMP	1022	1110	1345
21-BMP		7·2	0·9
BDP	<80	43	43

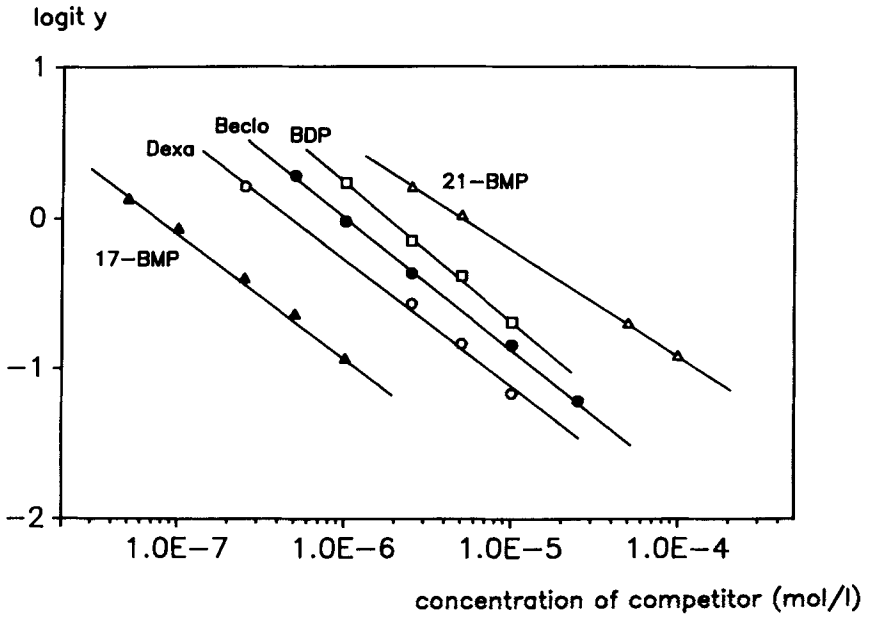

Figure 1. Competition curves of beclomethasone, its mono- and dipropionates and of the reference substance dexamethasone

Stability

Incubation of BDP with cytosol from human lung. In cytosol L containing the lysosomal fraction (supernatant after centrifugation at 1000 g) BDP was hydrolysed within 1 h until the limit of detection (Figure 2(a), Table 3). The 17-BMP is relatively stable in cytosol; within 6 h only 11 per cent B was formed. No 21-BMP was found.

In cytosol C without lysosomes (supernatant after 100 000 g, Figure 2(b), Table 3) BDP was hydrolysed significantly ($p < 0{\cdot}01$) more slowly: after 9 h incubation 1 per cent of BDP was present. The 17-BMP was hydrolysed

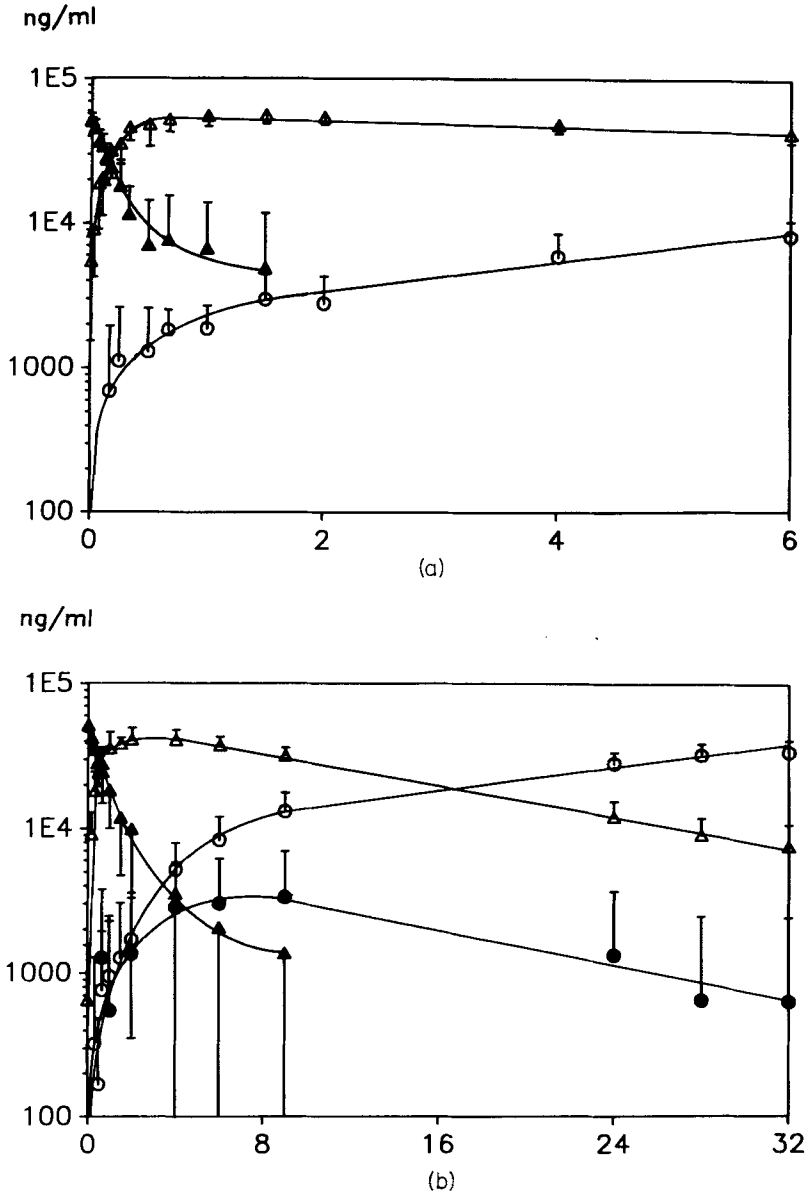

Figure 2. *In vitro* metabolism of beclomethasone dipropionate (BDP) in the cytosol of six human lung samples (mean ± SD), (a) 1000 g supernatant, (b) 100 000 g supernatant. ○ B, △ 17-BMP, ● 21-BMP, ▲ BDP

somewhat faster than in the presence of lysosomes, however the difference in $t_{½}$ was significant at the 10 per cent level only. The 21-BMP was detectable

Table 3. *In vitro* stability of BDP and its primary hydrolysis products in human lung cytosol, simulated gastric and intestinal fluid, human plasma, and buffer: summarized half-life times

Medium	Incubation with	Half-life times BDP	17-BMP	21-BMP
Cytosol L	BDP	10·4 min	14·3 h	
Cytosol C	BDP	39·0 min	11·6 h	8·3 h
Human plasma	BDP	4·7 h	5·4 h	31·0 h
Simulated gastric fluid	BDP	nh		
Simulated intestinal fluid	BDP	2·1 min	12·0 h	
TRIS-buffer	BDP	95·6 h		
Human plasma	17-BMP		2·5 h	3·7 h
Phosphate-buffer	17·BMP		5·4 h	ne
Human plasma	21-BMP			1·0 h
Phosphate-buffer	21-BMP			nh

nh: no hydrolysis.
ne: not estimated.

in 3 of the 6 lung tissues in concentrations near the limit of detection; in the other samples 21-BMP was present after 1 h of incubation. The $t_{½}$ of 21-BMP was found to 8·3 h in the interval between 9 and 32 h of the incubation period.

Hydrolysis of BDP in artificial gastric fluid and in artificial intestinal fluid. In artificial gastric fluid no hydrolysis or degradation of BDP was observed over 3 h. In artificial intestinal fluid BDP was rapidly hydrolysed to 17-BMP (Figure 3, Table 3). The 17-BMP was then slowly transformed to B following pseudo-first order kinetics. Within 3 h less than 14 per cent of 17-BMP was hydrolysed to B. No 21-BMP was detected. To differentiate enzymatic hydrolysis from the effect of pH, BDP was incubated at 37° in TRIS-buffer, pH 7·4. In this medium BDP showed a very limited hydrolysis so that the fast formation of 17-BMP in artificial intestinal fluid must be caused by hydrolases.

Incubation of human plasma with BDP, 17-BMP, and 21-BMP. In human plasma BDP was hydrolysed relatively slowly ($t_{½}$ = 4·7 h) and 17-BMP as well as 21-BMP were formed (Figure 4, Table 3). The 17-BMP was more rapidly hydrolysed than 21-BMP so that after a long incubation (36–48 h) only B and 21-BMP were present in plasma, BDP and 17-BMP concentrations being below the limit of detection.

Incubation of 17-BMP with plasma (Figure 5(a), Table 3) demonstrated that 21-BMP was formed by interesterification from 17-BMP and that B was formed finally from both monopropionates. The 17-BMP was converted to 21-BMP in buffer pH 7·4 at nearly the same rate as in fresh plasma (Figure 5(b), Table 3), indicating that the interesterification is a nonenzymatic process.

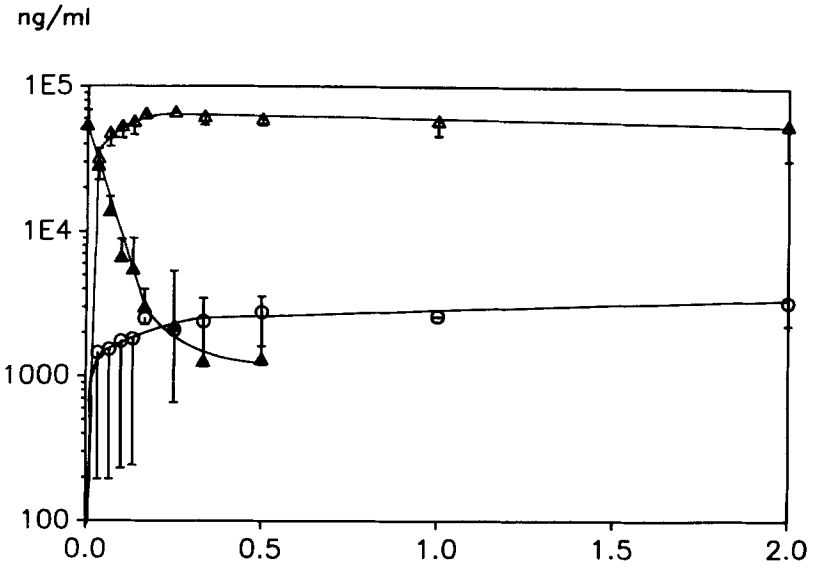

Figure 3. *In vitro* metabolism of beclomethasone dipropionate (BDP) in simulated intestinal fluid (analysis in triplicate, mean ± SD). ○ B, △ 17-BMP, ▲ BDP

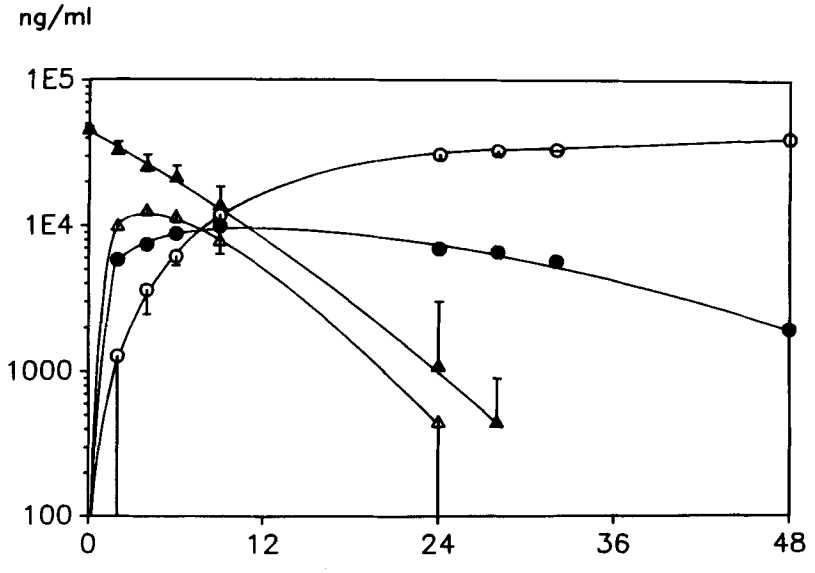

Figure 4. *In vitro* metabolism of beclomethasone, dipropionate (BDP) in human plasma (analysis in triplicate, mean ± SD). ○ B, △ 17-BMP, ● 21-BMP, ▲ BDP

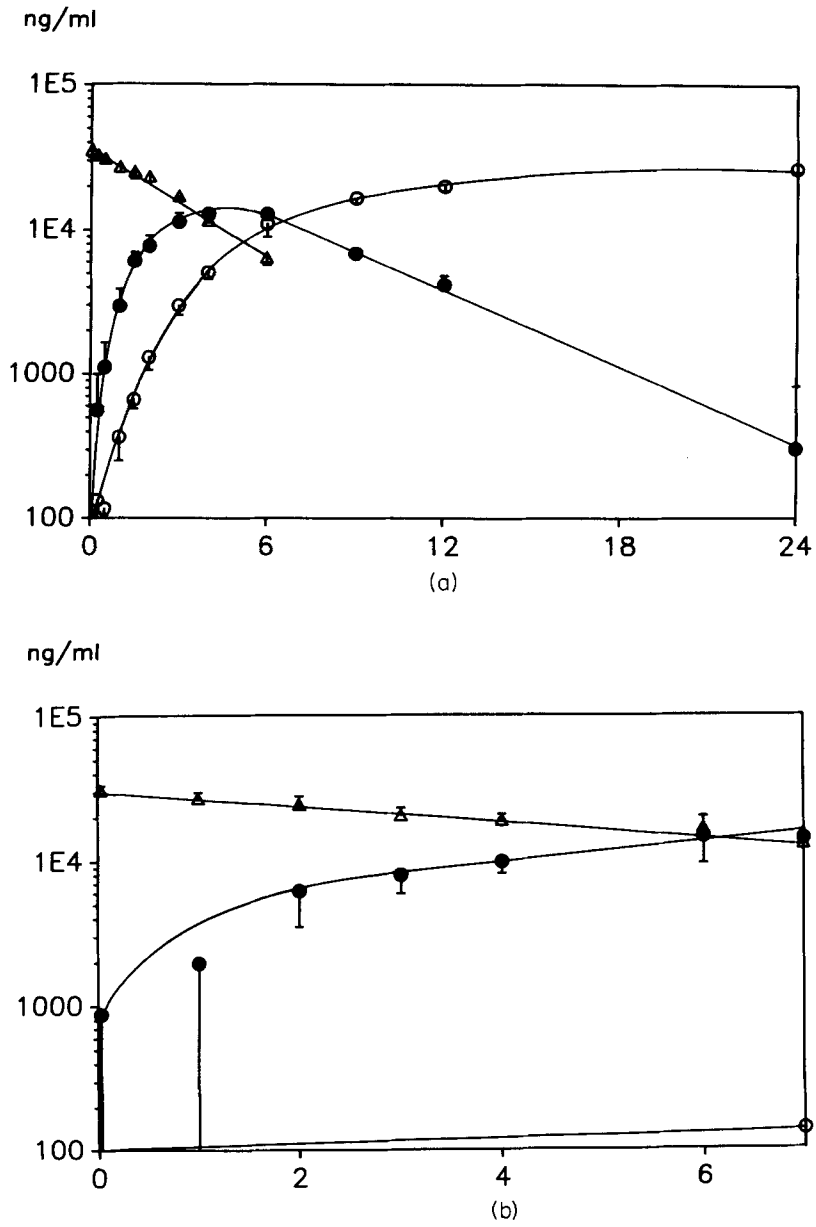

Figure 5. *In vitro* metabolism of beclomethasone-17-monopropionate (17-BMP) in (a) human plasma, (b) phosphate buffer pH 7·4 (analysis in triplicate, mean ± SD). ○ B, △ 17-BMP, ● 21-BMP

In human plasma 21-BMP was hydrolysed to B (Figure 6, Table 3); in buffer pH 7·4 no hydrolysis of 21-BMP was noted.

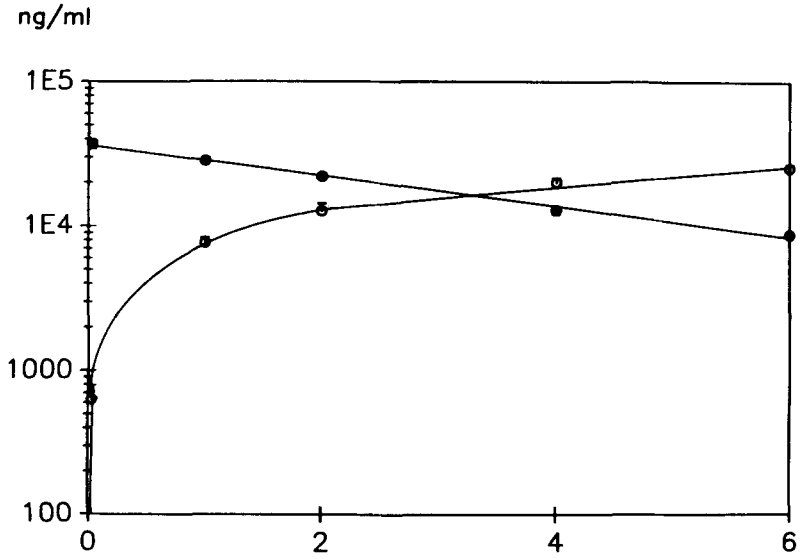

Figure 6. *In vitro* metabolism of beclomethasone-21-monopropionate (21-BMP) in human plasma (analysis in triplicate mean ± SD). ○ B, ● 21-BMP

DISCUSSION

Relative receptor affinities

Using a more sophisticated technique compared with our preceding experiments,[12] determinating impurities and hydrolysis products in additional experiments, and using a more effective inhibitor of ester hydrolases, we are able to correct values for RRAs given in Rohdewald *et al.*[12] The RRA for BDP, 49, is now much more precise than the estimated < 80, because the diester could be stabilized during the incubation by diisopropylfluorophosphate, whereas substantial hydrolysis could not be prevented by the phenylmethylsulfonylfluoride used previously.[12]

The two monopropionates of beclomethasone have totally different affinities: while the 21-monopropionate is practically inactive – in accordance with our findings on 21-esters of other glucocorticoids[11] – the 17-monopropionate possesses a very high affinity indeed. After correction for the presence of 21-monopropionate in the 17-ester the RRA of 17-BMP is 30 per cent higher than the value previously reported,[12] although the uncorrected RRAs are in close agreement.

Finally, the RRA of 73 for the glucocorticoid alcohol beclomethasone from replicate analysis is more accurate than the previous value of 59.[12]

The relevance of these differences in RRA, between BDP and its hydrolysis products, for therapeutic applications is obvious. BDP itself may be considered as a prodrug with intrinsic activity, being five times more potent than hydrocortisone.[12]

If the diester is converted to the 21-monopropionate by elimination of the 17-ester group, the prodrug becomes inactivated. If the 17-monopropionate is formed *in vivo*, the BDP is activated to the much more potent 17-BMP. Beclomethasone itself, the end product of hydrolysis of BDP, is 18 times less potent than the 17-ester, but still more effective than the parent compound BDP.

BDP is topically applied to the surface of the lung, hence the receptor affinity is closely related to the anti-inflammatory activity because absorption, distribution, and metabolism in the liver do not influence the local action.

Hydrolysis of BDP, activation or inactivation

Clearly, the process of activation or inactivation of BDP to 17-BMP or 21-BMP in bronchial secretion, lung tissue, and plasma will be very important for the local anti-inflammatory action, whereas the stability of BDP in the gastrointestinal tract and subsequent hydrolysis and metabolism in the liver will influence the systemic activity.

Pharmacokinetic data on beclomethasone and its propionate esters are lacking, the low amounts used do not give plasma concentrations detectable with HPLC-techniques. Therefore, several attempts have been made to get limited information concerning the stability of BDP in different tissues. The rapid hydrolysis of BDP to 17-BMP has been demonstrated before with human lung slices[7,14] and cytosol from human lung.[1,15]

Our experiments showed that lysosomal enzymes are responsible for this quick activation ($t_{½}$ BDP = 10·4 min), because after separation of lysosomes and in buffer alone, the rate of hydrolysis decreases. The stability of 17-BMP in cytosol from human lung is high, within 2 h 10 per cent only is hydrolysed to beclomethasone. The glucocorticoid alcohol itself was found to be stable in lung cytosol.

Therefore, following absorption of BDP from the bronchial epithelium, the intracellular formation of the highly active 17-BMP is to be expected and this active ester remains stable even in the presence of lysosomal enzymes.

From these findings it is reasonable to expect that mainly 17-BMP and only a small amount of BDP will enter the circulation following absorption from lung or gastrointestinal tract.

Ronca-Testoni[13] made absorption studies of BDP with rat everted intestine. He found that only B and 17-BMP are absorbed and concluded that the C21 esterified compounds are not absorbed or the absorbed material is deacylated at the C21 position during passage through the intestinal mucosa. However, our *in vitro* studies demonstrate that BDP is almost completely hydrolysed within 10 min incubation time using simulated intestinal fluid. These results suggest that BDP is nearly quantitatively hydrolysed before being absorbed by the intestinal mucosa.

Our *in vitro* studies showed an inactivation by interesterification of 17-BMP

to 21-BMP in plasma, about 10 per cent of 17-BMP being converted within the first hour.

However, hydrolysis by plasma enzymes *in vitro* is slow compared with the metabolic activity of human liver. Andersson and Ryrfeldt[1] found a rapid hydrolysis of BDP to 17-BMP in human liver homogenate; 17-BMP was biotransformed to unknown metabolites with short half-lives of 15 to 30 min. Although only *in vitro* experiments have been performed, one can conclude that the inactivation of 17-BMP by hydrolysis and metabolism caused by liver enzymes should be a much more effective inactivation step than the action of plasma enzymes.

Consequences for topical and systemic effects

The slow dissolution of the poorly water soluble beclomethasone dipropionate crystals in the aqueous mucus layer on the bronchial mucosa will be the rate-limiting step for the absorption of BDP from the lung. BDP, itself an active drug, then becomes activated to a more potent drug by hydrolysis of the 21-propionate. The resulting 17-monopropionate, formed mainly by the action of lysosomal enzymes inside the cells, produces intracellularly via receptor interaction lipocortin, the anti-inflammatory protein. The lipocortin blocks the phospholipase A_2 and consequently the release of all mediators like prostaglandins, leukotrienes, and PAF.

The high receptor affinity of 17-BMP means a prolonged synthesis of lipocortin; the low solubility of BDP causes the release of the glucocorticoid over a long period. As the sum of a slow release and a high receptor affinity, a relatively low, but long-lasting local concentration of the highly potent glucocorticoid – 17-BMP – produces the long-lasting local anti-inflammatory effect.

The systemic effect depends on the concentration of the glucocorticoid in the bloodstream leaving the lung. The rapid blood circulation in the lung dilutes the tissue concentration considerably. Therefore, the receptors of the cells in the hypothalamus are exposed to a much lower concentration of the glucocorticoid compared with the cells inside the bronchi, which are exposed to a very high concentration, and compared with the cells within the lung tissue, where intermediate concentrations of the glucocorticoid are found. This difference between the high topical concentration and the lower concentration on the hypothalamic cells, due to the dilution step of the circulation, is the principal benefit of steroid inhalation.

Orally or parenterally applied glucocorticoids must have identical concentrations within the bronchi as on the hypothalamic receptors, because they are transported via the circulation to the target cells and are not diluted during their way to the hypothalamus. The inactivation of the glucocorticoids by the liver is a subsequent step after the steroids have passed the receptors at the hypothalamus, therefore, glucocorticoids with the same receptor affinity, but different rates of metabolic inactivation in the liver, will cause the same

suppression of cortisol synthesis. In contrast, the same glucocorticoids given orally will have a different effect on the cortisol suppression, a significantly higher rate of metabolism in the liver will produce less cortisol suppression, but also less anti-inflammatory activity. The 17-BMP absorbed from the intestine will therefore influence the hypothalamic receptors according to its rate of inactivation in the liver.

Consequently, the oral intake of glucocorticoids during inhalation should be minimized, by the use of spacers or by mouth-rinsing following inhalation.

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