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Pulmonary and blood enzyme kinetics as predictors of pulmonary metabolism of esters

Paul A. Dickinson, Glyn Taylor *

Welsh School of Pharmacy, University of Wales College of Cardiff, Cathays Park, Cardiff CF1 3XF, UK

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Abstract

The enzymatic hydrolysis of four aliphatic esters of biphenylacetic acid by rat lung homogenate and blood was investigated. The log V_{max}/K_m ratios derived in both tissues were parabolically related to lipophilicity, similar trends were also observed for log V_{max} values against lipophilicity. K_m values could not be related to lipophilicity. The pulmonary and blood intrinsic clearances were linearly related, suggesting blood could be used to predict the pulmonary metabolism of esters. Pulmonary extraction ratios, predicted from the intrinsic clearance using the 'well-stirred' model, were negligible.

Keywords: Ester; Lung; Blood; Enzyme kinetics; 4-Biphenylacetic acid

1. Introduction

For several decades it has been recognised that the lungs contain non-specific esterases and as such may be a potential site for the hydrolysis of ester drugs (Short et al., 1972). It is also recognised that it may be desirable to modify the physicochemical characteristics of a drug by the production of ester prodrugs (Nielsen and Bundgaard, 1987). Indeed, ester prodrugs of xenobiotics intended for administration to, or whose site of action is in, the lungs have been synthesised (Brazzell and Kostenbauder, 1982; Andersson and Ryrfeldt, 1984; Ryrfeldt et al., 1988). Despite this history there are surprisingly few reports in the literature in which the pulmonary hydrolysis of esters has been investigated, in vitro. With even fewer workers reporting pulmonary hydrolytic activity against a series of esters (McCracken et al., 1993).

As a preliminary investigation into pulmonary esterase activity, prior to the in vivo investigation of pulmonary first-pass ester metabolism, the hydrolysis of four aliphatic esters of biphenylacetic acid was studied. The aims of this study were to investigate: whether the kinetic parameters of ester hydrolysis are dependent on the physicochemical nature of the esters; and whether enzyme kinetics derived in blood can be used to predict the pulmonary situation.

* Corresponding author. Tel. +44 222 874000, ext. 5822; Fax +44 222 874159.

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2. Materials and methods

2.1. Materials

All reagents were analytical grade, except acetonitrile which was HPLC grade. Albumin (bovine, fraction V powder, A6793) was obtained from Sigma (Poole, UK). 4-Biphenylacetic acid (BPAA) was donated by Cyanamid (Gosport, UK), ethyl biphenylacetate (EBPA), octyl biphenylacetate (OBPA) and dodecyl biphenylacetate (DBPA) were donated by Mr S. Jones and Dr K. Barrell, Welsh School of Pharmacy.

2.2. Synthesis of hexyl biphenylacetate

Thionyl chloride (10.9 ml, 0.15 mol) was added, dropwise with stirring, to biphenylacetic acid (10 g, 0.047 mol) in benzene (60 ml) in the presence of a catalytic amount of dimethyl formamide (two drops). The mixture was heated under reflux at 90°C for 4 h. The benzene was evaporated under reduced pressure and the resulting red solid was dissolved in toluene (50 ml) and evaporated to dryness under reduced pressure. This procedure was repeated twice to remove trace amounts of thionyl chloride. The resulting acid chloride and hexan-1-ol (6.03 ml, 0.048 mol) were then dissolved in dichloromethane (150 ml) and the mixture was cooled to 5°C. Triethylamine (13.4 ml, 0.096 mol) in dichloromethane (31 ml) was then added dropwise, with stirring, over a 20 min period. The resultant mixture was stirred at 5°C for 1 h after which the solvent was evaporated under reduced pressure. The resulting solid was redissolved in dichloromethane (100 ml) and washed with water (2×50 ml). The organic extract was dried (Na_2SO_4) and decolorised with carbon. The solvent was removed under vacuum to yield pale yellow crystals. The crystals were then recrystallised from ethanol.

2.3. Preparation of tissues

Five male Wistar rats (249–317 g) were anaesthetised, tracheostomised and exsanguinated. The lungs were perfused via the pulmonary artery with saline (0.9%), quickly excised, washed in 50 mM Tris-KCl buffer 1.15% pH 7.4 and weighed. They were then chopped, with scissors, and added to 10 ml of 50 mM Tris-KCl 1.15% buffer. The mixture was then coarsely homogenised (on ice) using an Ultra-Turrax T25 homogeniser (June and Kunkel, IKA-Labortechnick, Germany) for five 5 s bursts with 30 s interim cooling. The preparations were then further homogenised on ice using four passes of a Potter-Elvehjem homogeniser with interim cooling. The homogenates were then pooled and centrifuged at $800 \times g$ for 2 min, to remove any gross cellular debris. Blood was collected from an anaesthetised rat (225 g). Blood and homogenate were stored at 1–4°C and used within 4 days of collection. The protein concentration of each tissue was determined using a BCA™ protein assay kit (Pierce, IL, USA).

2.4. Determination of V_{max} and K_m in lung homogenate and blood

The incubations were performed in 13.5 ml polystyrene test tubes (Bibby Sterilin, Stone, UK) in a water bath with shaking at 37°C, under an atmosphere of air and darkness. All reactions were carried out at concentrations that were linear with respect to protein concentration and

Table 1
Incubation conditions for the determination of V_{max} and K_m

Ester	Lung		Blood	
	Time (min)	Protein concentration (mg 0.8 ml^{-1})	Time (min)	Protein concentration (mg 0.8 ml^{-1})
EBPA	8	1.07	10	0.0302
HBPA	12	2.14	15	0.0604
OBPA	15	2.14	10	0.1207
DBPA	150	3.74	60	0.3018

time. Four incubations were performed for each of the four esters at five concentrations, approx. 15–84 μM. Two controls at each concentration were prepared for the production of calibration curves. The incubation media consisted of magnesium chloride (6.25 mM) and microsomal protein in 50 mM Tris pH 7.4 buffer in a final volume of 0.8 ml. The esters were dissolved in ethanol before subsequent dilution with albumin (22% w/v) 50 mM Tris pH 7.4 buffer. The volume of ethanol in the final media was 8 μl. The incubation mixtures were prewarmed at 37°C for 5 min before addition of the microsomes. The enzyme activity was stopped by the addition of acetonitrile to the media. The protein concentration and incubation times used for each ester in lung homogenate and blood incubations are shown in Table 1. The final ester concentration was always greater than 70% of the initial concentration. The control tubes were incubated without microsomal preparation, which was added at the end of the time period immediately prior to the addition of acetonitrile. (No reaction was shown to occur without the presence of homogenate or blood, or when acetonitrile was added to the preparation.) The tubes were then centrifuged at $3000 \times g$ for 5 min and 100 μl aliquots of the supernatant subjected to HPLC analysis for the relevant ester or biphenylacetic acid.

The rate of the reaction (v) was measured as the disappearance of the ester (nmol) from the media/min per mg of protein. V_{max} and K_m were determined using a curve-fitting program (Minim; Purves, 1988), the line of best fit being described by $v = [s] \times V_{max}/(K_m + [s])$, where the ordinate is the reaction rate (v) and the abscissa is the initial ester concentration ([s]).

2.5. Assay of esters or biphenylacetic acid

Concentrations of ester or biphenylacetic acid were determined using isocratic reverse-phase HPLC assays with detection at 254 nm: Promis II Injection System (Spark Holland, Emmen, The Netherlands); Constametric 3000 solvent delivery system (Laboratory Data Control, Stone, UK); LDC Spectromonitor 5000; Spectra-Physics SP4270 integrator (San Jose, USA). A C_{18} Spherisorb S5-ODS column or C_4 Nucleosil NC-300-5C4 column (Hichrom, Reading, UK) was used at ambient temperature. The mobile phase for BPAA, EBPA, HBPA, OBPA and DBPA was 600, 630, 830, 830 and 750 ml of acetonitrile, respectively, with water to 1000 ml and 1 ml of trifluoroacetic acid. The flow rate was 2.0 ml min^{-1}. Dodecyl biphenylacetate concentrations were determined using the C_4 column, in all other cases the C_{18} column was utilised.

3. Results and discussion

3.1. Synthesis of hexyl biphenylacetate

The synthesis gave a yield of 13.1 g, equivalent to 93.8%. The uncorrected melting point was 38.6–39.5°C. Analysis – found: C, 80.03; H, 8.41. $C_{20}H_{24}O_2$ requires C, 81.04; H, 8.16%; ν_{max} (KBr) cm^{-1}: 1740 (C = O, ester); δ_H ($CDCl_3$) 0.00 (TMS), 0.50–1.50 (11H, m, aliphatic CH), 3.40 (2H, s, ($PhC\underline{H}_2$), 3.90 (2H, t, $CO_2C\underline{O}_2\underline{H}_2$), 6.90–

Table 2
V_{max}/K_m ratios and intrinsic clearances obtained for the hydrolysis of biphenylacetic acid esters by rat lung homogenate and blood, together with CLOGP values

Ester	Lung		Blood		CLOGP
	V_{max}/K_m (ml min^{-1} g $protein^{-1}$)	Cl_{int} (ml min^{-1} kg^{-1})	V_{max}/K_m (ml min^{-1} g $protein^{-1}$)	Cl_{int} (ml min^{-1} kg^{-1})	
EBPA	26.46 (±4.33)	8.799 (±1.440)	712.2 (±108.2)	452.4 (±68.7)	4.237
HBPA	7.190 (±0.968)	2.391 (±0.322)	239.4 (±22.5)	152.1 (±14.3)	6.353
OBPA	6.570 (±0.853)	2.184 (±0.284)	249.2 (±27.1)	158.3 (±17.2)	7.411
DBPA	0.216 (±0.027)	0.072 (±0.009)	13.42 (±1.57)	8.525 (±0.997)	9.527

7.40 (9H, m, (PhH). The product was 100% pure by HPLC and exhibited a retention time that would be predicted for HBPA.

3.2. Determination of V_{max} and K_m

The intrinsic clearances (Cl_{int}) of the esterases were calculated for each substrate and organ. They are the product of V_{max}/K_m ratio, protein yield (50.3 mg g^{-1} lung and 12.1 mg ml^{-1} blood) and lung weight (6.61 g lung kg^{-1}) or blood volume (52.5 ml blood kg^{-1} (Mehvar, 1991)). The V_{max}/K_m ratio and intrinsic clearance values for the hydrolysis of each ester in lung and blood preparations are given in Table 2, the computed log octanol-water partition coefficients (CLOGP) are also quoted (CLOGP program ver. 3.55, Medchem Project, Pomona College, Claremont, USA).

The rate of metabolism of the esters was measured by the disappearance of the parent compound from the incubate, however, the concurrent appearance of biphenylacetic acid occurred in all cases indicating that the disappearance of ester was due to hydrolysis.

The difference between the lowest and highest V_{max} values observed, for ester hydrolysis, in the pulmonary preparation was 26-fold, which was similar to the value obtained for the difference in V_{max} values for ester hydrolysis in blood. If the log of pulmonary V_{max} values are plotted against CLOGP there appears to be a parabolic relationship. A similar parabolic trend also exists between log V_{max} and CLOGP for the hydrolysis of esters in blood. The K_m values determined in either media showed no apparent relationship with lipophilicity. The difference between the lowest and highest K_m values, for both preparations, was a factor of five. The V_{max}/K_m ratio is the best descriptor of enzymatic activity when the substrate concentration is much lower than K_m and first-order kinetics apply. For the pulmonary derived V_{max}/K_m ratios there was a 120-fold difference between the ratios, while a 50-fold difference was observed between the ratios for the blood data. The V_{max}/K_m ratios, for pulmonary hydrolysis of esters, when plotted as log values against CLOGP suggest a parabolic relationship ($r = 0.985$, $CLOGP_o = 4.080$) (Fig. 1), which is also reflected in the log V_{max}/K_m values derived from the blood data ($r = 0.981$, $CLOGP_o = 4.240$) (Fig. 1). Both parabolic curves gave higher correlation coefficients than the linear curves for the same data ($r = 0.940$ and 0.930 for lung and blood, respectively). Although the above relationships have been discussed with regard to lipophilicity it should be noted that they apply equally to the number of carbon atoms in the alcohol moiety of the esters, which is the determinant of the CLOGP difference between the esters. As the CLOGP increases linearly with alcohol chain length it is impossible to differentiate whether lipophilicity of the compound or the size of the alcohol moiety is important in deciding the ki-

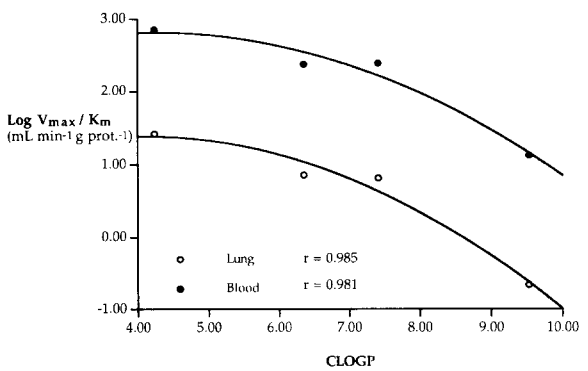

Fig. 1. Graph of log V_{max}/K_m vs CLOGP in rat lung homogenate and blood.

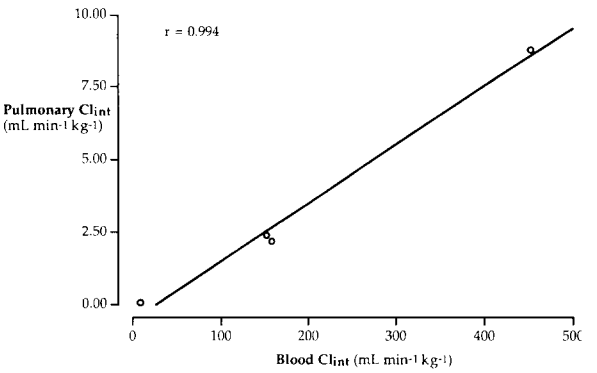

Fig. 2. Graph of pulmonary intrinsic clearance vs blood intrinsic clearance for the hydrolysis of biphenylacetic acid esters in rat.

netic parameters of enzymatic activity. To define the relative importance of these two factors it would be necessary to investigate esters with branched or aromatic alcohol moieties.

As the hydrolysis of only four esters has been studied the significance of the parabolic relationships is difficult to quantify. Considering previously published reports, the effect of acid moiety chain length, of esters with lower lipophilicity, on porcine liver esterase activity has been studied: showing a roughly parabolic relationship between apparent first-order rate constant and chain length (Kawaguchi et al., 1985). A parabolic relationship with log P was demonstrated, however, this was against the log K_m values for the hydrolysis of several compounds by rat liver (Durrer et al., 1991), which is in contrast to the results found here. Interestingly the log P_o was 3.8 which is less than the CLOGP of any of the compounds studied here. From the original QSAR theory (Hansch et al., 1968) a parabolic relationship may be expected to exist, unfortunately the lipophilicity of the esters studied here is probably to high to allow this to be investigated fully. For both V_{max}/K_m data sets the $CLOGP_o$ is approx. 4.16 (4.08–4.24) which is similar to the CLOGP of ethyl biphenylacetate and greater than that of methyl biphenylacetate (CLOGP = 3.708). This suggests that the ethyl ester will be hydrolysed more quickly than methyl ester. Whilst studying the hydrolysis of benzoic acid esters in human plasma, the opposite trend has been previously reported (Nielsen and Bundgaard, 1987). Again, the acid moiety studied was less lipophilic than that studied by our laboratory. It is possible that if a more hydrophilic acid had been chosen, relationships which more closely mimic those already published, would have been observed.

Intrinsic clearance is a representation of the organ's total ability to metabolise a compound. It can be seen that in all cases the blood has a much greater ability than the lungs to hydrolyse the esters, varying from a 50-fold greater ability to metabolise ethyl biphenylacetate up to a 120-fold greater ability to hydrolyse dodecyl biphenylacetate. If the intrinsic clearances obtained for pulmonary metabolism are plotted against the intrinsic clearances obtained for blood hydrolysis then a linear relationship is observed ($r = 0.994$, $p < 0.05$) (Fig. 2). This suggests that blood may be used to predict the in vitro metabolism of esters by the lungs, which would be desirable as blood can be easily obtained and without the need to kill animals.

The 'well-stirred' or venous equilibration hepatic model (Rowland et al., 1973) has been used to predict the pulmonary extraction ratio of compounds (e.g., Smith and Bend, 1980). The predicted pulmonary extraction ratios have been calculated, using this model, and are shown in Table 3. Pulmonary blood flow was taken to be 185 ml min^{-1} kg^{-1} (Mehvar, 1991). Maximum extraction ratios were calculated as protein binding was assumed to zero, which is obviously unlikely as BPAA has been shown to be extensively protein bound (Chiccarelli et al., 1980). The extraction ratio will probably decrease with an increase in plasma protein binding. The organ clearance shown is the product of extraction ratio and blood flow. The concept of organ extraction, for blood, is difficult to visualise and would depend on arbitrary selection of administration and sampling sites, accordingly only the intrinsic clearance is quoted, as the clearance of drug from the body by blood (Table 3).

It is evident that all the esters have very low

Table 3
Predicted pulmonary extraction ratios and lung and blood clearance

Esters	Maximum predicted pulmonary extraction ratio	Clearance (ml min^{-1} kg^{-1})	
		Lung	Blood
EBPA	0.045	8.32	452.4
HBPA	0.013	2.40	152.1
OBPA	0.012	2.33	158.3
DBPA	3.9×10^{-4}	0.07	8.525

predicted extraction ratios, with none of the esters showing an extraction ratio that could be expected to be measured in vivo. These results suggest that esters will not undergo first-pass extraction when passing through the lungs to any significant degree and that the lungs will not significantly contribute to the total body clearance of the esters studied.

Despite these low predicted pulmonary extraction ratios it is believed that the compounds are suitable for the investigation of pulmonary first-pass metabolism as extensive pulmonary metabolism of other esters has been demonstrated (Ryrfeldt and Bodin, 1975; Shargel and Dorrbecker, 1976). Also, the predictive nature of the model has not been fully established and warrants further investigation. It has previously been suggested that in vitro models may be more predictive in a comparative rather than quantitative manner (Mistry and Houston, 1987).

In conclusion, it appears that the kinetic parameters for the non-specific esterase catalysed hydrolysis of BPAA esters are predictable from the ester's physicochemical nature. Also the pulmonary enzyme kinetics for hydrolysis can be predicted from the kinetics measured in blood. Whether the kinetic parameters can be used to predict the in vivo situation remains to be ascertained.

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