PRIZIDILOL : ANALYTICAL METHODS AND *IN VITRO* METABOLISM BY HEPATIC ENZYMES

BY

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TABLE OF CONTENTS

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ABSTRACT

Two separate assays for prizidilol have been developed. One relies on separation by high performance liquid chromatography, and the other on separation by thin layer chromatography. Both of these methods have several advantages over the existing assay developed by JC Pearce for Smith Kline and French Ltd. (SK & F).

Firstly, both methods utilize pepsin to digest plasma protein since it is known that precipitation with trichloroacetic acid of the protein leads to losses by binding at different rates of prizidilol and of the internal standard. The SK & F assay might therefore be less sensitive and precise than necessary. Secondly, use of the complexing reagent, quinolin-3-al, leads to greater sensitivity of detection than the reagent, anisaldehyde, previously employed. The minimum level of quantitation of the developed assays are approximately 30 µg/l by HPLC and 15 µg/l by TLC, as against 50 µg/l by the earlier HPLC method originating from SK & F laboratories. Finally, the TLC method is much quicker to perform than the previous assay.

The biotransformation of prizidilol by hepatic cytochrome P-450, glutathione S-transferases and acetyltransferases has been investigated. The hepatic microsomal cytochrome P-450 enzyme system is capable of binding and metabolizing prizidilol. Prizidilol is shown to bind to two distinct sites on cytochrome P-450. At low concentrations (<23.7 µM) prizidilol binds to the type I site (active site) of the enzyme. In contrast, at higher concentrations (24 - 190 µM), prizidilol binds to the type II site (oxygen binding site) of the enzyme.

The apparent binding constants (K_s), maximum extents of binding (ΔA_{max}), Michaelis constants (K_m) and maximum velocity (V_{max}) for the interaction of prizidilol with hepatic microsomal cytochrome P-450 were assessed in differently pretreated rats. Rats were pretreated with inducing agents viz., phenobarbital, β-naphthoflavone and pregnenolone-16α-carbonitrile each of which specifically elevates the levels of a selected form of cytochrome P-450. Binding constants for binding to the type I site Michaelis constants were in all cases in the range of 1 to 5 μM. The binding constant for binding to the type II site was ca. 100 - 300 μM. The observed effect of induction on ΔA_{max} and V_{max} suggests that several of the multiple forms of cytochrome P-450 bind and metabolize prizidilol.

The biotransformation in vitro of prizidilol was shown to be inhibited by metyrapone, SK & F 525-A and $CO:O_2$ (80:20%), which are all effective inhibitors of cytochrome P-450. In contrast, the hepatic cytosol GSH S-transferases did not catalyze the metabolism of prizidilol: No loss of prizidilol was seen with liver cytosol plus GSH.

Acetylation was found to play a significant role in prizidilol metabolism. Enzymatic acetylation of prizidilol was found following incubation of the substrate and acetyl CoA with rat liver cytosol or partially purified N-acetyltransferase. The levels of cyclised acetylated product were significantly increased following incubation. The acetylation of prizidilol was inhibited by cuprous chloride and manganese sulfate, which inhibit acetyltransferase. The K_m for the acetylation of prizidilol was 0.78 μM and the maximum velocity of the reaction (V_{max}) was 0.98 μmol/mg protein/min. Limiting value of the apparent K_m for acetyl CoA was 22 ± 0.17 μM.

LIST OF FIGURES

PAGE

LIST OF TABLES

PAGE

1. INTRODUCTION

1.1 Hypertension

Hypertension, that is, an abnormal increase in blood pressure within arteries and arterioles, is a very common disorder in industrialized societies, generating a multitude of vascular complications which may be fatal.

According to the World Health Organization, normal blood pressure and hypertension may be defined as follows: 1. an adult is normotensive when his or her systolic arterial pressure is below 140 mm Hg and when his or her diastolic arterial pressure is below 90 mm Hg; 2. an adult is hypertensive when his or her systolic arterial pressure is above 160 mm Hg and/or when his or her diastolic arterial pressure is above 95 mm Hg; 3. when systolic arterial pressure lies between 140 and 160 mm Hg and diastolic arterial pressure between 90 and 95 mm Hg, the term 'borderline' hypertension is employed. There are several varieties of hypertension. The first is permanent hypertension where both systolic and diastolic arterial pressures are permanently above normal values; certain clinical and biological signs permit the classification of permanent arterial hypertension into categories of benign or malignant. The second is labile hypertension, characterized by an occasional elevation of systolic or diastolic arterial pressure, or both. Situations leading to these transient increases in arterial pressure appears to be mainly associated with psychic tension or physical effort. Some forms of hypertension are secondary to a definite cause, such as a disorder of a kidney or adrenal gland, and in certain instances may be corrected surgically. However, in the great majority of cases of hypertension, no precise causal anomaly can be detected. This form of hypertension, whose pathogenesis remains unclear, is

termed essential hypertension. A large number of different therapeutic agents is today available for the control of hypertension. Amongst other approaches clinical studies have shown the effectiveness of combined treatment with a vasodilator and a β-adrenoceptor antagonist in the long term control of essential hypertension (1, 2).

1.2 β-adrenoceptor blocking agents

The finding that the relative potency of a series of sympathomimetic amines varied with the effector organs or systems led Ahlquist (3) to conclude that there are two distinct types of adrenergic receptors viz. the alpha and the beta receptors. The beta adrenergic receptors can be subdivided pharmacologically into $beta_1$ (predominantly in heart) and $beta_2$ (predominantly in smooth muscle) on the basis of the relative selective effects of both agonists and antagonists on these receptors (4-7). The introduction of β-adrenoceptor blocking drugs into clinical practice has provided one of the most significant advances in the medical management of cardiovascular disease during the past 20 years. While the original investigators envisaged the use of these agents for the treatment of angina pectoris (8-10), hypertension (11-13) and arrhythmias (14, 15), their therapeutic benefits have extended well beyond the cardiovascular sphere. For instance, the β-blockers have been utilized in the treatment of porphyria, anxiety and other neuropsychiatric conditions. In short, few synthetic drugs have had such widespread applicability in human pharmacology (7).

1.3 Propranolol

β-adrenoceptor antagonists, of which propranolol hydrochloride (1-isopropylamino-3-(1-naphthyloxy) propan-2-ol hydrochloride) (Fig. 1) is the most widely used example specifically antagonizing catecholamine activation of β-adrenoceptors.

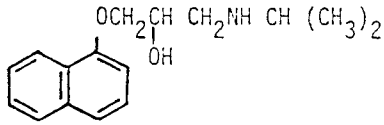


Fig. 1 Structural formula of propranolol

Propranolol, in common with all other β-antagonists, binds to β-adrenoceptors in a competitive manner. Propranolol is a non-selective β-antagonist although recent studies suggest that it might be slightly β_2 selective (16). The drug does not modify the effects of histamine, acetylcholine or α-adrenoceptor agonists. More importantly, it does not interfere with the inotropic actions of cardiac glycosides, calcium or glucagon (17). Propranolol (0.17 mg/kg i.v.) in normal supine subjects reduces heart rate, cardiac output, mean arterial pressure, and left ventricular work (18). The degree of drug-related alteration of these parameters depends on the extent of adrenoceptor activation; the greatest hemodynamic change is observed when propranolol is given during submaximal exercise (18). Under such circumstances, the following average reductions have been observed: heart rate, 20%; cardiac output, 20%; mean arterial pressure, 15%; left ventricular work, 34%; and myocardial consumption, 6% (18). Propranolol causes a reduction in the indices of left ventricular contractility such as peak left ventricular dp/dt max and velocity of fiber shortening, resulting in a reduction in peak systolic pressure at rest and on exercise. It also alters the pattern of regional myocardial contractile activity (19). In addition, propranolol reduces coronary blood flow by 15 to 25% and this is secondary to the reduction in myocardial oxygen consumption. Blood flow in the splanchnic and renal vascular beds is

also reduced secondary to systemic hemodynamic changes (20). Propranolol, in contrast with α-adrenoceptor antagonists, reduces the renal release of renin from the juxtaglomerular apparatus resulting from sympathetic nerve stimulation or catecholamine infusion (21). Hypertensive patients with elevated plasma renin activity generally show a satisfactory hypotensive response to propranolol (22), although this effect has also been noted in normoreninaemic hypertensive patients (23). During prolonged oral therapy with propranolol in hypertensive patients, the reduction in heart rate is maintained, but the peripheral vascular resistance, which initially rises, is likely to fall over a period of time despite a sustained satisfactory hypotensive response. The explanation for this is unknown and has been the subject of considerable debate (24).

In approximately 50% of patients with mild to moderate hypertension, a satisfactory hypotensive response is obtained following the administration of propranolol alone, and the fall in pressure generally occurs gradually over a period of several weeks (25). However, when employed in combination with a peripheral vasodilator such as hydralazine, an additive antihypertensive effect may be obtained and a satisfactory reduction in blood pressure can usually be achieved in 80% or more of patients (26). β-blocking agents have been used to prevent the reflex tachycardia induced by hydralazine (27). When vasodilators are given alone they tend to cause a reflex increase in cardiac function and in the circulating plasma renin level. Both these unwanted pharmacological effects are likely to be prevented by concurrent propranolol or other β-adrenoceptor blocker administration and several studies have shown that this is an effective form of antihypertensive therapy (28).

The widespread use of beta-blockers in recent years has been associated with an increasing incidence of adverse reactions, mostly due to excessive pharmacological action from inappropriate or excessive dosage (29). Special caution is needed in the elderly and patients with sinus bradycardia (<55 beats/min), heart block, cardiac enlargement (30), angina pectoris and bronchial asthma, airways obstruction. Patients with chronic lung disease are at risk of a reduced respiratory centre response to CO_2 when they are treated with propranolol (31). This may precipitate respiratory failure. Propranolol also may cause severe bronchospasm in patients with asthma (32). A common cardiovascular effect of β-blockade is the development of cold extremities and Raynaud's phenomenon. The mechanism is probably due to the combination of a reflex increase in α-adrenoceptor vasoconstriction due to reduced cardiac output and blockade of vascular β-adrenoceptors with unopposed α-adrenergic effect on small arterioles. The most common nonspecific effects are generalised fatigue and gastrointestinal disturbance.

1.4 Direct acting vasodilators

Drugs that reduce the arteriolar smooth muscle tone directly are known as vasodilators. All the vasodilator drugs act at receptor sites to reduce peripheral vascular resistance but share a tendency to produce reflex tachycardia and fluid retention. A rise in plasma renin is usual after vasodilator drugs, related to the control level and probably representing a reflex response to the fall in the blood pressure (33). The first effective vasodilator in the treatment of high blood pressure was hydralazine.

The effectiveness of hydralazine is limited by sympathetically mediated reflex tachycardia and an increase in cardiac output (34-36). Concomitant administration of a β-blocker (e.g. propranolol) increases the

clinical usefulness of hydralazine by blocking reflex tachycardia and by potentiating its hypotensive action, thus allowing the use of smaller doses of hydralazine (2).

1.5 Hydralazine

Hydralazine (1-hydrazinophthalazine, Fig. 2) containing a hydrazine group is a long-acting vasodepressor agent. Original observations in man suggested that this drug was capable of lowering blood pressure while at the same time increasing renal plasma flow (37).

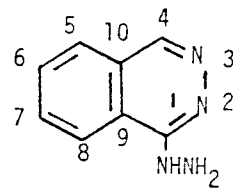


Fig. 2 Structural formula of hydralazine

Hydralazine is a peripheral vasodilator. This drug exerts its antihypertensive action by producing vasodilatation of the precapillary resistance vessels by direct relaxation of the arteriolar smooth muscle (38). Hydralazine has little or no effect on postcapillary venous capacitance vessels (39). The cellular mechanism by which hydralazine acts may be related to its ability to chelate trace metals, especially copper, which may be required for smooth muscle contraction (40, 41). Hydralazine also inhibits several enzyme systems including dopa decarboxylase and histidine decarboxylase (42, 43) and inactivates the two peptide pressor substances pherentasin and angiotensin (44). In addition, hydralazine reverses or blocks the vasopressor effect of many substances such as norepinephrine, epinephrine, histamine, vasopressin, barium salts, etc., suggesting a direct effect on the arteriole resembling that of the nitrites. However, it is likely that the reduction in blood pressure is sensed by the baroreceptors resulting in an increased sympathetic discharge from the vasomotor center. A direct sympathetic effect on cardiac output and heart rate, blocked by propranolol and preceding the fall in blood pressure induced by hydralazine has been demonstrated (45).

Hydralazine has two distinct effects on the cardiovascular system: a decrease in peripheral vascular resistance and an increased cardiac output. A decrease in smooth muscle tone occurs predominantly in the arterial (small arteries and arterioles) and not the venous circulation and leads to a reduction of total peripheral vascular resistance (45, 47). Cardiac output and heart rate elevations accompany the arterial vasodilation, but there is little orthostatic hypotension due to the lack of effects of hydralazine on capacitance vessels (48). Although peripheral vasodilation is produced throughout the vascular beds, with substantial increase in flow to the splanchnic, renal, coronary, and cerebral vascular beds, flow to cutaneous and muscle beds shows variable changes depending on the level of blood pressure reduction (49, 50).

A number of serious toxic side effects has limited the therapeutic usefulness of hydralazine. Nevertheless, the combination of hydralazine with other hypotensive agents (especially with a diuretic and a β-adrenoceptor antagonist) has proved effective (2). A β-adrenoceptor antagonist such as propranolol given simultaneously with hydralazine reduces the sympathetic mediated reflex increase in cardiac output and may alleviate certain of the haemodynamic side effects of hydralazine and potentiate its hypotensive activity. Hydralazine has also been employed successfully in the treatment of acute hypertensive emergencies (51) and congestive heart failure (52).

Because of the side effects associated with high doses of hydralazine it is recommended that the total daily dosage should not exceed 300 mg (53, 54). Many of the undesirable effects of hydralazine are due to vasodilatation and reflex haemodynamic changes which include tachycardia, palpitation, headache, flushing, nasal congestion, angina pectoris or

even myocardial infarction in patients with pre-existing coronary artery disease. An increased incidence of joint pain and a lupus erythematosus-type syndrome is found during hydralazine therapy, especially if the patient has a past history of rheumatoid problems. The incidence of this toxicity approaches 10 to 20% in individuals treated with 400 mg per day or more. With doses under 200 mg daily, toxicity has only rarely been reported in some series (54), but has occurred at doses of 100 mg daily in others (55). The lupus-like syndrome is almost always associated with circulatory antinuclear antibodies and is more common in Caucasians and slow acetylators of drug (54).

1.6 Prizidilol

The aim in the design of this drug molecule was to provide a vasodilator while attempting at the same time to avoid undesirable reflex effects such as tachycardia and increased cardiac output. The molecule closely resembles a combination of propranolol and hydralazine, as indicated by its structure which is shown in Fig. 3.

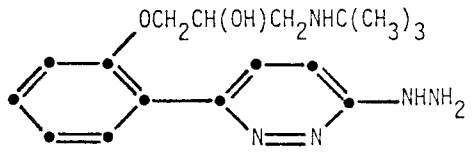


Fig. 3 Structural formula of prizidilol

This drug has a minimal effect on capacitance vessels. The diastolic blood pressure falls more than the systolic pressure, which suggests that the drug is acting predominantly by vasodilatation (56). Prizidilol is a potent and competitive β-adrenoceptor antagonist but, unlike propranolol, it does not appear to enter the central nervous system or to have significant local anaesthetic activity; prizidilol does have weak intrinsic sympathomimetic activity (less than oxprenolol) (56).

Experiments in cats, rats and dogs have confirmed that prizidilol (2 to 4 mg/kg i.v.) causes a pronounced peripheral vasodilatation, particularly of the arterioles, the renal, coronary and skeletal muscle vasculatures, with a redistribution of the cardiac output (56, 57). Acute, subacute and long-term chronic studies in mice, rats and baboons have not revealed any clear evidence of toxicity. High doses of prizidilol administered for long periods of time to various experimental animals, notably rodents and baboons, have produced brown discolouration of certain tissues and organs, particularly the liver and mesenteric lymph nodes but not the central nervous system. This pigmentation was not seen at lower dose levels, including intravenously, nor after short periods of exposure to the compound. It is only present in animals administered considerable quantities of the drug for extended periods of time (more than 100 mg/kg per day) (58).

Studies on healthy volunteers using isoprenaline infusion indicated that prizidilol causes beta-blockade in man and has a potency 10% that of propranolol on a miligram weight for weight basis (59). The clinical evidence is good in that both properties of the drug, namely the beta blocker activity and hydralazine effect, are manifest at the same dose (59). Prizidilol did not have significant effect on glucose tolerance, serum insulin, lipid profile, 24-hour urine electrolyte excretion, aldosterone and plasma renin activity, cerebral blood flow and renal blood flow (56, 60, 61). One-hundred-and-seventeen healthy subjects had taken part in studies with prizidilol. One case of cardiac arrhythmia was reported after administration of prizidilol but no other adverse effects have been noted. Forty-two patients had received the drug for up to sixteen weeks. Drop-out rate was low and adequately accounted for. None of the drop-out events recorded appear to have

been directly attributable to administration of prizidilol. Isolated fall in platelet count did not appear to be attributable to the administration of prizidilol (56).

Records of 97 patients with mild to moderate essential hypertension treated for up to 48 weeks demonstrated the antihypertensive efficacy of prizidilol. Three patients withdrew from the study because of side effects probably related to the drug (bronchoconstriction-2, nausea and vomiting-1) (61-63). It would appear that patients who are slow acetylators may require a comparatively smaller dose of prizidilol for satisfactory control of blood pressure than fast acetylators. This observation needs to be confirmed in chronic studies (56).

2. ANALYSIS

The published techniques for the quantitative determination of propranolol and for hydralazine are outlined in the following sections since they were felt to be relevant, at least potentially, to the analysis of prizidilol. Indeed, the standard SK & F (unpublished) method for the drug (81) is derived from an assay for hydralazine (73).

2.1 Discussion

2.1.1 Propranolol assay

The original assay method for propranolol was based on fluorimetry (64). More recently, the techniques of high performance liquid chromatography (HPLC) (65) and thin-layer chromatography (TLC) have been applied to the assay of propranolol (66), although both techniques still rely on fluorometric detection. The HPLC assay has been compared directly with the spectrofluorometric assay and the former gives better results only when a plasma blank is not available for each subject (67). Other assay techniques that have been described are: gas chromatography-mass spectrometry (GC-MS) (68); gas chromatography with a ^{63}Ni electron-capture detector (69); immunoassay based on specific antisera to propranolol or its isomers (70) and radioreceptor assay (71).

2.1.2 Hydralazine assay

Three different methods have been used to measure hydralazine in the plasma of human subjects; spectrophotometry (72, 73), radioisotope dilution analysis (74) and gas chromatography (75). These methods have a common denominator. Since hydralazine can not be extracted from biological material without decomposition, all methods rely on the formation of a derivative of hydralazine prior to extraction and

quantification. The spectrophotometric method, where hydralazine is derivatized with p-hydroxy or p-methoxybenzaldehyde to form the hydrazone (Fig. 4), lacks sufficient sensitivity to measure hydralazine plasma levels following low doses of hydralazine. In the gas chromatographic method, hydralazine is treated with nitrous acid to form tetrazolophthalazine. This method is of high sensitivity, providing a detection limit of about 0.063 M (10 µg/l) hydralazine in plasma. The use of 4-methylhydralazine as internal standard guarantees the necessary reproducibility of assay.

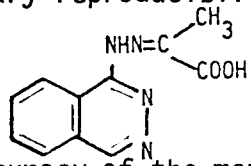


Fig. 4 Structural formula of pyruvic acid hydrazone

The accuracy of the method is questionable, however, since hydralazine readily forms hydrazones with endogenous ketones in vivo and in vitro. These hydrazones are unstable under the acid condition which had been employed for hydralazine assay, but are partially reconverted to parent drug. As a result the values for hydralazine were found to be incorrectly high (76, 77). This is because acid labile conditions of the derivation procedure and only an "apparent hydralazine" level is obtained. Two modifications of specific high-performance liquid chromatographic assay have been reported for hydralazine and its metabolite (78, 79), hydralazine pyruvic acid hydralazone, 5-triazolo-3,4a-phthalazine, 3-hydroxymethyl and 3-methyl-5-triazolo-3,4a-phthalazine in human plasma.

2.1.3 Prizidilol assay

Assay for prizidilol in blood serum is challenging because the compound is poorly stable at room temperature at neutral pH and decomposes very rapidly above pH 10 (239). Although it is a base (pKa 5.4 and 9.85), extraction into organic solvents under alkaline conditions is ineligible unless the hydrazino- moiety is previously reacted, for instance, with

an aromatic aldehyde. Furthermore, the drug forms at least two metabolites in man of unknown activity: the "acetyl metabolite", by acetylation of the hydrazino- moiety, and the "pyruvic acid hydrazone". These revert back to the parent drug when heated at 70^{o} at pH 1 for one hour to the extent of 22-29% and 69-78% respectively (239). The conditions quoted are those used in the standard assay (81). Lastly, if plasma protein is precipitated using trichloroacetic acid, the recoveries of the anisaldehyde hydrazones of prizidilol and of the internal standard (81) are respectively 37% and 32% after one minute and 31% and 21% after 10 minutes (239).

In view of the foregoing difficulties, it seemed improbable that the standard method for the assay of prizidilol (81; see section 2.2.2) would be either specific or accurate. Since it included a deliberate (but not wholly successful) attempt to hydrolyse the acetyl and pyruvyl metabolites entirely, it was unclear what relationship the concentration measured in a sample would bear to the concentration of prizidilol actually present. Furthermore, although recommendations were made to separate protein precipitated by trichloroacetic acid from supernatant as quickly as possible so as to minimise drug loss of drug and internal standard, the loss would nevertheless be high. A belief current among analysts, but based admittedly on anecdotal evidence, is that assays with low drug recoveries tend to be unpredictably inaccurate; this is not supported in this thesis or the work of Pearce (81) but an attempt to correlate results from five different laboratories each using essentially Pearce's technique was only partially successful (239). There was therefore some justification for attempts to devise new analytical methods for prizidilol.

The approach adopted for the extraction was made with the problems discussed above in mind. In a single step, plasma protein was digested using pepsin (so a precipitation step was unnecessary) and the

derivatisation was performed using quinolin-3-al (arbitrarily chosen) under mild conditions (pH 3, 45^{o} for 45 min) which did not lead to detectable hydrolysis of either authentic acetyl or pyruvyl metabolites. The reaction mixture was alkalinised and the quinolinal hydrazones were extracted into dichloromethane, which was separated and evaporated to dryness (see section 2.2.3).

Two approaches for detection of prizidilol were pursued simultaneously, by HPLC and by TLC, since it was not clear which method would be superior. The HPLC technique (see sections 2.2.4) was a trivial modification of that of Pearce (81), but the TLC method was novel in several ways: firstly, the sensitivity of detection was increased by choice of a suitable solvent for sample application (for which the prizidilol derivative has a low Rf value) and maximising the proportion of extract applied; secondly, interference by other compounds present in the extract was minimised by using a pre-washing solvent (for which also the prizidilol derivative has a low Rf value); thirdly, accuracy and reproducibility were improved by using the internal standard (uncommon procedure in TLC). Although the TLC assay was more difficult to develope than the HPLC version, TLC densitometry should have the following advantages: it is cheap in materials; the stationary phase can be pre-washed and is disposable; it is a batchwise rather than sequential assay and would therefore normally be more rapid in the analysis of large numbers of samples than HPLC.

2.2 Methods and results

2.2.1 Validation of results

The analyst, when examining an assay for prospective use, is interested in 4 parameters: accuracy, reproducibility, sensitivity and specificity.

In this thesis, accuracy and reproducibility have been determined by the repeated assay of samples spiked with several known amounts of prizidilol so that peak heights of the added internal standard and those corresponding to different concentrations of prizidilol were obtained. The peak height ratios (prizidilol/internal standard) were calculated and calibration curves were obtained. From these the recovered concentrations could be calculated and compared to the known (because added) concentrations in the samples analysed. From these data, it would, when necessary, be possible to estimate how accurate a concentration estimate would be, were a sample containing an unknown amount of drug to be analysed. This point needs to be emphasised since, if a calibration curve is linear (as in the HPLC methods discussed in this thesis) the same parameters for coefficients of correlation and variance will be obtained if the peak height ratios are used instead of the recovered concentrations; but, if the calibration curve is not linear (as in the TLC method), this is not the case.

The sensitivity (or limit of detection) was calculated for each assay method semiempirically. The extreme scatter due to baseline "noise" was determined experimentally in blanks at the positions where prizidilol would be expected to appear then, knowing the heights of the internal standard peaks and using the calibration curves, it was possible to calculate the concentration of prizidilol in samples which would give rise to peaks twice as high as the baseline scatter for the method employed. The estimates derived would be different from those made using variances calculated during regression analysis but they are probably of more practical utility, especially when a nonlinear calibration curve is encountered or a linear curve for which the conventionally-used linear regression is, strictly speaking,

invalid because of inhomogeneity of variance.

The specificity was not examined because tbe presence of interfering drugs was not anticipated, the assays were validated using pooled human serum samples and the chromatographic nature of the assays makes it unlikely that interfering drugs or endogenous substances reacting with quinolin-3-al would cause problems. However, had the drug been assayed in subjects participating in pharmacokinetic trials, the matter would have been pursued further, but prizidilol was withdrawn from clinical testing before this stage was reached.

2.2.2 SK & F method (81)

2.2.2.1 Materials and equipment

2.2.2.1.1 For derivatization and extraction

Trichloroacetic acid (5% w/v aqueous solution) reagent grade

Anisaldehyde (1% v/v methanolic solution); a fresh solution was prepared on each day of analysis

Sodium hydroxide solution (4M)

Dichloromethane (HPLC grade)

Methanol (HPLC grade)

Glass distilled water

12 ml polypropylene tubes and stoppers

15 ml glass culture tubes with screw cap

Blood cell suspension mixer

Vortex mixer

Vortex evaporator was set at 25^{o} and 375 mm Hg with cold trap containing propan-2-ol and solid CO_2

Centrifuge

Shaking water-bath was set at $70^{o}C$

2.2.2.1.2 For high performance liquid chromatography (HPLC)

a) Solvent system: Methanol
Distilled water
n-Dibutylamine
Orthophosphoric acid (90% w/w)

b) Instrumentation:
Pump: Waters 6000A or equivalent
Detector: Perkin Elmer LC-75 or equivalent
Injector: Waters WISP, Rheodyne 7125 or equivalent
Column: Zorbax-CN 5 µm packed in a stainless steel column 150 mm x 4.6 mm i.d.
Integrator: Perkin Elmer Sigma 10 or equivalent.

2.2.2.1.3 Standard solutions

Prizidilol dihydrochloride (SK&F 92657-A_2) 2.55 mg (= 2.0 mg base) dissolved in 200 ml of methanol.

SK&F 93238 trihydrochloride (SK&F 93238-A_3) 5.44 mg (= 4.0 mg base) dissolved in 200 ml of methanol.

Fresh solutions were prepared on each day of analysis.

2.2.2.2 Methods

2.2.2.2.1 Extraction and derivatization procedure

1. 1 ml of plasma was measured (by pipette) into a 12 ml

polypropylene tube, to which was added 100 µl of the internal standard solution (equivalent to 2 µg SK&F 93238) and mixed by vortex. The sample was allowed to stand for 5 minutes at room temperature.

2. To this was added 2 ml trichloroacetic acid (5% w/v aqueous solution) which was mixed by vortex and centrifuged for 5 minutes at 2500 rpm.

3. The supernatant was poured off into a 15 ml screw-cap glass tube, and 100 µl anisaldehyde solution (1% v/v in methanol) was added. This was then incubated in a shaking water-bath maintained at 70°C for one hour.

4. The tubes were cooled to room temperature and the pH then adjusted to 12 with ca. 200 µl of 4 M sodium hydroxide solution.

5. 4 ml dichloromethane was added and rotated on a blood cell suspension mixer for 10 minutes. The sample was centrifuged for 5 minutes at 1500 rpm, and the aqueous layer aspirated and discarded.

6. The organic layer was transferred to a 12 ml polypropylene tube and evaporated to dryness in a vortex evaporator for one hour at 25°C.

7. The residue was reconstituted in 100 µl methanol/water/glacial acetic acid (75 : 25 : 0.1). At this stage the samples could be stored overnight at -20°C.

2.2.2.2.2 Preparation of HPLC solvent

All solvents were filtered through 0.5 µm Millipore filters prior to mixing.

375 ml methanol and 125 ml distilled water were mixed and degassed under reduced pressure. 0.32 ml n-dibutylamine and 0.14 ml orthophosphoric acid was added and mixed.

2.2.2.2.3 Conditions

Column:	Zorbax-CN
Solvent:	Methanol/water (75 : 25), 0.004 M dibutylamine phosphate (pH 3)
Flow:	1.5 ml/minute
Temperature:	ambient
Wavelength:	326 nm
Absorbance:	0.02 AUFS

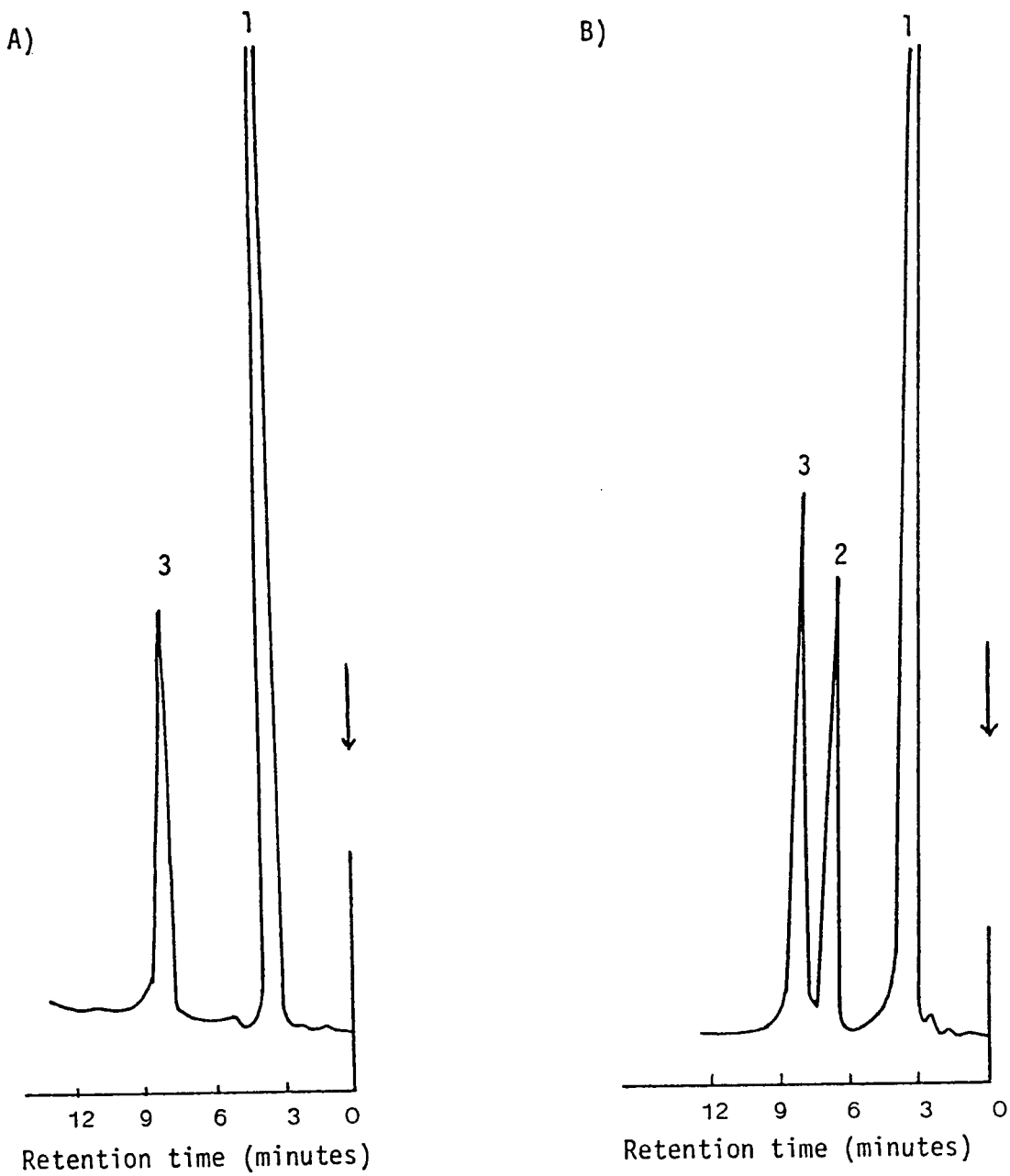
The column was allowed to equilibrate with the solvent for at least 2 hours. After use, the column was flushed with methanol/water (75 : 25) for a minimum of one hour.

2.2.2.2.4 Chromatography

5 - 20 µl aliquots of the extracts were injected to obtain the chromatogram (see Fig. 6).

Retention times for prizidilol hydrazone and SK&F 93238 hydrazone were ca. 7 minutes and 8 minutes respectively.

Fig. 6 SK & F method: typical HPLC chromatograms



A) Control serum sample spiked with internal standard

B) Serum sample containing prizidilol (0.50 mg/l) and internal standard
1. Anisaldehyde
2. Prizidilol anisaldehyde hydrazone
3. SK & F 93238 anisaldehyde hydrazone

arrow denotes the time injection.

2.2.2.2.5 Standard curve

1. Tubes (where appropriate, in replicate) were labelled S1000, S800, S600, S400, S200, S100, S50, and B.
2. Into tubes S1000 were put 100 µl standard solution; into S800, 80 µl; into S600, 60 µl; into S400, 40 µl; into S200, 20 µl; into S100, 10 µl; S50, 5 µl and into B zero.
3. The methanol was evaporated off using the rotary evaporator.
4. Just before analysis 1 ml serum was added to each tube and mixed.
5. This made standards at each of the following concentrations of prizidilol base; 1 µg/ml (S1000), 800 ng/ml (S800), 600 ng/ml (S600), 400 ng/ml (S400), 200 ng/ml (S200), 100 ng/ml (S100), 50 ng/ml (S50) and zero (B).
6. Standards were worked up according to section 2.2.2.2.1 "Extraction and derivatization procedure" steps 2 to 7.

2.2.2.3 Validation of the method

The procedure was followed as exactly as possible and a calibration curve was obtained by plotting peak height ratios against concentration of samples spiked with known amounts of prizidilol and internal standard. The data obtained are presented in Table 1. The calibration curve was linear in the range 0.05 - 1 mg/l (Fig. 7).

Plot of peak height ratio (prizidilol/internal standard) against spiked prizidilol concentration (Fig. 7): Slope = 3.58 (SD = 0.06); y-intercept = 0.04 (SD = 0.04); r = 0.9960.

Plot of recovered against spiked prizidilol concentrations: Slope = 1.00 (SD = 0.02); y-intercept = 0.00 (SD = 0.01); r = 0.9960.

Spiked conc. (mg/l)	Peak height ratio	Mean	Recovered concs. (mg/l)	Mean	CV (%)
0.05	0.28	0.237	0.068	0.056	34
	0.25		0.070		
	0.18		0.035		
0.1	0.41	0.367	0.105	0.092	14
	0.35		0.088		
	0.34		0.085		
0.2	0.69	0.723	0.183	0.193	8
	0.70		0.186		
	0.80		0.214		
	0.72		0.191		
0.4	1.66	1.453	0.454	0.396	12
	1.26		0.342		
	1.45		0.395		
	1.44		0.393		
0.6	2.21	2.274	0.608	0.626	9
	2.38		0.656		
	2.14		0.588		
	2.16		0.594		
	2.48		0.685		
0.8	2.79	2.818	0.770	0.778	5
	2.92		0.807		
	2.68		0.739		
	2.88		0.795		
1.0	3.55	3.618	0.983	1.001	1
	3.63		1.005		
	3.66		1.013		
	3.63		1.005		

Table 1. Validation of SK & F standard assay (81): replicate determinations of prizidilol in spiked human serum.
CV = coefficient of variation; conc. = concentration.

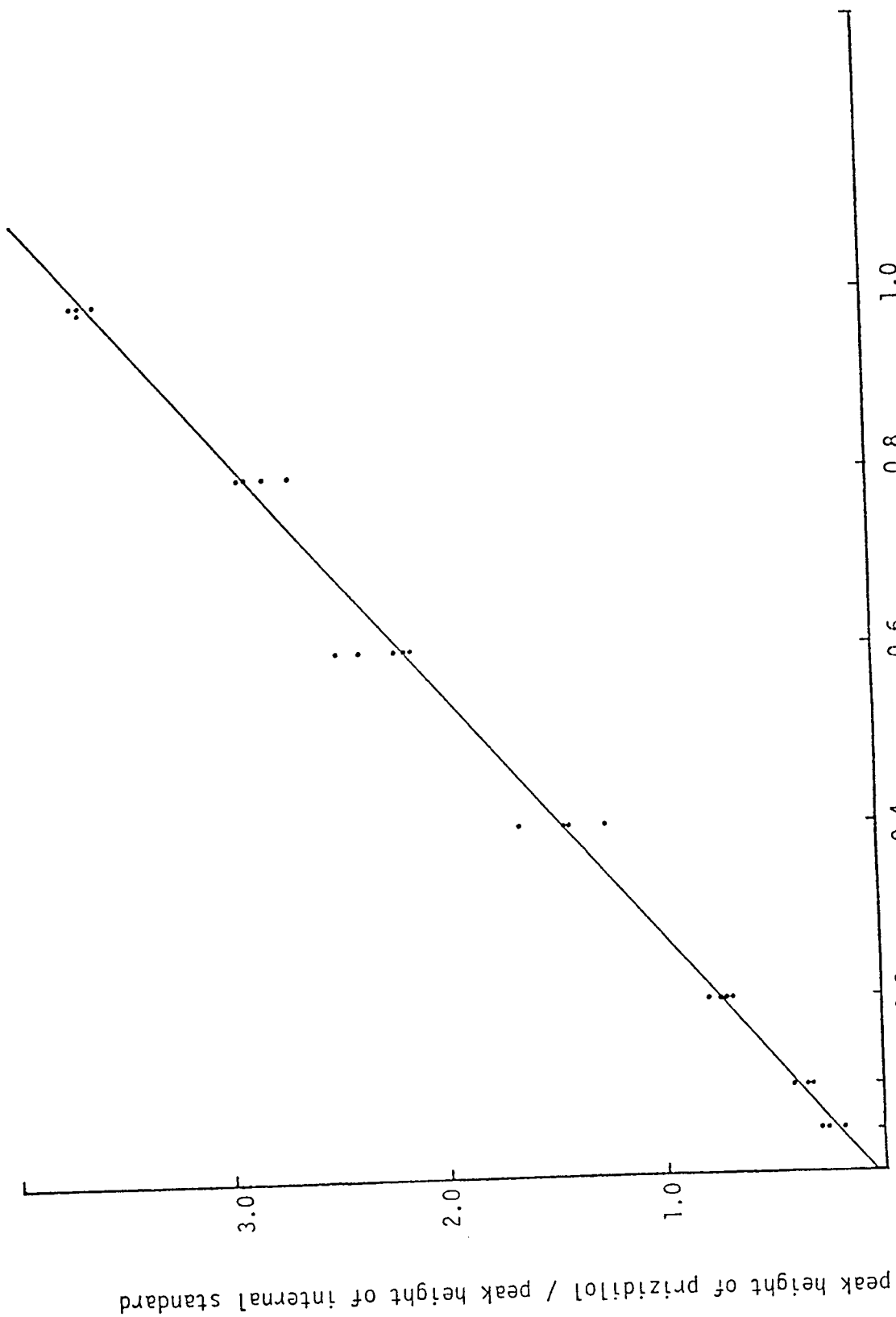


Fig. 7 SK & F method, calibration curve for the determination of prizidilol. internal standard (SK & F 93238-A3)

$y = 3.58x + 0.04$ $r = 0.9960$

Overall CV = 11%.

(SD = standard deviation: r = correlation coefficient; CV = coefficient of variation).

Minimum detectable concentration was about 50 µg/l.

2.2.3 New extraction of prizidilol, for both HPLC and TLC assays

2.2.3.1 Materials and equipment

10 ml glass tubes with liquid-tight closures

Pipettes and micropipettes

Volumetric flasks

A rotary evaporator with bath set at 25°C for evaporation of methanol solution was used

Tipping table for producing gentle mixing of phases (about 1 oscillation/sec)

Centrifuge

Water bath at 45-47°C

Distilled water

Human serum

Methanol (HPLC grade)

Dichloromethane

Propan-2-ol

85% orthophosphoric acid (20% v/v aqueous solution)

Sodium hydroxide solution (3.5 M)

Quinolin-3-al (2% w/v propan-2-ol solution)

Pepsin: EC 3.4.2.3.1 from porcine stomach mucosa, crystallised and lyophilised 3000 units/mg
(2% w/v aqueous solution)

N.B. All glassware coming in contact with samples containing prizidilol and/or the internal standard was silanised.

2.2.3.2 Standard solutions

Prizidilol stock solution: 2.55 mg prizidilol dihydrochloride

hydrate (= 2.0 mg base) dissolved in 200 ml methanol (10 µg/ml). Internal standard solution: 1.36 mg SK&F 93238-A_3 4-(prop-1-oxy) prizidilol trihydrochloride (= 1.0 mg base) dissolved in 50 ml methanol (= 20 µg/ml).

2.2.3.3 Extraction procedure

1. Pooled human serum was spiked to give samples containing various concentrations of prizidilol (see Tables 2, 3 and 4). Blanks were also analysed, but no interfering peaks were observed. Replicate analyses were performed on each sample.
2. To each 1 ml spiked serum (or blank) was added, with mixing, a reproducible volume (about 25 µl) of internal standard solution, then,
3. with mixing between each step, were added 100 µl phosphoric acid, 100 µl pepsin and 20 µl quinolin-3-al solutions.
4. Each tube was heated at 45^{o} for 45 min, before cooling to below 25^{o}.
5. To each reaction mixture, with mixing, was added 250 µl sodium hydroxide solution, then
6. 6 ml dichloromethane as soon as was practicable.
7. Each tube was inverted about once a second for 15 min.
8. Phases were separated by centrifugation at about 2500 g and each organic layer collected into a clean, appropriately-labelled tube by filtration through a cotton-wool plug soaked in dichloromethane.
9. The organic filtrate was evaporated to dryness under nitrogen at about 25^{o}.
10. The dried extracts were stored, when necessary, at -20^{o} in the dark.

2.2.4 New HPLC assay

2.2.4.1 Materials and equipment

Spectraphysics model 3500 high-pressure liquid chromatograph fitted with a model 770 spectrophotometric detector and Phillips PM8100 recorder. Samples were injected into a 10 µl loop injector using a Glenco gas syringe.

Reversed phase column used was 30 cm x 3.9 mm i.d. 5 micron Bondapak C18 from Waters Associates. (The mode of reversed-phase chromatography utilizes, in this case, hydrophobic octadecyl residues bonded to silica as stationary phase and a polar mobile phase; the less polar the solute, the more it binds hydrophobically to the stationary phase and therefore the greater its retention).

Methanol

Distilled water

Triethylamine

Orthophosphoric acid (85%)

Mobile phase: Filtered and degassed mixture of 590 ml water, 750 ml methanol, 800 µl triethylamine and enough phosphoric acid (about 400 µl) to bring the pH to 4.7.

2.2.4.2 Operating conditions

Detection wavelength: 334 nm (see Fig. 8)

Sensitivity: range 0.01 AUFS, recorder input 1 mV

Recorder chart speed: 5 mm/min

Flow rate: 1.6 ml/min

Pressure about 250 p.s.i. indicated

Temperature: ambient (20^{o} - 25^{o})

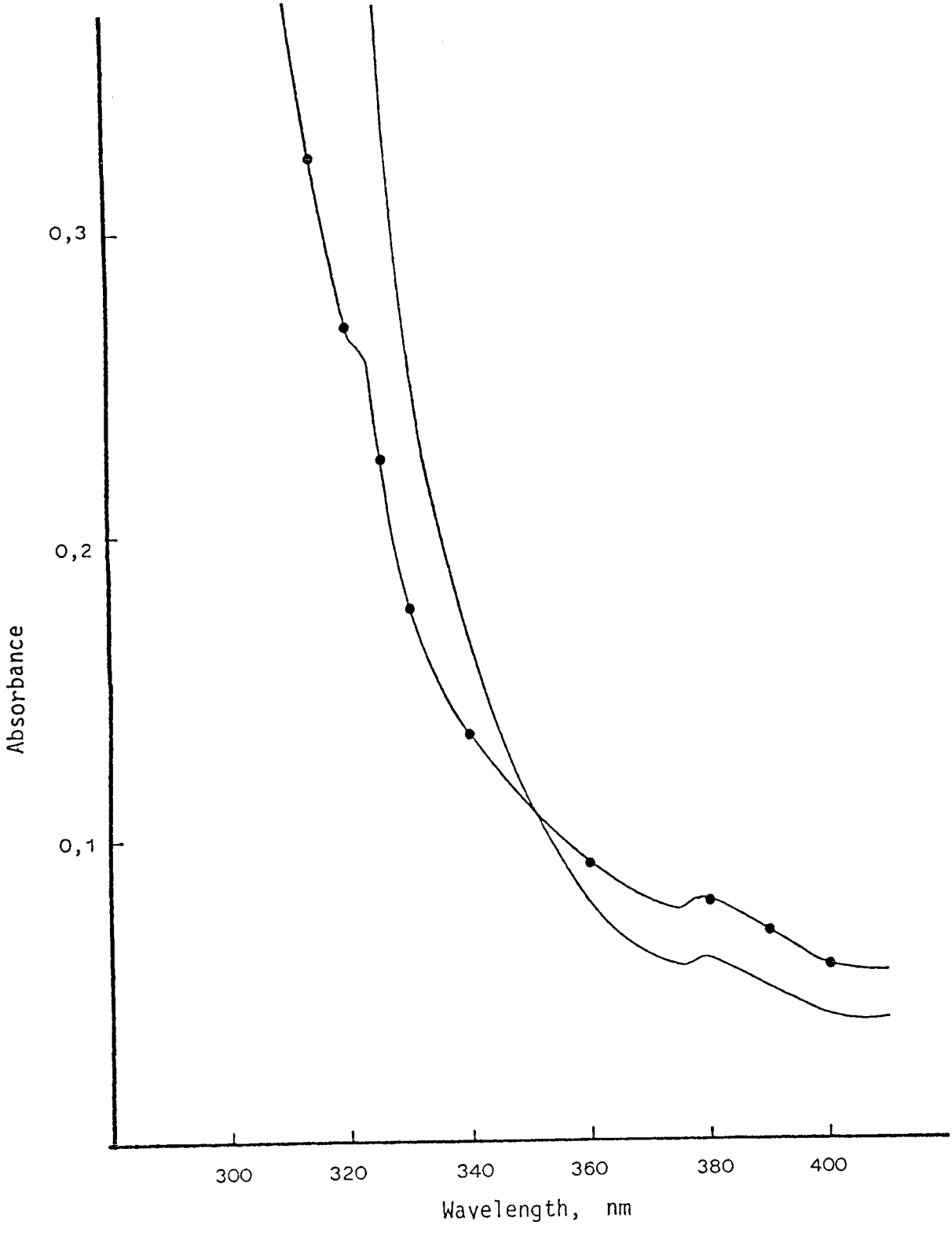


Fig. 8 Absorption spectra of derivatised prizidilol and internal standard in mobile phase.

2.2.4.3 Analysis

Each dried extract (section 2.2.3.3.10) was redissolved in 50 µl mobile phase.

10 µl portions were injected to obtain chromatograms (Figs. 9 and 10).

Retention times were about 5 min for the prizidilol hydrazone and 15 min for the internal standard hydrazone.

The wavelength used was chosen empirically to minimise interference from other compounds in the extract.

2.2.4.4 Validation of the method

A calibration curve was obtained (Fig. 11) by plotting peak height ratio (prizidilol/internal standard) against concentrations of samples spiked with known amounts of prizidilol and internal standard. The data obtained are presented in Table 2. The calibration curve was linear in the range 0.05 - 1 mg/l.

Plot of peak height ratios against concentration:

Slope = 3.54 (SD = 0.04); y-intercept = 0.05 (SD = 0.02); r = 0.9984.

Plot of recovered against spiked prizidilol concentrations:

Slope = 1.00 (SD = 0.01); y-intercept = 0.00 (SD = 0.01); r = 0.9984

Overall CV = 9%.

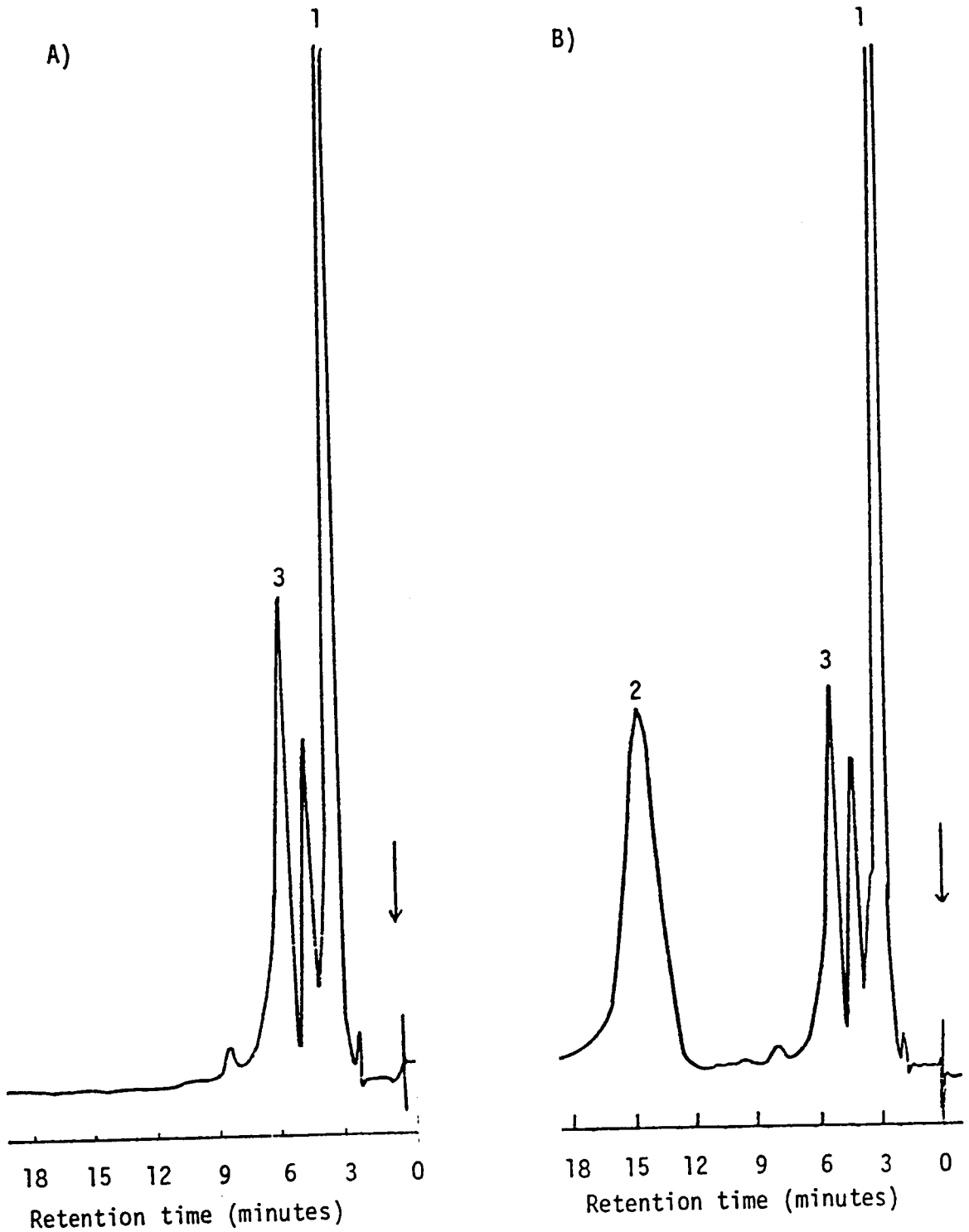
(SD = standard deviation; r = correlation coefficient; CV = coefficient of variation).

Minimum detectable concentration about 30 µg/l.

2.2.4.5 Comments

Although the coefficients of variation and correlation were seemingly improved for this HPLC method over the standard HPLC technique, and the minimum detectable limit was lower, both methods were disappointingly

Fig. 9 The HPLC method: typical chromatograms

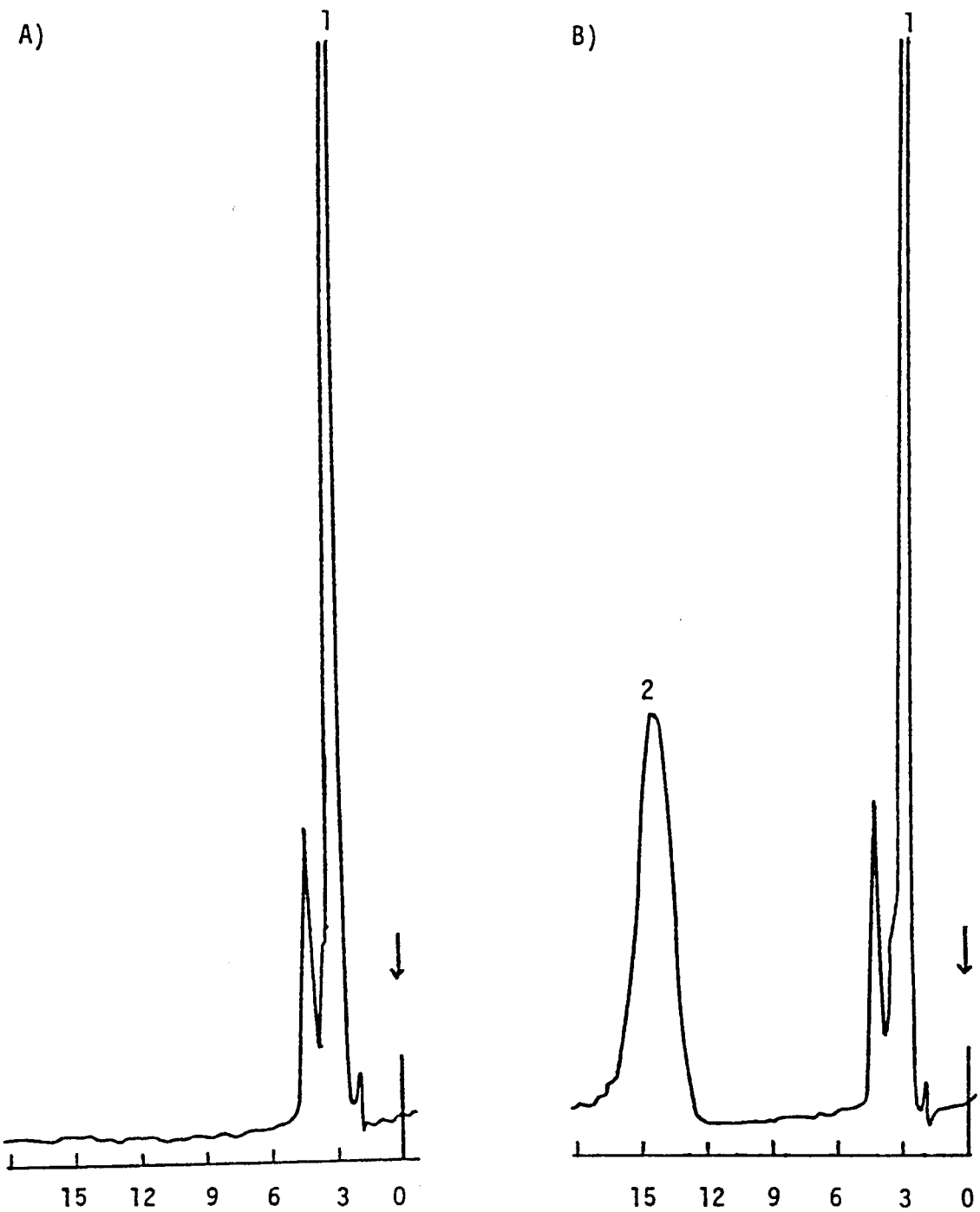


A) Serum sample containing prizidilol

B) Serum sample containing prizidilol and internal standard
1. quinolin-3-al
2. SK & F 93238 quinolin-3-al-hydrazone
3. prizidilol quinolin-3-al-hydrazone

The arrow denotes the time injection.

Fig.10 The HPLC method: typical chromatograms



A) Control serum

B) Serum sample spiked with internal standard
   1. quinolin-3-al
   2. SK & F 93238 quinolin-3-al hydrazone

The arrow denotes the time of sample injection.

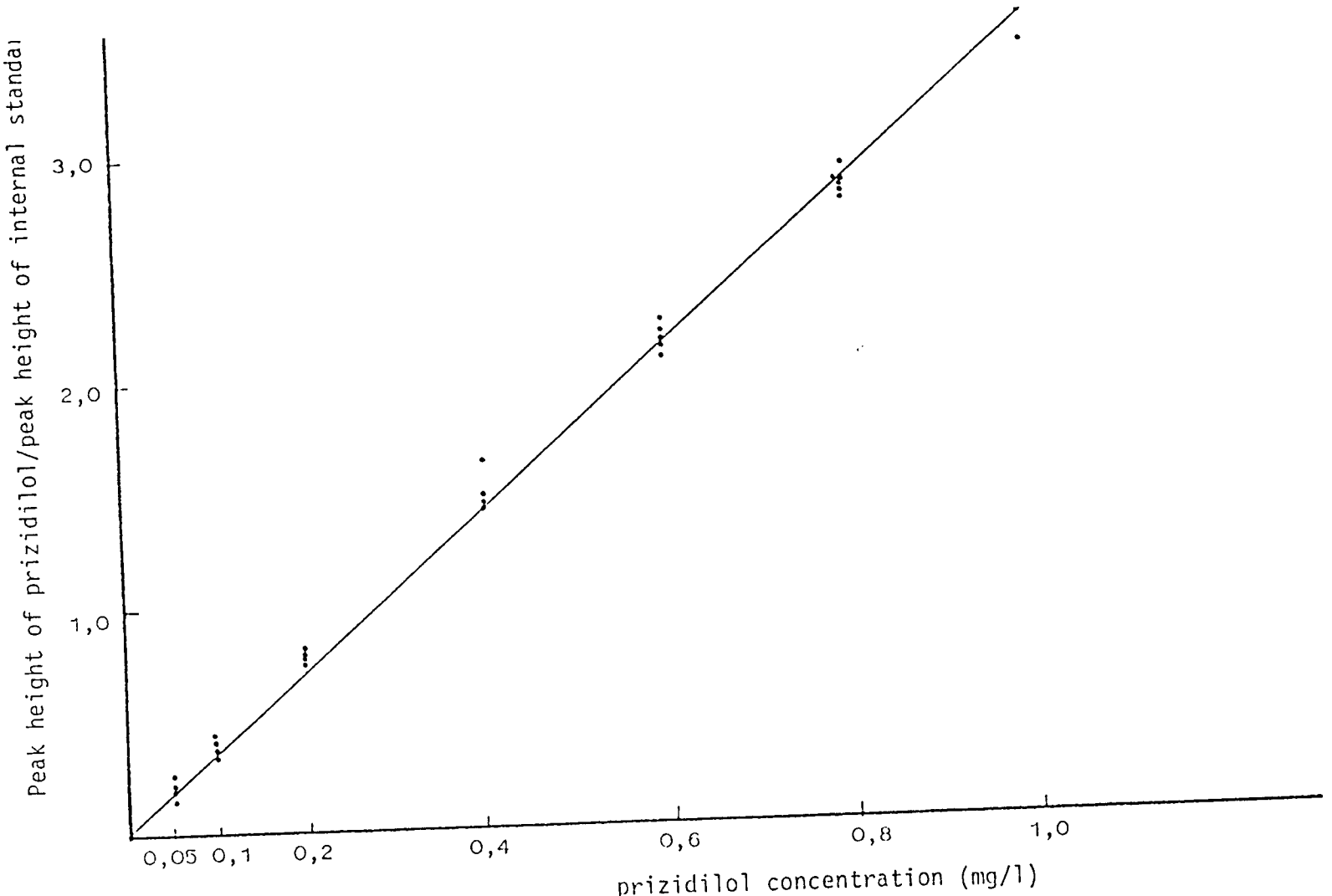


Fig. 11 The HPLC method, calibration curve for the determination of prizidilol.
internal standard (SK & F 93238-A_3)
$y = 3.54x + 0.05$ $r = 0.9984$

Spiked conc. (mg/l)	Peak height ratio	Mean	Recovered conc. (mg/l)	Mean	CV (%)
0.05	0.21	0.203	0.045	0.043	33
	0.27		0.061		
	0.15		0.028		
	0.18		0.036		
0.1	0.40	0.385	0.098	0.094	11
	0.35		0.084		
	0.42		0.104		
	0.37		0.090		
0.2	0.83	0.803	0.220	0.212	8
	0.81		0.214		
	0.80		0.211		
	0.77		0.203		
0.4	1.50	1.520	0.409	0.415	8
	1.45		0.395		
	1.48		0.403		
	1.65		0.451		
0.6	2.09	2.172	0.576	0.599	3
	2.25		0.621		
	2.17		0.598		
	2.21		0.610		
	2.14		0.590		
0.8	2.84	2.823	0.788	0.783	3
	2.84		0.788		
	2.78		0.771		
	2.76		0.765		
	2.92		0.810		
	2.80		0.776		
1.0	3.61	3.625	1.005	1.010	3
	3.46		0.963		
	3.69		1.028		
	3.65		1.016		
	3.70		1.031		
	3.64		1.014		

Table 2. Validation of new HPLC assay: replicate determinations of prizidilol in spiked human serum.
CV = coefficient of variation; conc. = concentration.

slow to perform and column cleaning procedures were too often necessary.

2.2.5 TLC assay

2.2.5.1 Materials and equipment

2.2.5.1.1 a) Materials

Disposable 5 µl micropipettes for TLC sample application; Drummond microcaps used.

Hairdrier; any source of cool air (below $30^{o}C$).

Vacuum dessicator; needed to be large enough to hold at least one 10 x 20 cm TLC plate.

Water pump; to deliver a vacuum of about 15 torr when connected to the vacuum dessicator.

Tank for TLC elution; needed to be large enough to hold at least one 10 x 20 cm TLC plate.

Glass rod with round end; the diameter of the rod should be at least a third of that of the glass tubes.

Funnels should have a volume of about 10 ml.

Cotton wool.

Silica plate for TLC, 10 cm x 20 cm, Merck HPTLC-precoated glass plates for nano-TLC silica gel 60F-254, Cat. No. 5642.

b) Solvent:

Acetic acid

Butan-2-one

Acetone

Ammonia solution (25% in water; S.G. = 0.9)

All analytical grade or purer.

c) Instrumentation:

A Zeiss KM3 Chromatogram spectrophotometer was used to measure the fluorescence of the spots on the thin-layer plates. The following operating conditions were used:

1. Indicator unit

   Model selector set to "R"

   Recording mode selector to KOMP A

   Damping switch set to "1"

   High voltage selector to step 1

2. Recorder unit

   Chart paper speed 5 cm/min.

   Scanning speed 120 mm/min.

   Measuring range 500 mV

3. Illuminator and monochromator

   Mercury source was provided

   Exciting wavelength set to 366 nm

   Slit width set to 0.04 mm

   Blank barrier filter was used

   Slip-in diaphragm 3.5 mm was used

   The amplification was adjusted to obtain approximately 95% full-scale deflection on the recorder when the blank spot in the chromatogram was being scanned.

2.2.5.1.2 Standard solutions

Tubes (where appropriate, in replicate) were labelled S1000, S750, S500, S250, S100, S50, B, into tubes S1000 put 100 µl "stock", into S750, 75 µl; into S500, 50 µl; into S250, 25 µl; into S100, 10 µl; into S50, 5 µl; and into B zero.

The solvent was evaporated off by using the rotary evaporator and the residue then re-dissolved in 1 ml serum to each tube and mixed. This makes two standards at each of the following concentrations of prizidilol base: 1 mg/l (S1000), 750 µg/l (S750), 500 µg/l (S500), 250 µg/l (S250), 100 µg/l (S100), 50 µg/l (S50) and zero (B).

2.2.5.1.3 Operating solutions

"Spotting solvent": 500 µl acetic acid mixed with 500 µl butan-2-one.

"Chromatographic solvent": 15 ml acetone + 5 ml butan-2-one + 1 ml ammonia solution.

"Washing solvent": 20 ml acetone + 200 µl acetic acid.

The latter three solvent mixtures were constituted just before use.

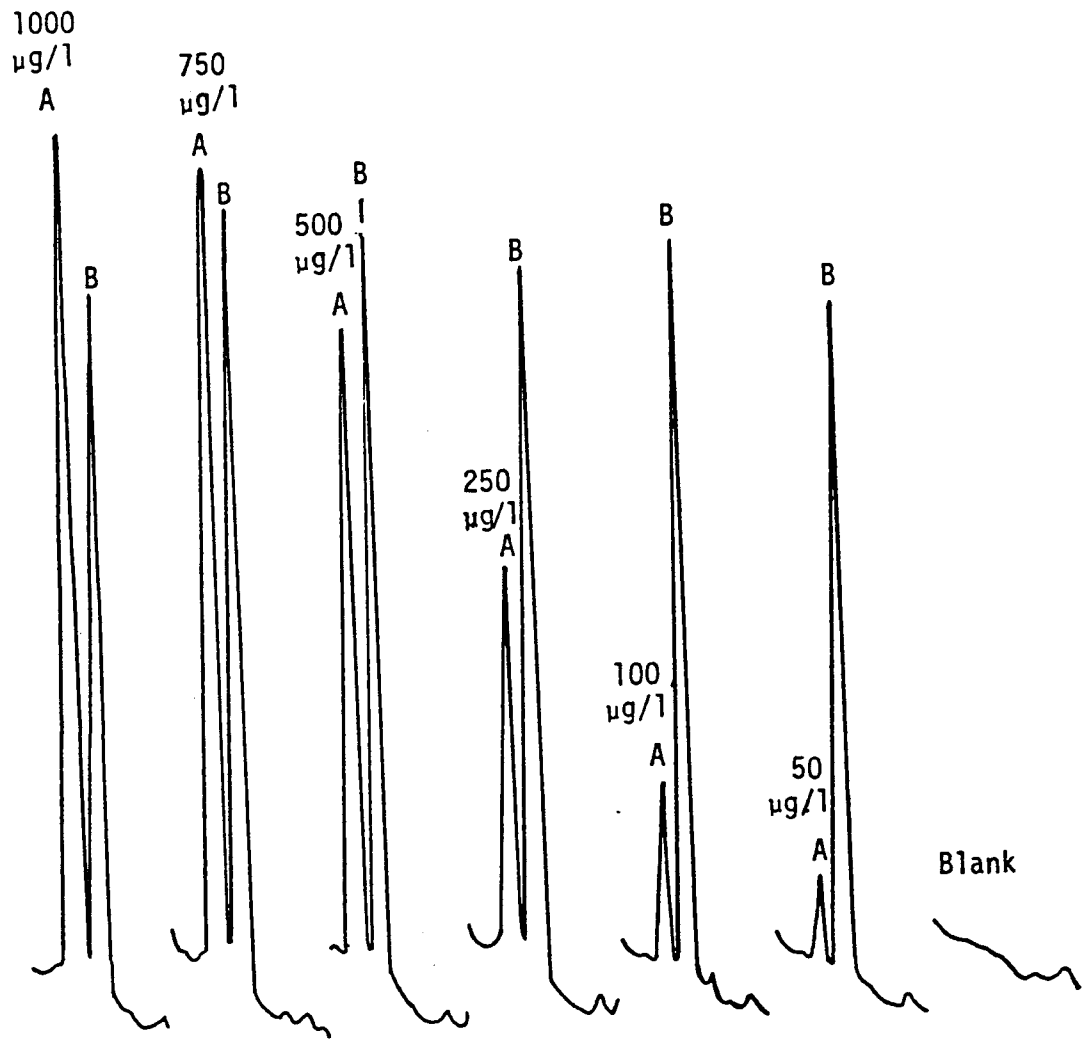
2.2.5.2 Analysis

1. Each dried extract (section 2.2.3.3.10) was dissolved in 10 µl "spotting solvent" by smearing this over the inside of the tube using the glass rod.
2. The solution was drained to the bottom of the tube by brief centrifugation.
3. The rod was rinsed with ethanol and dried between samples.
4. Each silica TLC plate was marked lightly in pencil with a line drawn 1 cm from an edge arbitrarily assigned as the bottom of the

plate. The line (the origin) was then subdivided at 1 cm intervals and each subdivision labelled lightly to identify the sample to be applied there.

5. For each sample, a 5 µl aliquot was applied to the appropriately-labelled position.
6. The spots were dried using cool air from a hairdrier,
7. then developed to the top of the plate (9 cm distance) with the "washing solvent".
8. The plate was then dried in vacuo (about 20 Torr) for not less than 15 min.
9. Meanwhile, the freshly-constituted "chromatographic solvent" was put into the chromatography tank and allowed to equilibrate with the atmosphere therein, a process aided by occasional vigorous shaking.
10. The plate was then developed to the top immediately after removal from the dessicator.
11. The plate was again dried using cool air from the hairdrier positioned at least 60 cm from the plate.
12. Using the mercury arc lamp of the densitometer, selecting a scanning wavelength of 366 nm and with the instrument in absorption mode, the instrument zero was set while illuminating a clean portion of plate. Recorder full-scale deflection was then set while scanning a spot corresponding to the highest prizidilol concentration anticipated.
13. Typical chromatograms are shown in Fig. 12.
14. Each sample development track in turn was scanned and the peak heights corresponding to prizidilol and the internal standard were measured.

Fig. 12 TLC method: typical chromatograms



Chromatograms of spiked serum

A) prizidilol quinolin-3-al hydrazone

B) SK & F 93238 quinolin-3-al hydrazone.

2.2.5.3 Validation of the assay

Calibration curves were obtained (Figs. 13 and 14) by plotting peak height ratios (prizidilol/internal standard) against concentration of samples spiked with known amounts of prizidilol and internal standard (500 ng for data in Fig. 13 and Table 3; 550 ng for data in Fig. 14 and Table 4). Note that neither calibration curve is linear; this is normal for TLC absorptiometry.

Plots of peak height ratio (y) against concentration (x)

Figure 13 and Table 3: $y = 1.43\ (1 - e^{-1.58x})$

Figure 14 and Table 4: $y = 1.54\ (1 - e^{-1.32x})$

Plots of recovered against spiked prizidilol concentrations:

Fig. 13 and Table 3:

Slope = 1.01 (SD = 0.01); y-intercept = 0.00 (SD = 0.01); r = 0.9979

Overall CV = 6%.

Fig. 14 and Table 4:

Slope = 1.00 (SD = 0.02); y-intercept = 0.00 (SD = 0.01); r = 0.9963

Overall CV = 8%.

(SD = standard deviation; r = correlation coefficient; CV = coefficient of variation)

Minimum detectable concentration about 15 µg/l.

2.2.5.4 Comments

As might be expected when the calibration curve is (nearly) a rising exponential, the precision of the TLC assay is not superior to that of the HPLC assay at high concentrations, but comparison of the results shown in Tables 3 and 4 with those in Tables 1 and 2 show a definite advantage for the former method at low concentrations. This observation has one obvious consequence, that if a sample to be analysed is thought

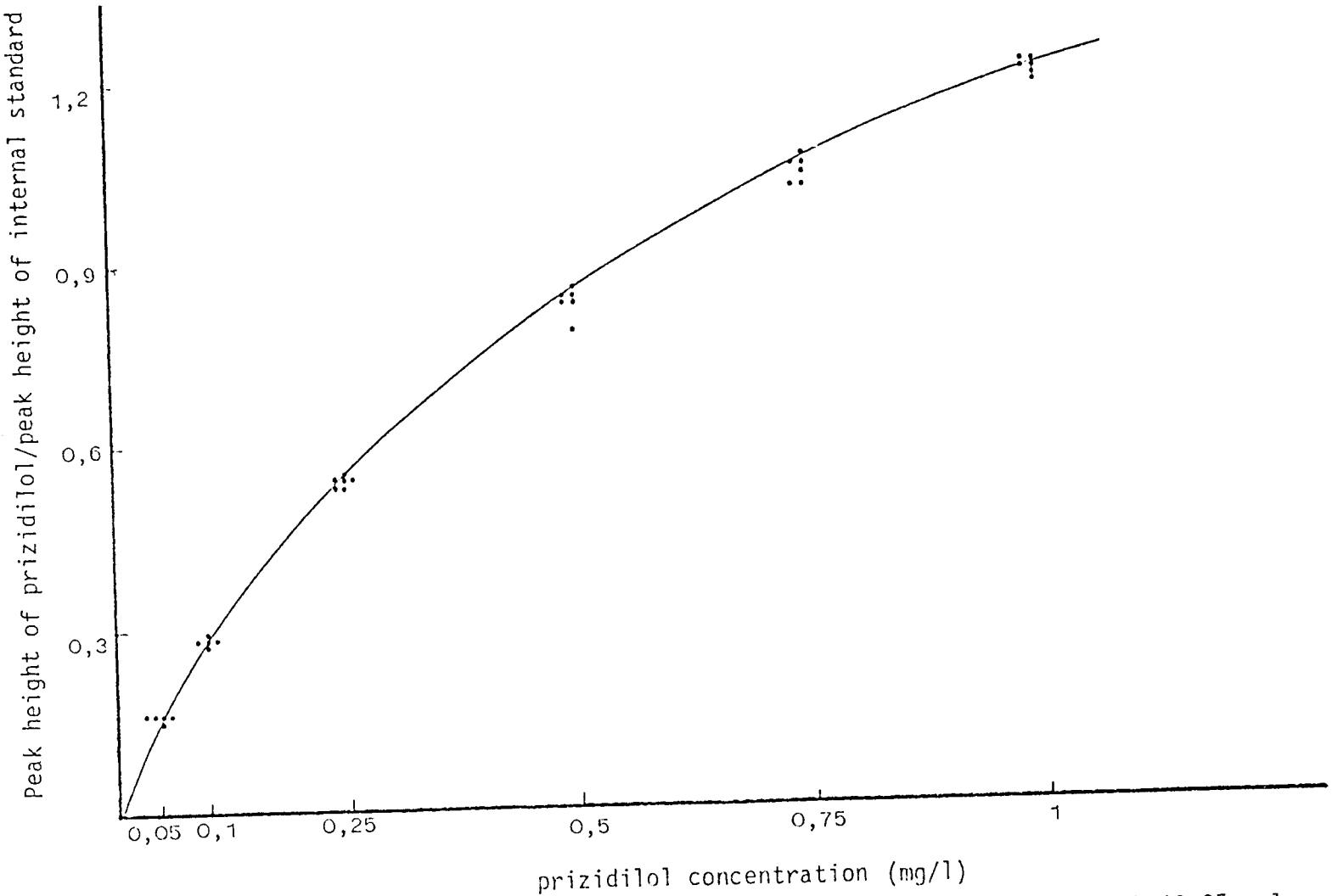


Fig. 13 TLC method: calibration curve of the determination of prizidilol (0.05 - 1 mg/l) internal standard (0.5 µg) in serum

$y = 1.43\ (1-e^{-1.58x})$ $r = 0.9979$

Spiked conc. (mg/l)	Peak height ratio	Mean	Recovered conc. (mg/l)	Mean (SD)	CV (%)
0.05	0.16 0.15 0.16 0.16 0.16	0.157	0.047 0.041 0.046 0.048 0.048	0.045 (0.006)	11
0.1	0.26 0.27 0.26 0.28 0.27	0.269	0.099 0.102 0.101 0.105 0.100	0.101	3
0.25	0.55 0.53 0.53 0.55 0.55 0.56	0.544	0.265 0.257 0.251 0.268 0.267 0.271	0.263 (0.016)	6
0.5	0.85 0.79 0.84 0.83 0.84 0.83	0.829	0.514 0.452 0.497 0.492 0.498 0.491	0.491 (0.023)	5
0.75	1.04 1.01 1.05 1.05 1.01 1.06	1.038	0.728 0.698 0.752 0.752 0.697 0.770	0.733 (0.036)	5
1	1.22 1.19 1.19 1.21 1.21 1.21	1.204	1.053 0.985 0.997 1.026 1.035 1.044	1.023 (0.037)	4

Table 3. Validation of new TLC assay: replicate determinations of prizidilol in spiked human plasma.
CV = coefficient of variation; conc. = concentration.

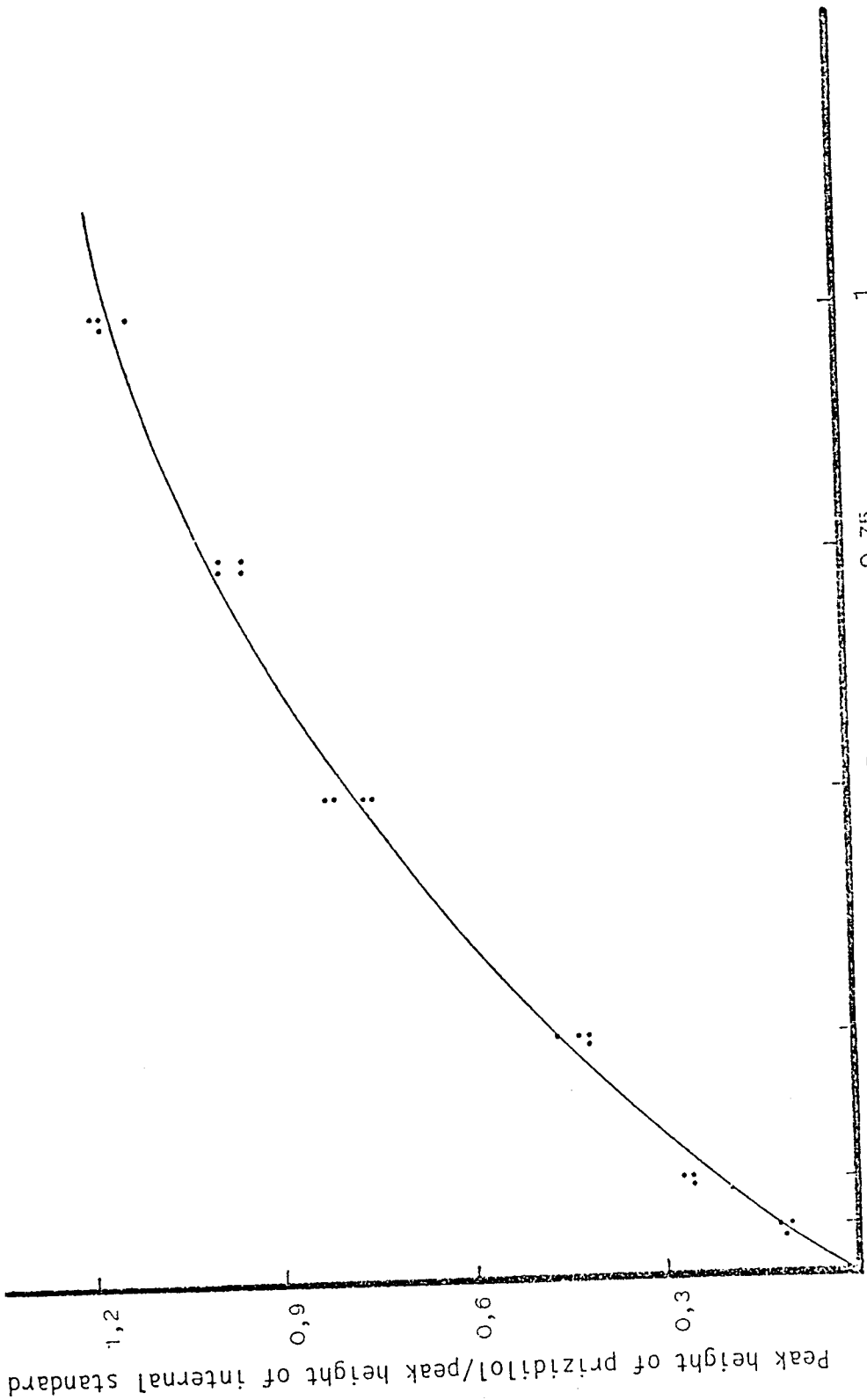


Fig. 14 TLC method: calibration curve for the determination of prizidilol (0.05 - 1.0 mg/l) internal standard (0.55 µg) in serum

$y = 1.54 (1 - e^{-1.32x})$ $r = 0.9963$

Spiked conc. (mg/l)	Peak height ratio	Mean	Recovered conc. (mg/l)	Mean (SD)	CV (%)
0.05	0.12	0.113	0.052	0.048	9
	0.11		0.044		
	0.11		0.047		
	0.11		0.048		
0.1	0.22	0.218	0.106	0.105	8
	0.21		0.098		
	0.23		0.110		
	0.22		0.107		
0.25	0.48		0.269	0.241	9
	0.42	0.438	0.231		
	0.41		0.225		
	0.44		0.241		
0.5	0.76	0.784	0.500	0.521	9
	0.75		0.483		
	0.81		0.541		
	0.82		0.560		
0.75	0.95		0.700	0.724	5
	0.99	0.967	0.752		
	0.99		0.749		
	0.94		0.696		
1	1.18	1.165	1.054	1.015	5
	1.11		0.940		
	1.16		1.029		
	1.17		1.036		

able 4. Validation of new TLC assay: replicate determination of prizidilol in spiked human plasma
CV = coefficient of variation; conc. = concentration.

to be likely to contain more than, say, 800 µg/l prizidilol, it is advisable to apply to the TLC plates more dilute solutions of standards and unknowns than is indicated in section 2.2.5.2.1 . It is also clear that extrapolation would be particularly hazardous.

A curiosity of the statistics is that, while the coefficients of variation for the TLC assay were generally similar to or lower than those for the HPLC assay, the correlation coefficients were not better. Examination of the data, however, suggests that this may be because, in the former assay, the variances were not homogeneous throughout the concentration range so that to apply the standard linear regression equations is incorrect, strictly speaking, and will lead to a pessimistic estimate for r.

The TLC method was adopted for the studies on drug metabolism because, although the procedures (section 2.2.5.2) are relatively lengthy, up to 19 samples could be washed and eluted simultaneously. The advantages suggested for TLC in section 2.1.3 (last paragraph) were justified in practice.

2.5.5 Extraction efficiency

Samples were prepared containing 1 mg/l or 0.5 mg/l prizidilol in serum and were analysed as described in sections 2.2.3.3 and 2.2.5.2. The results are presented in Tables 5 and 6. The extraction efficiency from plasma was about 97% which suggests that any protein binding effect is negligible during the extraction.

Prizidilol conc. mg/l	Peak height of prizidilol / peak height of I.S. (y)	Y Estimate	Mean ± S.D. C.V.
0	0 0 0 0		
0.05	0.122 0.105 0.111 0.114	0.183	0.113 ± 0.007 6.2%
0.1	0.220 0.206 0.227 0.222	0.238	0.218 ± 0.009 4.1%
0.25	0.479 0.423 0.414 0.438	0.402	0.438 ± 0.028 0.5%
0.5	0.763 0.745 0.805 0.823	0.676	0.784 ± 0.036 4.6%
0.75	0.948 0.988 0.986 0.944	0.950	0.967 ± 0.024 2.5%
1.0	1.176 1.114 1.163 1.167	1.224	1.155 ± 0.028 2.4%

Table 4 Data from calibration curve for the validation of TLC method for prizidilol in serum.

$y = 1.10x + 0.13$
$r = 0.9867$

Table 5 Analytical recovery of prizidilol from serum extraction

prizidilol (1.0 mg/l) in 1 ml serum / SK&F 93238-A_3 (internal standard) (1.25 mg/l)	prizidilol (1.0 mg/l) in 1 ml serum / SK&F 93238-A_3 (1.25 mg/l)
0.956	1.010
0.943	1.010
0.931	1.018
0.979	0.991
0.989	0.967
0.988	0.920
0.976	0.969
0.976	
Mean S.D. 0.964 ± 0.021	0.984 ± 0.037
% Recovery 97.9%	

Table 6 Analytical recovery of prizidilol from serum extraction

prizidilol (0.5 mg/l) in 1 ml serum / SK&F 93238-A_3 (internal standard) (1.43 mg/l)	prizidilol (0.5 mg/l) in 1 ml serum / SK&F 93238-A_3 (1.43 mg/l)
0.375	0.384
0.379	0.381
0.373	0.384
0.356	0.352
0.373	0.406
Mean ± S.D. 0.371 ± 0.008	0.381 ± 0.019
% Recovery 97.4%	

3. METABOLISM OF PROPRANOLOL, HYDRALAZINE AND PRIZIDILOL

Prizidilol is a molecular moiety representing a combination of propranolol and hydralazine. In this chapter the metabolism of propranolol and of hydralazine, and the pharmacokinetics of prizidilol itself - as far as they are known - are reviewed.

3.1 Metabolism of propranolol

Propranolol is virtually completely absorbed after oral administration (86). Peak plasma concentrations of propranolol are seen at approximately 2 hours (range 1 to 4 hours) in fasting patients (87-90). Administration of food does not significantly change the time for achievement of peak levels in healthy individuals given a single tablet of propranolol with a standardised meal, though systemic availability of the drug was increased by feeding (91). There appears to be no relationship between the half-life of gastric emptying and the time at which peak plasma propranolol concentration is attained (92).

Propranolol is widely distributed through body tissues after administration. The apparent volume of distribution derived from the β portion of the concentration time curve ($V_d\beta$) is approximately 200 litres (93, 94). This exceeds the physiological body space and it indicates that propranolol is concentrated in extravascular sites (94). This proposal is supported by experimental data in animals showing high concentrations of propranolol in lung, liver, kidney, brain and heart after oral and intravenous administration of propranolol (95, 96).

Propranolol is extensively bound (85 to 96%) to protein in plasma in man (97-99). Since only 62% is bound to human serum albumin (5 g/100 ml), other proteins are also important in determining the degree of binding of this drug (97). Propranolol is bound to lipoproteins independent of serum propranolol concentration, and also to α_1 acid glycoprotein. The latter protein is reported to be responsible for 75% of binding of propranolol to

plasma proteins at therapeutic propranolol concentrations (98). Red blood cells also bind significant quantities of the drug (99). Red cells have been shown to partition about 5:1 with free drug in plasma (100). Blood/plasma ratios of approximately 0.85 have been found in monkey, dog and rat. In man, values between 0.85 and 1.5 have been reported (101, 102).

Propranolol is almost entirely eliminated following metabolism by various enzyme systems in the liver (103). The systemic clearance after intravenous administration was about 1 litre/min with values varying from 0.6 to 1.5 litre/min (89, 94, 104, 105). The plasma elimination half-life was between 2 and 3 hours but values of 4 hours have been reported (89, 104).

Shand et al have shown that drug concentrations declined biexponentially after intravenous administration in man. The early α-phase had a half-life of 10 minutes and the later β-phase of 2.3 hours (94). A biphasic curve has also been observed in rat (106).

Early studies of healthy young volunteers established that propranolol was eliminated almost entirely as metabolites. Only 1 to 4% of a radiolabelled dose was recovered as the parent drug in the urine and faeces (86). In 1976, Bond characterized the metabolites of propranolol as acidic (30%) and amphoteric (70%), with only minute amounts of basic products. Bond isolated two of the main metabolites, 4-hydroxy propranolol and propranolol glucuronide (107). Since that early report, at least 19 metabolites have been identified (108-110).

The documented pathways for the metabolism of propranolol are shown in Fig. 15. The four primary pathways involve O-dealkylation, side chain oxidation, glucuronic acid conjugation and ring oxidation. The metabolic degradation of propranolol in man involves first, oxidative deamination to give N-desisopropylpropranolol (II) which is then metabolized either to

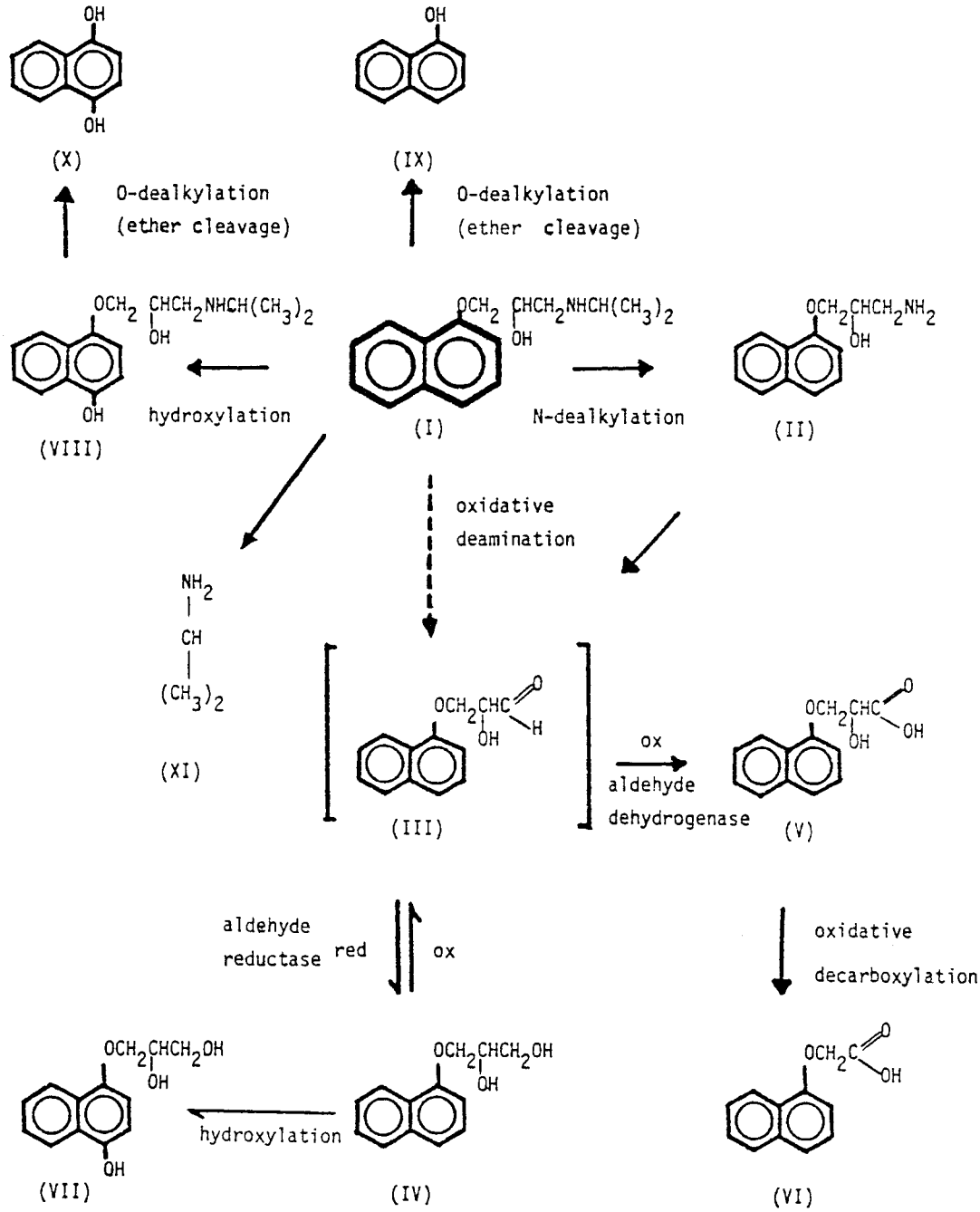


Fig. 15 Schematic representation of propranolol metabolism in man and dog. I, propranolol; II, desisopropylpropranolol; III, proposed aldehyde; IV, glycol; V, naphthoxylactic acid; VI, naphthoxyacetic acid; VII, OH-glycol; VIII, 4-hydroxy-propranolol; IX, α-naphthol; X, dihydroxynaphthalene; XI, isopropylamine
[Based on Walle and Gaffney (116)]

naphthoxylactic acid (V) by aldehyde dehydrogenase or to propranolol glycol by an aldehyde reductase. Small amounts of naphthoxyacetic acid (VI) are formed from naphthoxylactic acid (V). Ring hydroxylation of propranolol (I) in the 4-position, gives rise to 4-hydroxypropranolol (VIII) and also to the 4-hydroxypropranolol glycol (VII). In addition, significant quantities of α-naphthol (IX) and dihydroxynaphthalene (X) are formed as well as methoxy metabolites, these latter suggesting that propranolol passes through the catechol-O-methyl transferase pathway (111). Most of these metabolites are subsequently conjugated and excreted as glucuronide or sulfate conjugates. The 4-hydroxy metabolite of propranolol (VIII) which is formed only after oral administration of propranolol has equivalent β-antagonist activity to propranolol (86, 112). The isopropylamine metabolite (XI) has sympathomimetic activity, and it has been speculated that the glycol metabolite (IV) may contribute to propranolol's effects on the central nervous system (113, 114). Propranolol has varied pharmacological effects, and it is possible that several of the metabolites contribute to these properties (115); 4-hydroxypropranolol (VIII), which can be a major metabolite depending on route of administration of propranolol (112), as mentioned above, exhibits β-antagonistic activity. Hayes and Cooper (95) and others (116) detected 4-hydroxypropranolol only after oral dosing; they proposed that hydroxylation occurs only when the concentration of propranolol in the portal vein exceeds that necessary to saturate the other metabolic pathways.

3.2 Metabolism of hydralazine

Hydralazine is well absorbed from the gastrointestinal tract, approximately 87% of circulating hydralazine being bound to plasma protein (117). Peak plasma concentrations occur after three to four hours oral administration (118).

This drug appears to be rapidly and extensively metabolized (84). About 90% of the absorbed hydralazine is excreted in urine. Less than 5% of the parent drug is eliminated unchanged (53). After ingestion, considerable gut wall and first pass liver metabolism occurs to produce a phthalazine derivative (II) (117, 119). The pathways for the metabolism of hydralazine are shown in Fig. 16. The major metabolites are products of acetylation, hydroxylation and conjugation reaction (17, 120).

For the most part, the acetylated product (Acetyl HP, VIII) undergoes cyclization to form a triazolophthalazine (TP, IV) derivative. TP is devoid of any antihypertensive activity (121). In man, TP, PZ (III), HTP (VI) and HTP glucuronide have been identified in the urine of patients given hydralazine (122). The triazolo metabolites of hydralazine (TP, MTP (IV) and HTP) are separable by gas and high performance liquid chromatography (123, 124); PYR (VIII) has been shown to be a conjugate of hydralazine with pyruvate (125). In the urine of rats administered ^{14}C-hydralazine hydrochloride HP-glucuronide or the glucuronide of 7-hydroxy-hydralazine, N-acetyl HP, hydroxy HP and PYR have been identified. The major metabolite in rats is 3-methyl-5-triazolo (3,4a) phthalazine (MTP) (124, 125).

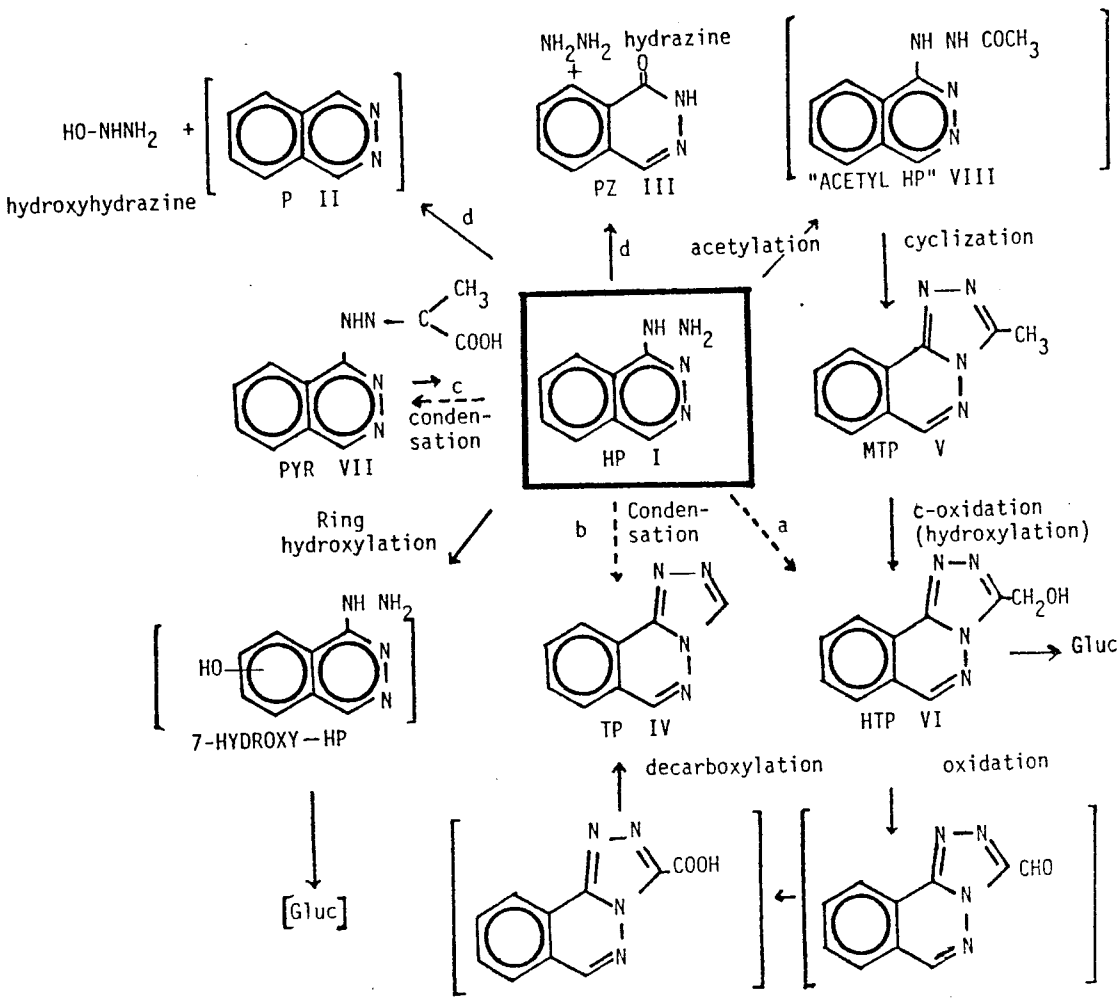


Fig. 16 Metabolism of hydralazine. Compounds in brackets have not been isolated as metabolites. Dotted lines represent suggested pathways. CO_2 was formed in vivo from (methyl-^{14}C) -MTP. a: HTP may also be formed directly by reaction of HP with glycolic acid. b: HP may also be formed by reaction with formic acid. c: HP may also form hydrazones with other endogenous carbonyl containing compounds such as pyridoxal, glucose, ascorbic acid, and macromolecules. The hydrazones can be hydrolyzed. d: PZ (which can exist as tautomers) may be formed nonenzymatically (e.g., via paroxidation). P may also be formed nonenzymatically. In these cases the nature of the eleaved N-N product is not known.

I HP: Hydralazine
II P: phthalazine
III PZ: phthalazinone
IV TP: s-triazolo (3,4-a) phthalazine
V MTP: s-methyl-s-triazolo (3,4-a) phthalazine
VI HTP: 3-hydroxy-methyl-s-triazolo (3,4-a) phthalazine
VII PYR: pyruvic acid hydrazone
VIII Acetyl HP: acetylated hydralazine.

(Based on Israili and Dayton, (120))

In comparison to normal patients, "fast acetylators" exhibit smaller plasma concentration-time curves for hydralazine and less incidence of lupus (viz. the development of antinuclear antibodies). "Fast acetylators" thus require more hydralazine for adequate blood pressure control (126).

3.3 Metabolism of prizidilol

The concentration of prizidilol in the plasma of rats and baboons has been estimated after oral administration of the compound. At the time of maximum plasma concentration (2 to 3 hours after oral dosing) direct proportionality between dose and plasma concentration is seen in baboons but not in rats (127).

Studies of the metabolism of ^{14}C-prizidilol support the existence of a first pass effect for this compound in rats, since 89% of an oral dose was excreted in the faeces, whereas only 48% of an IV dose was excreted by this route during the 24 hours after dosing. No barrier to absorption was observed in rats even at the highest dose which produced individual plasma concentrations as high as 55 mg/l (127).

Whole body autoradiography of rats indicated that, 30 minutes after a dose of ^{14}C-prizidilol, radioactivity was found mainly in the liver and kidney and in tissues containing collagen and elastin such as the cartilage of the ear and aortic wall (Table 7). At no time was measurable radioactivity detected in the brain after administration of ^{14}C-prizidilol (127) (Table 7).

The urinary metabolites of ^{14}C-prizidilol have been examined in rats, cats and baboons. In rat urine, the parent compound and four metabolites which are more polar than the parent compound have been

Table 7: Prizidilol residue in the tissues of male rats after intravenous administration of ^{14}C-prizidilol.

Rats received an intravenous dose of ^{14}C-prizidilol (2 μCi/animal, 20 mg/kg). Results are expressed as μg prizidilol residue/g wet weight of tissue ± S.D. (n=3). The weight of prizidilol residue was calculated using the assumption that the residue has same molecular weight as parent drug. Values were considered non-significant, if counts less than twice background were obtained. Adapted from Clancy A. et al (127)

Tissue	Time After Administration (Days)		
	7	14	28
Kidney	4.19 ± 0.43	2.23 ± 0.06	0.73 ± 0.25
Spleen	2.05 ± 0.97	1.22 ± 0.03	1.18 ± 0.25
Aorta	3.16 ± 0.70	4.10 ± 1.38	5.05 ± 0.81
Liver	4.69 ± 1.96	1.18 ± 0.22	0.84 ± 0.36
Lung	2.43 ± 0.29	1.88 ± 0.64	0.76 ± 0.28
Heart	1.24 ± 0.11	1.08 ± 0.36	0.95 ± 0.18
Ear	5.02 ± 1.82	2.40 ± 0.70	2.70 ± 0.94
Small Intestine	0.52 ± 0.11	0.44 ± 0.04	0.35 ± 0.04
Large Intestine	0.82 ± 0.36	0.44 ± 0.02	0.20 ± 0.04
Carcase	0.38 ± 0.02	0.31 ± 0.05	0.21 ± 0.04
Testes	0.58 ± 0.08	0.46 ± 0.06	0.41 ± 0.28
Eye	not significant	not significant	not significant
Brain	not significant	not significant	not significant
Blood	not significant	not significant	not significant

observed up to five hours after dosing. Preliminary results from the cat and baboon suggest a similar pattern of urinary metabolites, but without better chemical characterisation than Rf values, it is not possible to state that these three species metabolize prizidilol in a similar manner (127).

Collection of urine and faeces, and biliary cannulation in rats dosed with prizidilol has indicated that both the kidney and liver play a role in eliminating the parent drug and/or its metabolites. No $^{14}CO_2$ was found in expired air, suggesting that ^{14}C-prizidilol does not contribute labelled CO_2 to the metabolic pool (127).

A bolus injection of 20 mg/kg IV prizidilol had no effect on any of the biochemical assays for liver function, including alanine and aspartate aminotransferases unless the concentration of prizidilol in the blood approached 250 μM. Such a high concentration of the parent drug is not approached following IV infusion or oral administration of approximately 10 mg prizidilol base to a human subject. Therefore, at the doses likely to be given to human subjects, it appears that aminotransferase and other liver function tests are not affected. Male rats, starved overnight, received 900 mg/kg prizidilol in an attempt to effect liver aminotransferase activity. Liver samples were taken between two and three and a half hours after dosing, to correspond with the maximum blood concentration of prizidilol. In these experiments, the hepatic aminotransferase activity control and treated rats did not differ significantly (127).

4. HEPATIC DRUG METABOLIZING ENZYMES

The aim of this portion of my thesis was to investigate aspects of the hepatic metabolism of prizidilol. In particular, the metabolism of this drug by three liver enzyme systems which are known to interact with xenobiotics was investigated. The enzyme systems which were studied were the cytochrome P-450 enzyme system (128, 129), the glutathione-S-transferases (130, 131) and the acetyltransferases (132, 133).

4.1 General introduction on drug metabolism

Drug metabolism is the process whereby a wide range of xenobiotics, viz., drugs, carcinogens and environmental pollutants, undergo enzymatic biotransformation leading to the production of a variety of metabolites. In mammals, the metabolic transformation of these compounds occurs primarily in the liver, although most organs and tissues have some capability for xenobiotic transformation. The enzymes involved in the metabolism of xenobiotics are known as drug metabolizing enzymes.

Drug metabolism is considered to occur in two distinct stages. The two phases of drug metabolism are shown in Fig. 17. The enzymes catalyzing Phase I and Phase II reactions are summarized in Table 8.

Fig. 17 The general pathway for the metabolism of xenobiotics.

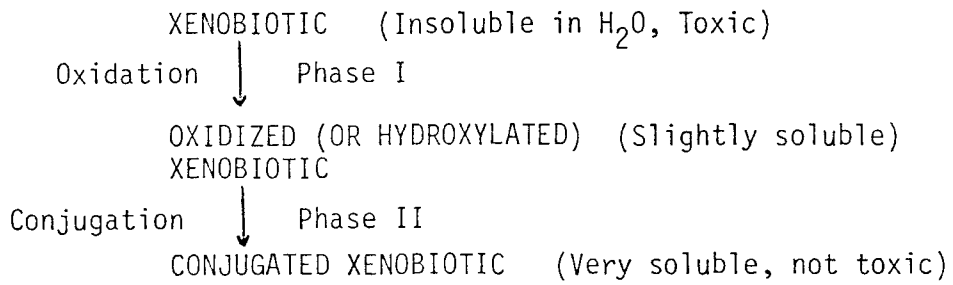


Table 8 : Hepatic metabolism of xenobiotics (134-136).

Reaction	Enzyme	Major subcellular location of enzyme
Phase I		
Oxidation of aliphatic and aromatic groups, N-oxidation, sulphoxidation, oxidative dealkylation, epoxidation	Cytochrome P-450	Endoplasmic reticulum, nuclear envelope
Reduction of azonitro-compounds	Flavin enzymes or cytochrome P-450	Endoplasmic reticulum
Reduction of carbonyl-compounds and halo-compounds	Cytochrome P-450	Endoplasmic reticulum, nuclear envelope
Oxidation and reduction of alcohols, aldehydes and carboxylic acids	Alcohol dehydrogenase, aldehyde dehydrogenase	Cytosol and various organelles
Oxidation of amines	e.g. Monoamine oxidases	Mitochondria
Hydrolysis of esters	Esterases	Cytosol and various organelles
Phase II		
Conjugation with glucuronic acid	UDP-glucuronyl transferases	Endoplasmic reticulum, nuclear envelope
Conjugation with glutathione	Glutathione-S-transferases	Cytosol, Endoplasmic reticulum
Conjugation via acetylation	Acetyl transferases	Cytosol
Conjugation with sulphate, glycine, other amino acids; and methylation	Various transferases	Cytosol and various organelles
Hydration of epoxides	Epoxide hydrase	Cytosol and Endoplasmic reticulum

Phase I involves the oxidative conversion of the lipophilic or fat-soluble compound into more hydrophilic or water soluble products by reactions such as dealkylation, deamination and hydroxylation. Phase II involves the conjugation of the oxidized intermediate or the parent compound with a small, polar organic molecule such as glutathione, glucuronic acid or sulfate.

The overall process of drug metabolism converts a lipophilic xenobiotic which would be stored in the fat depots of the body into a hydrophilic conjugate which is readily excreted into the urine via the kidney or into the bile. This overall process generally results in detoxication, since it converts a hydrophobic compound, the storage of which in the body could be associated with toxic effects into a conjugate which, with few exceptions, is devoid of physiological effect.

Phase I of drug metabolism is catalyzed predominantly by the cytochrome P-450 enzyme system of the hepatic endoplasmic reticulum (Table 8). This enzyme system is frequently studied in vitro using the subcellular particles known as hepatic microsomes. Microsomes are vesicles which form spontaneously from segments of endoplasmic reticulum following the disruption of the endoplasmic reticulum by homogenization of the liver tissue. Hepatic microsomes provide an advantageous system for studies of drug metabolism since they are easily prepared and collected, and since in contrast to the purified reconstituted cytochrome P-450 enzyme system, the various components are present in their physiological relationship, essentially as found in vivo. Microsomes are thus a useful tool with which to study phase I of drug metabolism.

4.2 Cytochrome P-450

4.2.1 Introduction

Cytochrome P-450 is a group of haem-containing isoenzymes, so named because of a striking spectral feature of the CO-ferrocytochrome P-450 complex, i.e. its absorbance at 450 nm. This group of enzymes is of great importance in phase I of drug metabolism, because of its extremely broad substrate specificity. For example, substrates for one or more forms of cytochrome P-450 include the following: aliphatic hydrocarbons, polycyclic hydrocarbons, barbiturates, halogenated hydrocarbons, amphetamines, alkanes, fatty acids and polychlorinated biphenyls (See Table 9). In addition to the broad substrate specificity of cytochrome P-450, this group of enzymes also catalyzes a wide variety of reactions. Oxidative reactions catalyzed by this cytochrome include C-oxidation, N-oxidation, S-oxidation, O-dealkylation, N-dealkylation and deamination. In addition, cytochrome P-450 also catalyzes a number of reductive reactions (Tables 9, 10).

The overall oxidative reaction catalyzed by the microsomal cytochrome P-450 enzyme system is accepted to be of the following form, even though in many cases, the final stable product may not be hydroxylated.

$$SH + NADPH + H^+ + O_2 \xrightarrow{\text{Cytochrome P-450}} SOH + NADPH^+ + H_2O \quad \text{(Equation 1)}$$

where SH represents the hydrophobic substrate, and SOH its hydroxylated product. As shown in Equation 1, the rate of metabolism of a compound by cytochrome P-450 can be assessed by measuring the rate of oxidation of NADPH. According to this equation one mole of NADPH is oxidized per mole of substrate hydroxylated. Thus, monitoring the oxidation of NADPH as a function of time provides a facile spectral assay for the rate of metabolism of diverse substrates by cytochrome P-450.

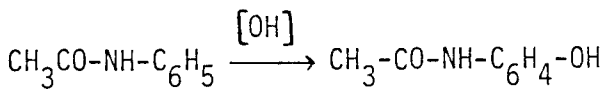
Table 9 : Metabolism of drugs by cytochrome P-450 in liver microsomes (134)

Type	Substrate	Product
Oxidation		
Aromatic hydroxylation	Aniline	p-Aminophenol
Aliphatic hydroxylation	Hexobarbital	Hydroxyhexobarbital
Arene oxide formation	Bromobenzene	Bromobenzene epoxide
N-Dealkylation	Aminopyrene	4-Aminoantipyrine
N-Hydroxylation	2-Acetylaminofluorene	N-Hydroxy-2-acetyl aminofluorene
O-Dealkylation	p-Acetanisidine	p-Hydroxyacetanilide
S-Dealkylation	6-Methylthiopurine	6-Thiopurine
N-Oxidation	Dimethylaniline	Dimethylaniline N-oxide
S-Oxidation	Chloropromazine	Chloropromazine sulfoxide
Oxidative Dechlorination	Halothane	Trifluoroethanol
Reduction		
Deamination	Amphetamine	Phenylacetone
Desulfuration	O-Ethyl-O-(4-nitrophenyl) phenylphosphonothionate	O-Ethyl-O-(p-nitrophenyl) phenylphosphate
Dechlorination	Carbon tetrachloride	Chloroform
Dealkylation of metaloalkanes	Tetraethyl lead	Triethyl lead
Reductive dechlorination	Halothane	2-Chloro-1,1,1-trifluoroethane 2-Bromo-1,1,1-trifluoroethane

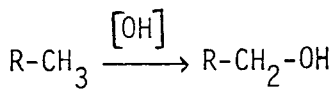
Table 10

Reactions catalyzed by the cytochrome P-450 drug metabolizing system (136)

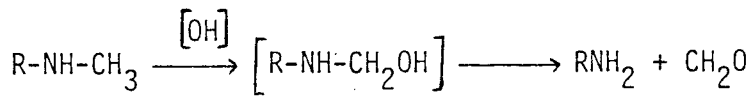
Aromatic hydroxylation



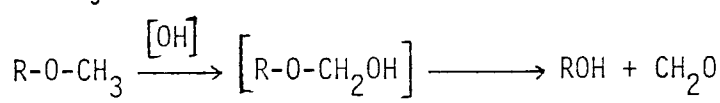
Aliphatic hydroxylation



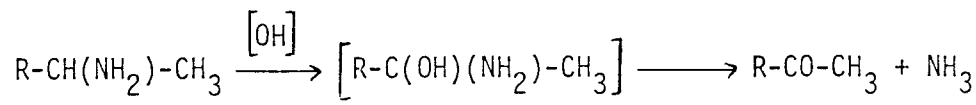
N-Dealkylation



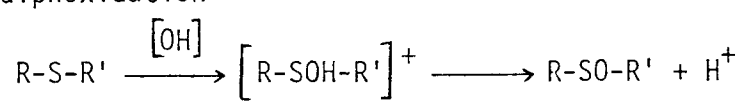
O-Dealkylation



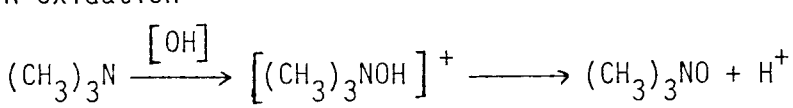
Deamination



Sulphoxidation



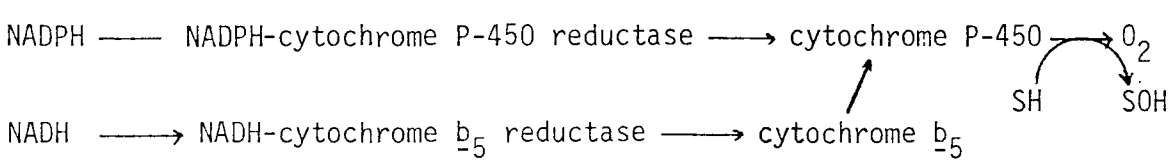
N-Oxidation



In practice, in experiments conducted with hepatic microsomes, the theoretical stoichiometry of 1:1 may not be observed due to oxidation of NADPH by side reactions. Following inhibition of cytochrome-mediated reactions by CO, corrections for background rates of NADPH oxidation in the presence of substrate and $CO:O_2$ (80:20) can be made, resulting in a closer experimental approximation to the theoretical stoichiometry of one mol of NADPH oxidized per mol substrate hydroxylated (see Equation 1) (137).

Cytochrome P-450 haemoproteins are not in themselves capable of metabolizing xenobiotics, but require one or more carriers to transfer electrons one at a time from the preferred two electron donor NADPH to the cytochrome P-450 substrate complex. The overall scheme for electron transport for cytochrome mediated reaction is outlined below (Fig. 18) (138). Although NADPH is a far more efficient electron donor than is NADH, it has been found that NADH is an essential electron donor for the metabolism of certain compounds by cytochrome P-450. For these compounds, cytochrome $\underline{b}_5$ is an obligate electron carrier in their metabolism by cytochrome P-450.

Fig. 18 Pathways for electron transfer to cytochrome P-450 (138). Straight arrows indicate electron flow, and curved arrows indicate reactions occurring.



4.2.1.1. Mechanism for cytochrome P-450 catalyzed oxidative metabolism.

The accepted mechanism for the metabolism of compounds by cytochrome P-450 is depicted diagrammatically in Fig. 19 and may be summarized as follows:

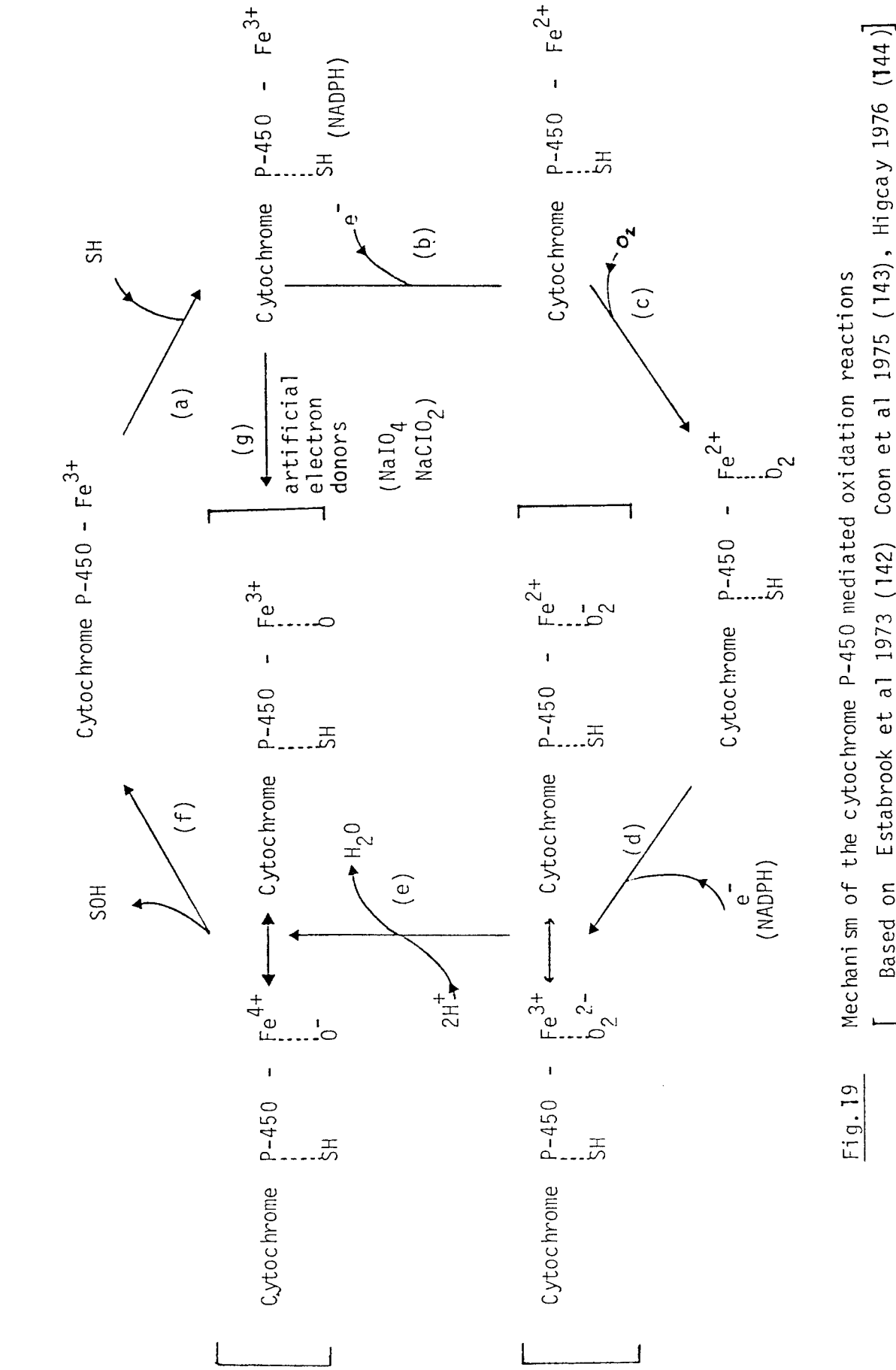


Fig.19 Mechanism of the cytochrome P-450 mediated oxidation reactions
[Based on Estabrook et al 1973 (142) Coon et al 1975 (143), Higcay 1976 (144)]

a) The substrate binds to active site of cytochrome P-450 resulting in the formation of a high spin ferricytochrome P-450 enzyme-substrate complex.

b) The ferricytochrome P-450 enzyme-substrate complex is reduced to the ferrous form by one electron from NADPH via NADPH-cytochrome P-450 reductase.

c) Molecular oxygen binds to the ferrous ion of the reduced complex.

d) The ternary complex is further reduced with a second electron from NADPH via the appropriate reductase. The superoxide ferrous enzyme-substrate intermediate which is formed is in resonance with the hydroperoxoferric enzyme-substrate complex.

e) The O-O bond of the hydroperoxoferric enzyme-substrate complex is heterolytically cleared to yield water and a ferric enzyme-mono-oxygen species which is in resonance with the ferryl ion-oxene complex.

f) The hydroxylated product is released, and cytochrome P-450 is regenerated in the low spin ferric state.

Artificial electron donors such as $NaIO_4$, $NaClO_2$, H_2O_2 and organic hydroperoxides can support the hydroxylation of a variety of substrates by purified cytochrome P-450 in the absence of NADPH, NADPH-cytochrome P-450 reductase and molecular oxygen (139, 140). These artificial electron donors are thought to function by directly providing cytochrome P-450 with the active oxygen species necessary for hydroxylation (Fig. 19). In addition, various model systems have been shown to catalyze reactions typical of cytochrome P-450. These systems usually comprise haem, a ligand or not, and an electron donor such as thiol or NADPH, and have been shown to exhibit aniline hydroxylase and other activities typical of cytochrome P-450 (139, 141).

4.2.1.2 Cytochrome P-450 catalyzed reductive metabolism

Cytochrome P-450 has recently been shown to participate in the reductive metabolism of xenobiotics such as halocarbons (145-147), polyhalogenated compounds (148) and organic nitro and azo compounds (145). Such a reductive function of cytochrome P-450 is unexpected in view of high affinity of the enzyme for molecular oxygen. However, the oxygen concentration in cells is quite low (147) especially in the centre of the liver lobules, and this allows reducible lipophilic compounds with relatively high oxidation potentials to compete effectively with oxygen for electrons at the active site of cytochrome P-450. Under these conditions, the reduced form of cytochrome P-450 can transfer one or two electrons directly and sequentially to the substrate, resulting in substrate reduction and concomitant oxidation of ferrocytochrome P-450 to the ferric state (147, 149, 150). Cytochrome P-450 has been shown to catalyze the reduction of halothane to a number of products, a process which is thought to be associated with the production of halothane hepatitis (151).

4.2.1.3 Binding of compounds to cytochrome P-450

The first step in the oxidative and reductive metabolism of a compound by cytochrome P-450 (see Fig. 20) is the binding of the substrate to ferricytochrome P-450. Cytochrome P-450 is perhaps unique in that one can easily ascertain whether or not a compound is a substrate simply by observing the type of visible spectrum that it produces with the enzyme. One can readily obtain spectral binding constants in order to characterize substrate binding. This favourable situation occurs because the spin state and coordination chemistry of the ferric ion of the haem group of cytochrome P-450 are extremely sensitive to ligands and to conformational changes in the enzyme caused by binding to the active site or to other sites on the enzyme.

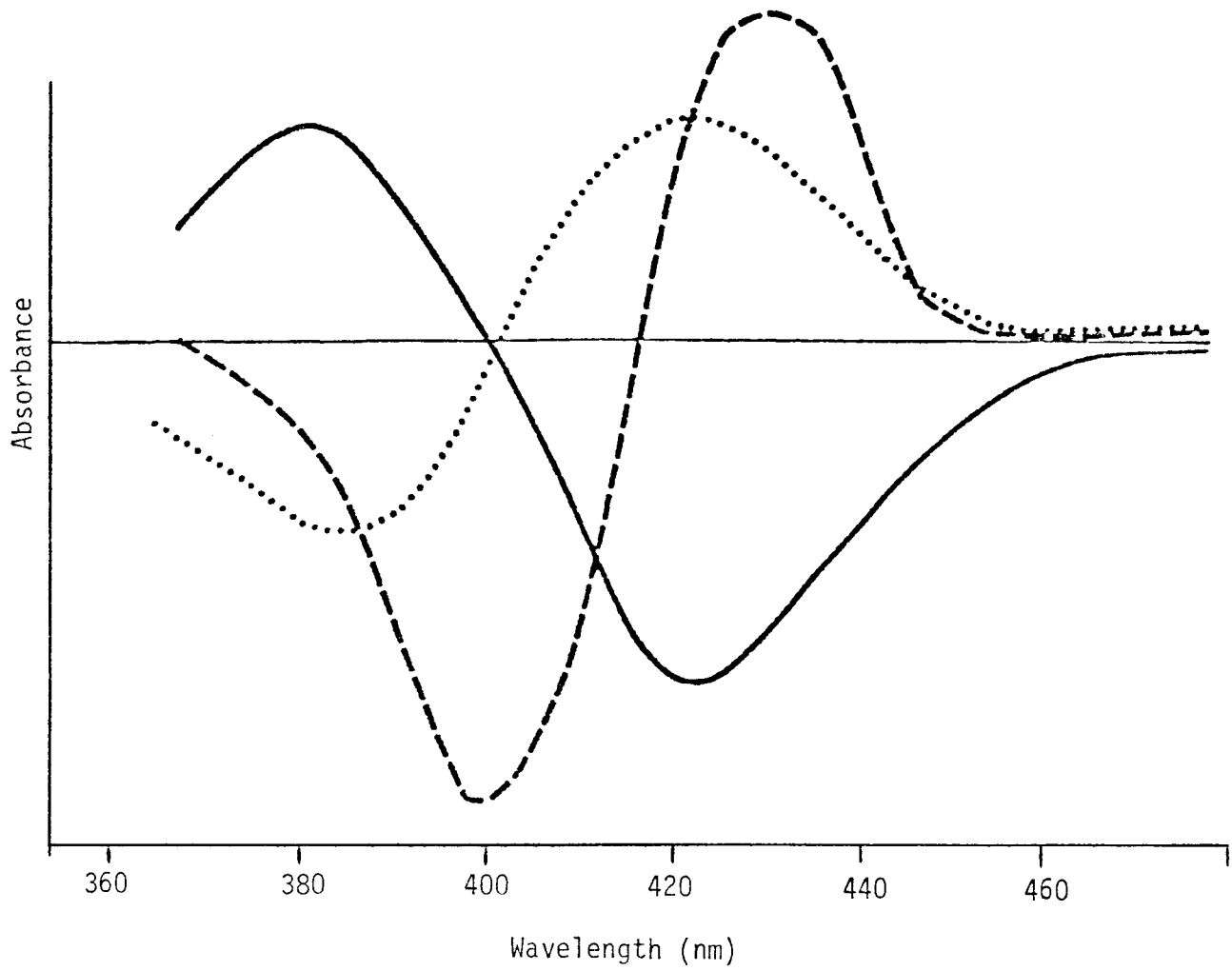


Fig. 20 Difference spectra with hepatic microsomes.

Sample cuvette contains hepatic microsomes and compound. Reference cuvette contains microsomes alone. Spectral changes represented: type I ———; type II – – – –; and type IR .....

[Based on Schenkman, 1968 (140), 1970 (157)]

Since 1966, it has been appreciated that various substrates and inhibitors combine with ferricytochrome P-450 to produce characteristic difference spectra with cytochrome P-450 in isolated hepatic microsomes (152-155). The changes in the absorbance spectrum caused by the binding of compounds to ferricytochrome P-450 are shown in Fig. 20. Three binding sites on ferricytochrome P-450 are distinguishable spectrally: the type I, IR and II difference spectra. The type I difference spectrum is a spectral manifestation of the formation of a substrate - ferricytochrome P-450 complex - since compounds which bind to the type I binding site of cytochrome P-450 are with few exceptions metabolized by cytochrome P-450. The type I difference spectrum has been shown to arise from substrate-induced conformational changes in the protein which alter the environment and the spin state of the haem of ferricytochrome P-450 from a low spin to a high spin state (140). The type IR spectrum reflects the conversion of the haem iron atom of ferricytochrome P-450 from a high spin to a low spin state (156). This change results in a difference spectrum which is the exact reverse of the type I difference spectrum (Fig. 20). Compounds displaying type IR difference spectra are not bound in the region of the haem moiety and consequently are not metabolized by cytochrome P-450 (157). The binding of type IR compounds decreases the rate of reduction of the cytochrome P-450 complex: the decrease in activity appears to be directly related to the magnitude of type IR spectral change (158). There are some compounds e.g. hexobarbital, which at low concentrations bind to hepatic cytochrome P-450 with the production of a type I difference spectrum, but at higher concentrations (5-fold increase) elicit a type IR spectrum.

In contrast to type I and type IR spectral changes, the type II spectral change results from direct liganding of the added xenobiotic to the central iron atom of the haem of cytochrome P-450, leading to the formation of a low spin ferrihaemochrome. (Fig. 20). Type II compounds compete with oxygen as a ligand for the haem iron ion of ferrocytochrome P-450, and thereby inhibit cytochrome P-450 catalyzed oxidative metabolism (159). Examples of type II binding compounds which inhibit cytochrome P-450 dependent metabolism are CO and metyrapone (160).

4.2.1.4 Inhibitors

SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate), metyrapone [2-methyl-1,2-bis (3'-pyridyl)-1-propanone] and CO are considered as examples of inhibitors of cytochrome P-450. Fig. 21 (145) shows a hypothetical model of the microsomal hydroxylase that results from inhibition experiments with SKF 525-A, CO and metyrapone. The substrate and oxygen binding sites are also shown therein.

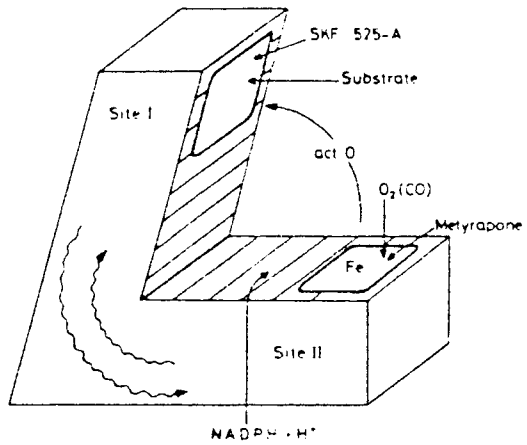


Fig. 21 A hypothetical model of the microsomal hydroxylase ('The mouse trap') [Based on Netter et al 1969 (151)]

SKF 525-A exerts a competitive inhibitory effect on the oxidative N-demethylation of ethylmorphine. Both SKF 525-A and its metabolite are generally considered to be competitive inhibitors of the microsomal monooxygenase. As can be seen in Fig. 21, SKF 525-A competes with substrates for binding to the type I site. SKF 525-A itself, when bound to ferricytochrome P-450, causes a type I spectral change.

CO and metyrapone bind to the type II binding site, and both interfere with the iron-catalyzed activation of oxygen. The curved arrows in Fig. 21 serve to symbolize the establishment of actual physical contact between the active oxygen and a type I substrate. This concept might also be useful in the explanation of competitive inhibition of the oxidation of type I substrates (e.g. p-nitroanisole) by metyrapone. Metyrapone binds near the haem (site II), but in some instances may partially block the type I site.

4.2.1.5 Induction and multiple forms of cytochrome P-450

In rat liver endoplasmic reticulum at least ten to fifteen distinct forms of cytochrome P-450 have been identified on the basis of electrophoretic mobility and immunochemical evidence (see e.g. 161, 162). It is expected that ultimately approximately 30 forms will be identified (163). The different forms of cytochrome P-450 have distinct amino acid compositions, minimum molecular weights, spectral characteristics and sensitivities to inhibitors. In addition, the isozymes of cytochrome P-450 differ in their substrate specificities (164-166). For example, different forms of cytochrome P-450 may catalyze one type of reaction (e.g. biphenyl hydroxylation) at two or more different sites on one substrate (167), or they may catalyze different reactions (e.g.

N-demethylation and c-hydroxylation) on one substrate. However, there are certain activities that are common to several of the multiple forms of cytochrome P-450.

The levels of cytochrome P-450 can be increased in vivo by a wide variety of inducing agents (Table 11) (168-172). The inducing agents often selectively increase the levels of one or more forms of cytochrome P-450 in the mixture of cytochrome P-450 isozymes which is present in the liver. The ability of an inducing agent to increase a particular form of cytochrome P-450 provides a useful tool for studies of the role of distinct isozymes of cytochrome P-450 in the metabolism and toxicity of xenobiotics. The nomenclature and properties of some of the inducible forms of cytochrome P-450 are summarized in Table 11.

The most widely used inducing agent for cytochrome P-450 is phenobarbital (PB). This agent causes an increase in the isoenzyme termed "the major phenobarbital-inducible form of cytochrome P-450" having a molecular weight of 51000 daltons (173, 174). This form of cytochrome P-450 catalyzes the biotransformation of a wide variety of compounds including N,N-dimethylaniline, parathion, benzamphetamine and N,N-dimethylphentermine (172, 175). Phenobarbital drastically increases the ability of the liver to metabolize certain xenobiotics because it causes proliferation of the smooth endoplasmic reticulum in the hepatocyte, increases liver weight and elevates the levels of NADPH-cytochrome c reductase, cytochrome b_5, cytochrome P-450, epoxide hydratase and glucuronyl transferase per mg of microsomal protein (168, 169, 175).

Pregnenolone-16α-carbonitrile (PCN) has been demonstrated to act as an enzyme inducer for cytochrome P-450 (176). The species of cytochrome P-450 induced by PCN resembles the phenobarbital type spectrally, but is a distinct isozyme. For example, while PB was most effective in inducing

TABLE 11

DIFFERENT FORMS OF CYTOCHROME P-450 PURIFIED FROM RAT LIVER MICROSOMES

Preparation	Treatment of Animals	CO Maximum	Subunit Molecular weight	Comments
$P\text{-}450_b$	Aroclor 1254 PB	450	52,000	Major PB-inducible form
Fraction C	PB	450	52,000	Identical to $P\text{-}450_b$
Fraction B	PB	449.6	53,000	Major PB-inducible form; probably identical to $P\text{-}450_b$, although the end terminals of these two preparations have been found to be different
Peak I_b, phosphocellulose column	PB	450.7	50,000	Probably identical to $P\text{-}450_b$
P-450	PB	451	50,000	Probably identical to $P\text{-}450_b$
P-450	PB	Not available	48,500	Probably identical to $P\text{-}450_b$
$P\text{-}450_c$	Aroclor 1254 3-MC	447	56,000	Major 3-MC-inducible form
Fraction B	3-MC	447.6	56,000	Major 3-MC-inducible form; probably identical to $P\text{-}450_c$, although the end terminals of these two preparations have been found to be different.
Peak III, DE-52 column	3-MC	448	54,000	Probably identical to $P\text{-}450_c$
P-448	3-MC	448	56,500	Probably identical to $P\text{-}450_c$
H-II fraction	3,4,5,3',4'-Pentachlorobiphenyl	447	53,500	Probably identical to $P\text{-}450_c$
Peak III, phosphocellulose column	PCN	450	51,000	Major PCN-inducible form
$P\text{-}450_a$	Aroclor 1254 PB 3-MC	452	48,000	
Fraction D	PB	449.2	54,000	
50 mM phosphate fraction, CM-sephadrex	Cholestyramine	Not available	50,000	
Fraction A	None	450	52,200	
Fraction B	None	450.8	52,400	

*The abbreviations used are PB, phenobarbital; 3-MC, 3-methylcholanthrene: PCN, pregnenolone 16α-carbonitrile.
[Based on Lu AYH and West SB (1980) (172)]

N-demethylation of benzamphetamine, PCN maximally increases the activity for the N-demethylation of ethylmorphine and aminopyrine (172, 177, 178). The form of cytochrome P-450 induced by PCN has a molecular weight of 54000 daltons (173, 174). PCN induction results in an increase in the levels of both cytochrome P-450 and NADPH-cytochrome c reductase relative to the levels of microsomal protein and in the proliferation of the smooth surface endoplasmic reticulum in hepatocytes (175).

The polycyclic hydrocarbons, β-naphthoflavone (βNF) and 3-methylcholanthrene induce cytochrome P-448 (P_1-450) by a common mechanism (179). Cytochrome P-448 is distinct from the forms of cytochrome P-450 which are elevated by pre-treatment of rats with PB or PCN. The molecular weight of cytochrome P-448 is 55000 daltons. This form is predominantly responsible for the metabolism of polycyclic aromatic hydrocarbons such as 3,4-benzpyrene, 7-ethoxycoumarin, 7-ethoxyresofurin, zoxazolamine, benzanthracene, naphthalene and phenanthrene and is therefore known as aryl hydrocarbon hydroxylase (169). Polycyclic hydrocarbons do not alter the levels of the microsomal electron transfer proteins cytochrome b_5 and NADPH-cytochrome c reductase or cause proliferation of the smooth surface endoplasmic reticulum, but they do increase the levels of some phase II enzymes viz. epoxide hydrase and UDP-glucuronyl transferase.

The multiple forms of cytochrome P-450 have been named in terms of their electrophoretic mobility. For example, rat liver microsomal cytochrome P-448 which is induced by 3-methylcholanthrene is known as LM_2. However, this system of nomenclature is confusing, since in different species LM_2 would designate different isozymes, e.g. in rabbit, LM_2 would be the major form induced by phenobarbital (172). Therefore, in this thesis, the terminology of "phenobarbital inducible form of cytochrome P-450" will be utilized.

4.2.2 Experimental

4.2.2.1 Materials

The inducing agents sodium phenobarbital and β-naphthoflavone were obtained from Maybaker Ltd., Port Elizabeth, South Africa and Aldrich Chemical Company, Milwaukee, Wisconsin, USA, respectively. Pregnenolone-16α-carbonitrile was a gift from G.D. Searle and Co., Chicago, Illinois, USA. NADPH, and the components of the NADPH-generating system (NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from Miles Laboratories, Cape Town, South Africa. Cylinders of pure gases (O_2 and CO) were obtained from Afrox Ltd., Cape Town, South Africa. Metyrapone [2-methyl-1,2-bis (3-pyridyl)-1-propane] and SK & F 525-A (β-diethylaminoethyl-2,2-diphenyl valerate) were gifts from Ciba-Geigy Limited, Basle, Switzerland and Smith, Kline and French Ltd., Isando, Transvaal, South Africa, respectively. Sodium dithionite was obtained from Merck Chemicals, Darmstadt, Germany. All other chemicals were of the highest purity commercially available. Water was glass distilled and deionized.

4.2.2.2 Methods

4.2.2.2.1 Experimental animals

Male Long Evans rats weighing between 190 g and 120 g were used for all experiments. The animals were allowed free access to Epol Laboratory Chow (protein minimum 20%, fat 2.5%, fibre maximum 6%, calcium 1.4%, phosphorus 0.7%) unless otherwise indicated and were allowed free access to water at all times.

4.2.2.2.2 Treatment of animals

Different forms of cytochrome P-450 were induced by intraperitoneal injection of sodium phenobarbital (80 mg/kg/day in 0.9% saline for three consecutive days) or pregnenolone-16α-carbonitrile (50 mg/kg/day in corn oil for three consecutive days), the last injection 16 hrs prior to sacrifice, or by one injection of β-naphthoflavone (80 mg/kg/day in corn oil 40 hrs prior to sacrifice). All animals were fasted for 16 hrs prior to sacrifice by cervical fracture.

4.2.2.2.3 Preparation of hepatic microsomes

Immediately after the animals were sacrificed, microsomes were prepared from fresh rat liver homogenated at $4^{o}C$ by differential ultracentrifugation by a modification of the method of Holtzman and Carr (180). The livers from three to eight rats were homogenized in 3 ml of 0.15 M KCl-0.02 M Tris-HCl, pH 7.4 per gram wet weight of liver. The cell debris, nuclei and mitochondria were removed by centrifugation at 10000 g for 25 min in a Beckman J-21B centrifuge. The microsomes were sedimented from the supernatant by centrifugation at 105000 g for 55 min in a Beckman model L ultracentrifuge. The microsomal pellet was resuspended in 0.15 M KCl-0.02 M Tris-HCl and resedimented by centrifugation at 105000 g for 45 min. The microsomes were finally suspended in 0.02 M Tris-HCl, pH 7.4 at a concentration of 2 mg microsomal protein/ml and were used within 5 hours of preparation (181). The protein concentration of the microsomal suspension was determined by the method of Lowry (182) as modified by Chaykin (183) using bovine serum albumin as a standard.

4.2.2.2.4 Spectral assays on hepatic microsomes

4.2.2.2.4.1 Difference spectra

Two Teflon-stoppered 4 ml cuvettes each containing 2.5 ml of microsomal suspension (2 mg protein/ml) were equilibrated to 25°. A small volume (5-100 µl) of an aqueous solution of prizidilol was added to the sample cuvette below the surface of the microsomal suspension using a Hamilton microsyringe. Equal volumes of water were added to reference cuvettes. The cuvettes were then stoppered and the suspension shaken by hand. The magnitude of the difference spectrum was measured as the difference in absorbance between the trough at ca. 396 nm and the peak at ca. 431 nm at high concentrations of prizidilol (24 - 189 µM) or the trough at ca. 416 nm and the peak at ca. 380 nm at low concentrations of prizidilol (1 - 23 µM). Correction was made for the intrinsic differences in absorbance at these wavelengths for cuvettes containing microsomal suspension only.

4.2.2.2.4.2 Cytochrome P-450 assay

The concentration of cytochrome P-450 in hepatic microsomes was determined from measurement of the difference spectrum of CO-ferro-cytochrome P-450 versus ferrocytochrome P-450, as described by Omura and Sato (184). Two Teflon-stoppered 4 ml cuvettes each containing 2.5 ml of microsomal suspension (2 mg protein/ ml) were equilibrated to 25°. The sample cuvette was bubbled with CO (30 ml/min) for 30 sec. A few grains of sodium dithionite were added to each cuvette, and the absorbance difference spectrum was measured from 500 nm to 410 nm. The extinction coefficient used for the difference between the absorbance at 450 nm and 490 nm was 91 mM^{-1} cm^{-1}.

4.2.2.2.5 Spectrophotometry

All spectral measurements were performed using a Beckman 5230 recording spectrophotometer. In all cases, cuvettes with a pathlength of 1 cm were used in the compartment adjacent to the photomultiplier.

4.2.2.2.6 Metabolism of prizidilol

4.2.2.2.6.1 NADPH oxidation

The metabolism of prizidilol was measured by monitoring the rate of hepatic microsomal NADPH consumption in the presence of prizidilol in the presence and absence of $CO:O_2$ (80:20; v/v). Two Teflon-stoppered 4 ml cuvettes each containing 2.5 ml of microsomal suspension (2 mg protein/ml) were equilibrated to 25^{o}. Varying amounts of a solution of prizidilol (14.2 µM or 142 µM) were added to the sample cuvette. The reaction was initiated with the addition of 50 µl of NADPH (0.12 mM final concentration) to the sample cuvette. Background rates were measured with the presence of prizidilol and $CO:O_2$ (80:20). The decrease in absorbance at 340 nm was recorded. The reported rates for the oxidation of NADPH were calculated using $E_{340\ nm}$ of 6.2 mM^{-1} cm^{-1} (185).

4.2.2.2.6.2 Incubation mixtures

Prizidilol disappearance was assayed in incubation mixtures containing prizidilol (varying concentrations), NADPH generating system [NADPH (0.4 mM), glucose-6-phosphate (7.5 mM), glucose-6-phosphate dehydrogenase (0.5 units per ml), $MgCl_2$ (5 mM), EDTA (0.2 mM), nicotinamide (1 mM)] and hepatic microsomes (2 mg protein/ml) in 0.02 M Tris-HCl, pH 7.4. Where inhibitors of cytochrome P-450 were present, they were added to the microsomal suspension prior to the introduction of the prizidilol.

Incubations were carried out at 37^{o} in a B & T Laboratory Thermal Equipment shaking water bath with shaking at 60 oscillations per min.

The reaction was stopped by the addition of 100 µl of orthophosphoric acid solution (see 2.2.2.1.1.) and 100 µl pepsin solution. Then 100 µl of internal standard (2 µg/100 µl in water) and 50 µl of quinolin-3-al were added. The above samples were mixed vigorously with a vortexing mixer. Prizidilol was extracted and assayed as described in sections 2.2.3.2.1 and 2.2.3.2.3 respectively.

4.2.2.3 Statistical analyses

Student's t test for unpaired data (SD based on n-1) was utilized to calculate significant differences between means. $P < 0.01$, being significant, $p < 0.05$ being probably significant. Each piece of data represents the results of a single incubation mixture, not one of several determinations on a single incubation.

4.2.3 RESULTS

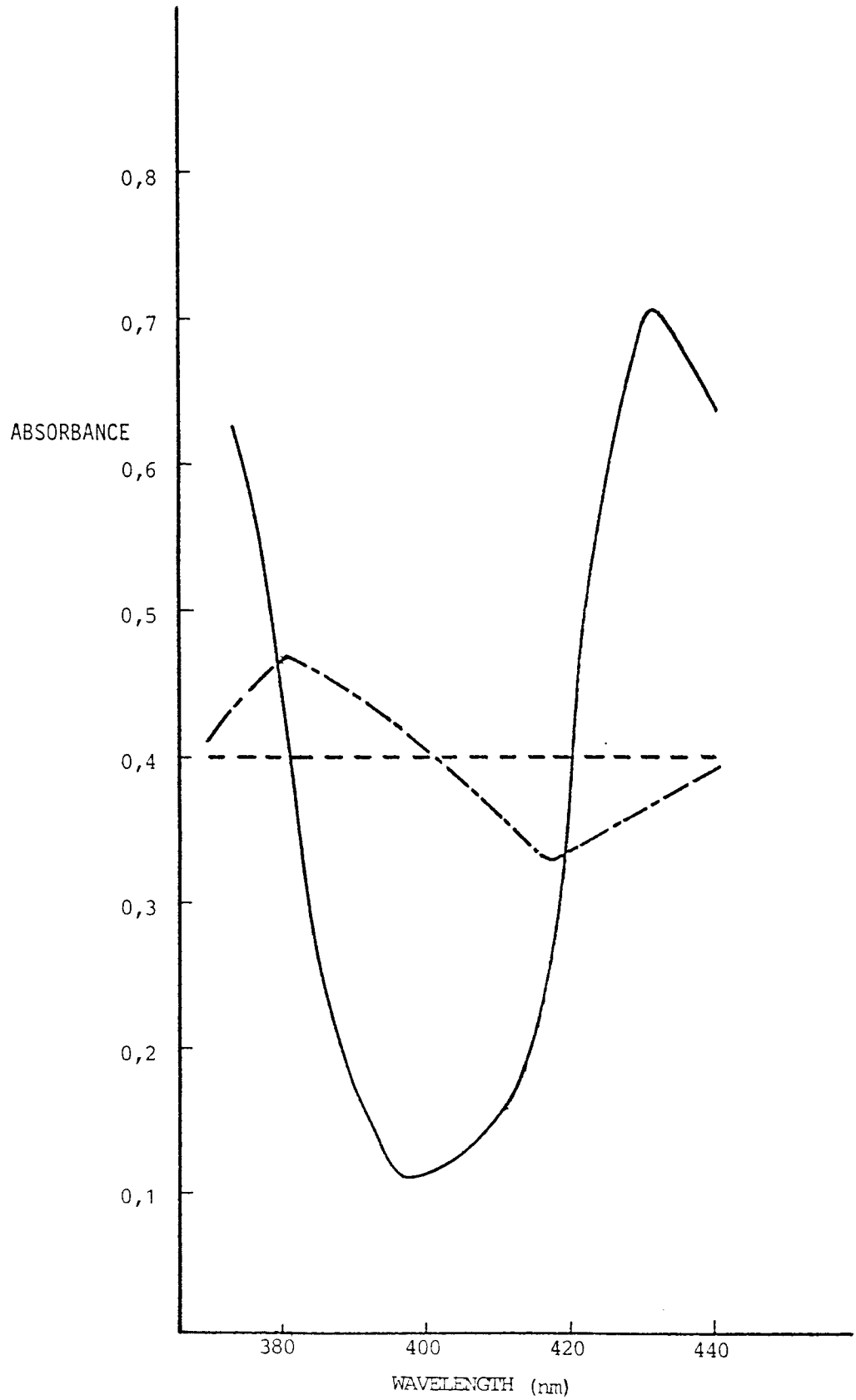
4.2.3.1 The binding of prizidilol to hepatic microsomal cytochrome P-450

Prizidilol exhibited two distinct types of difference spectra with hepatic microsomal cytochrome P-450. With low concentrations (1 - 23 μM) of prizidilol, a type I difference spectrum (λ_{min} = 416 nm: λ_{max} = 380 nm) was obtained. In contrast, high concentrations (24 - 189 μM) of prizidilol resulted in the production of a type II difference spectrum (λ_{min} = 396 nm; λ_{max} = 431 nm). (See Fig. 22). The effects of induction of different forms of cytochrome P-450 on the binding of prizidilol to hepatic cytochrome P-450 was assessed:

Hanes plots for the binding of prizidilol to cytochrome P-450 in hepatic microsomes from uninduced, phenobarbital, pregnenolone-16α-carbonitrile and β-naphthoflavone induced rats were biphasic, with K_s values for low concentrations (1 - 23 μM) (type I binding) and high concentrations (24 - 189 μM) (type II binding) of prizidilol being calculable (Figs. 23-26).

The effect of induction of different forms of cytochrome P-450 on the binding constant (K_s) and the maximum extent of binding (ΔA_{max}) for the binding of prizidilol to hepatic microsomal cytochrome P-450 in the type I and type II manner is presented in Tables 12 and 13 respectively. Pretreatment of rats with β-naphthoflavone, pregnenolone-16α-carbonitrile or phenobarbital did not result in significant alterations in the values of K_s, ΔA_{max} or ΔA_{max}/nmol microsomal cytochrome P-450 for the binding of prizidilol (low concentrations) to the type I site (Table 12). For the binding of the high concentration of prizidilol to the type II site, the value of K_s was significantly decreased following β-naphthoflavone or phenobarbital pretreatment,

Fig. 22 Difference spectra of hepatic microsomes (2 mg protein/ml) from phenobarbital treated rats at 30° (-----) no addition; (——) 190 µM prizidilol added to sample cuvette (—·——); 23 µM prizidilol added to sample cuvette.



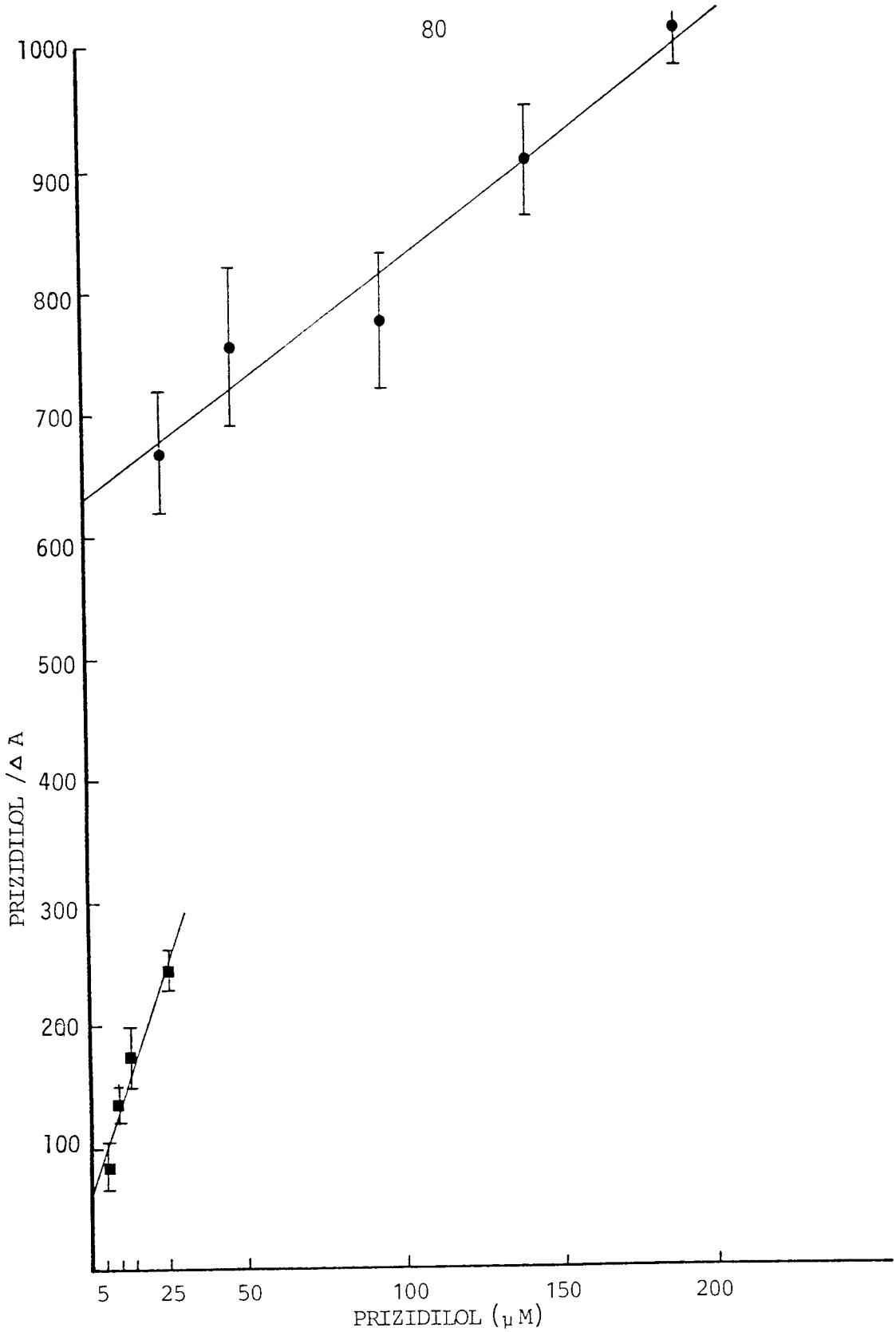


Fig. 23 Hanes plot for the binding of prizidilol to cytochrome P-450 in hepatic microsomes from untreated rats.

$\Delta A = A_{431}$ nm - A_{396} nm for high concentrations of prizidilol (>24 μM)(●)
K_S = 327 μM ΔA = 0.54

$\Delta A = A_{380}$ nm - A_{416} nm for low concentration of prizidilol (1-23 μM) (■)
K_S = 8.6 μM ΔA = 0.14

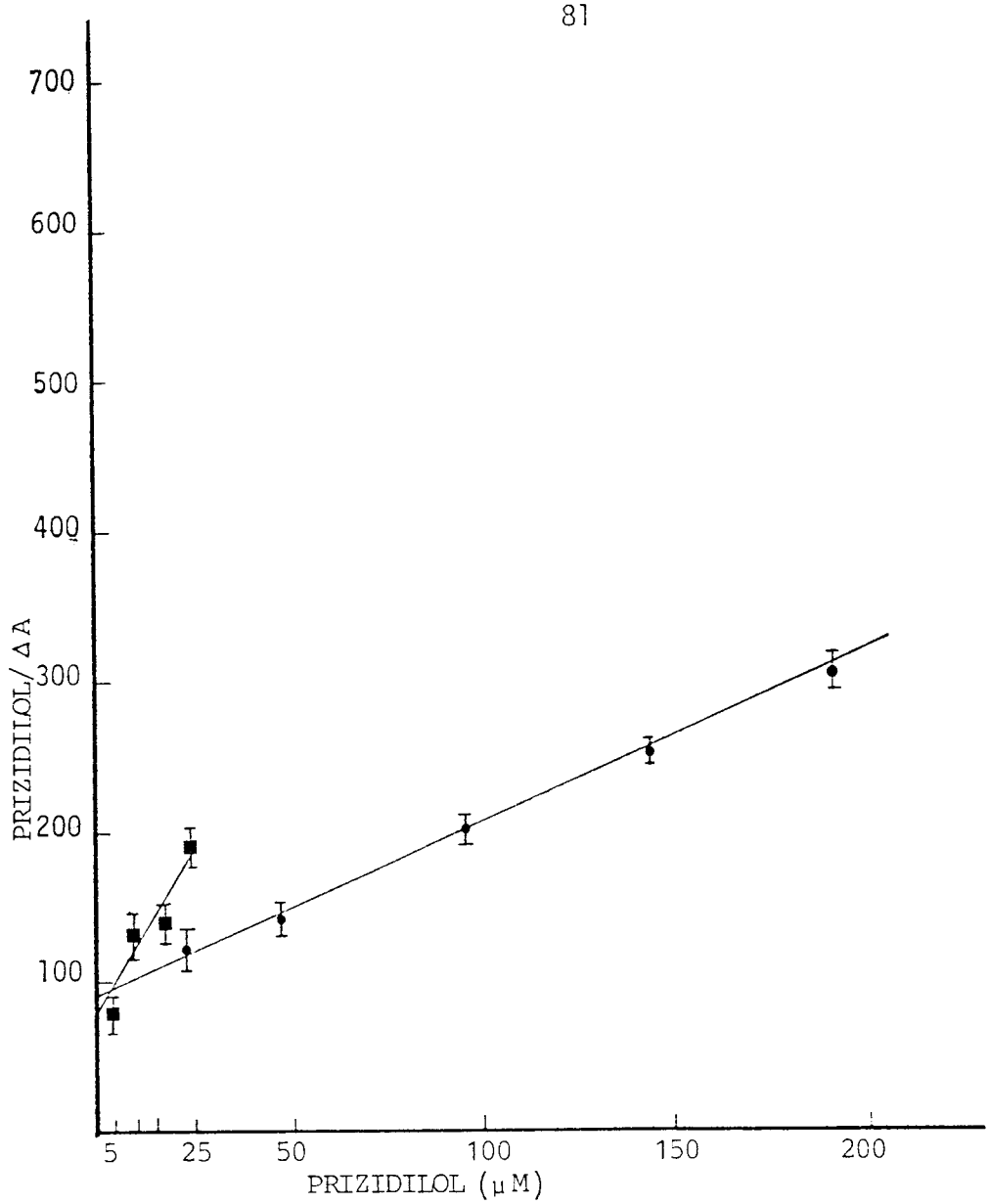


Fig. 24 Hanes plot for the binding of prizidilol to cytochrome P-450 in hepatic microsomes from phenobarbital pretreated rats.

$\Delta A = A_{431}$ nm - A_{396} nm for high concentration of prizidilol (>24 μM) (●)

K_S = 78.7 μM ΔA = 0.92

$\Delta A = A_{380}$ nm - A_{416} nm for low concentration of prizidilol (1-23 μM) (■)

K_S = 7.58 μM ΔA = 0.20

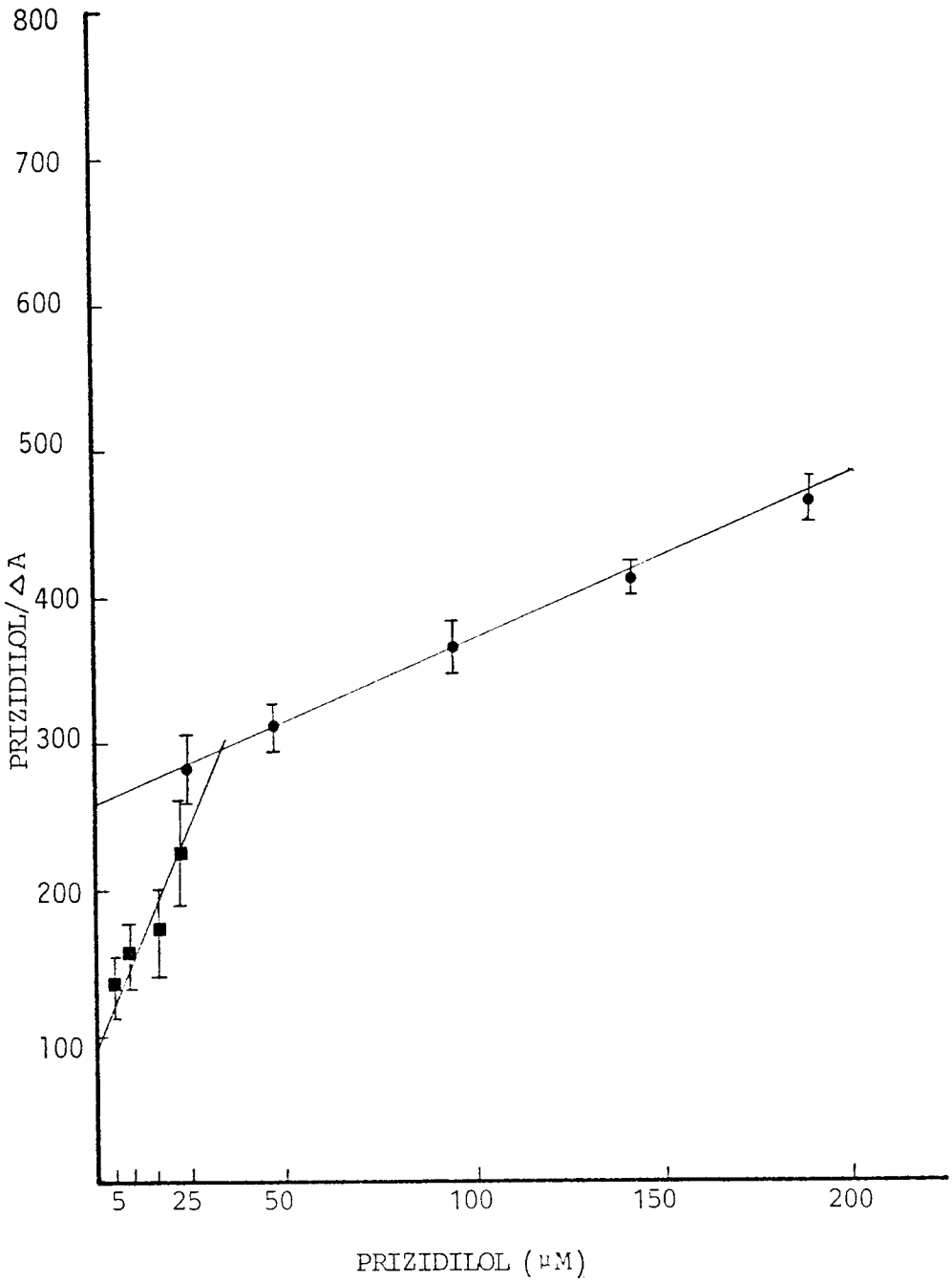


Fig. 25 Hanes plot for the binding of prizidilol to cytochrome P-450 in hepatic microsomes from pregnenolone-16α-carbonitrile pretreated rats.

$\Delta A = A_{431}$ nm - A_{396} nm for high concentration of prizidilol (>24 μM) (●)
K_S = 232 μM ΔA = 0.78

$\Delta A = A_{380}$ nm - A_{416} nm for low concentration of prizidilol (1-23 μM) (■)
K_S = 8.8 μM ΔA = 0.10

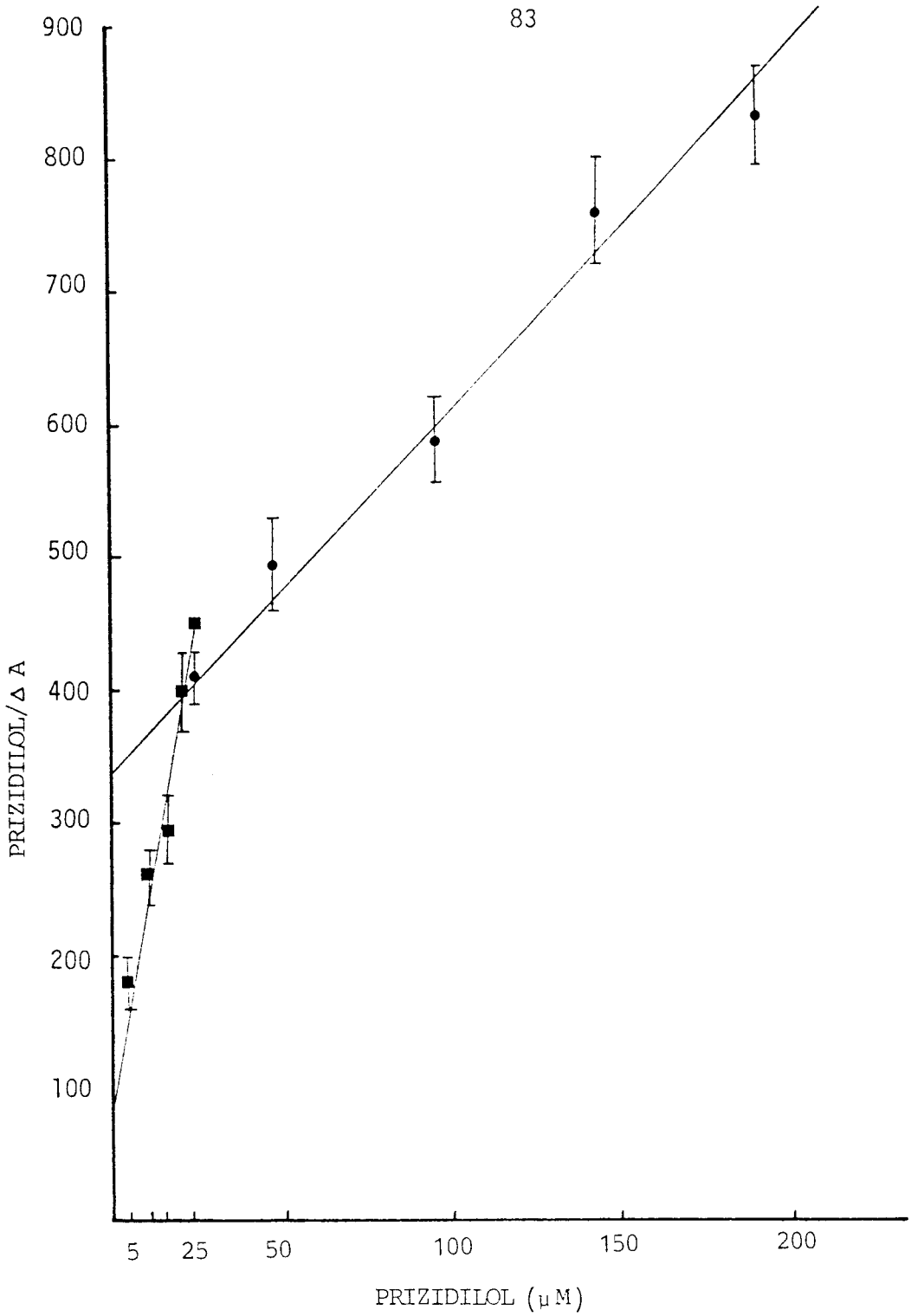


Fig. 26 Hanes plot for the binding of prizidilol to cytochrome P-450 in hepatic microsomes from β-naphthoflavone pretreated rats

$\Delta A = A_{431}$ nm - A_{396} nm for high concentrations of prizidilol (>24 μM) (●)

K_S = 120 μM ΔA = 0.38 .

$\Delta A = A_{380}$ nm - A_{416} nm for low concentrations of prizidilol

K_S = 5.6 μM ΔA = 0.07 (1-23 μM) (■)

Table 12 Effect of induction on the binding of low concentrations of prizidilol (1-23 μM) to the type I site of hepatic microsomal cytochrome P-450.

Inducing agent	Cytochrome P-450 (nmol/mg microsomal protein)	K_s (μM)	ΔA_{max}	ΔA_{max}/nmol cytochrome P-450
none	1.21 ± 0.11	5.2 ± 1.6	0.15 ± 0.09	0.13 ± 0.07
βNF	1.44 ± 0.19	3.5 ± 3.0	0.06 ± 0.02	0.04 ± 0.01
PCN	1.52 ± 0.22 †	7.3 ± 1.4	0.10 ± 0.01	0.06 ± 0.01
PB	2.61 ± 0.29 *	5.7 ± 1.3	0.20 ± 0.07	0.07 ± 0.02

Results are means ± S.D. for determinations in triplicate on two or more separate groups of three animals each.
The abbreviations used are:
βNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.

$\Delta A = A_{380\ nm} - A_{416\ nm}$

*Differs from corresponding value for microsomes from uninduced rat $p < 0.01$.

†Probably differs from corresponding value for microsomes from uninduced rats $p < 0.05$.

Table 13 Effect of induction on the binding of high concentrations of prizidilol (24-189 μM) to the type II site of hepatic microsomal cytochrome P-450.

Inducing agent	Cytochrome P-450 (nmol/mg microsomal protein)	K_s (μM)	ΔA_{max}	ΔA_{max}/nmol cytochrome P-450
none	1.21 ± 0.11	300 ± 81	0.48 ± 0.08	0.37 ± 0.04
βNF	1.44 ± 0.19	136 ± 23 †	0.46 ± 0.12	0.30 ± 0.04
PCN	1.52 ± 0.22 †	224 ± 10	0.85 ± 0.11 †	0.50 ± 0.11
PB	2.61 ± 0.29 *	76 ± 5 *	0.91 ± 0.07 *	0.37 ± 0.01

Results are means ± S.D. for determinations in triplicate on two or more separate groups of three animals each.
The abbreviations used are:
βNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.

$\Delta A = A_{431\ nm} - A_{396\ nm}$

*Differs from corresponding value for microsomes from uninduced rat $p < 0.01$.

†Probably differs from corresponding value for microsomes from uninduced rats $p < 0.05$.

but not pregnenolone-16α-carbonitrile treatment. The value of ΔA_{max} was significantly increased following pregnenolone-16α-carbonitrile or phenobarbital treatment but not β-naphthoflavone treatment (Table 13). For the binding of prizidilol to the type II site, there were no significant changes in ΔA_{max}/nmol cytochrome P-450, following any type of pretreatment.

4.2.3.2 Prizidilol stimulation of hepatic microsomal CO-inhibitable NADPH oxidation

The rate of NADPH oxidation in the presence of a xenobiotic is in theory a valid method for measuring the metabolism of that compound by hepatic microsomal cytochrome P-450. In practice, it is found that significant background rates of NADPH oxidation occur. Therefore, the observed rates of NADPH oxidation are corrected for the rate in the presence of the drug and $CO:O_2$ (80:20). In the latter situation, the CO will inhibit all cytochrome P-450 dependent metabolism and allow measurement of a background rate which can be subtracted for the observed rate in the presence of drug and absence of CO. Thus the rate of prizidilol stimulated CO-inhibitable NADPH oxidation should provide an accurate reflection of the metabolism of prizidilol by hepatic microsomal cytochrome P-450.

Prizidilol stimulated CO-inhibitable NADPH oxidation in hepatic microsomes from uninduced and pretreated rats (Fig. 27) (Tables 14 and 15). The effect of pretreatment of rats with inducing agents for cytochrome P-450 on the initial rates of hepatic microsomal oxidation of NADPH in the presence of prizidilol, with and without $CO:O_2$ (80:20), is shown in Tables 14 and 15. With a low concentration of prizidilol (14.2 μM), pretreatment of rats with β-naphthoflavone or pregnenolone-16α-carbonitrile did not significantly alter the rate of CO-inhibitable NADPH oxidation per mg microsomal protein. But microsomes from rats pretreated with phenobarbital exhibited significantly increased

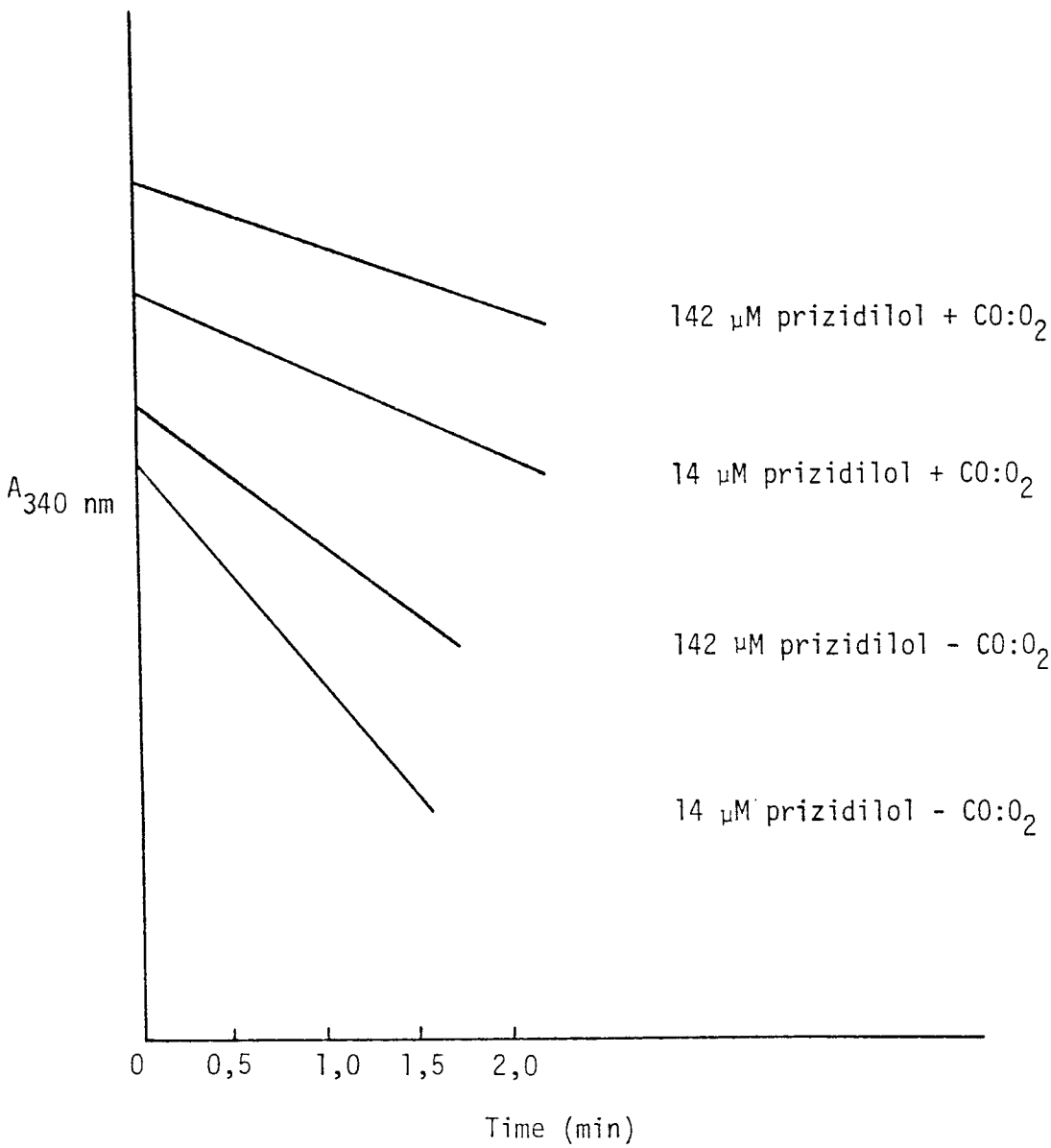


Fig. 27 Prizidilol stimulated NADPH oxidation with hepatic microsomes from pretreatment of rat with phenobarbital in the presence and absence of $CO:O_2$.

Table 14 Effect of induction of different forms of cytochrome P-450 on the hepatic microsomal NADPH oxidation of prizidilol at low concentration (14 μM).

Inducing agent	Initial rate of microsomal NADPH oxidation			
	nmol/min/mg microsomal protein			nmol/min/nmol cytochrome P-450
	In absence of CO : O_2	In presence of CO : O_2 (80 : 20 v/v)	CO - Inhibitable NADPH oxidation	CO - Inhibitable NADPH oxidation
None	2.61 ± 0.19	1.29 ± 0.12	1.32 ± 0.07	1.04 ± 0.09
βNF	3.19 ± 0.18 †	1.82 ± 0.19	1.37 ± 0.11	0.92 ± 0.09
PCN	3.52 ± 0.25 †	1.92 ± 0.24	1.60 ± 0.18	1.07 ± 0.05
PB	4.88 ± 0.21 *	2.27 ± 0.17 *	2.60 ± 0.19 *	1.11 ± 0.12

Values reported are means ± S.D. for experiments performed in duplicate or more with two different preparations of hepatic microsomes.
Experimental details are given in Section 4.2.4.2.
Abbreviations used are:
βNF, β-naphthcflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.

*Differs from corresponding value for microsomes from uninduced rat $p < 0.01$.

†Probably differs from corresponding value for microsomes from uninduced rats $p < 0.05$.

Table 15 Effect of induction of different forms of cytochrome P-450 on the hepatic microsomal NADPH oxidation of prizidilol at high concentration (142 μM).

Inducing agent	Initial rate of microsomal NADPH oxidation			
	nmol/min/mg microsomal protein			nmol/min/nmol cytochrome P-450
	In absence of CO : O_2	In presence of CO : O_2 (80 : 20 v/v)	CO - Inhibitable NADPH oxidation	CO - Inhibitable NADPH oxidation
None	2.64 ± 0.14	1.12 ± 0.11	1.51 ± 0.04	1.25 ± 0.14
βNF	2.46 ± 0.29	1.61 ± 0.24	0.85 ± 0.05 *	0.56 ± 0.03 *
PCN	2.62 ± 0.16	1.65 ± 0.21 †	0.97 ± 0.13 †	0.67 ± 0.15 †
PB	3.63 ± 0.14 *	2.08 ± 0.07 *	1.54 ± 0.16	0.63 ± 0.02 *

Values reported are means ± S.D. for experiments performed in duplicate or more with two different preparations of hepatic microsomes.
Experimental details are given in Section 4.2.4.2.
Abbreviations used are:
βNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.

*Differs from corresponding value for microsomes from uninduced rat $p < 0.01$.

†Probably differs from corresponding value for microsomes from uninduced rats $p < 0.05$.

rates of NADPH oxidation per mg microsomal protein. No type of pretreatment altered the rates of CO-inhibitable NADPH oxidation per nmol cytochrome P-450 (Table 14).

With a high concentration of prizidilol (142 μM), the rate of prizidilol stimulated CO-inhibitable NADPH oxidation per mg protein was not affected by pretreatment of the rats with phenobarbital, but was significantly diminished following pre-treatment with pregnenolone-16α-carbonitrile or β-naphthoflavone. CO-inhibitable NADPH oxidation per nmol cytochrome P-450 was significantly diminished by all pretreatments (Table 15). For microsomes from untreated rats the rates of CO-inhibitable NADPH oxidation were identical for the low and high concentrations of drug. However, CO-inhibitable NADPH oxidation per mg protein and per nmol cytochrome P-450 was significantly lower with 142 μM prizidilol versus 14 μM prizidilol for all types of pretreatment (Tables 14 and 15).

4.2.3.3 Hepatic microsomal metabolism of prizidilol

The disappearance of prizidilol in incubations containing NADPH-generating system and hepatic microsomes from uninduced or pretreated rats is shown in Fig. 28. The disappearance of prizidilol with microsomes from phenobarbital-pretreated rat was linear for 10 minutes. With microsomes from pregnenolone-16α-carbonitrile or β-naphthoflavone treated and control rats, prizidilol was lost rapidly for a short period (up to 3 min) and subsequently (between 3 and 20 min), the loss of prizidilol was linear. The time periods used for incubations to determine K_m and V_{max} values were 10 min for microsomes from phenobarbital treated rats and 20 min in all other cases.

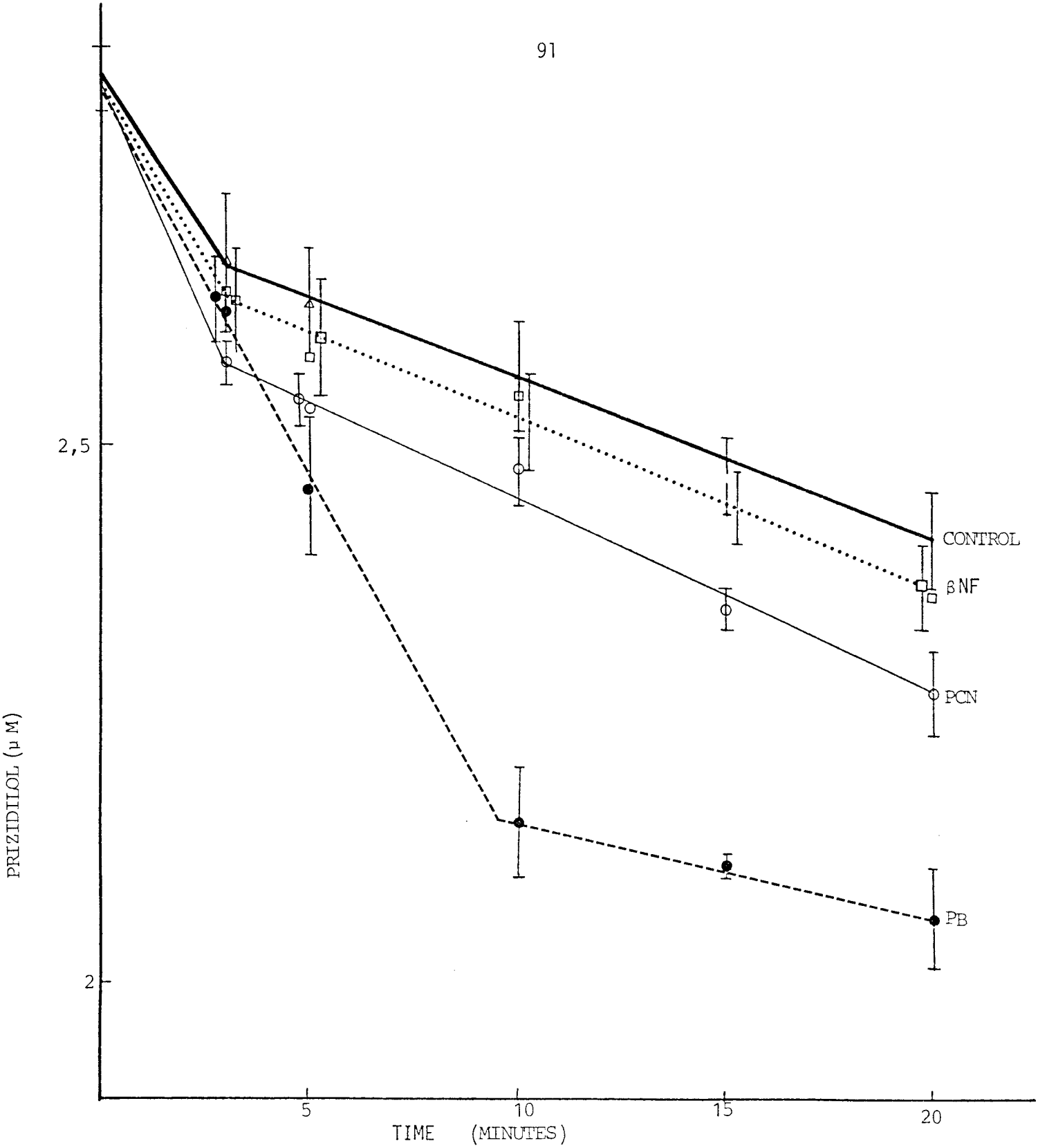


Fig. 28 Effect of incubation with NADPH-generating system plus hepatic microsomes (2 mg protein/ml) from untreated or pretreated rats on prizidilol concentration as a function of time. Abbreviations used are βNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.

4.2.3.3.1 Inhibition of the metabolism of prizidilol in hepatic microsomal cytochrome P-450.

The effect of inhibitors of cytochrome P-450 on the disappearance of prizidilol in incubation mixtures containing NADPH-generating system and hepatic microsomes from rats pretreated with phenobarbital is given in Table 16. Metyrapone (1 mM), SKF 525-A (200 mM) and $CO:O_2$ (80:20 v/v) all effectively inhibited the metabolism of prizidilol by hepatic microsomes. As inhibitors of prizidilol disappearance, metyrapone and SKF 525-A were equivalent while CO was slightly less effective.

4.2.3.3.2 Prizidilol disappearance

Hanes plots for the disappearance of prizidilol from incubations of NADPH-generating system plus microsomes from rats pretreated with phenobarbital, pregnenolone-16α-carbonitrile, β-naphthoflavone and uninduced rat are shown in Figs. 29-32. Pretreatment of rats with phenobarbital increased the values of K_m and V_{max} (nmol/min/mg microsomal protein) relative to microsomes from untreated rats (Table 17). Pretreatment of rats with β-naphthoflavone or pregnenolone-16α-carbonitrile did not significantly alter the values of K_m or V_{max} (nmol/mg microsomal protein/min) (Table 17). The values of V_{max}/ cytochrome P-450/min were not affected by the pretreatment of rats with any of the inducing agents (Table 17).

Table 16 Effect of inhibitors of cytochrome P-450 on prizidilol metabolism by hepatic microsomes from phenobarbital induced rats.

Inhibitor	Prizidilol concentration after 20 min incubation (mM)	Percent (%) inhibition of prizidilol disappearance
None	2.01	0
$CO:O_2$(80:20 v/v)	2.48	53.5 ± 1.8*
Metyrapone (1 mM)	2.68	78.5 ± 12.7*
SKF 525-A (200 mM)	2.66	76.5 ± 17.7*

Values reported are means ± S.D. for experiments performed in duplicate with two separate preparations of hepatic microsomes. Experimental details are given in Section 4.2.3.3.1. Initial concentration of prizidilol averaged 2.84 mM.

* Differs significantly from values for incubations in the absence of inhibitor $p < 0.01$.

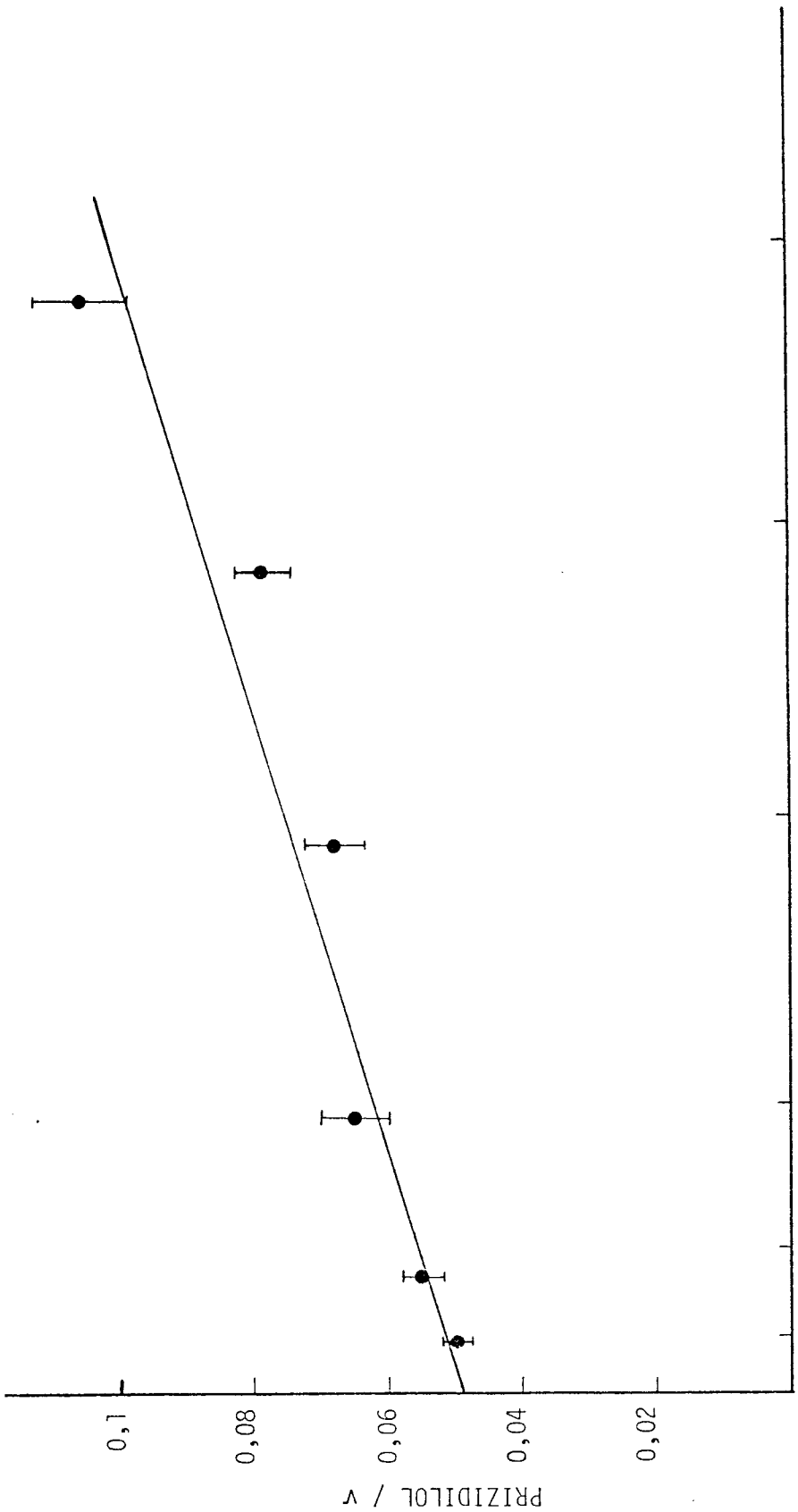


Fig. 29 Hanes plot for prizidilol disappearance with hepatic microsomes from phenobarbital pretreated rats plus NADPH-generating system.
v = rate of prizidilol disappearance in pmol/min/mg microsomal protein
K_m = 4.1 µM V_{max} = 32 pmol/min/mg microsomal protein
= 12 pmol/min/nmol cytochrome P-450.

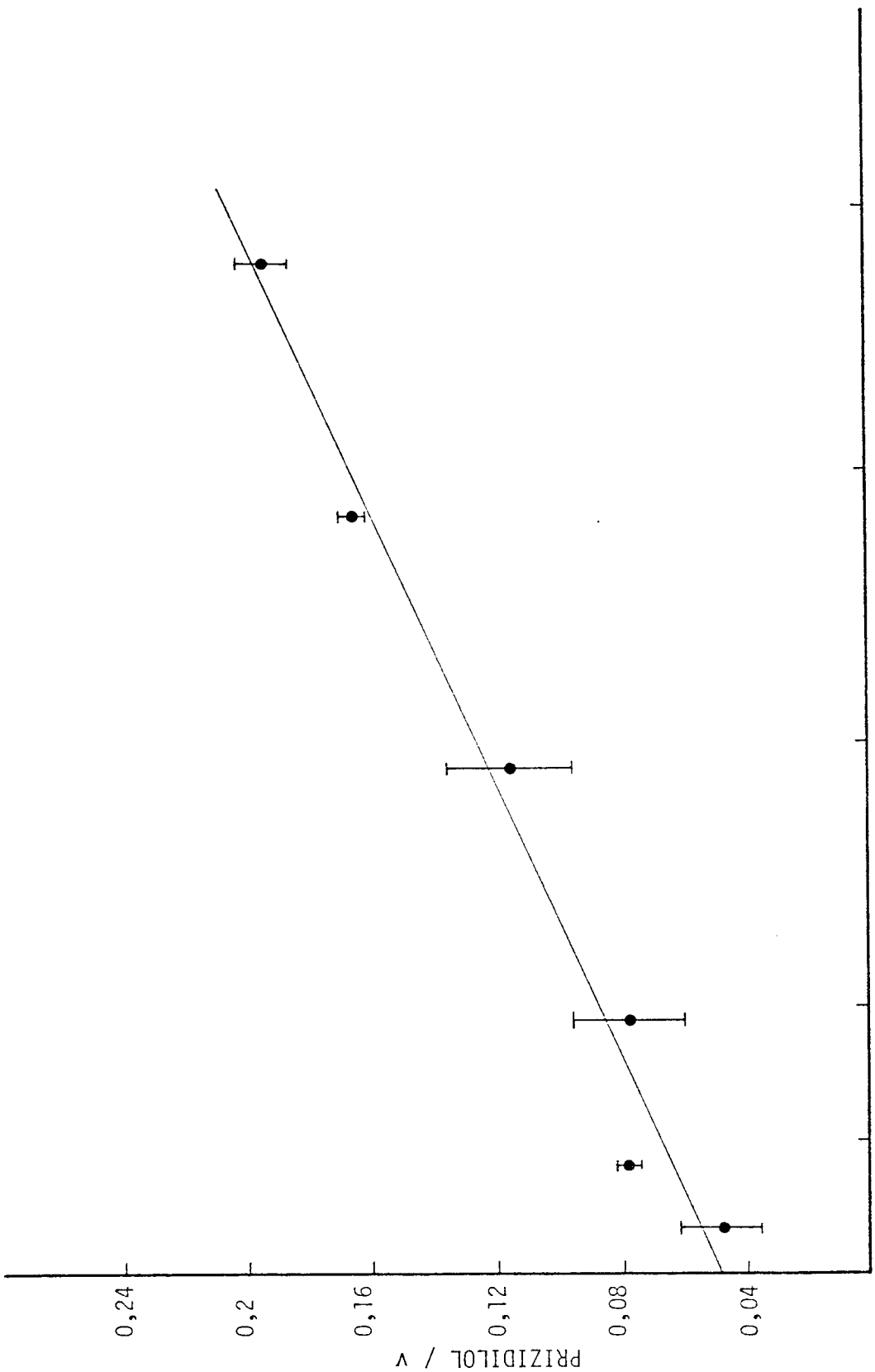


Fig. 30 Hanes plot for prizidilol disappearance with hepatic microsomes from Pregnenolone-16α-carbonitrile pretreated rats plus NADPH-generating system.
v : rate of prizidilol disappearance in pmol/min/mg microsomal protein
K_m = 1.37 μM V_{max} = 10.8 pmol/min/mg microsomal protein
= 7 pmol/min/nmol cytochrome P-450.

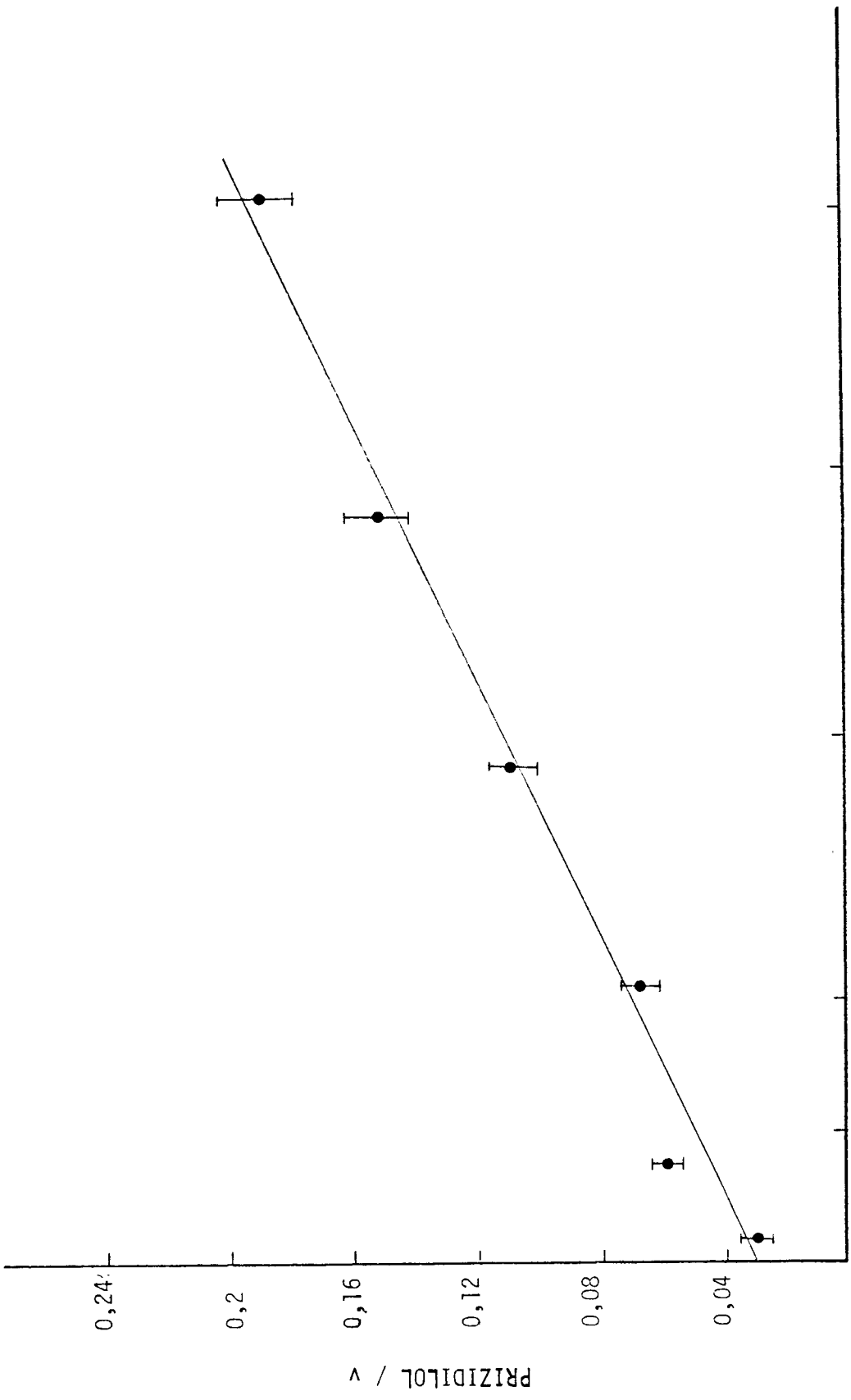


Fig. 31 Hanes plot for prizidilol disappearance with hepatic microsomes from β-naphthoflavone pretreated rats plus NADPH-generating system.
v : rate of prizidilol disappearance in pmol/min/mg microsomal protein
K_m = 0.82 µM V_{max} = 10.4 pmol/min/mg microsomal protein
= 7.2 pmol/min/nmol cytochrome P-450.

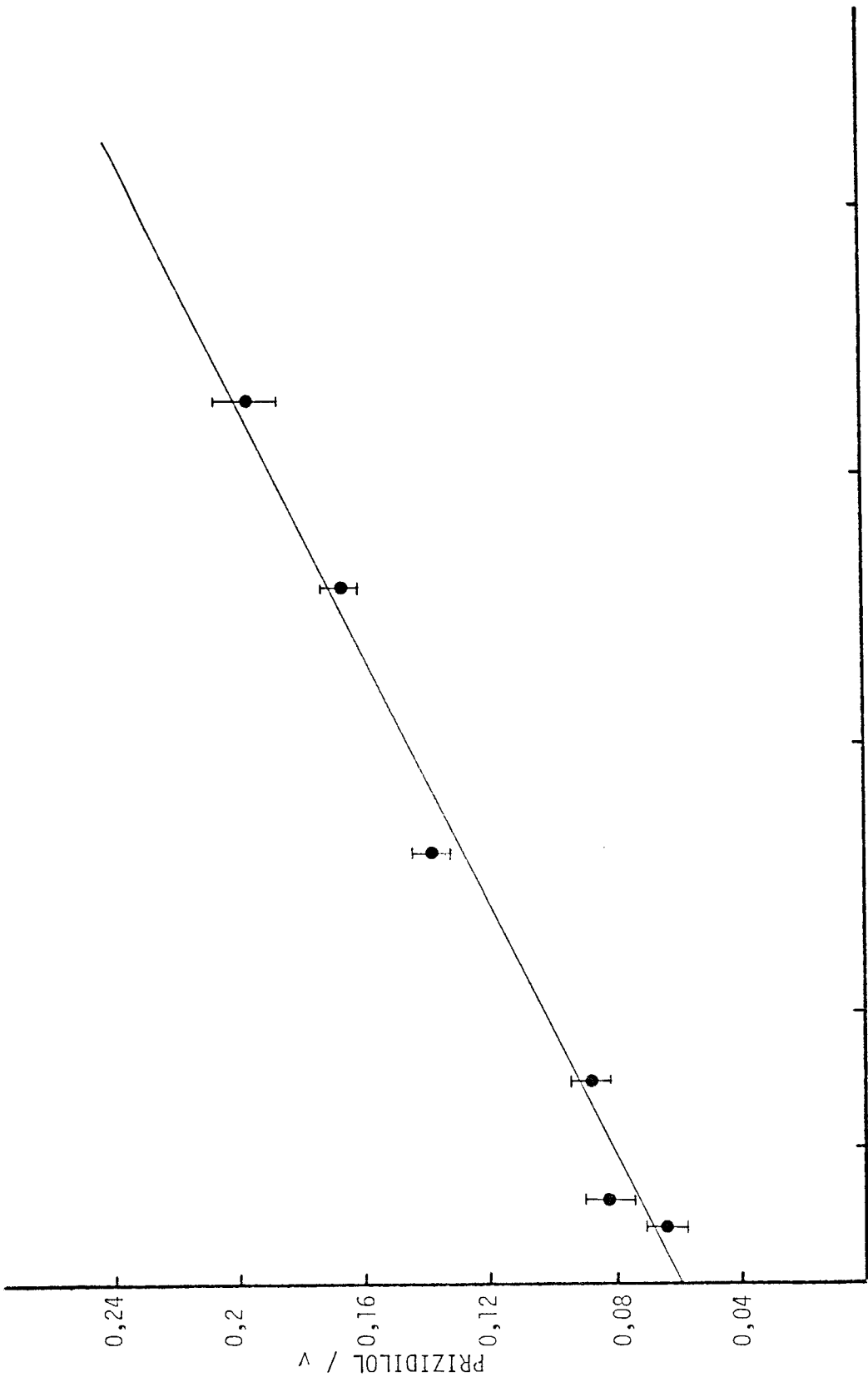


Fig. 32 Hanes plot for prizidilol disappearance with hepatic microsomes from untreated rats plus NADPH-generating system.
v : rate of prizidilol disappearance in pmol/min/ mg microsomal protein
K_m = 1.61 µM V_{max} = 10 pmol/min/mg microsomal protein
V_{max} = 7.2 pmol/min/nmol cytochrome P-450.

Table 17 Effect of induction of different forms of cytochrome P-450 on the metabolism of prizidilol by microsomal cytochrome P-450.

Induction	K_m (μM)	V_{max} (pmol/min)	
		pg microsomal protein	pmol cytochrome P-450
None	1.30 ± 0.45	8.6 ± 2.0	8.2 ± 1.4
βNF	1.04 ± 0.19	9.6 ± 1.6	7.0 ± 0.6
PCN	1.64 ± 0.51	10.4 ± 0.2	7.2 ± 3.2
PB	3.81 ± 0.55*	28.4 ± 7.2 †	11.4 ± 3.4

Results are means ± S.D. for determinations in triplicate on two or more separate groups of three animals each.
Abbreviations used are:
βNF, β-naphthoflavone; PCN, pregnenolone-16α-carbonitrile; PB, phenobarbital.

*Differs from corresponding value for microsomes from uninduced rats $p < 0.01$.

†Probably differs from corresponding value for microsomes from uninduced rats $p < 0.05$.

4.3 GSH S-Transferases

4.3.1 Introduction

Glutathione (GSH) S-transferase B, the major GSH S-transferase of rat liver, was first described in 1959 (186). The GSH S-transferases catalyze the conjugation of GSH with a large number of compounds bearing an electrophilic carbon to form a corresponding thioether. A number of substrates for the GSH S-transferases are shown in Fig. 33. The thioether adducts are subsequently acted upon by other enzymes which remove glutamate and glycine and which acetylate the amino group of the resultant thioether of cysteine. The N-acetyl cysteine thioether derivative is known as the mercapturic acid derivative of the substrate and is usually a non-toxic, water soluble product which is readily excreted. This pathway is shown in Fig. 34. Mercapturic acids are generally the major products of drugs primarily metabolised by GSH conjugation.

In the early nineteen sixties, the GSH S-transferases were implicated in the metabolism of insecticides (187). The GSH S-transferases catalyse two main types of reaction with these insecticides which include organophosphorus triesters, organothiocyanates and chlorinated hydrocarbons, i.e. the formation of S-alkyl or S-aryl conjugates, both of which result in detoxication, the former appearing to be the major metabolic pathway *in vivo* (188). The insect GSH S-transferases exhibit multiplicity with insecticides as substrates (189), i.e. several enzymes may be active with a specific compound. Each enzyme may also display activity with several different types of compounds as do the transferase

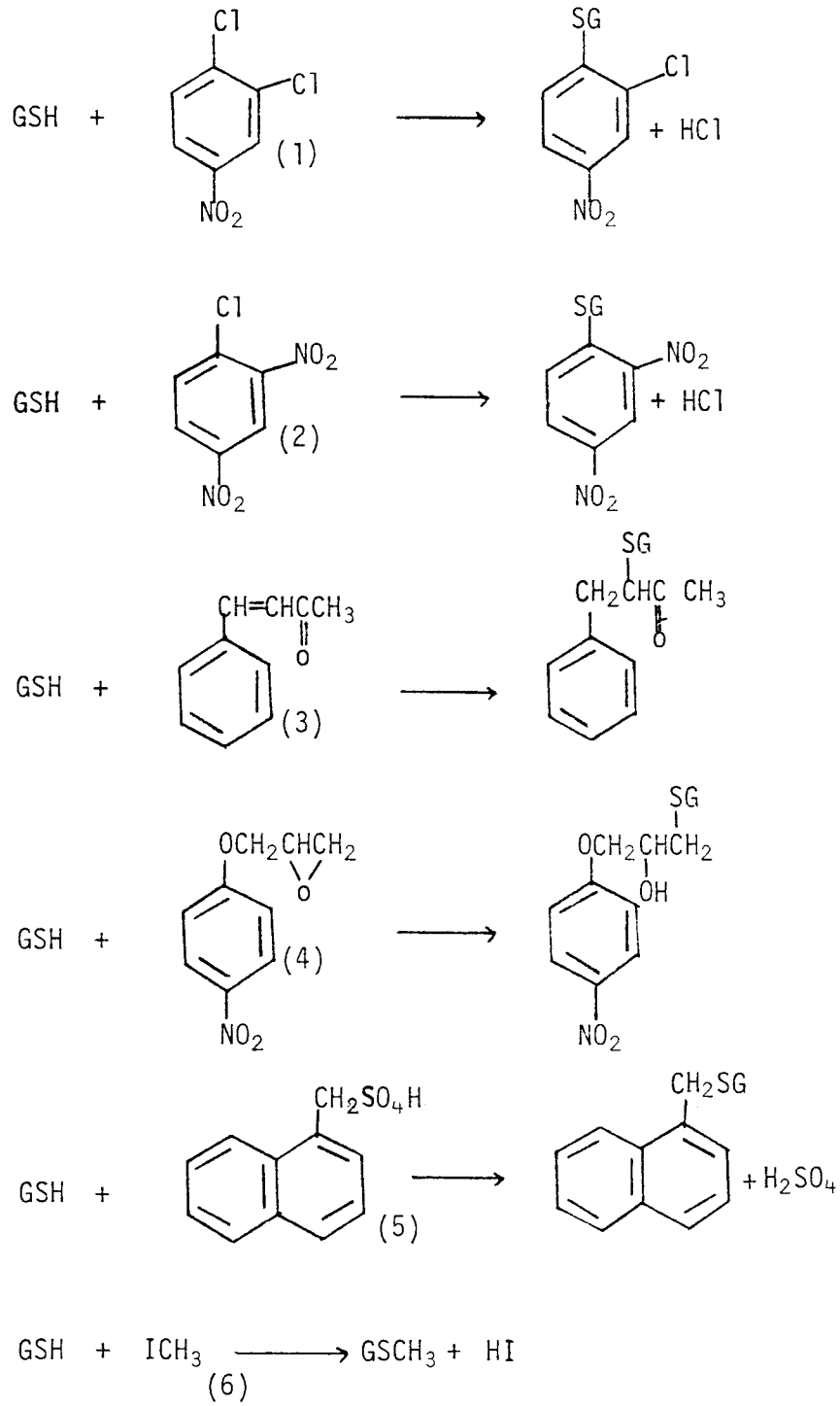


Fig. 33 Substrates for the GSH S-transferases.
The reactions shown are between GSH and (1) 1.2-dichloro-4-nitrobenzene (186); (2) 1-chloro-2,4-dinitrobenzene (both are aryl substrates) (190); (3) trans-4-phenyl-3-butan-2-one (an alpha, beta-unsaturated ketone) (191); (4) 1,2-epoxy-3-(p-nitrophenoxy) propane (an epoxide) (192); (5) menapthyl sulphate (a sulphate ester) (193) and (6) iodomethane (an alkyl substrate).

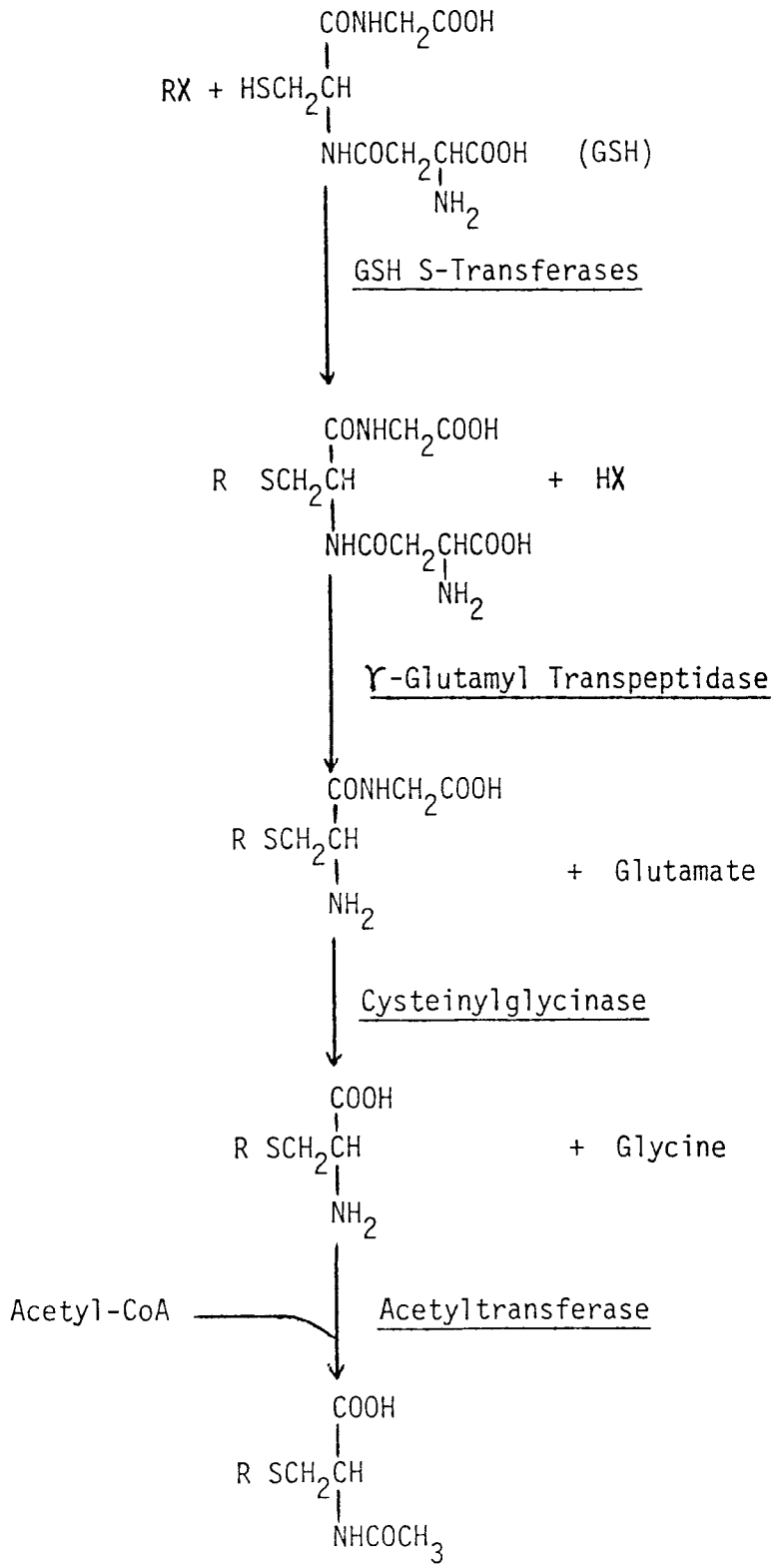


Fig. 34 Metabolic pathway for the biosynthesis of the mercapturic acid. RX represents an electrophilic compound. [Based on Boyland and Chasseaud 1969 (186)]

enzymes of rat liver (184). Multiple forms of GSH S-transferase exist in all species studied. For example, rat liver has seven isoenzymes, which vary in amino acid composition and isoelectric point but are similar in size and overlap in substrate specificity. They are thought to be the product of at least 6 genes.

4.3.1.1 Rat liver cytosol GSH S-transferases

Generally, hepatic tissue has greater GSH S-transferase activity than extrahepatic tissue. However, GSH S-transferase activity was also detected in kidney, small intestine mucosa, testis, ovary and adrenal gland. Lesser quantities were present in salivary gland, gastric mucosa of colon, pancreas, pituitary, lung, thyroid, bladder, heart and seminal vesicles (195).

The GSH S-transferases represent approximately 10% of soluble rat liver protein. The major transferase of rat liver is GSH S-transferase B which accounts for 5% of the total cytosolic protein (196).
The properties of seven rat cytosol GSH S-transferases are listed in Table 18. In one form of nomenclature, the isozymes are designated as E,D,C,B,A and AA, in order of their elution from carboxymethyl-cellulose cation exchange columns (197, 198). The separation depends on the observation that each GSH S-transferase has a distinct isoelectric point within the range pH 7 to 10. An additional transferase, GSH S-transferase M, which binds to diethylaminoethyl-cellulose (DEAE-cellulose) at pH 8.0 and catalyzes the reaction of GSH to 1-menapthyl sulfate, has been purified to homogeneity (199).

Table 18 Multiple forms of the rat liver GSH S-transferases - their properties and substrate specificities.

PROPERTY	AA	A	B	C	D	E	M
M Weights	45000	46000	47000	47000	-	40000	-
s/u	25000 YcYc	23000 YbYb	22 + 25000 YaYc	23500 YbYb	-	-	-
Reaction with antibody	none	A, C	B	A, C	-	B	-
Isoelectric point	10	8.9	9.8	8.0	-	7.3	-
Relative concentration	0.14	0.22	1.0	0.52	0.02	0.1	-
Substrate specific activities (μmol/min/mg)							
CDNB	14	62	11	10	-	0.01	-
DCNB	0.008	4.3	0.003	2.0	-	0	0.004
IM	1.4	0	0.59	0	-	8.9	0
EA	0.3	0	0.26	0.11	-	0	-
pNBC	0.09	11.4	0.1	10.2	-	4.1	0.5
tPBO	0	0.02	0.001	0.40	-	0	-
SO							
MS	-	0	0.004	-	-	-	0.1
BSP		0.53	0.006				
Prostaglandin A_1		0.013	0.005	0.021			
Δ^5-Androstene-3, 17, dione	.001	0.01	1.87	0.005			

Abbreviations used are:

GSH	-	reduced glutathione
CDNB	-	1-chloro-2.4-dinitrobenzene
DCNB	-	2.4-dichloronitrobenzene
PNBC	-	p-nitrobenzyl chloride
SO	-	styrene oxide
EA	-	ethacrynic acid
IM	-	iodomethane
MS	-	1-menapthyl sulphate
tPBO	-	trans-4-phenylbut-3-en-2-one
BSP	-	sulphobromopthalein or disodium phenolterabromophthalein sulphonate

[Based on Jakoby et al 1976 (213) Jakoby and Habig 1980 (214) and Habig and Jakoby 1981 (215)]

Rat GSH S-transferases A and C are structurally and immunologically similar, and they differ only in isoelectric point. None of the other rat liver GSH S-transferases appear to share immunological identity. The specific activity of GSH S-transferases B and AA with the substrate CDNB (1-chloro-2.4-dinitrobenzene) is several times greater than that with DCNB (2,4 dichloronitrobenzene) while GSH S-transferases A and C both have considerable activity with DCNB. GSH S-transferases A and C differ in their ability to catalyze the reaction of GSH with the substrate trans-4-phenyl-3-bute-2-one, the specific activity of GSH S-transferase C being 20-fold higher than A (200). The amino acid composition of rat GSH S-transferases A and C are similar (201), while the other transferases showed much less similarity (202).

4.3.1.2 Human GSH S-transferases

Five basic GSH S-transferases have been isolated from human liver. These are designated alpha, beta, gamma, delta and epsilon in order of increasing isoelectric point (pI 7.8 - 8.8) (203). These proteins have similar amino acid compositions and molecular weights (48000 daltons), and each is a homodimer of two identical subunits. The delta protein accounts for the major portion of the hepatic transferases. All five basic human transferases react similarly with monospecific antibody prepared against each of the other four proteins (204). In addition to the basic transferases, acidic transferases have been demonstrated in human liver (205, 206). Significant differences in catalytic properties of cationic, anionic and neutral forms have been noted (207, 208). GSH S-transferase activity has also been documented in human erythrocytes (209), lymphocytes, peripheral leucocytes (210) and placenta (211, 212).

4.3.2 Experimental

4.3.2.1 Materials

All reagents used were of analytical grade and were purchased as detailed below: Human serum albumin (crystalline) from Miles Laboratories (Pty), Ltd., Goodwood, South Africa. Glutathione (Type IV) was obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

4.3.2.2 Methods

4.3.2.2.1 Preparation of cytosol

Male Sprague-Dawleys rats (190-210 grams) were sacrificed by cervical fracture. The liver was rapidly removed and following initial fragmentation with scissors, homogenization was carried out in 0.01 M sodium phosphate buffer pH 7.4/0.25 M sucrose; sufficient buffer was added to yield a 25% (w/v) homogenate. The initial centrifugation was at 27000 g for 30 minutes in a Sorvall RC-5 super speed centrifuge. The supernatant fraction was carefully removed without disturbing the floating lipid layer and centrifuged at 100000 g for 120 minutes in a Beckman model L5.35 ultracentrifuge. The supernatant was removed as before and stored on ice. The cytosol (post-microsomal supernatant) was finally added to the test tube at a concentration of 3.5 mg protein per ml.

4.3.2.2.2 Prizidilol disappearance in rat liver cytosol

Prizidilol (1.7 μM) was added to cytosol (3.5 mg/ml protein) and mixed vigorously with a vortexing stirrer. The reaction was initiated with GSH (10 mM) then allowed to proceed at 37^{o}C for 15 or 30 minutes

with shaking at 60 oscillations per minute. The estimation of prizidilol was described in sections 2.2.3.2.1 and 2.2.3.2.3.

4.3.3 Results

The effect of incubation of prizidilol with rat liver cytosol and GSH is reported in Table 19. There is not a statistically significant decrease in the concentration of prizidilol after incubation of prizidilol with cytosol for 15 or 30 minutes. These results indicate that rat liver cytosol GSH S-transferases do not effectively catalyze the metabolism of prizidilol, and that prizidilol does not react non-enzymically with GSH (<0.7 nmol/ml/30 min).

Table 19 The disappearance of prizidilol with rat liver cytosol

Composition of incubation mixture	prizidilol concentration (μM)			% loss at	
	0 min	15 min	30 min	15 min	30 min
Rat liver cytosol + GSH + prizidilol	1.67 ± 0.02	1.66 ± 0.05	1.60 ± 0.07	0.6	4.2
Buffer + GSH + prizidilol	1.71 ± 0.03	1.65 ± 0.05	1.66 ± 0.11	3.5	2.9
Buffer + prizidilol	1.69 ± 0.02	1.63 ± 0.04	1.62 ± 0.05	3.5	4.1

Results are means ± S.D. for assays performed in triplicate or more on each of three separate groups of three untreated animals.
Experimental details are given in Section 4.3.2.2.2.
The abbreviation used is GSH, glutathione.

4.4 Acetyltransferases

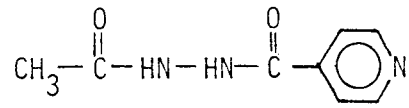
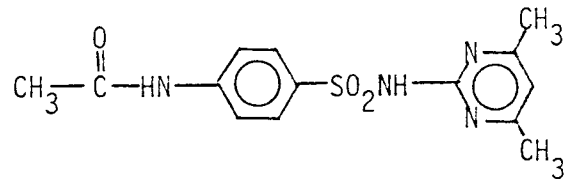
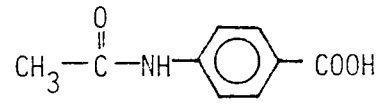
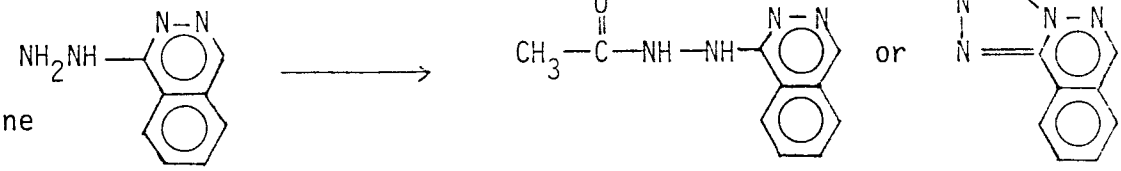
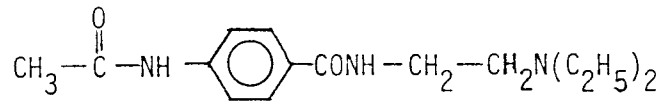
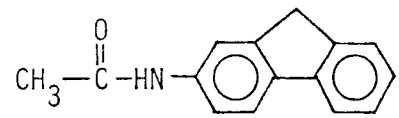
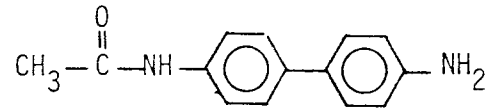
4.4.1 Introduction

Acetylation is a major route for the metabolism of aromatic amines in many different species (216). The liver cytosolic N-acetyltransferases catalyze the acetylation of a number of commonly used drugs and foreign compounds such as isoniazid, sulfamethazine, p-aminobenzoic acid, hydralazine and diaminodiphenylsulphone, in man and certain other mammalian species (217). Selected substrates for the acetyl transferases are shown in Table 20.

The activity of the acetyltransferases varies widely between species. For example, the rabbit has a relatively high capacity to acetylate sulfanilamide on the N4-position. The human has a moderate capacity for this process, while other species, including the dog and fox, are unable to acetylate sulfanilamide in that position but can acetylate the sulfonamido group and excrete N-acetylsulfonamide. These results indicate the existence of more than one form of the N-acetyltransferases (218).

The N-acetylation of xenobiotics in man exhibits a hereditary polymorphism, which is readily observable in the rate of drug acetylation (219). For this reason, people can be classified as either rapid or slow acetylators of isoniazid and certain other arylamine and hydralazine drugs. No biochemical basis for the acetylation polymorphism has as yet been found, viz. the characteristics of N-acetyltransferase prepared from rapid and slow acetylator livers appear to be identical (220, 221).

Table 20 Some xenobiotics which are biotransformed by acetylation via the N-acetyltransferases.

Substrate	Acetyl derivative
Isoniazid	
Sulfamethazine	
p-Aminobenzoic Acid	
Hydralazine	
Procainamide	
Aminofluorene	
Benzidine	

[Based on Hearse DJ and Weber WW I973 (217)]

The N-acetyltransferases are cytosolic enzymes which are found in many tissues of a number of species (222, 223). The cellular distribution differs from species to species. For example, in rabbit the enzyme appears in the reticuloendothelial cells and not in hepatic cells (224), while in rat liver, activity is found both in the reticuloendothelial (225) and hepatic cells (226). In the gastro-intestinal tract, enzyme activity occurs in mucosal elements but not in connective or muscle tissue. Activity is also present in cells of peripheral blood, but not in plasma, fat or skeletal muscle (227).

N-acetyltransferase activity exhibits a broad temperature optimum; the temperature optimum for the purified enzyme from slow and fast acetylators being from 25^{o} to 45^{o} C, with a peak at 37^{o}C. The pH profile of the activity of human liver N-acetyltransferases purified from both slow and fast acetylators was biphasic with peaks at pH 6.6 and pH 7.2. In both cases, the specific activity was relatively greater at the lower pH (228). N-acetyltransferase activity is inhibited by Cu^{++}, Hg^{++}, Zn^{++}, Cd^{++}, Ca^{++}, Mn^{++} and Fe^{++} (228).

4.4.2 Experimental

4.4.2.1 Materials

Acetyl Coenzyme A was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Cuprous chloride was purchased from May and Baker Ltd., Poole, England. Manganese sulfate and ammonium sulfate were obtained from BDH Ltd., London. N-acetyltransferase was purified from rat liver cytosol (100000 g supernatant) by differential precipitation with ammonium sulfate and chromatography as described in section 4.4.2.2.2.

4.4.2.2 Methods

4.4.2.2.1 Determination of activity of N-acetyltransferases of cytosol

Rat liver cytosol (post-microsomal supernatant) was prepared according to section 4.3.2.2.1. The protein concentration of the cytosol was measured by the method of Lowry et al (182). The N-acetyltransferase activity of cytosol was measured by the rate of disappearance of prizidilol from incubation mixtures containing prizidilol (3.7 μM), acetyl CoA (1 mM) and cytosol (0.8 mg protein/ml). Incubations were carried out at 37°C in a B & T Laboratory Thermal Equipment shaking water bath, with shaking at 60 cycles per minute.

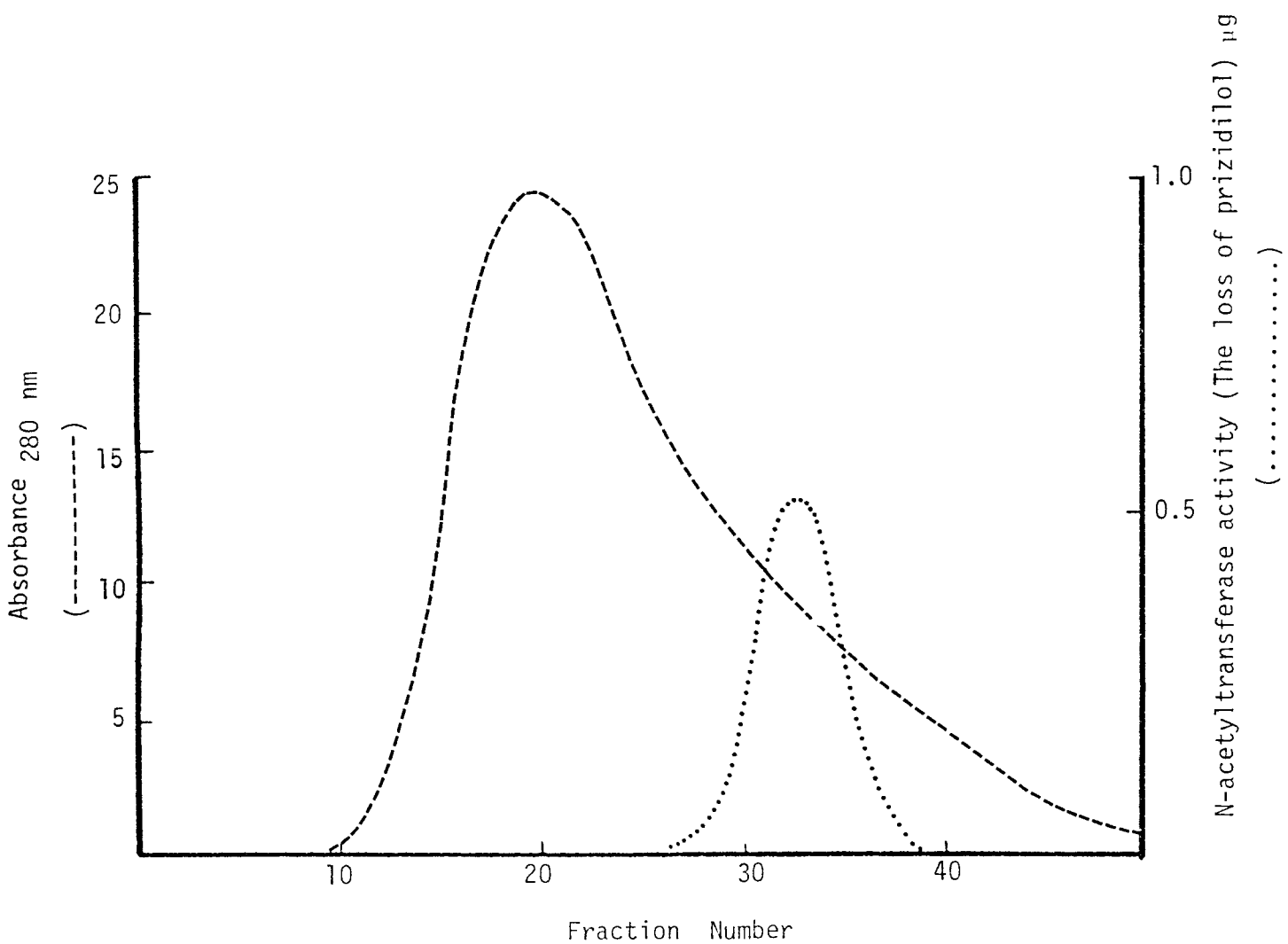
4.4.2.2.2 Purification of rat liver N-acetyltransferases

100 ml of rat liver cytosol was made to 50% saturation with respect to ammonium sulfate by the addition of a saturated solution of this salt. After 10 min, the precipitate was removed by centrifugation and the supernatant fraction was adjusted to 70% saturation with respect to ammonium sulfate. The precipitate obtained was dissolved in 30 ml of 0.01 M phosphate buffer (pH 7.0). This solution was further fractioned by gel filtration on a Sephadex G-100 column (2.5 cm x 50 cm) using 0.005 M phosphate buffer, pH 7.2. The protein concentration of this preparation was measured by the method of Lowry et al (182). The fractions from this column with activity for the acetylation of prizidilol were combined. Figure 35 shows protein concentration and the activity of N-acetyltransferase in each fraction.

4.4.2.2.3 Determination of activity of the purified N-acetyltransferases

Purified N-acetyltransferases were assayed for prizidilol acetylating activity by measuring the rate of disappearance of prizidilol from incubation mixtures. In each experiment, time-activity curves were

Fig. 35 The protein concentration (Absorbance $_{280\ nm}$) and N-acetyltransferase activity of fractions from the purification of rat liver N-acetyltransferase on Sephadex G-100.



The protein concentrations were measured at Absorbance $_{280\ nm}$. N-acetyltransferase activity were measured by the loss of prizidilol. Each fraction contained 5 ml.

constructed, and enzyme activity was determined from the linear portion of the curve (see e.g. Fig. 36). All incubations were carried out at 37^{o} in 3 ml stoppered vials with shaking at 60 cycles per minute. The reaction mixture consisted of enzyme solution (2 mg protein concentration/2.5 ml) acetyl CoA (1 mM) and prizidilol (3.7 μM). Control vials contained no acetyl CoA. The reaction was initiated by the addition of the enzyme preparation and the vials (two for each time point) were capped and incubated for 30 min. The reaction was terminated by the addition of 100 μl of orthophosphoric acid solution (section 2.2.2.1.1).

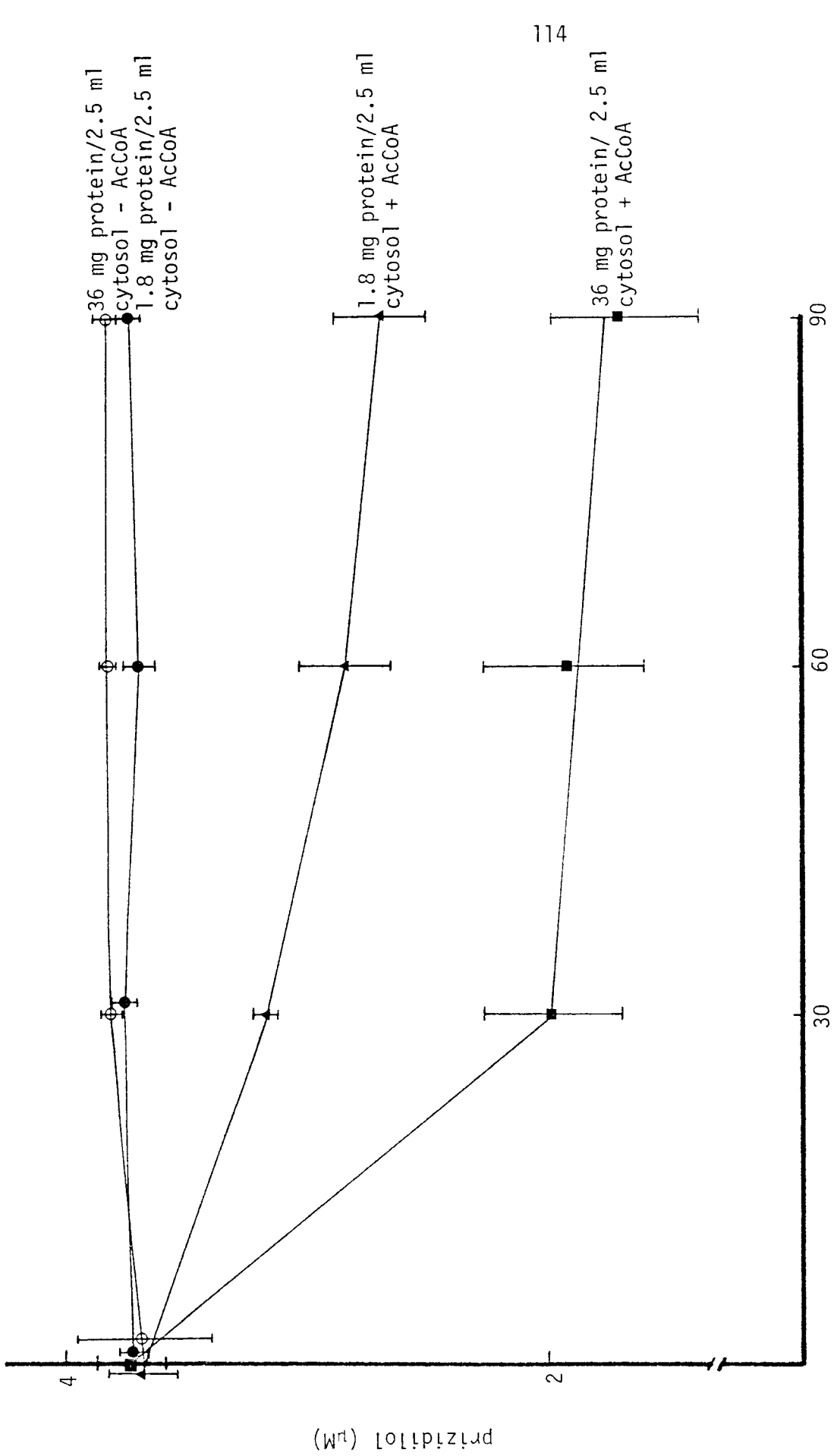


Fig. 36 The effect of time on the levels of prizidilol in incubation mixtures containing cytosol (concentrations as shown) and acetyl CoA (1 mM). Abbreviation used is: AcCoA, acetyl CoA.

4.4.3 RESULTS

4.4.3.1 The enzymatic acetylation of prizidilol by rat liver cytosol

The biotransformation of prizidilol by the rat cytosolic acetyltransferases was assayed by the disappearance of prizidilol from reaction mixtures after incubation in the presence of acetyl CoA (1 mM), post mitochondrial supernatant or partially purified enzyme and prizidilol (0-4 μM) in 0.01 M phosphate buffer, pH 7.0. The effect of time on the loss of prizidilol is shown in Fig. 36 and summarized in Table 21.

There was no significant loss of prizidilol when acetyl CoA (Table 21) (Fig. 36) or cytosol (data not shown) was omitted from incubation mixtures. These results suggest that the disappearance of prizidilol reflects its acetylation and that a non-enzymatic acetylation was not occurring. The disappearance of prizidilol with cytosol was linear for 30 minutes (Fig. 36), and this time period was used for further incubations to study the acetylation of prizidilol with hepatic cytosol acetyltransferases.

The loss of prizidilol per 30 min was shown to increase linearly with respect to the concentration of cytosol added, up to 5 mg protein/2.5 ml incubation mixture (Fig. 37).

4.4.3.2 The acetylation of prizidilol by the purified rat liver N-acetyltransferase

The loss of prizidilol as a function of time, at various concentrations of partially purified acetyltransferase is shown in Fig. 38. The reaction was linear for at least 30 min for all concentrations

Table 21 The effect of time on the levels of prizidilol disappearance in cytosol

Volume of cytosol	Concentration of Acetyl CoA	$0_{(min)}$	$30_{(min)}$	$60_{(min)}$	$90_{(min)}$
2 ml	1 mM	3.77 ± 0.13	1.98 ± 0.30*	1.97 ± 0.36*	1.69 ± 0.34*
2 ml	0	3.70 ± 0.31	3.79 ± 0.04	3.82 ± 0.03	3.83 ± 0.04
0.1 ml	1 mM	3.70 ± 0.13	3.19 ± 0.04*	2.87 ± 0.22*	2.68 ± 0.26*
0.1 ml	0	3.73 ± 0.05	3.77 ± 0.02	3.69 ± 0.07	3.73 ± 0.04

Values reported are means ± S.D. for experiments performed in duplicate with the preparation of cytosol (2 mg protein/2.5 ml). Experimental details are given in Section 4.4.2.2.1.

*Differs significantly from identically comprised sample at 0 time, $p < 0.01$.

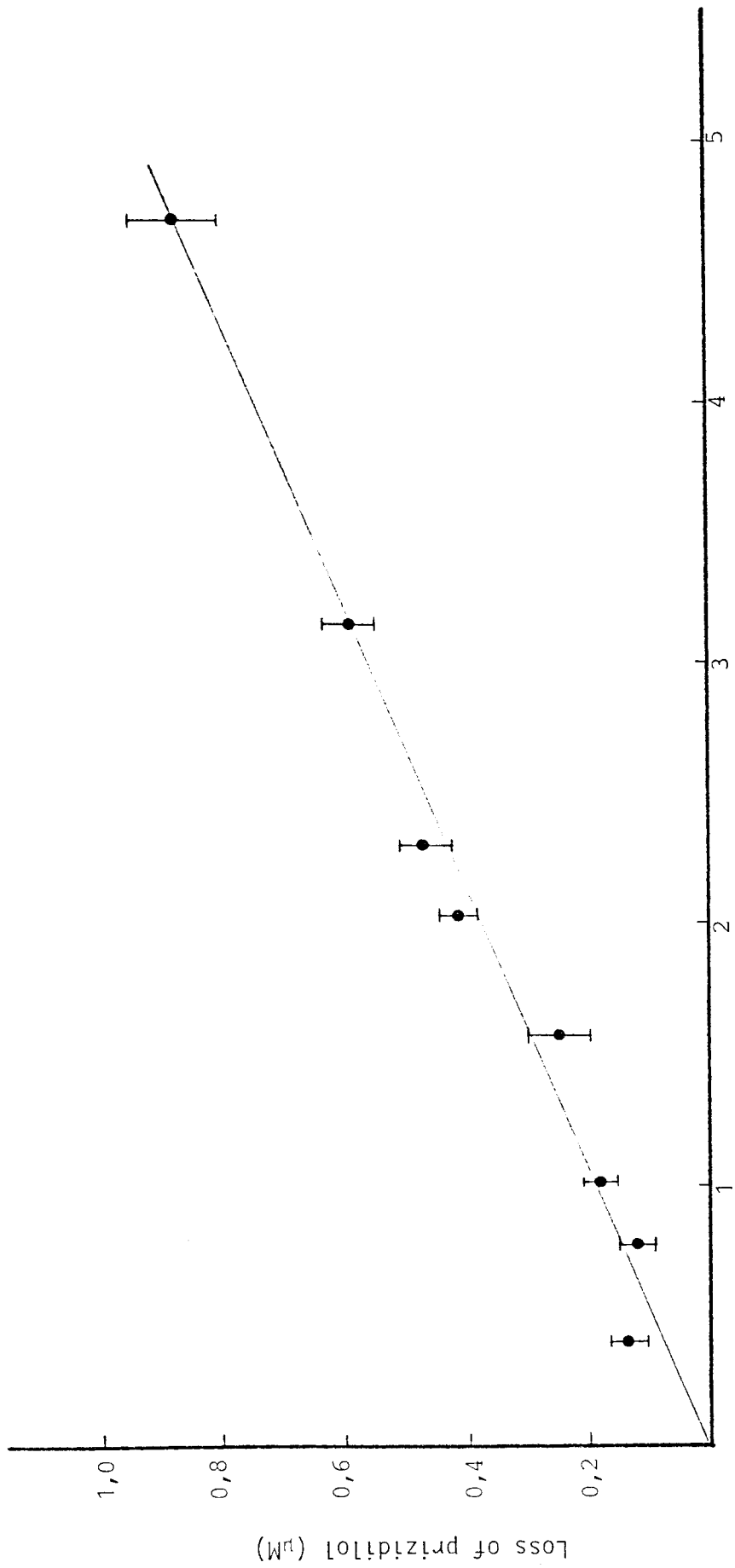


Fig. 37 Effects of various concentration of cytosol on the loss of prizidilol.
The concentration of acetyl CoA was 1 mM.
y = 0.173x + 0.01 r = 0.9905

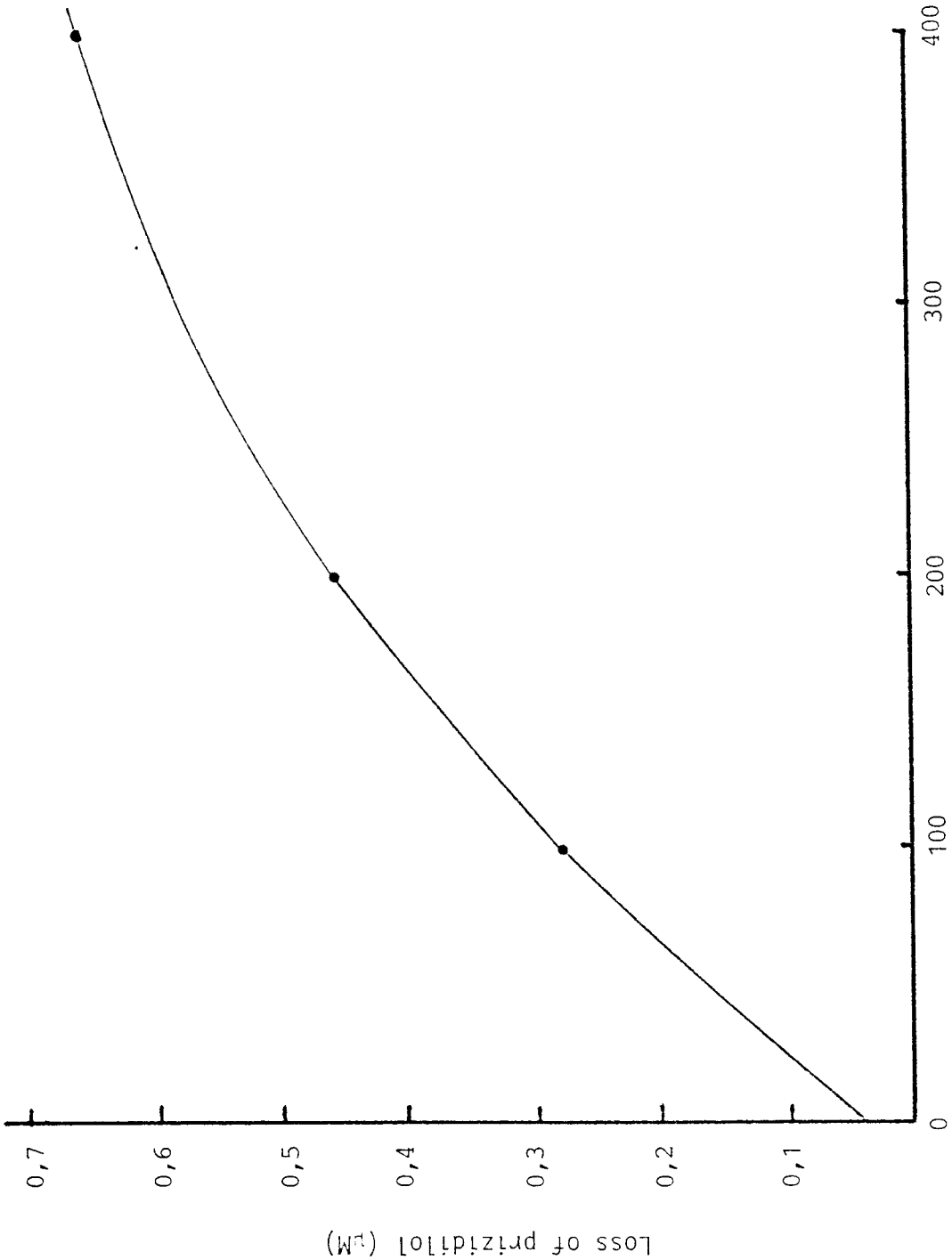


Fig. 38 Effects of various concentrations of purified N-acetyltransferases (12 mg protein/ml) on the loss of prizidilol. The concentration of acetyl CoA was 1 mM.

of enzyme studied. This time period was used in all further incubations, e.g. to determine Michaelis constant (K_m) and maximal velocity (V_{max}) for the metabolism of prizidilol by the acetyltransferases.

There was no loss of prizidilol from reaction mixtures which contained the enzyme but not acetyl CoA (Fig. 39, Table 22). There was no loss of prizidilol when acetyl CoA and prizidilol were incubated together in the absence of purified N-acetyltransferase, suggesting that a non-enzymic acetylation was not occurring (Fig. 40, Table 23). There was also no disappearance of substrate in reaction mixtures containing prizidilol and a preparation of acetyltransferase that had been heated for 10 min at 90^{o} before incubation (Fig. 40, Table 23). The effect of enzyme concentration on the loss of prizidilol is shown in Figs. 38, 41. The plot approximated linearly for up to 200 µl of enzyme solution.

The effects of inhibitors of N-acetyltransferase on prizidilol disappearance are given in Fig. 40 and Table 23. Cuprous chloride (0.14 mM) and Manganese sulfate (0.14 mM) were all effective inhibitors of the metabolism of prizidilol by N-acetyltransferases. Table 23 shows that cuprous ion inhibited N-acetyltransferase activity towards prizidilol by 69%, whereas manganese ion inhibited this process by 51%.

The effect of acetyl CoA concentration and prizidilol concentration on the acetylation of prizidilol by purified rat liver cytosol acetyltransferase is shown in Fig. 39 and Table 22. K_m and V_{max} values were calculated from Hanes plots of these data (Figs. 42 and 43).

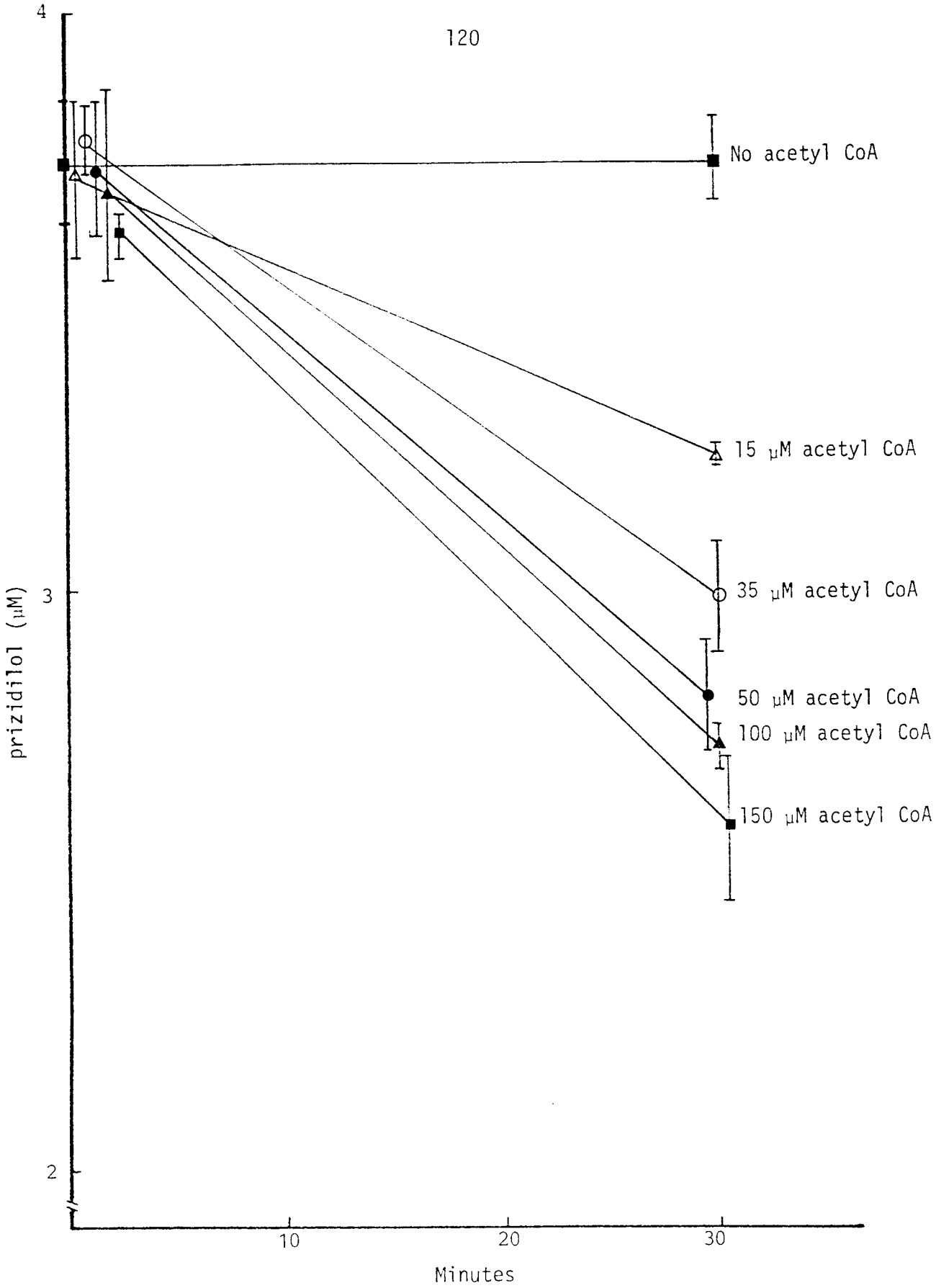


Fig. 39 Effects of various concentrations of acetyl CoA on the disappearance of prizidilol. The volume of purified N-acetyltransferase (7.7 mg protein/ml) was 300 μl.

Table 22 Effects of various concentrations of acetyl CoA on the disappearance of prizidilol

Acetyl CoA (μM)	0 min	30 min	Loss
0	3.73 ± 0.11	3.73 ± 0.02	0
15	3.72 ± 0.14	3.24 ± 0.02	0.48
35	3.80 ± 0.06	3.00 ± 0.11	0.80
50	3.77 ± 0.12	2.88 ± 0.10	0.89
100	3.79 ± 0.18	2.77 ± 0.04	1.02
150	3.75 ± 0.20	2.65 ± 0.13	1.10

Values reported are means ± S.D. for experiments performed in duplicate with the preparation of 300 μl purified N-acetyltransferase of cytosol (7.7 mg protein/ml)
Experimental details are given in Section 4.4.3.2.

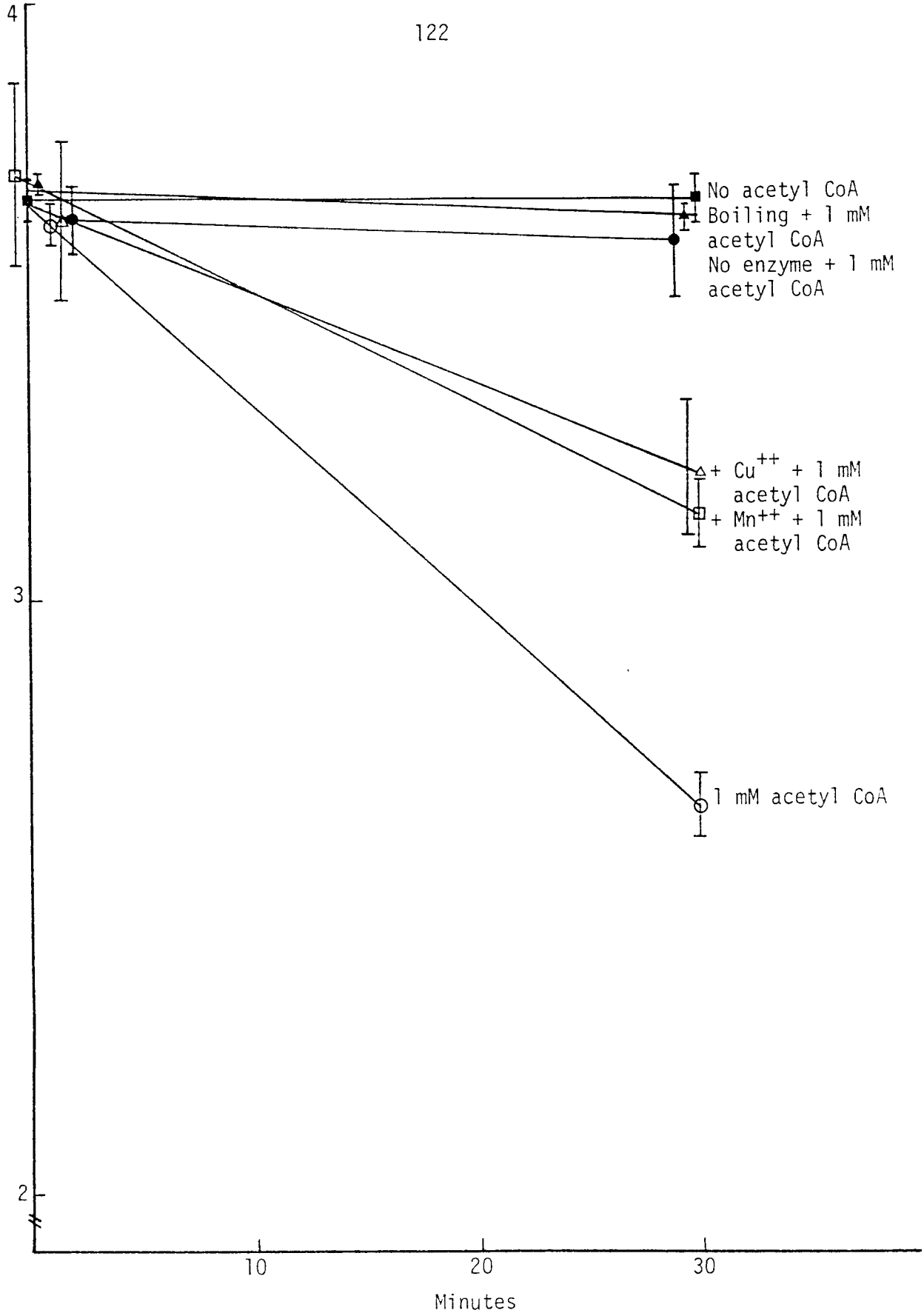


Fig. 40 Effect of inhibitors of N-acetyltransferase on the disappearance of prizidilol. The volume of purified N-acetyltransferase (12 mg protein/ml) was 200 µl.

Table 23 Effect of inhibitors of N-acetyltransferase on the disappearance of prizidilol

	0 min	30 min	Loss	% inhibition of prizidilol disappearance
prizidilol + Acetyl CoA	3.65 ± 0.05	2.65 ± 0.05	1.00	
prizidilol + Acetyl CoA + Mn^{++}	3.69 ± 0.23	3.20 ± 0.37	0.49	51%
prizidilol + Acetyl CoA + Cu^{++}	3.63 ± 0.19	3.32 ± 0.06	0.31	69%
prizidilol + No acetyl CoA	3.66 ± 0.04	3.67 ± 0.05	0	
prizidilol + No enzyme	3.68 ± 0.03	3.63 ± 0.02	0.03	
prizidilol + Boiled enzyme	3.63 ± 0.06	3.59 ± 0.10	0.04	

Values reported are means ± S.D. for experiments performed in duplicate. Incubation mixtures (3 ml) contain prizidilol (3.7 μM), Acetyl CoA (1 mM), Cu^{++} (0.14 mM), Mn^{++} (0.14 mM) purified N-acetyltransferases (12 mg protein/ml) as indication, incubated at 37°C for 30 minutes.

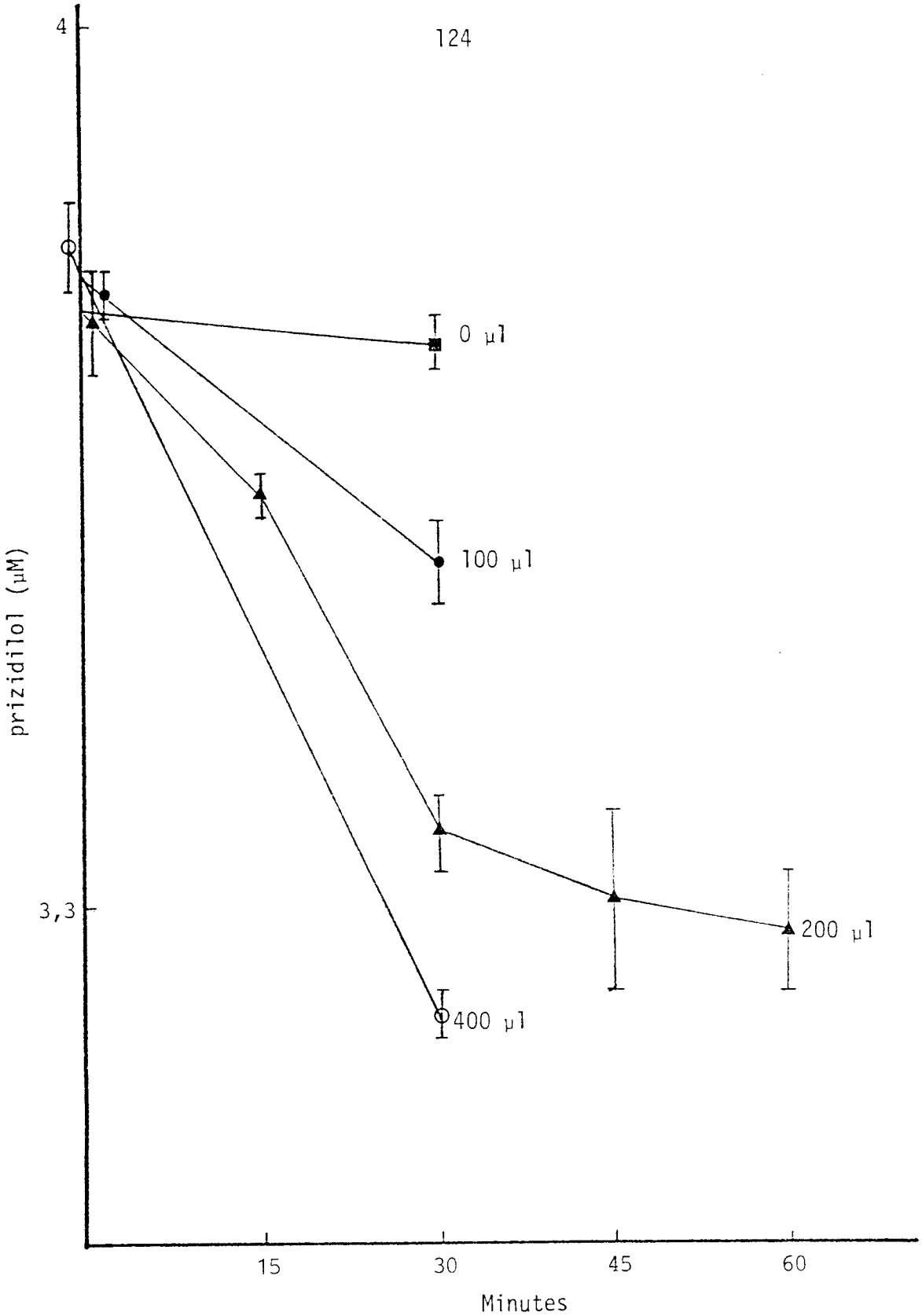


Fig. 41 Effects of various concentrations of purified N-acetyl-transferases (12 mg protein/ml) on the disappearance of prizidilol. The concentration of acetyl CoA was 1 mM.

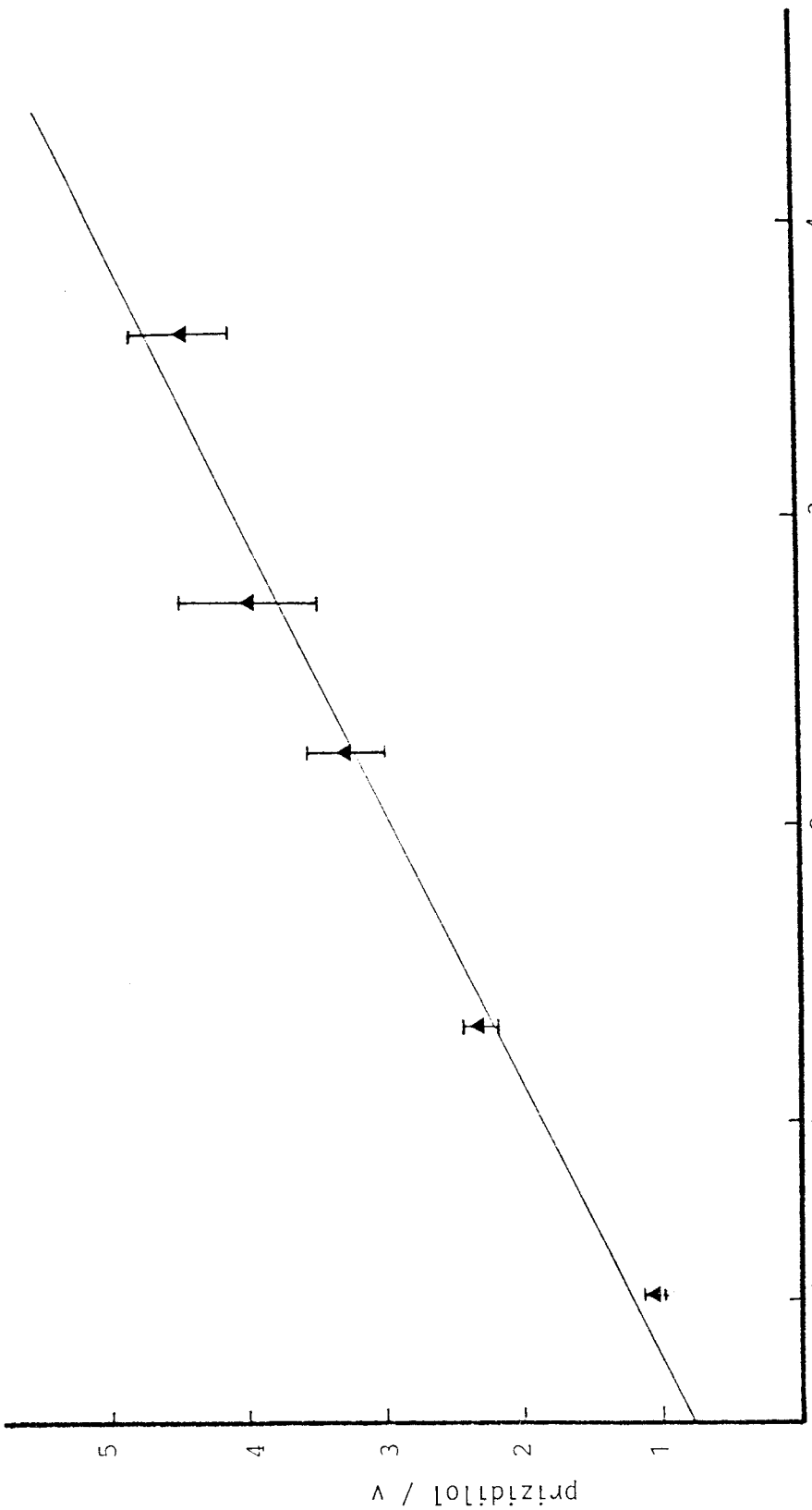


Fig. 42 Hanes plot of prizidilol metabolism by N-acetyltransferase in cytosol from phenobarbital pretreated rats. Each bar represents the range of 2 experiments. K_m = 0.78 µM V_{max} = 0.98 µmol/mg protein/min.

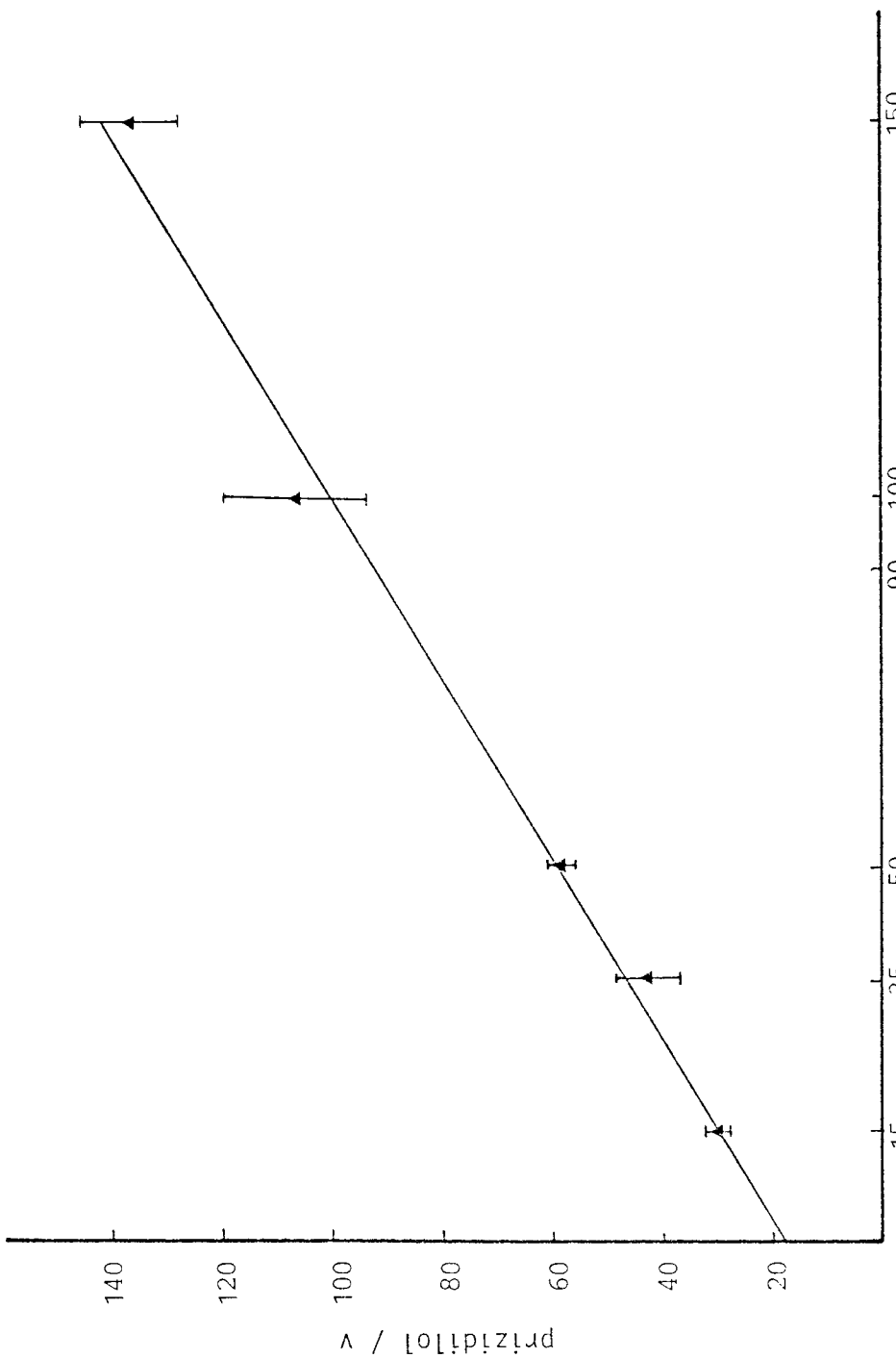


Fig. 43 Initial velocity pattern with acetyl CoA as the variable substrate in borate buffer 0.1 M pH 8.0, prizidilol concentration 3.7 mM. The prizidilol disappearance was calculated. Each bar represents the range of 2 experiments. v = rate of prizidilol disappearance in μmol/30 min/2 mg purified N-acetyltransferase protein.

K_m = 22 μM V_{max} = 1.2 μmol/mg protein/min.

The value of the K_m for prizidilol was 0.78 μM; the maximum velocity of the reaction V_{max} was 0.98 μmol/min; and the K_m for acetyl CoA was 22 ± 0.17 μM.

4.4.3.3 Metabolite identification

The levels of prizidilol were decreased following the incubation of prizidilol with reaction mixtures containing liver cytosol or partially purified N-acetyltransferase and acetyl CoA. An increase in the production of cyclised acetylated prizidilol was found in the TLC method for the determination of quinolin-3-al derivatived prizidilol, SKF 93238 and cyclised acetylated prizidilol. Unfortunately, the yield of cyclised acetylated prizidilol was very low and was even affected by the background, so using isotopically labelled acetyl CoA will be recommended to confirm the metabolite identification.

5. DISCUSSION

The results presented herein demonstrate that hepatic cytochrome P-450 and cytosolic N-acetyltransferases but not GSH S-transferases are involved in the metabolism of prizidilol.

That cytochrome P-450 catalyzes the metabolism of prizidilol is supported by the following evidence:

1. Prizidilol bound to the active site of the enzyme as evidenced by the production of a type I difference spectrum with a low concentration (<23.7 μM) of prizidilol and displayed a type II difference spectrum with a high concentration (24 - 190 μM) of prizidilol.
2. Prizidilol stimulated CO-inhibitable NADPH oxidation with hepatic microsomes, which is a measure of cytochrome P-450 dependence metabolism.
3. The inhibitors of cytochrome P-450 - CO, metyrapone and SKF 525-A inhibited the disappearance of prizidilol in the presence of hepatic microsomes and NADPH-generating system.

With the aid of inducing agents such as phenobarbital, β-naphthoflavone, and phenobarbital which elevate the levels of specific forms of hepatic cytochrome P-450, the role of different forms of the enzyme in the binding and metabolism of prizidilol in hepatic microsomes was investigated. With a low concentration of prizidilol, multiple forms of cytochrome P-450 appear to bind and metabolize prizidilol. The form of cytochrome P-450 elevated by β-naphthoflavone or pregnenolone-16α-carbonitrile did not appear to bind or metabolize prizidilol extensively, while the phenobarbital inducible form did. β-Naphthoflavone and pregnenolone-16α-carbonitrile did not affect the values of K_s and did

not alter or decrease ΔA_{max} and ΔA_{max} per nmol cytochrome P-450 for the binding of prizidilol.

Pretreatment of rats with β-naphthoflavone or pregnenolone-16α-carbonitrile did not result in significant alterations in the values of K_s, ΔA_{max} or ΔA_{max}/nmol microsomal cytochrome P-450 for the binding of prizidilol (1 - 23 μM) to the type I site (Table 12).

In addition the rates of the disappearance of prizidilol per 2 mg microsomal protein and per nmol cytochrome P-450 were not affected following β-naphthoflavone or pregnenolone-16α-carbonitrile induction.

The phenobarbital inducible form of cytochrome P-450 appears to play a minor role in the metabolism of prizidilol. Phenobarbital did not affect K_s, ΔA_{max} or ΔA_{max} per nmol cytochrome P-450 for the binding of prizidilol to the active site (Table 14). Phenobarbital significantly increased prizidilol (14 μM), stimulated hepatic microsomal NADPH oxidation and V_{max} for prizidilol metabolism per mg protein, but did not affect either parameter per nmol cytochrome P-450 (Tables 14 and 17).

It is proposed that one or more of the forms of cytochrome P-450 present in microsomes from untreated and phenobarbital treated rats, function efficiently in the metabolism of prizidilol.

Prizidilol appears to inhibit its own metabolism by hepatic microsomal cytochrome P-450; prizidilol bound to the type II site of the enzyme (Table 13, Fig. 22). The binding of prizidilol to this site competes directly with the binding of the required co-factor oxygen; the binding constant for this interaction ranges from 70 to 300 μM. Concentrations

of prizidilol in this range significantly inhibited the ability of prizidilol to stimulate hepatic microsomal NADPH oxidation (Table 14 and 15).

The effect of inducing agents on the binding of prizidilol to the type II site are as follows: β-naphthoflavone and phenobarbital significantly decreased the value of K_s and pregnenolone-16α-carbonitrile and phenobarbital significantly increased the values of ΔA_{max}. No type of pretreatment affected ΔA_{max}/nmol cytochrome P-450 (Table 13). For all types of pretreatment high concentration of prizidilol diminished CO-inhibitable NADPH oxidation.

The GSH S-transferases in rat liver cytosol did not decrease the levels of prizidilol after 30 minutes on the incubation of prizidilol with cytosol, indicating that liver cytosolic GSH S-transferases do not effectively catalyze the metabolism of prizidilol.

The cytosolic N-acetyltransferases appear to catalyze the metabolism of prizidilol. This proposal is supported by the following evidence:

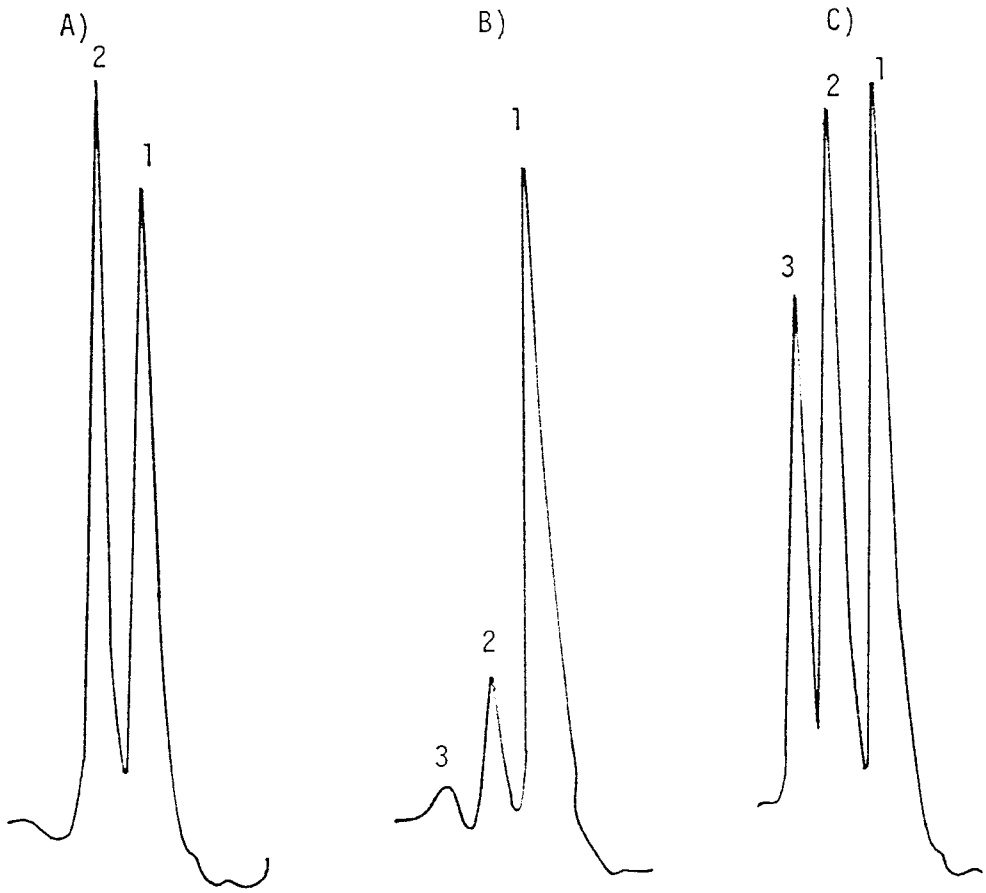
1. The disappearance of prizidilol with cytosol or purified cytosolic N-acetyltransferases from phenobarbital-pretreated rats required acetyl CoA and active transferase.
2. The inhibitors of acetyltransferases, cuprous and manganese ions inhibited the disappearance of prizidilol in the presence of cytosol or purified cytosolic acetyltransferases plus acetyl CoA.
3. K_m values of acetyl CoA for the acetylation of prizidilol are essentially as found for the acetylation of other xenobiotics by these enzymes.

The metabolism of prizidilol by the acetyl transferases leads to the disappearance of prizidilol in incubation mixtures, but the products of the acetylation reaction need further investigation. It is shown that small amounts of a product which chromatographs identically to cyclic acetyl prizidilol is produced in incubation mixtures containing prizidilol, acetyl CoA and partially purified N-acetyltransferases (Fig. 44).

If extrapolation of the results presented herein can be made, it is proposed that the metabolism of prizidilol by hepatic cytosol acetyltransferases would greatly exceed the metabolism of this drug by hepatic microsomal cytochrome P-450.

The rate of metabolism of prizidilol by hepatic cytochrome P-450 as assessed by its V_{max} value which was ca 20 pmol/mg protein/min. In comparison the V_{max} value for the acetylation of prizidilol by hepatic cytosolic N-acetyltransferases was 0.5 μmol/mg protein/min. A comparison of these two values demonstrates that the V_{max} for acetylation is approximately 20 000 times greater than the V_{max} value for metabolism of prizidilol by cytochrome P-450. Since the K_m values for these two processes are approximately 1 μM of the V_{max}, values differ by the amount mentioned above. It would appear that acetylation greatly exceeds cytochrome P-450-dependent metabolism of prizidilol. In addition, the levels of N-acetyltransferase in the liver in total amount would greatly exceed the levels of cytochrome P-450 in the liver, making this difference in acetylation for cytochrome P-450 metabolism rate even more striking.

Fig. 44 Possible identification of cyclised prizidilol derivative, from the action of partially purified N-acetyltransferases on prizidilol.



A) Before the acetylation reaction of prizidilol

B) After 60 min acetylation reaction of prizidilol
   1. SK & F 93238 quinolin-3-al hydrazone (Internal standard)
   2. prizidilol quinolin-3-al hydrazone
   3. cyclised acetylated prizidilol

C) Serum sample containing 1) prizidilol, 2) SK & F 93238 and 3) cyclised acetylated prizidilol.

The metabolism of prizidilol by hepatic cytochrome P-450 appears to be characterized by a very high affinity, relative to other substrates for this enzyme, as well as a very low V_{max} value compared to other substrates. The K_m value, which is in the μM range compared to the K_m value for drugs such as diphenylhydantoin (229). In contrast, however, the K_m value for prizidilol is far lower than the K_m's for a number of volatile anaesthetic agents such as halothane (230), enflurane (231), methoxyflurane (231) and isoflurane (232). For a lot of compounds the K_m values are in the mM range. The V_{max} values for the metabolism of prizidilol by hepatic microsomal cytochrome P-450 are extremely low compared to the V_{max} values for the metabolism of other drugs; for example, the volatile anaesthetic agents have V_{max} values in the range of 1-20 nmol of product form/mg protein/min. In contrast the V_{max} value for prizidilol is in the pmol range. These results indicate that if other drugs were present, in addition to prizidilol, the metabolism of prizidilol by hepatic microsomal cytochrome P-450 would be extremely slow. The interaction of prizidilol with the hepatic cytosol N-acetyltransferases, appears to be similar to the interaction of a number of other drugs. Both the K_m's for acetyl CoA and for prizidilol are similar to the corresponding values found for the metabolism of other drugs by the N-acetyltransferases, for example the K_m for the acetylation of prizidilol with respect to acetyl CoA concentration was found to be 22 μM. The corresponding values for the metabolism of, for example, p-aminosalicylic acid, sulfamethazine, hydralazine, p-phenetidin and aniline are in the range of 20-39 μM (218). The K_m value for prizidilol for the N-acetyltransferases was found to be approximately 1 μM. Corresponding values for hydralazine varied from species to species and ranged from 2 μM in the rat to 18 μM in the human (218).

In contrast INH exhibited markedly higher K_m values in the range of 60-182 as a function of species (233). Therefore, it appears that prizidilol binds relatively tightly to the N-acetyltransferases compared to a number of other drugs and that it would be metabolized at an equivalent concentration at a more rapid rate by this group of enzymes.

Of the three enzyme systems studied for the metabolism of prizidilol, it would appear that the GSH S-transferases play no role in prizidilol metabolism. The cytochrome P-450 enzyme system could play an intermediate role in prizidilol metabolism while the N-acetyltransferases are proposed to play the most important role in the transformation of this drug. These conclusions rest upon the observed K_m and V_{max} values for interaction with the enzyme systems in question. On the basis of our results, it is proposed that the major metabolite of prizidilol *in vivo* would therefore be an acetylated derivative. In preliminary experiments it was found that a product moving on thin layer chromatography identically to the cyclised acetylated derivative of prizidilol, was found in small quantities. It is thought that this is one possible metabolite from the acetyltransferases. However, it appears to be a minor metabolite. It could be speculated that the major metabolite of prizidilol *in vivo* will prove to be the N-acetylated product or some further metabolite thereof.

6. GENERAL SUMMARY AND CONCLUSIONS

The hepatic microsomal cytochrome P-450 enzyme system has been shown to metabolize a number of volatile anaesthetic agents, e.g. enflurane (231, 232, 234), fluroxene (235), halothane (230), trichloroethylene (236) and a number of other drugs (229), and is also capable of metabolizing propranolol (233, 237), hydralazine (238). We have demonstrated that cytochrome P-450 also metabolizes the combination drug prizidilol. Prizidilol bound to two distinct sites on cytochrome P-450. At low concentrations (<23.7 μM) viz. the type I site (or substrate binding site), and at higher concentrations (24 - 190 μM) the type II site (or oxygen binding site) of the enzyme. Binding constants for the binding of prizidilol for both sites are reported.

Prizidilol stimulated CO-inhibitable NADPH oxidation which is a decreased measure of cytochrome P-450 dependent metabolism. Prizidilol levels in the presence of hepatic microsomes plus NADPH generating system. The latter process was inhibited by metyrapone, SK & F 525-A and $CO:O_2$ (80:20 v/v), which are known inhibitors of cytochrome P-450 (151). The role of different forms of cytochrome P-450 in the metabolism of prizidilol was assessed by studying the effects of different inducing agents on the ability of prizidilol to stimulate hepatic microsomal CO-inhibitable NADPH oxidation and on the disappearance of prizidilol for hepatic microsomal incubation mixtures. The inducing agents used included phenobarbital, β-naphthoflavone and pregnenolone-16α-carbonitrile. Time courses and Hanes plots of the loss of prizidilol allowed calculation of K_m and V_{max} values for all types of pretreatments. It was concluded, based on the observed effects of induction on K_m and V_{max} values, that several of the multiple forms of cytochrome P-450 bind and metabolize prizidilol.

The hepatic cytosol GSH S-transferases did not catalyze the metabolism of prizidilol. No loss of prizidilol was seen with liver cytosol plus GSH. Acetylation was found to play a significant role in prizidilol metabolism. Enzymatic acetylation of prizidilol was found following incubation of the substrate and acetyl CoA with rat liver cytosol or partially purified N-acetyltransferases. The levels of cyclised acetylated product were significantly increased following incubation. The acetylation of prizidilol was inhibited by cuprous chloride and manganese sulfate, which inhibit acetyltransferase. The K_m for the acetylation of prizidilol was 0.78 µM and the maximum velocity of the reaction (V_{max}) was 0.98 µmol/mg protein/min. Limiting value of the apparent K_m for acetyl CoA was 22 ± 0.17 µM.

Future directions of research will include the following:

(a) Analysis of prizidilol metabolism by human liver microsomes - comparison to the rat and inter-individual human differences;

(b) Investigation of the metabolism of prizidilol in isolated rat and human hepatocytes in order to assess in a more physiologically relevant system, the biotransformation of this drug, and

(c) Characterization of the acetylated derivatives of prizidilol.

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