Characterization of Pulmonary Alveolar Esterases of the Primate *Cercopithecus pygerythrus*

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Received December 4, 1996; accepted December 6, 1996

Purpose. To evaluate and classify the hydrolases of the primate lung.
Methods. Homologous series of aromatic, aliphatic, and choline ester substrates were assayed with the pH-stat method to obtain the Michaelis-Menten kinetic constants, V_{max} and K_m, for the enzymes in pulmonary alveolar tissue with esterase activity. Polyacrylamide gel electrophoresis was employed to determine the number of such hydrolytic enzymes. Inhibition studies with selective esterase inhibitors were carried out to classify enzymes as either arylesterases, carboxylesterases, or cholinesterases.
Results. Aromatic, aliphatic, and choline ester drugs were hydrolyzed by alveolar tissue of the primate lung. The catalytic enzymes were more specific for aromatic esters since these were metabolized at faster rates than the other substrates. Aromatic ester hydrolysis was also inhibited by triorthocresylphosphate (TOCP), a potent inhibitor of carboxylesterases. Inhibitors of arylesterases and cholinesterases had minimal effect on the enzymic hydrolysis of all substrates. Polyacrylamide gel electrophoresis demonstrated three enzymes to have esterolytic activity, two (MWs 269 and 281 kDa) of which were sensitive to TOCP and are therefore carboxylesterases. The third enzyme (MW 34 kDa), was unaffected by esterase inhibitors and, thus, cannot be classified as an esterase.
Conclusions. Primate pulmonary alveolar tissue contains two isozymes of carboxylesterases.

KEY WORDS: carboxylesterases; ester prodrugs; pulmonary esterases; primate; vervet monkey.

INTRODUCTION

The therapeutic efficacy of the β_2-sympathomimetic bronchodilators is limited by muscle tremor (1) and cardiovascular (2) side-effects. A method which has been used to overcome these adverse effects utilizes the prodrug approach.

Most bronchodilator prodrugs are esters since the lung is rich in esterase enzymes (3) compared to skeletal (4) and cardiac muscle (3) in which bronchodilators exert their unwanted effects. Esterases are difficult to classify since they exhibit overlapping substrate specificities (5). The International Enzyme (E.C) Commission identifies three major groups—arylesterases [E.C 3.1.1.2], carboxylesterases [E.C 3.1.1.1] and cholinesterases. Cholinesterases are further subdivided into acetylcholinesterases [E.C. 3.1.1.7], and cholinesterases [E.C 3.1.1.8]. A parallel and overlapping classification is based on their interaction with organophosphate esters (6). This system also distinguishes three groups: A-esterases hydrolyze organophosphate esters; B-esterases are irreversibly phosphorylated and inhibited; and C-esterases do not react with organophosphate esters at all. A-esterases include arylesterases whereas B-esterases include carboxylesterases and cholinesterases. C-esterases correspond to acetylesterases [E.C 3.1.1.6].

Pulmonary esterases of mammals such as rats (3), mice (7), hamsters (8) and rabbits (9), have been characterized. The types of esterases which populate the lungs of primates have, however, not been fully determined. Esterases also exhibit species variation (10,11). When the cascade ester D2438, a terbutaline prodrug, was administered orally to dogs, both sustained action and prolonged terbutaline plasma concentration profiles were observed. This could not be achieved in man—an effect ascribed to variations in the esterases of man and dog (11). Biological activation of ester prodrugs, as measured in common laboratory animals may, therefore, not predict their behaviour in man. A primate lung model could, however, prove more useful for studying the pharmacokinetics of such pharmaceutical entities.

The objective of this study was, therefore, to characterize pulmonary esterases of the vervet monkey, *Cercopithecus pygerythrus*, using substrates and inhibitors specific for the different classes of esterases.

MATERIALS AND METHODS

Chemicals

Phenylbutyrate was synthesized from phenol and butyrylchloride, and phenylpropionate from phenol and propionylchloride as described previously (12). Acetylcholine, butyrylcholine, fast red TR, *alpha*-naphthylacetate, neostigmine, physostigmine, and propionylcholine were purchased from Sigma Chemical Company (St Louis, MO). Butyrylchloride, ethylacetate, ethylbutyrate, ethylpropionate, *para*-hydroxymercuribenzoate (*p*-HMB), phenol, phenylacetate, propionylchloride and triorthocresylphosphate (TOCP) were obtained from Fluka A-G (Buchs, Switzerland). Sterile pyrogen-free isotonic saline was bought from Sabax (Pty) Ltd. (Cape Town, South Africa). Acrylamide, ammonium persulphate, bisacrylamide, glycine, *N,N,N',N'*-tetramethylethylenediamine (temed), and tris (hydroxymethyl) aminomethane (Tris) were purchased from Merck South Africa (Pty) Ltd. (Midrand, Transvaal).

Enzyme Source

Fresh vervet monkey lungs were obtained from the Provincial Animal Centre, Kuils River, South Africa. The lungs were cleansed by perfusion with cold isotonic saline, cut into thin slices and homogenized with one to two volumes of isotonic saline in a chilled Potter-Elvejhem homogenizer with a teflon pestle. The homogenates were centrifuged for 30 min. at 4°C and 9000 *g*. The postnuclear supernatant was removed, transferred to freeze-drying flasks, frozen and lyophilized overnight at −40°C, and then stored at −20°C.

Prior to kinetic analysis, the freeze-dried tissue was reconstituted in isotonic saline (0.5 mg tissue/ml) and 10 ml of this mixture served as enzyme source for the hydrolysis reaction.

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0724-8741/97/0200-0203$12.50/0

Kinetic Procedure

The hydrolysis of the ester substrates was measured titrimetrically by means of a pH-stat (autotitrator TT, pH meter 28, autoburette ABU 12, titrigraph SBR 3, and titration assembly, Radiometer, Copenhagen, Denmark) with 0.01 M NaOH at a constant pH of 7.4 and at 37°C without addition of buffer (12,13). Nonenzymatic hydrolysis was determined in isotonic saline. In the case of inhibition studies, the inhibitor was incubated with the reconstituted lung tissue at room temperature for 10 min. The hydrolytic reaction was initiated by addition of substrate, and the inhibited rate followed over the same time-course as the uninhibited rate.

pH Optima

The effects of hydrogen ion concentration on pulmonary hydrolase activity were studied by monitoring the hydrolysis kinetics of an aliphatic ester, ethylbutyrate (4 mM), an aromatic ester, phenylbutyrate (4 mM), and a choline ester, butyrylcholine (10 mM) over a pH range of 4 to 10. The pH of the reaction medium was adjusted to the required value with the pH-stat without the use of buffers. Enzymatic rates were corrected for spontaneous hydrolysis by following the breakdown of the substrates in the absence of lung tissue over the same pH range.

Analytical Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gels (10%) were used for the separation of pulmonary alveolar proteins. Gel and electrode buffers were prepared according to Ljungquist and Augustinsson (14). The lyophilized tissue was solubilized in saline (10 mg/ml, pH 7.4). A 1:1 dilution with 0.125 M Tris-HCl buffer pH 6.8 containing 20% glycerol was made and 150 μl (28.3 μg protein) of this mixture was applied to the gel. Protein concentration was determined according to the method of Bradford (15) using bovine serum albumin as standard. In the case of inhibition studies, the inhibitor was incubated with the protein sample for 10 min. at room temperature before the sample was mixed with the buffer solution. Electrophoresis was carried out at room temperature with a constant current of 60 mA for 1 hour.

After electrophoresis, the gels were stained for esterase activity by incubating them at room temperature for 10 min. in a solution containing 20 mg *alpha*-naphthylacetate (dissolved in 1 ml acetone), 50 mg diazonium salt, fast-red TR, in 100 ml of 0.05 M Tris-HCl buffer (pH 8). The liberated *alpha*-naphthol reacted with fast-red TR to give a reddish-brown colour in regions with esterase activity.

Molecular Weight Estimation

Polyacrylamide gel electrophoresis for the estimation of molecular weights was done using the nondenatured protein molecular weight marker kit (Sigma Chemical Co., St Louis, MO). A 10% slab gel was used and electrophoresis was conducted as described above. The gel was stained with a solution of 0.125% Coomassie Blue R-250, 50% methanol, and 10% acetic acid in water and destained first with a 50% methanol, 10% acetic acid solution in water for an hour, and then with an aqueous solution of 7% acetic acid and 5% methanol until fully clarified.

Data Analysis

The Michaelis-Menten constants (K_m and V_{max}) were calculated as described by Wilkinson (16). These constants were used to calculate the intrinsic free clearance (Cl'_{int}), which is the fractional loss of substrate per mass of lung tissue (17). In terms of the enzyme kinetic parameters, $Cl'_{int} = V_{max}/K_m$.

The extent of inhibition was expressed in terms of an I_{50} value which is the concentration of inhibitor that will produce a fifty percent reduction in the enzymatic rate. Each concentration was assayed thrice and the hydrolysis of each substrate repeated three to five times. The Student's *t*-test was used to analyse the data and a p value less than 0.05 was considered significant.

RESULTS

Substrate Specificity

Pulmonary alveolar esterases of the vervet monkey were found to catalyze the hydrolysis of aliphatic, aromatic, and choline ester drugs (Table 1). The enzymes were more specific for the aromatic esters, since these were metabolized at faster rates than the corresponding aliphatic and choline esters. Choline ester drugs had the lowest hydrolysis rates.

All enzymatic rates were corrected for nonenzymatic hydrolysis. Nonenzymatic hydrolysis for ethylacetate at low concentrations (0.2 to 1 mM) was higher than the enzymatic breakdown over the same concentration range. Only at concentrations above 1 mM ie., at 4 and 10 mM, could enzymatic hydrolysis be detected. Since substrate inhibition was detected above 10 mM, a V_{max} and K_m could not be calculated for this compound. Kinetic parameters could also not be calculated for acetylcholine since hydrolysis of the compound in saline (K_m 7.64 ± 6.37 mM) and lung tissue medium (K_m 2.76 ± 0.75 mM) did not differ significantly.

The alkyl substituents of all ester drugs were found to affect enzymatic hydrolysis. Butyryl esters had higher Cl'_{int}'s suggesting a more rapid metabolism than propionyl and acetyl (phenylacetate) esters (Table 1). The latter had the lowest clearance rate.

Inhibition by Esterase Inhibitors

The I^{*}_{50} values and percentage inactivation obtained with three esterase inhibitors for the enzymatic hydrolysis of the

Table 1. Michaelis-Menten Constants for Hydrolysis of Choline, Aliphatic, and Aromatic Esters by Lyophilized Pulmonary Alveolar Tissue of *Cercopithecus pygerythrus*

Substrate	V_{max} ± SEM ($\mu M\ min^{-1}\ mg^{-1}$)	K_m ± SEM (mM)	Cl'_{int} ($min^{-1}\ mg^{-1}$)
Propionylcholine	0.81 ± 0.1	3.22 ± 1.12	0.0003
Butyrylcholine	1.26 ± 0.85	1.45 ± 0.41	0.0009
Ethylpropionate	19.06 ± 1.75	2.34 ± 0.45	0.0082
Ethylbutyrate	47.27 ± 1.32	0.95 ± 0.09	0.05
Phenylacetate	23.21 ± 1.21	1.57 ± 0.27	0.015
Phenylpropionate	38.85 ± 0.86	0.82 ± 0.08	0.048
Phenylbutyrate	42.25 ± 2.55	0.19 ± 0.06	0.22

Table 2. Percentage Inhibition and I_{50} Constants of Esterase Inhibitors for the Hydrolysis of Butyrate Analogues of Choline, Ethyl, and Phenyl Esters by Lyophilized Pulmonary Alveolar Tissue of *Cercopithecus pygerythrus*

Substrates	Inhibitors	Percent inhibition	I_{50} (mM)
Butyrylcholine (5 mM)	TOCP (0.01 mM)	—	—
	Neostigmine (2 mM)	7.4	27.13
	p-HMB (0.1 mM)	9.3	1.07
Ethylbutyrate (1 mM)	TOCP (0.01 mM)	—	—
	Neostigmine (2 mM)	—	—
	p-HMB (0.1 mM)	—	—
Phenylbutyrate (1 mM)	TOCP (0.01 mM)	60.9[a]	0.02
	Neostigmine (2 mM)	20.4[a]	9.8
	p-HMB (0.1 mM)	7.0	1.42

[a] $p < 0.05$.

butyryl analogues of aliphatic, aromatic and choline esters, are shown in Table 2.

The hydrolysis of butyrylcholine was unaffected by TOCP whereas *p*-HMB and neostigmine caused minimal inhibition ($p > 0.05$). None of the three inhibitors had any effect on the catalytic breakdown of ethylbutyrate. The hydrolysis of phenylbutyrate was, however, inhibited by all three esterase inhibitors with the organophosphate (TOCP) being the most potent.

pH Optima

Symmetrical pH-activity profiles were obtained with ethylbutyrate and phenylbutyrate (Figure 1). In both cases optimum hydrolysis was detected between pH 7 and 8. Cleavage of the choline ester, butyrylcholine, was observed only at pH 6 and 7.4, and the rates were significantly less ($p < 0.05$) than those of the other two butyrate esters at these pH values. At pH values of 8 and above, butyrylcholine was found to hydrolyse spontaneously.

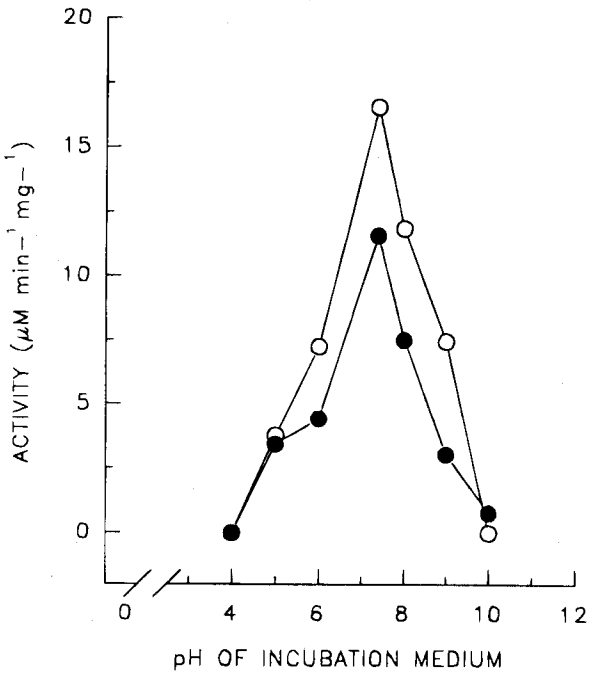

Fig. 1. Effect of pH on the esterase activity of primate pulmonary alveolar tissue. (○) Phenylbutyrate; (●) ethylbutyrate.

Electrophoretic Resolution

Esterase activity of the primate lung resided in three proteins with estimated molecular masses of 34, 269, and 281 kDa (Figure 2). The 34 kDa protein only gave a faint band with *alpha*-naphthylacetate, suggesting little hydrolase activity. TOCP (0.01 mM) completely inhibited the two high molecular weight enzymes but not the 34 kDa one (Figure 3). Physostigmine and *p*-HMB had little, if any, effect on the catalytic activity of the three hydrolases.

DISCUSSION

All ester substrates, except acetylcholine, were enzymatically hydrolyzed by saline-reconstituted lyophilized lung tissue. This suggests that a specific acetylcholinesterase is absent from alveolar tissue of the vervet monkey lung. Rabbit lung homogenate was also found to be devoid of this enzyme (9).

Hydrolysis rates for propionyl- and butyrylcholine were low compared to the rates for corresponding aliphatic and aromatic esters. Neostigmine, a specific cholinesterase inhibitor, caused minimal inhibition of butyrylcholine hydrolysis. Its high

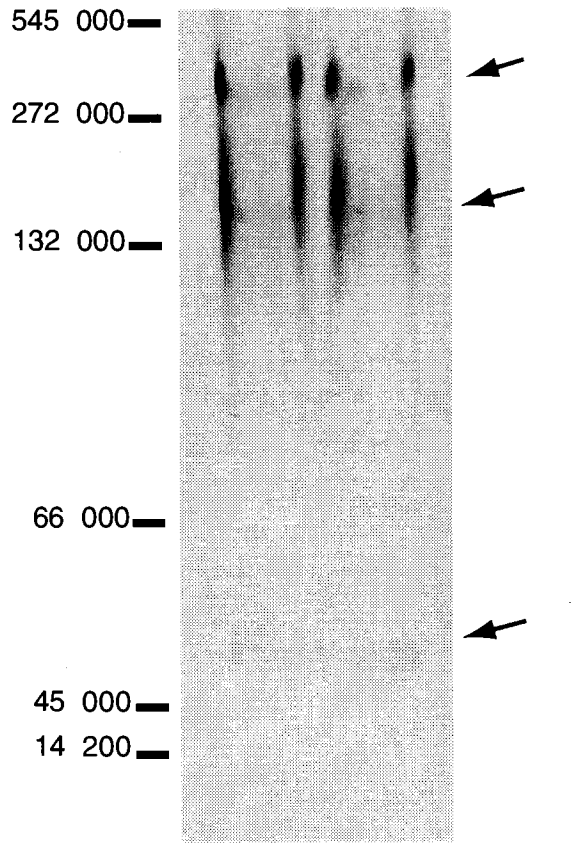

Fig. 2. Electropherogram of pulmonary alveolar enzymes of the vervet monkey with esterase activity. Arrows indicate the positions of the three enzymes.

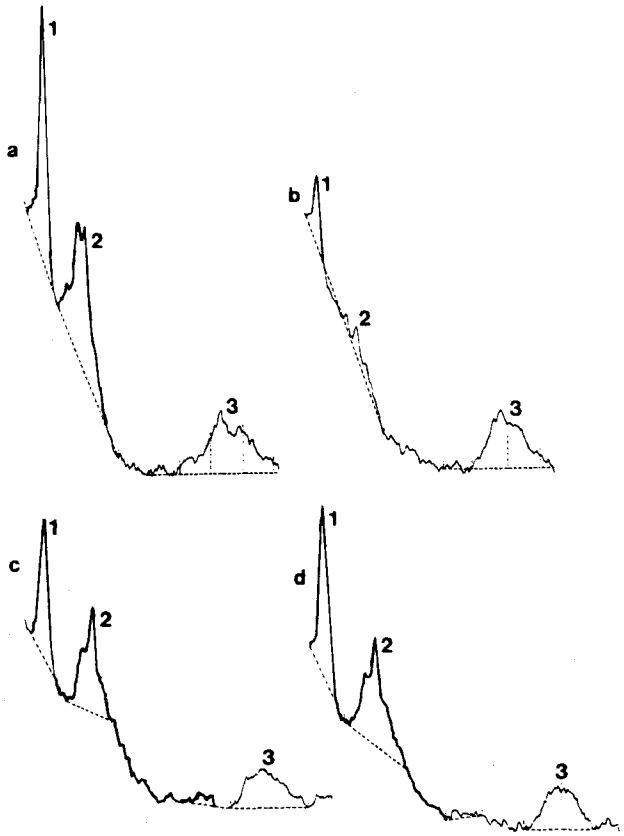

Fig. 3. Densitometer scans of an electropherogram of the three pulmonary enzymes with esterase activity after incubation with a) saline (control), b) TOCP, c) physostigmine, and d) *p*-HMB. Peak 1, 281 kDa; Peak 2, 269 kDa; Peak 3, 34 kDa.

I_{50} of 27.13 mM suggests a low specificity for the hydrolyzing enzyme. These findings, therefore, suggest an absence of cholinesterases from the alveolar tissue of primate lung. Murine pulmonary alveolar cells were also devoid of cholinesterase enzymes (7).

The B-esterase (cholinesterase and carboxylesterase) inhibitor, TOCP, had no effect on the enzymatic hydrolysis of butyrylcholine, whereas *p*-HMB, an arylesterase inhibitor effected only a slight reduction (9.3%) in butyrylcholine hydrolysis. This suggests that the degradation of the choline esters in the primate lung was not catalyzed by an esterase enzyme.

Hydrolysis rates for the aliphatic esters were higher than for the corresponding choline ester drugs. They were, thus, more specific as substrates for primate pulmonary esterases. None of the esterase modulators were found to affect the hydrolysis of ethylbutyrate. This suggests that the enzyme which catalyzed the breakdown of the aliphatic esters, was not an esterase. Hydrolase activity is not unique to esterases since carbonic anhydrase (18) and amidases (19) have been demonstrated to possess esterase activity.

The aromatic ester drugs proved to be the best substrates for primate pulmonary esterases. Their enzyme kinetic rates were higher than for corresponding aliphatic and choline esters. Since inhibition of the hydrolysis rate of phenylbutyrate with *p*-HMB was minimal (7%), it appears unlikely that an arylesterase catalyzed the degradation of the aromatic esters. The anticholinesterase, neostigmine, also had a low specificity (I_{50}, 9.8 mM) for the catalyzing enzyme. TOCP, however, caused a significant reduction in the hydrolysis rate of phenylbutyrate. Its low I_{50} of 0.02 mM suggests that it is highly specific for the hydrolyzing enzyme. Since cholinesterases could not be detected in the primate lung, the B-esterase which promoted the hydrolysis of the aromatic esters, must be a carboxylesterase.

Carboxylesterases, therefore, appear to be the only type of esterases in primate pulmonary alveolar tissue and are specific for aromatic ester drugs. Although aliphatic and choline esters were also degraded by lung tissue medium, the catalyzing enzymes were insensitive to esterase inhibitors, and hence, cannot be classified as esterases.

pH optima were obtained for the enzymes which cleaved ethylbutyrate (aliphatic ester) and phenylbutyrate (aromatic ester), but not butyrylcholine. The optimum pH for both enzymes was between 7 and 8, which is similar to that reported for enzymes with esterase activity (20).

The results obtained from kinetic assays of the lung tissue medium were supported by electrophoretic analysis of the lung tissue for enzymes with esterase activity. Three such enzymes could be identified, none of which were significantly inhibited by the cholinesterase inhibitor, physostigmine, or the arylesterase inhibitor, *p*-HMB. TOCP, almost completely inactivated the two high molecular mass enzymes (269 and 281 kDa) but not the low molecular mass (34 kDa) one. The two high molecular mass enzymes are, therefore, carboxylesterases. The low molecular mass enzyme, although capable of catalyzing the hydrolysis of ester-type drugs, cannot be classified as an esterase due to its insensitivity to esterase inhibitors. This enzyme probably mediated the breakdown of the aliphatic and choline esters in the vervet monkey lung.

An interesting observation was that the number of carbon atoms in the acyl chain of the drugs affected their rates of hydrolysis. Considering the propionyl and butyryl derivatives of the three types of esters, lengthening the substrates by one carbon atom caused a 3 to 5 fold increase in the intrinsic free clearance rate (Cl'_{int}). A similar finding was reported for the hydrolysis of 4-nitrophenylacetate and 4-nitrophenylbutyrate by rat lung carboxylesterases (21).

The information generated by this study, may prove useful for the design of bronchoactive ester prodrugs. When a rapid onset of action is required, the prodrug can be targeted for selective hydrolysis by the carboxylesterases. Such a compound would be aromatic and may include an acyl chain containing at least four carbon atoms. When a more sustained release of the active drug is required, the prodrug would incorporate an alkyl (or choline) group with an acyl chain of appropriate length for prolonged activation.

ACKNOWLEDGMENTS

The authors wish to thank Professor David T. Okpako for editorial comments and Mr. Gary Gabriels for technical assistance. This work was supported by grants from the Foundation for Pharmaceutical Research and the University of the Western Cape.

REFERENCES

1. W. van den Berg, J. G. Leferink, R. A. Maes, J. K. Fokkens, J. Kreukniet, and P. L. Bruynzeel. *Eur. J. Resp. Dis.* **65**:181–193 (1984).

2. J. S. Finch. *Ann. Allergy.* **47**:402–404 (1981).
3. N. W. McCracken, P. G. Blain, and F. M. Williams. *Biochem. Pharmacol.* **45**:31–36 (1993).
4. F. Crespi, E. Pagliacci, G. M. Rocchini, R. Bianchi, M. Salmona, S. Garattini, and E Mussini. *Eur. J. Drug Metab. Pharmacokinet.* **3**:175–177 (1979).
5. F. M. Williams. *Clin Pharmacokinet.* **10**:392–403 (1985).
6. W. N. Aldridge. *Biochem. J.* **53**:110–117 (1953).
7. R. Schiff, M. A. Brunstetter, R. L. Hunter, and C. E. Cross. *Cytochem.* **18**:167–177 (1970).
8. C. Meban. *Acta anal.* **108**:184–192 (1980).
9. A. E. Vatter, O. K. Reiss, J. K. Newman, K. Lindquist, and E. Groeneboer. *J. Cell. Biol.* **38**:80–98 (1968).
10. T. Minagawa, Y. Kohno, T. Suwa, and A. Tsuji. *Biochem. Pharmacol.* **49**:1361–1365 (1995).
11. L. A. Svensson. *Acta Pharm. Suec.* **24**:333–341 (1987).
12. H. M. J. Leng. M. Pharm. thesis. University of the Western Cape, Bellville, SA, 1987.
13. C. W. Chong, L. W. Dittert, H. B. Kostenbauder, and J. V. Swintovsky. *J. Pharm. Sci.* **56**:1647–1652 (1967).
14. Å. Ljungquist and K-B Augustinsson. *Eur. J. Biochem.* **23**:303–313 (1971).
15. M. M. Bradford. *Anal. Biochem.* **72**:248–254 (1976).
16. G. N. Wilkinson. *Biochem. J.* **80**:324–332 (1961).
17. A. Rane, G. R. Wilkinson, and D. G. Shand. *J. Pharmacol. Exp. Ther.* **200**:420–424 (1977).
18. M. K. Ghosh and A. K. Mitra. *Pharm. Res.* **7**:251–255 (1990).
19. W. Junge and K. Krisch. *Mol. Cell Biochem.* **1**:41–52 (1973).
20. T. L. Huang, A. Székács, T. Uematsu, E. Kuwano, A. Parkinson, and B. D. Hammock. *Pharm. Res.* **10**:639–648 (1993).
21. R. Gaustad, K. Sletten, D. Løvhaug and F. Fonnum. *Biochem. J.* **274**:693–697 (1991).