International Journal of Pharmaceutics, 8 (1981) 167–174
Elsevier/North-Holland Biomedical Press

ENZYMATIC HYDROLYSIS BY MOUSE SKIN HOMOGENATES: STRUCTURE–METABOLISM RELATIONSHIPS OF PARA-NITROBENZOATE ESTERS *

ANDRÉ PANNATIER, BERNARD TESTA ** and JEAN-CLAUDE ETTER

School of Pharmacy, University of Lausanne, Place du Château 3, CH-1005 Lausanne (Switzerland)

(Received January 22nd, 1981)
(Accepted February 25th, 1981)

SUMMARY

The hydrolysis of 8 alkyl esters of *p*-nitrobenzoic acid has been investigated in the presence of skin preparations from untreated mice, using as analytical technique a sensitive and specific gas chromatographic method. The hydrolysis reaction was shown to be enzyme-mediated, negligible or no abiotic hydrolysis taking place under the conditions of the study. The cutaneous esterase involved in the reaction was found to be located in the cytosol and to be stable to heat pretreatment up to 50°C. The initial rate of hydrolysis increased with increasing lipophilicity of the substrates and decreased with increasing steric bulk on the alpha-carbon; a highly significant QSAR equation was derived.

INTRODUCTION

The skin as a covering organ has a specific pathology which may require treatment by topical application of drugs. In addition, the skin (like, for example, the lungs and the gastrointestinal tract) is an interface between an organism and its environment, and as such may be used as a portal of entry of drugs into the general circulation. This latter aspect is particularly important in view of current efforts towards new drug delivery systems (Shaw and Chandrasekaran, 1978), as exemplified by the prolonged duration of action of nitroglycerine administered percutaneously in ointment form and of scopolamine liberated from a transdermal therapeutic device.

* Presented as a poster communication at the 7th European Workshop on Drug Metabolism, Zurich, October 5–10, 1980.

** To whom correspondence should be addressed.

0 378-5173/81/0000–0000/$02.50

Drug metabolism by cutaneous tissues is gaining constantly increasing interest due to its pharmacokinetic, pharmacological, therapeutic and toxicological implications. The ability of the skin to metabolize xenobiotics is still largely unexplored, much of our current knowledge on cutaneous biotransformation being restricted to steroids and polycyclic aromatic hydrocarbons (for reviews, see Pannatier et al, 1978; Vainio, 1980). This lack of knowledge hinders a proper understanding of the fate of topically applied drugs, and stands as an obstacle to a fairly rational cutaneous or transcutaneous therapy. In two preceding publications (Pannatier et al., submitted), we have investigated oxidative O-dealkylase activity in mouse skin microsomes and shown the involvement of cytochrome P450. The present paper examines *p*-nitrobenzoate ester hydrolase activity in mouse skin. This activity is shown to display the properties of a soluble (cytosolic) enzyme, and to be strongly influenced by the chemical structure of the substrates.

MATERIALS AND METHODS

Substrates

The methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *sec*-butyl, and isobutyl *p*-nitrobenzoate esters have been synthetized from the acid and the corresponding alcohol in the presence of concentrated sulfuric acid, and recrystallized twice from ether or absolute ethanol in the presence of activated charcoal. The tertiobutyl derivative has been synthetized from *p*-nitrobenzoyl chloride (Organikum, 1977) and recrystallized according to Vogel (1967). The identity of the synthesized chemicals has been ascertained by NMR (60 MHz spectrometer Varian EM360). The purity of the compounds has been verified by GC (for conditions, see later), the free acid contaminant being undetectable even at the highest sensitivity (contamination less than 0.04%).

Reagents and solvents

Tetrabutylammonium hydrogenosulfate (TBA), methyl iodide, ethyl iodide and dichloromethane, all of quality "purissimum", were obtained from Fluka AG (Buchs, Switzerland). Ethyl acetate of "pro analysi" quality was obtained from Merck (Darmstadt, G.F.R.).

Quantitative determination of substrates and metabolite

The instrument used was a Hewlett-Packard 5710A gas chromatograph equipped with FID and a HP 3380A integrator. The glass column (6 ft., i.d. 4.5 mm) was packed with Chromosorb W(HP) 80–100 mesh coated with 3% Ultraphase (Pierce Eurochemie, Rotterdam, The Netherlands). The operating conditions were as follows: injector and detector temperature 250°C, oven temperature 140°C; flow-rates: nitrogen 20 ml/min, hydrogen 30 ml/min, air 240 ml/min.

The metabolite, *p*-nitrobenzoic acid, is alkylated before GC analysis by solid–liquid phase-transfer catalysis (Arbin et al., 1979). To a solution of *p*-nitrobenzoic acid in ethyl acetate are added: 50 µl of a 0.1 M solution of TBA in CH_2Cl_2, 40 µl of the appropriate alkyl iodide, and 0.05 g of $NaHCO_3$. The mixture is shaken for 10 min and centrifuged for 5 min; 1 µl of the supernatant is then injected into the chromatograph. This method allows quantitative derivatization of the free acid by using either methyl iodide or ethyl

TABLE 1

ANALYTICAL CONDITIONS FOR *p*-NITROBENZOATE ESTERS (PNB ESTERS)

Substrate	Retention time of substrate (min)	Internal standard	Alkylating agent
Methyl PNB	3.5	*n*-Propyl PNB	C_2H_5I
Ethyl PNB	4.8	*n*-Propyl PNB	CH_3I
n-Propyl PNB	7.6	Isopropyl PNB	CH_3I
Isopropyl PNB	5.7	*n*-Propyl PNB	CH_3I
n-Butyl PNB	12.2	*n*-Propyl PNB	C_2H_5I
Isobutyl PNB	9.9	*n*-Propyl PNB	C_2H_5I
sec-Butyl PNB	9.0	*n*-Propyl PNB	C_2H_5I
tert-Butyl PNB	7.1	Isopropyl PNB	CH_3I

iodide. Furthermore, this method proved to be suitable for the analysis of *p*-nitrobenzoic acid in the presence of any of its esters, no transesterification (reagent-catalyzed hydrolysis, followed by derivatization) taking place.

An internal standard was used for the quantitative determination of derivatized metabolite and unmetabolized substrates. The alkylating reagent and the internal standard were chosen in each case so that a complete separation of the 3 peaks was ensured (Table 1). For each substrate, a linear calibration ($r^2 = 0.999$) was obtained in the concentration range investigated (125–2500 ng/μl in the injected solution). For the derivatized *p*-nitrobenzoic acid, the callibration is also linear ($r^2 = 0.999$) in the concentration range considered (12.5–250 ng/μl).

Incubations

The animals used, the preparation of cutaneous homogenates, and the isolation of microsomes, have been described in a previous publication (Pannatier et al., in press). The incubation medium consisted of 1 ml isotonic KCl, 2 ml isotonic phosphate buffer, pH 7.4, and 2 ml of cutaneous homogenate (corresponding to 1 g tissue). The substrate (2.5 μmol in 10 μl acetone) was incorporated with a microsyringe, and the incubations were carried out at 37°C in duplicate.

The incubations were stopped using 0.6 ml 0.5 M HCl and extracting for 10 min with 5 ml of ethyl acetate containing the internal standard. After centrifugation, the organic layer was dried (Na_2SO_4) and concentrated to about 150 μl at 40°C under a flux of nitrogen. The reagents and ethyl acetate (up to a total volume of 400 μl) were then added, and the reaction of derivatization carried out as described above. Under these conditions, the extraction of substrate and metabolite are quantitative (101.6%, S.D. 3.4%, n = 10). The reaction mixture from each incubation flask was injected 2–4 times into the chromatograph.

RESULTS

Hydrolysis of the methyl and ethyl esters

The hydrolysis of these two esters as a function of time is shown in Fig. 1. The more lipophilic ethyl ester is metabolized faster than the methyl ester, and after 45 min the total amount of incubated ethyl *p*-nitrobenzoate has been hydrolyzed. In contrast, only 85% of the amount of the methyl ester has been hydrolyzed after 45 min, but the reaction appears to continue. Fig. 1 also shows that for both substrates the reaction rate is constant during the first 15 min. These results indicate a considerable hydrolytic activity in the skin preparations. A number of experiments have been undertaken with the goal: (a) to prove the enzymatic nature of the reaction; and (b) to investigate the nature of the enzyme.

In order to prove the enzymatic nature of the hydrolysis reaction, the methyl and ethyl esters have been incubated in a medium devoid of skin homogenate. The results (Table 2) show that the ethyl ester undergoes no detectable hydrolysis under these conditions. As regards the methyl ester, 0.6% of the substrate undergoes chemical hydrolysis under the conditions of the study (pH 7.4, 37°C, 45 min); this value is negligible when compared to the extent of hydrolysis in the presence of skin homogenate (see Fig. 1).

The enzymatic nature of the hydrolytic reaction is further substantiated by results of experiments in which the cutaneous 10,000 *g* preparations have undergone a heat treatment for 10 min prior to their addition to the incubation mixture. The results (Table 2) show that a pretreatment at 50°C has little effect on the biological activity (6% loss). After treatment at 60°C, however, over 80% of the activity is lost, while still higher temperatures completely destroy the activity. The jump from 50 to 60°C thus produces a sudden and extensive loss of activity, a behavior compatible with enzyme denaturation.

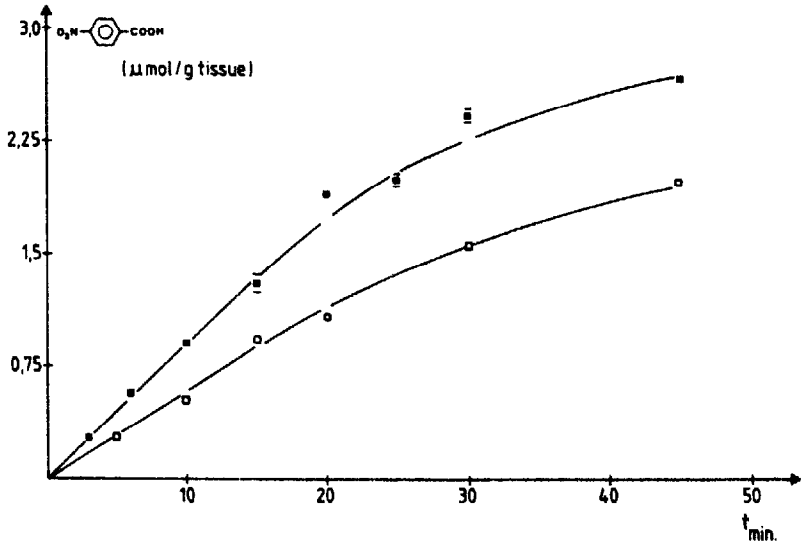

Fig. 1. The hydrolysis of methyl (□) and ethyl (■) *p*-nitrobenzoate by mouse skin 10,000 *g* preparations at 37°C as a function of time. The points are the average of two independent studies; mean errors are drawn when larger than symbol.

TABLE 2

CONTROL STUDIES: INCUBATION OF *p*-NITROBENZOATE ESTERS IN THE ABSENCE OF SKIN HOMOGENATE (STUDY A), IN THE PRESENCE OF HEAT-PRETREATED CUTANEOUS 10,000 *g* PREPARATIONS (STUDY B), AND IN THE PRESENCE OF CYTOSOL OR MICROSOMES AFTER 100,000 *g* CENTRIFUGATION (STUDY C)

Study	Cutaneous preparation	Substrate (2.5 μmol)	μmol metabolite/45 min (±S.D.)
A	None [a]	Methyl ester	0.015 ± 0.001
		Ethyl ester	ND [b]
B	10,000 *g*	Ethyl ester	2.51
	10,000 *g* 50°C/10 min	Ethyl ester	2.36 ± 0.04
	10,000 *g* 60°C/10 min	Ethyl ester	0.46 ± 0.04
	10,000 *g* 70°C/10 min	Ethyl ester	0.028 ± 0.002
	10,000 *g* 90°C/10 min	Ethyl ester	ND [b]
C	Cytosol [c]	Ethyl ester	2.43 ± 0.12
	Microsomes [c]	Ethyl ester	0.086 ± 0.005

[a] Replaced by an equal volume of phosphate buffer pH 7.4.
[b] Not detectable.
[c] Corresponding to 2 g tissue.

Furthermore, the relative heat stability of the esterase is suggestive of a soluble enzyme. Indeed, membranal polyenzymatic systems are more readily inactivated by heat than soluble enzymes, as shown by the complete inactivation of cutaneous mono-oxygenases at 50°C (Pannatier et al., in press). To prove the soluble nature of the esterase, the cutaneous cytosol and microsomes have been separated by centrifugation at 100,000 *g*. The results (Table 2) show full activity in the cytosol, and residual activity in

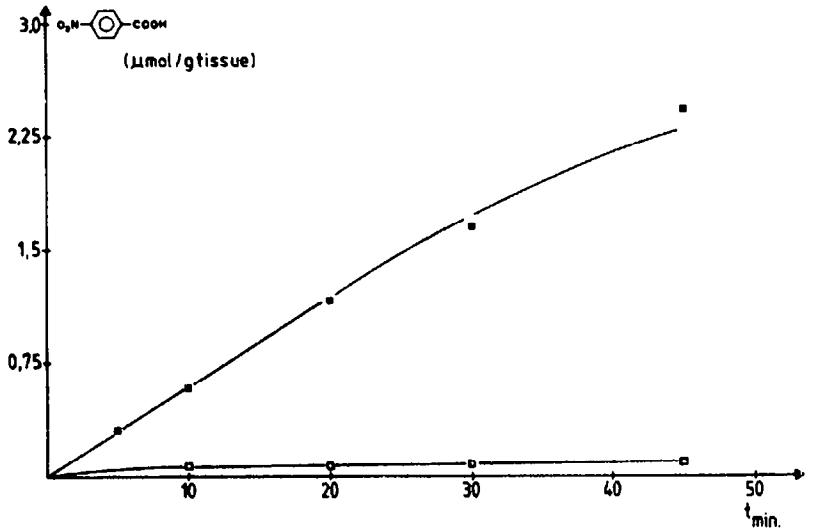

Fig. 2. The hydrolysis of ethyl *p*-nitrobenzoate by the cytosol (100,000 *g*) supernatant (·) and microsomes (□) obtained from mouse skin homogenates.

the microsomes. This is also illustrated in Fig. 2. The residual activity in microsomes does not appear to be time-dependent, and is probably due to contamination.

Influence of the alkyl substituent on the rate of hydrolysis of p-nitrobenzoate esters

The alkyl *p*-nitrobenzoate esters (alkyl with 3 and 4 carbon atoms, linear and branched series) were incubated with mouse skin 10,000 *g* supernatant preparations as described and the extent of hydrolysis measured after 5, 10, 15, 20, 30 and 45 min. The rate of hydrolysis was found to be constant for an initial period of 10–45 min depending on the substrate.

Table 3 lists the initial rate of hydrolysis of the 8 substrates. These results are also displayed in Fig. 3, where the rate of hydrolysis is plotted against the number of carbon atoms of the alkyl substituent. The relationship approaches linearity for the esters of primary alcohols (primary C_α atom), whereas the esters of secondary alcohols (secondary C_α) and of *tert*-butanol (tertiary C_α) are more resistant towards hydrolysis.

Fig. 3 thus suggests the hydrolysis of the *p*-nitrobenzoate esters to be markedly influenced by the structural properties of the substrates. To gain a quantitative insight into these influences, statistical correlations have been sought using the QSAR (quantitative structure–activity relationships) methodology. These theoretical techniques have already found extensive applications in drug metabolism studies (for reviews, see Hansch, 1972; Testa and Jenner, 1980). The biological activity and the structural parameters used in the present study are given in Table 3. Eqn. 1 has been obtained by multiple linear regression:

$$\log RH = 0.273(\pm 0.140)\, f - 0.516(\pm 0.108)\, I - 0.866(\pm 0.246) \quad (1)$$

$$n = 8;\ r^2 = 0.968;\ s = 0.076$$

TABLE 3

INITIAL RATE OF CUTANEOUS HYDROLYSIS AND STRUCTURAL PROPERTIES OF *p*-NITROBENZOATE ESTERS (PNB ESTERS)

Substrate	f [a]	I [b]	log RH [c]	
			exp. [d]	calc. [e]
Methyl PNB	0.701	1	−1.26 ± 0.01	−1.19
Ethyl PNB	1.220	1	−1.01 ± 0.01	−1.05
n-Propyl PNB	1.739	1	−0.82 ± 0.02	−0.91
Isopropyl PNB	1.739	2	−1.38 ± 0.01	−1.42
n-Butyl PNB	2.258	1	−0.80 ± 0.04	−0.77
Isobutyl PNB	2.258	1	−0.84 ± 0.02	−0.77
sec-Butyl PNB	2.258	2	−1.22 ± 0.01	−1.28
tert-Butyl PNB	2.258	3	−1.85 ± 0.01	−1.80

[a] Hydrophobic fragmental constant (Rekker and de Kort, 1979) of the alkyl substituent.
[b] Indicator variable for the branching of the alpha-carbon atom: 1 = primary; 2 = secondary; 3 = tertiary.
[c] RH = initial rate of hydrolysis in μmol/g tissue/min.
[d] Experimental value and mean deviation.
[e] Calculated according to Eqn. 1.

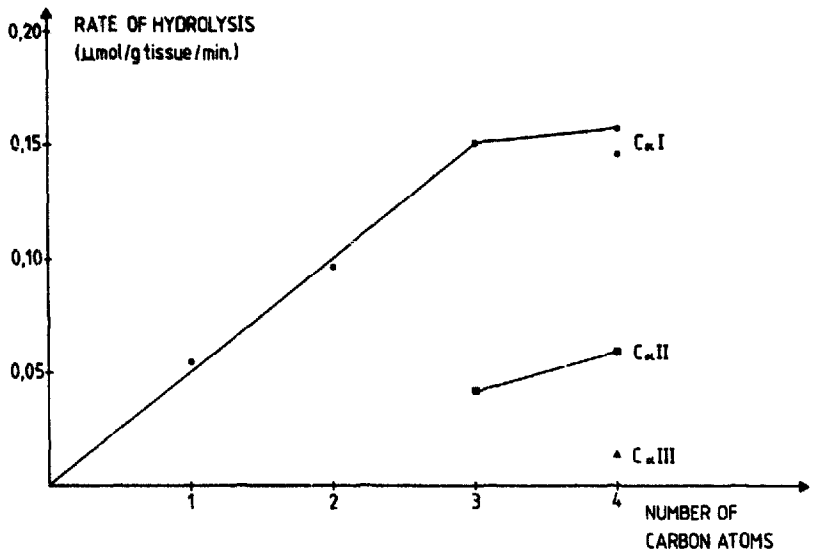

Fig. 3. A plot of the initial rate of hydrolysis of the 8 substrates by mouse skin 10,000 *g* supernatant preparations as a function of the number of carbon atoms of the alkyl substituent and of the degree of branching of the alpha-carbon atom (C_α primary, •; secondary, ■; tertiary, ▲).

This equation is statistically highly significant, as assessed by the high correlation coefficient (*r*) and the low standard deviation of the equation (s). Each independent variable in this equation is also statistically highly significant, as assessed by a Student's *t*-test analysis (not shown) and by the 95% confidence intervals given in parenthesis.

Eqn. 1 indicates in a quantitative fashion how the rate of hydrolysis of *p*-nitrobenzoate esters by mouse skin preparations increases with the lipophilicity of the substrate, and decreases with increasing branching of the alpha-carbon atom.

DISCUSSION

Using alkyl *p*-nitrobenzoate esters as substrates, we have been able in the present study to demonstrate the considerable hydrolytic activity of skin preparations from untreated mice. Using ethyl *p*-nitrobenzoate as a substrate, it was found that the mouse liver 10,000 *g* supernatant is approximately 10 times more active than the skin on a weight of tissue basis. This ratio of esterase activity can be contrasted with the cytochrome P450-mediated 0-dealkylase activity, which we found to be 500 higher in the liver than in the skin, again on a weight of tissue basis (Pannatier et al., in press).

The QSAR approach applied in the present study has yielded Eqn. 1, which indicates how the rate of cutaneous hydrolysis increases with increasing lipophilicity, and decreases with branching of C_α. This latter effect indicates that the enzymatic reaction of hydrolysis is highly sensitive to steric hindrance in the proximity of the ester linkage. Such a result may be of value in the design of topically applied prodrug esters.

The standard deviation of Eqn. 1 is found to be 0.076, i.e. larger than the mean devia-

tion of experimentally determined log RH, which is 0.015. We interpret this discrepancy as meaning that Eqn. 1 does not incorporate all the structural features influencing the biological activity. In particular, the bilinear effect seen in Fig. 3 for the primary alkyl esters suggests that the f versus log RH relationship should not be linear beyond C_8. But only additional studies with higher homologs could solve this problem. In any case, the 8 observations available do not allow more than 2 independent variables in a regression equation if chance correlations are to be avoided. Care must therefore be taken not to interpret Eqn. 1 beyond the explored range of structural properties.

In view of the current and developing interest in topical therapy, it is hoped that studies such as the present one may help to gain a better understanding of cutaneous drug disposition, and may allow rational design of relevant drugs and prodrugs.

REFERENCES

Arbin, A., Brink, H. and Vessman, J., Alkylation of carboxylic acids by solid–liquid phase-transfer catalysis for determination by gas chromatography. J. Chromatogr., 170 (1979) 25–33.

Hansch, C., Quantitative relationship between lipophilic character and drug metabolism. Drug Metab. Rev., 1 (1972) 1–14.

Organikum, Organisch-Chemisches Grundpraktikum, VEB Deutscher Verlag der Wissenschaften, Berlin, 1977, p. 505.

Pannatier, A., Jenner, P., Testa, B. and Etter, J.C., The skin as a drug-metabolizing organ. Drug Metab. Revs., 8 (1978) 319–343.

Pannatier, A., Testa, B. and Etter, J.C., In vitro studies on aryl ether 0-dealkylase activity in the skin of untreated mice. Xenobiotica, in press.

Pannatier, A., Testa, B. and Etter, J.C., Effect of topically applied phenobarbital on mouse skin 0-dealkylase activity and cytochrome P-450. Submitted for publication.

Rekker, R.F. and de Kort, H.M., The hydrophobic fragmental constant; an extension to a 1000 data point set. Eur. J. Med. Chem., 14 (1979) 479–488.

Shaw, J.E. and Chandrasekaran, S.K., Controlled topical delivery of drugs for systematic action. Drug Metab. Rev. 8 (1978) 223–233.

Testa, B. and Jenner, P., A structural approach to selectivity in drug metabolism and disposition. In Jenner, P. and Testa, B. (Eds.), Concepts in Drug Metabolism, Part A, Dekker, New York, 1980, pp. 53–176.

Vianio, H., Role of extrahepatic metabolism. In Jenner, P. and Testa, B. (Eds.), Concepts in Drug Metabolism, Part A, Dekker, New York, 1980, pp. 251–284.

Vogel, A.I., Textbook of Practical Organic Chemistry, 3rd edn., Longmans, Green, London, 1967, p. 263.