THE FIRST-PASS EXTRACTION OF PINDOLOL IN COMPARISON WITH PROPRANOLOL IN RAT LIVER

HELEN MARGARET BASSET

B.Sc. (Cape Town)

B.Sc. (Hons.) (Medical Science) (Stellenbosch)

A thesis presented in fulfilment of the degree of Master of Science (Medicine) of the University of Cape Town.

September, 1979.

CONTENTS

ACKNOWLEDGEMENTS

I would like to acknowledge the generous financial support of Sandoz Ltd. without which this work would not have been possible. In particular, my thanks are due to Dr P. Goodson and Dr J.P. Glynn for their personal interest and help.

I have thoroughly enjoyed my work in the Department of Pharmacology and thank Professor P. Folb for the opportunity to work in his department. His encouragement and advice were of great benefit to me.

I am grateful to Dr J. Cridland for many suggestions and discussions. To him I owe a great deal for help with the assays when I was close to despair.

I want to thank Professor R. Kirsch for the use of the liver perfusion facilities in the liver laboratory of the Department of Medicine. Professor Kirsch's advice on the perfusion model and his willingness to help in all aspects of this work are greatly appreciated.

A very special expression of thanks is due to Mr M. Parker who did so much of the hard work involved in setting up the perfusions. The success of my perfusions was in no small way due to his expertise. He was also responsible for patiently teaching me the perfusion operation technique and in this respect I am also grateful to Miss P. Mason.

I am particularly indebted to Dr A. Robins for the many hours and nights he spent meticulously going through this thesis. His enthusiasm and interest were an invaluable asset to me. I am also grateful to him for his help with the statistics. Thanks are due to his wife for the many cups of coffee.

I was fortunate to work in the laboratory with Mrs D. Herbert, Miss N. de Villiers, Mrs D. Thompson and Mr J. Holmes. I thank them for their patience when I was in the way and wanted to use the shaker or change around the spectrofluorimeter! Mrs Herbert's help with practical

problems and the ordering of equipment and glassware is appreciated. I would like to thank Mrs C. van Diemel and Mr C. Makana for washing my glassware.

Mr Holmes was always willing to help with any practical problems in the laboratory and I would like to thank him for his help with the perfusion apparatus. In this respect Mr M. Wells was also of great assistance.

Mrs C. Kingdon had the arduous task of typing this manuscript and I would like to thank her for a really excellent job.

SUMMARY

Reports in the literature have shown that in man propranolol has a larger first-pass effect (70%) than pindolol (13%). The aim of this research was to make a direct comparison of first-pass extraction and other pharmacokinetic parameters of these drugs under identical experimental conditions by means of an isolated rat liver perfusion model.

Livers were removed from Long-Evans rats and these were perfused with a recirculating perfusate medium of 20% rat blood - 80% KREBS buffer. Pressure was held constant at a physiologically acceptable level of 16 cm of water so that, as in the *in vivo* situation, each liver determined its own individual flow rate. 5 mg of either propranolol or pindolol were injected as a single bolus into the reservoir (simulating intravenous administration) and samples taken at 1, 3, 5, 10, 20, 30, 40 and 60 minutes from the portal venous inflow into the liver and the hepatic venous outflow from the liver.

Whole blood-KREBS concentrations for both propranolol and pindolol were obtained by spectrofluorometric assays. Logarithmic drug concentrations were plotted against time and the best lines fitted. Points were then derived and transposed on to linear graph paper so as to calculate the areas under the drug concentration-versus-time curves.

Propranolol and pindolol differed in the following respects:

(i) The first-pass effect (hepatic extraction ratio) of propranolol (0,71) and its hepatic clearance (2,47 ml/g/min) were highly significantly greater than the corresponding values (0,50 and 1,56 ml/g/min respectively) for pindolol ($p<0,01$) even when perfusions were strictly controlled for flow.

(ii) The hepatic intrinsic clearance of propranolol (10,44 ml/g/min) was very highly significantly greater than that of pindolol (3,42 ml/g/min) ($p<0,001$).

(iii) The correlation between perfusate flow rate and extraction ratio was significant in the case of the pindolol perfusions ($r = -0,88$; $p<0,01$) but not in the propranolol group ($r = -0,74$; $p<0,10$).

(iv) Significant correlations were obtained between flow rate and hepatic clearance in both the propranolol ($r = 0,97$; $p<0,001$) and the pindolol ($r = 0,87$; $p<0,05$) groups of perfusions.

(v) The portal venous logarithmic concentration-time graphs conformed to a biexponential two-compartment system in the case of propranolol but a monoexponential one-compartment model in the case of pindolol.

(vi) The α-phase $t_{\frac{1}{2}}$ of propranolol was rapid ($t_{\frac{1}{2}\alpha} = 1$ minute) and flow-dependent whereas the $t_{\frac{1}{2}\beta}$ was relatively slow ($t_{\frac{1}{2}\beta} = 5,1$ minutes) and non-flow-dependent. Its $t_{\frac{1}{2}\beta}$ was longer than that for pindolol ($t_{\frac{1}{2}\beta} = 4$ minutes), the latter being flow-dependent.

(vii) The apparent volume of distribution of propranolol (21,23 ml/g) was larger than that for pindolol (8,72 ml/g) ($p<0,001$).

These results supported the "venous-equilibration" model of hepatic clearance. The pharmacokinetic characteristics of propranolol identified it as a high clearance, flow-limited drug whereas pindolol emerged as an intermediate, partly flow-limited drug.

It was proposed that the higher first-pass extraction of propranolol was predominantly the result of avid, high affinity binding to liver sites. It was the extent of this binding which created a two-compartment system for propranolol - with the exceedingly high extraction ratio in the α-phase (0,99 after 1 minute of perfusion) - and accounted for the much larger apparent volume of distribution of propranolol.

The differences in the pharmacokinetics of the two beta-blockers may be related to the higher lipid-solubility and resultant greater tissue penetrability of propranolol. The larger first-pass extraction and hepatic intrinsic clearance

of propranolol compared to those of pindolol may reflect not drug metabolism *per se* but rather the hepatic uptake of unchanged drug and its partitioning into specific liver sites.

CHAPTER 1

1. INTRODUCTION

Pindolol ('Visken', Sandoz) is a potent β-receptor antagonist or β-blocker. Its indole nucleus, unique among β-blockers, is the only feature that differentiates it from propranolol ('Inderal', I.C.I.) which has a naphthalene ring structure (Fig. 1). This relatively small modification in aromatic ring structure between the two compounds accounts for their different pharmacodynamic and pharmacokinetic properties.

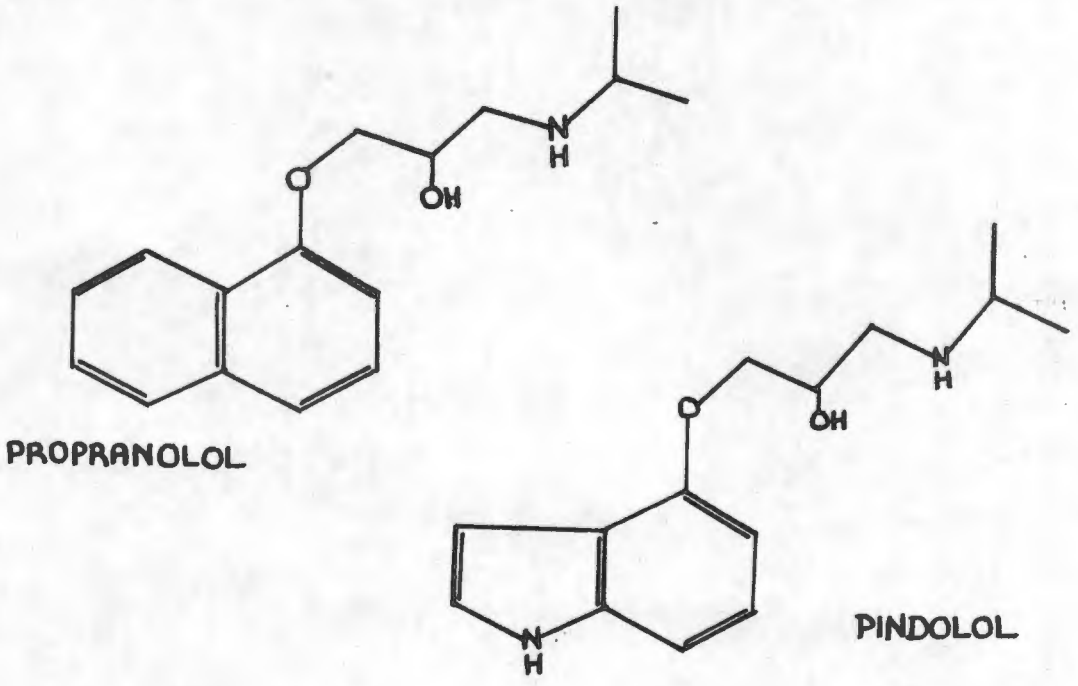


Fig. 1. Structures of Pindolol and Propranolol

β-blockers are used extensively in the treatment of hypertension, angina pectoris and cardiac arrythmias (12, 37, 55, 69). Pindolol is at least five to ten times more potent than propranolol on a weight-to-weight basis and, unlike propranolol, it possesses intrinsic agonist activity (21, 47, 48, 96).

All β-adrenoreceptor blocking agents can be characterised as weak bases. In the acidic or slightly alkaline gastro-intestinal fluid, they exist predominantly in an ionised, easily soluble form because of the secondary amino group in the side-chain which has a pk_a value of 9,5 - 9,7. They are

rapidly and well absorbed from the gastro-intestinal tract and peak blood concentrations are reached one to two hours after ingestion (12, 37, 55).

However, it is now well established that the important factor is not the absolute amount of drug absorbed but rather the amount which becomes systemically available thereafter. In its passage from the gut to the systemic circulation the drug traverses the liver via the portal system. It is in the liver that some of the drug is extracted and metabolised. This extraction occurs during the drug's first passage through the liver and before it enters the circulation for general distribution (Fig. 2). This phenomenon is referred to as first-pass metabolism, first-pass extraction or the first-pass effect.

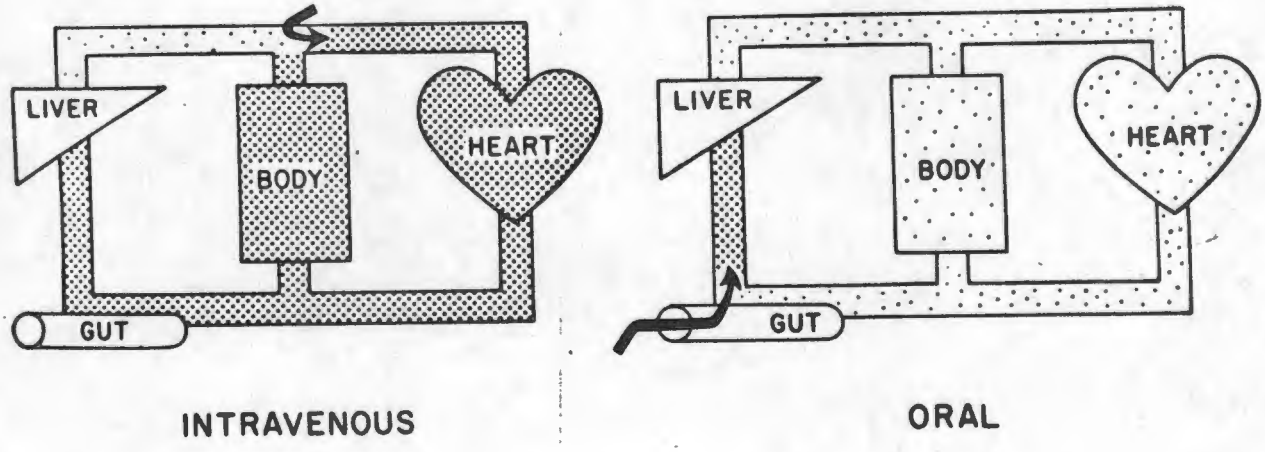


Fig. 2. "First-pass extraction". A diagrammatic representation of oral and intravenous administration. The shading represents drug concentration and the arrows represent route of administration. Since the major organ of elimination is the liver, the drug can be extracted from portal venous blood prior to reaching the systemic circulation during oral administration (after Nies, A.S. and Shand, D.G. (69)).

A large first-pass extraction would result in a low systemic bioavailability of the drug after oral administration despite complete absorption. The lower bioavailability of a drug with a high first-pass extraction can be partly compensated for by administering a higher dosage. However, a large genetically determined inter-individual variation in first-pass extraction can create wide differences in plasma levels of the drug, even in patients receiving identical doses, particularly when the first-pass extraction is large.

In general, many other factors may also affect the

first-pass extraction and hence bioavailability of a drug. These will be discussed in a later section.

Several groups of workers (3, 21, 56) had found that pindolol had a first-pass effect in man that was apparently much lower than that established for propranolol (18, 31, 46, 50).

This project was set up to test whether propranolol differed in its first-pass effect from that of pindolol in an isolated rat liver perfusion model. There are no data available which make a direct comparison of the first-pass effect of these two drugs using identical experimental conditions.

1.2. Review of the Literature

1.2.1. General Introduction to Pharmacokinetics

In this project several pharmacokinetic parameters are derived in order to study the differences between pindolol and propranolol. In view of the fact that pharmacokinetics are a complex and mathematical field, the introduction that follows is confined only to those aspects which bear directly on the subject of my research.

All the abbreviations used in the following chapters are shown in Appendix B.

1.2.2. First-order Elimination Kinetics

In first-order elimination kinetics the rate of elimination is proportional to the amount of drug in the body (D_B) at any time, t.

$$\frac{-dD_B}{dt} \propto D_B$$

and

$$\frac{dD_B}{dt} = k_{el} D_B$$

where k_{el} = overall first-order elimination rate constant which has the dimensions of $\frac{1}{time}$. (If the drug is eliminated only by the liver then k_{el} represents the hepatic rate constant.)

A plot of the logarithm of concentration-versus-time is linear in first-order kinetics.

1.2.3. Elimination Half-life ($t_{\frac{1}{2}}$)

This is the time required for the plasma concentration of drug to fall by 50%.

$$t_{\frac{1}{2}} = \frac{0,693}{k_{el}} \quad \text{(for first-order kinetics)} \qquad \text{(equation 1)}$$

where k_{el} = first-order elimination constant.

1.2.4. Apparent Volume of Distribution (V_d)

Once a drug has been absorbed and reaches the systemic circulation, it distributes into organs and tissues. The apparent volume of distribution is that volume in which the drug would appear to be distributed during the steady state if it existed throughout that volume at the same concentration as in the plasma.

For drugs which are eliminated by first-order processes, the volume of distribution is calculated as follows:

$$V_d = \frac{D}{c_o} \qquad \text{(equation 2)}$$

where D = dose

and c_o = theoretical plasma concentration at zero time when distribution is complete.

1.2.5. Compartmental Drug Disposition

Pharmacokinetics are most frequently explained by the one-compartment and two-compartment open models. In the one-compartment model, drug entering the body is distributed instantaneously into the available space and is eliminated directly from this single pool of drug. Assuming first-order kinetics plasma concentrations-versus-time plotted on a semi-logarithmic scale depicts a straight line (Fig. 3).

An open two-compartment model is needed to explain the pharmacokinetics of most drugs. Drug entering the body instantaneously distributes into a space, termed the central compartment, which consists of blood and other readily accessible fluids and tissues (e.g. liver and lung). Drug distribution is much slower into a second compartment (the peripheral compartment) which consists of poorly perfused organs and tissues (e.g. adipose and muscle). Elimination occurs from

the central compartment. Plasma concentration-versus-time plotted on a semi-logarithmic scale produces a curve which can be broken down into two exponential components.

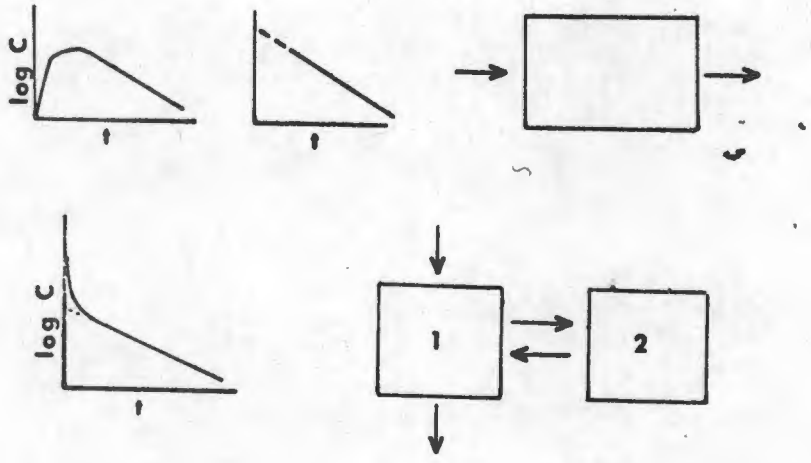


Fig. 3. Pharmacokinetic models. Accompanying semi-logarithmic plots of plasma concentration (C) versus-time (t) are consistent with an open one-compartment (above) and two-compartment model (below) (after Gibaldi, M. (86)).

The α-phase represents the distribution phase and reflects primarily distribution from the central to the peripheral compartment although elimination begins from the first moment when drug is injected. When distribution is complete and equilibrium reached, the two lines meet and thereafter elimination dominates. However, although the β component reflects elimination kinetics it also reflects distribution from the peripheral to the central compartment (Fig. 3) (134).

Multicompartment models produce curves that can be broken down into more than two components

1.3. The Kinetics of Hepatic Elimination

1.3.1. Hepatic Extraction Ratio (E)

The hepatic extraction ratio (E) can be estimated from the arterial-venous concentration difference across the liver at steady state. However, after a single dose of drug is administered, a constant extraction ratio is only achieved once distribution equilibrium is complete. A valid estimation of the extraction ratio can nevertheless be obtained using the areas under the inflow and outflow concentration-versus-time curves.

Thus

$$E = \frac{AUC_{PV} - AUC_{HV}}{AUC_{PV}} \qquad \text{(equation 3)}$$

This corresponds to

$$E = \frac{AUC_{s} - AUC_{HV}}{AUC_{s}}$$

where AUC_{PV} = area under the portal vein concentration-versus-time curve = area under the systemic concentration-versus-time curve after intravenous administration. (This represents inflow into the liver.)

AUC_{HV} = area under the hepatic vein concentration-versus-time curve. (This represents outflow from the liver.)

The hepatic extraction ratio of a single dose of intravenously administered drug is equivalent to the extraction of the drug during its first pass through the liver after oral administration (46, 94). Thus the extraction ratio derived from equation 3 is equal to the first-pass extraction.

1.3.2. Hepatic Clearance of Total Drug (Cl_H)

The efficiency of any organ in removing a drug irreversibly from the perfusing blood is described by the term clearance. Hepatic clearance (Cl_H) is the volume of blood from which drug is completely removed in unit time. It can be calculated from the product of the hepatic blood flow and the hepatic extraction ratio (i.e. the difference in the arterial-venous concentration across the liver at steady state).

$$Cl_H = Q\left[\frac{C_s - C_v}{C_s}\right] = Q\,E \qquad \text{(equation 4)}$$

where Q = total hepatic blood flow

C_s = mixed portal venous and arterial concentration of total drug = systemic concentration of total drug

C_v = hepatic venous concentration of total drug

E = steady state extraction ratio

Hepatic clearance can also be calculated according to the following equations if extrahepatic elimination is negligible and elimination is first-order:

$$Cl_H = Q\,E = \frac{D_{iv}}{AUC_s} \qquad \text{(equation 5)}$$

where D_{iv} = intravenous dose of drug
AUC_s = area under the drug concentration time curve in the systemic blood.

In addition,

$$Cl_H = \frac{V_d \cdot 0{,}693}{t_{\frac{1}{2}}} \qquad \text{(equation 6)}$$

where V_d = apparent volume of distribution
$t_{\frac{1}{2}}$ = elimination half-life.

1.3.3. Bioavailability (f)

The fraction of drug which escapes hepatic extraction or first-pass extraction is an index of the bioavailability (f) of a drug if absorption is complete.

$$f = 1 - E \qquad \text{(equation 7)}$$

since

$$E = \frac{AUC_s - AUC_{HV}}{AUC_s} \qquad \text{(equation 3)}$$

$$f = 1 - \frac{AUC_s - AUC_{HV}}{AUC_s}$$

$$f = \frac{AUC_{HV}}{AUC_s} \qquad \text{(equation 8)}$$

(after administration of a single intravenous dose)

1.3.4. First-pass Extraction - a Compartmental Model

Ordinary compartmental pharmacokinetics are not sufficient to predict the pharmacokinetics of drugs subject to first-pass extraction. Gibaldi has thus proposed a new approach (27, 94), the essential features of which are shown in Fig. 4.

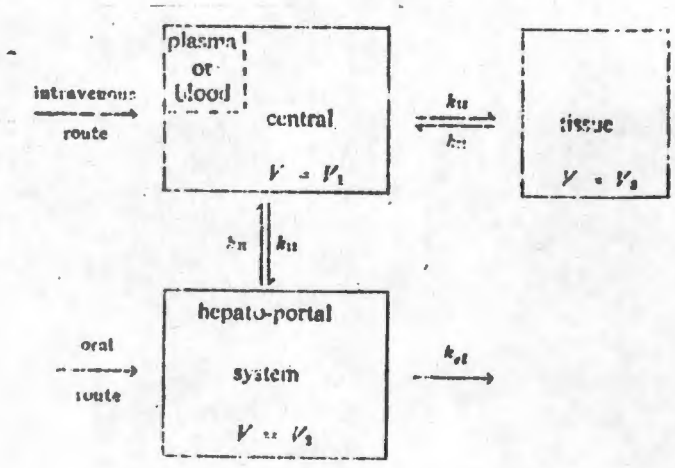


Fig. 4. First-pass pharmacokinetic model, a three-compartment open model (after Gibaldi, M. et al (27)).

In this model elimination occurs from a compartment which is distinct from that containing the vascular sampling site. Hence, although the plasma concentration - time data simply suggest a two-compartment model as described in section 1.2.5., an additional rapidly accessible compartment is proposed. This compartment from which elimination occurs is analogous to the hepato-portal system. Drug introduced into the portal vein (oral administration) will go directly into this compartment. Using this model, Gibaldi has derived a number of equations to calculate drug clearance and bioavailability.

f can be calculated from drug concentration - time data obtained after intravenous administration of a drug (27, 77, 94, 95).

$$f = 1 - \frac{D_{iv}}{Q \cdot AUC_s}$$

where $$\frac{D_{iv}}{Q \cdot AUC_s} = E$$ (from equation 5)

The above equation indicates that oral bioavailability can be calculated even after intravenous administration and thus $\frac{D_{iv}}{Q \cdot AUC_s}$ is equal to the first-pass extraction of a drug if elimination occurs only from the hepato-portal system.

1.4. Models of Hepatic Drug Clearance

Two models have been put forward to explain and predict drug clearance. Although the two models have different assumptions, in practice the differences in hepatic clearance and extraction that each predictsare not very large. Moreover, the broad principles involved in hepatic clearance are not critically dependent on the model chosen (131).

One of the models is the "parallel tube" model. This involves complex pharmacokinetics and enzyme kinetics. It is discussed in detail by Brauer (119) and Keiding et al (99).

The most commonly used model and the one relevant to this thesis is the "venous-equilibration" model (also known as the perfusion limited model and the "well-stirred" model) (18, 19, 20, 85).

1.5. The "Venous-equilibration" Model of Hepatic Drug Clearance

From equation 4, hepatic drug clearance depends on two biological variables: (i) hepatic blood flow, and (ii) the extraction ratio which must reflect the intrinsic ability of the liver to metabolise drug. Hepatic drug clearance also depends on a third variable, the binding of drug to plasma proteins and cellular components of blood.

Blood and plasma concentration/time profiles are frequently used to characterise drug kinetics and are usually interpreted by compartmental analysis. However, these analyses do not indicate the relationship between the three biological variables mentioned above. The "venous-equilibration" model of hepatic elimination incorporates the biological variables mentioned and thus further clarifies drug elimination and disposition. It is based on the model of Rowland et al (20), Branch et al (19) and Gillette (85).

One of the critical assumptions of the model is that drug distributes so rapidly as it passes through the liver that drug concentration within the liver is in equilibrium with that in the emergent venous blood. Other assumptions are: (i) elimination is first-order, (ii) absorption is complete, (iii) all or most of the drug is eliminated by the

liver (50), and (iv) that the volume of distribution does not change (125).

The equations and inter-relationships predicted by the model are described in the following sections and are found in the following references (17, 18, 19, 22, 24, 25, 32).

1.5.1. Intrinsic Clearance of Total Drug (Cl_{int})

The extraction ratio depends on both liver blood flow and on the overall inherent ability of the liver to extract and metabolise the drug by rate-limiting processes. The term intrinsic clearance of total drug (Cl_{int}) has been introduced to describe this ability of the liver and it is independent of any modifying effects of flow. Thus intrinsic clearance is defined as "the maximal ability of the liver to irreversibly remove drug from the blood by all pathways in the absence of any flow limitations"(17).

In effect, then, intrinsic clearance is a composite of several different processes: (i) the partitioning of the drug from the blood into the liver, (ii) liver size, and (iii) the intrinsic overall rate of elimination by biochemical processes, i.e. $\frac{V_{max}}{k_m}$ (1) when metabolism is first-order and flow is not rate-limiting.

Intrinsic clearance is a distinctive characteristic for any particular drug at a given dose in a given situation.

1.5.2. Relationship between Hepatic clearance, Intrinsic clearance, Extraction ratio and Hepatic blood flow rate

If it is assumed that drug binding to blood constituents remains constant and that there is no extrahepatic clearance (i.e. hepatic clearance Cl_H = systemic clearance Cl_s) the following relationship can be derived after single dose intravenous administration.

$$Cl_H = Cl_{int} \cdot \frac{AUC_{HV}}{AUC_s} \qquad \text{(equation 9)}$$

Now $$Cl_H = Q\ E \qquad \text{(equation 4)}$$

---

(1) V_{max} = maximum rate of the reaction (metabolism)
k_m = Michaelis-Menton constant

and $$\frac{AUC_{HV}}{AUC_s} = f = 1 - E \qquad \text{(equations 7 and 8)}$$

$$\therefore \quad Q\,E = Cl_{int}\,(1 - E)$$

$$\therefore \quad E = \frac{Cl_{int}}{Q + Cl_{int}} \qquad \text{(equation 10)}$$

$$\therefore \quad Q\,E = Q\left[\frac{Cl_{int}}{Q + Cl_{int}}\right] \qquad \text{(equation 11)}$$

$$\therefore \quad Cl_{int} = \frac{Q\,E}{1 - E} \qquad \text{(equation 12)}$$

1.5.3. Calculation of Intrinsic Clearance from changes in Flow and Extraction ratio

Equation 11 can be rearranged to give:

$$\frac{1}{E} = 1 + \frac{Q}{Cl_{int}} \qquad \text{(equation 13)}$$

Thus a plot of $\frac{1}{E}$ against Q should yield a straight line with an intercept of one and a slope of $\frac{1}{Cl_{int}}$

1.5.4. Calculation of Intrinsic Clearance after Intravenous administration of Drug

If extrahepatic clearance is negligible, then equation 5 can be used to describe hepatic drug clearance.

$$Cl_H = \frac{D_{iv}}{AUC_s}$$

Incorporating equation 9:

$$\frac{D_{iv}}{AUC_s} = Cl_{int}\,\frac{AUC_{HV}}{AUC_s}$$

and $$Cl_{int} = \frac{D_{iv}}{AUC_{HV}} \qquad \text{(equation 14)}$$

Thus Cl_{int} can be calculated from the intravenous dose administered divided by the area-under-the-concentration-time-curve in the hepatic vein. AUC_{HV} will therefore be independent of flow.

1.5.5. Calculation of Intrinsic Clearance after Oral administration of Drug

A formula for intrinsic clearance has been derived after oral administration of the drug if absorption is complete and there is no extrahepatic clearance.

$$Cl_{int} = \frac{D_o}{AUC_o} \qquad \text{(equation 15)}$$

where D_o = oral dose administered

AUC_o = area-under-the-concentration-time-curve in the systemic circulation after oral administration.

Interestingly enough, this relationship indicates that AUC_o is independent of flow and depends only on the dose and Cl_{int}, regardless of whether the drug has a low or high clearance (assuming absorption is complete and there is no extrahepatic clearance). The AUC_{HV} after intravenous administration will be equal to AUC in the systemic circulation after oral administration of the same dose.

$$Cl_{int} = \frac{D_{iv}}{AUC_{HV}} = \frac{D_o}{AUC_o}$$

1.5.6. The effect of changes in flow on hepatic drug clearance

The relationship between hepatic drug clearance and flow according to the "venous-equilibration" model is described by equation 11.

$$Cl_H = Q\,E = Q\left[\frac{Cl_{int}}{Cl_{int} + Q}\right]$$

Fig. 5 shows the relationship between flow and actual clearance (as distinct from intrinsic clearance); the latter is calculated from the product of liver blood flow and extraction ratio. It will be seen (at the extreme right of the curve) that when flow is infinitely large, the actual clearance approaches the intrinsic clearance (i.e. its maximum possible clearance) and flow does not therefore affect clearance substantially. However, where flow is small compared to intrinsic clearance (Cl_{int}), as in the extreme left portion of the curve, the actual clearance is quite definitely affected by changes

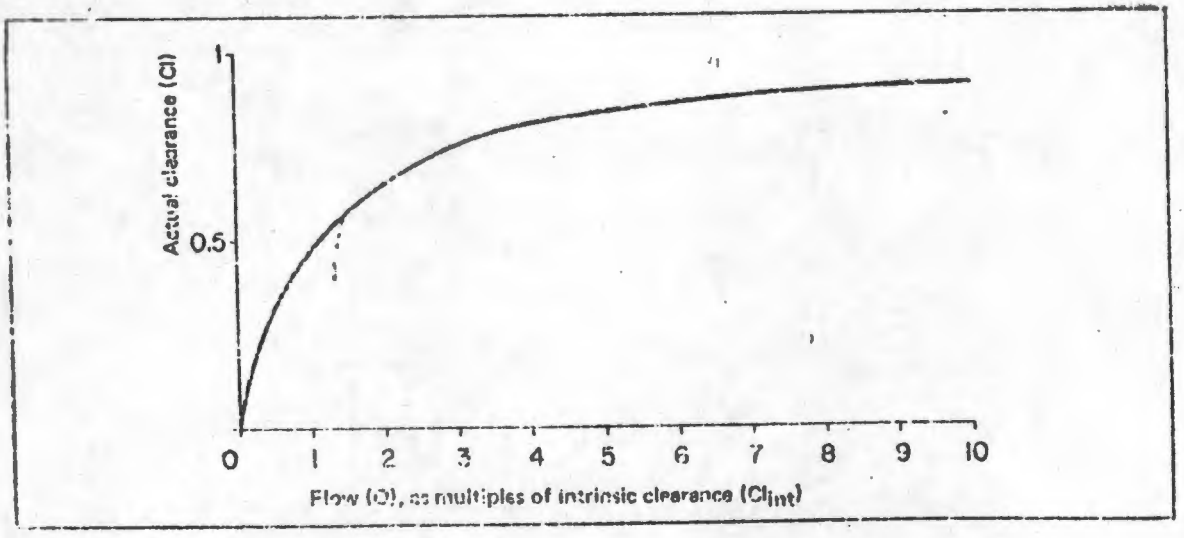


Fig. 5. Theoretical relationship between liver blood flow and actual drug clearance. Both flow and actual clearance have been calculated as multiples of intrinsic hepatic clearance using equation 12 in the text (after Branch et al (19)).

in flow. In the intermediate portion of the curve the effect of flow on actual clearance is less marked.

Because in vivo liver blood flow is not infinitely variable, only a portion of the curve in Fig. 5. will be applicable for a drug depending on the Cl_{int} characteristic for that particular drug.

When Cl_{int} is equal to liver blood flow according to equation 10 ($E = \frac{Cl_{int}}{Q + Cl_{int}}$), the extraction ratio is 0,5. With intrinsic clearances lower than blood flow the extraction is less than 0,5; at intrinsic clearances higher than blood flow hepatic extraction exceeds 0,5.

Fig. 6 shows the set of curves representing the actual clearance versus liver blood flow for drugs which have different intrinsic clearances corresponding to extraction ratios from 0,1 to 0,9 at normal liver blood flow. Clearly for drugs with low Cl_{int} values and low E(<0,5) actual clearances are independent of blood flow, while the clearances of drugs with high Cl_{int} value and high E(>0,5) are flow dependent.

Fig. 7 demonstrates the relationship between liver blood flow and extraction ratio (E). It is apparent that where drugs have high extraction ratios increasing the blood flow

produces minimal decreases in E; where drugs have a low extraction ratio increasing the blood flow results in substantial decreases in E.

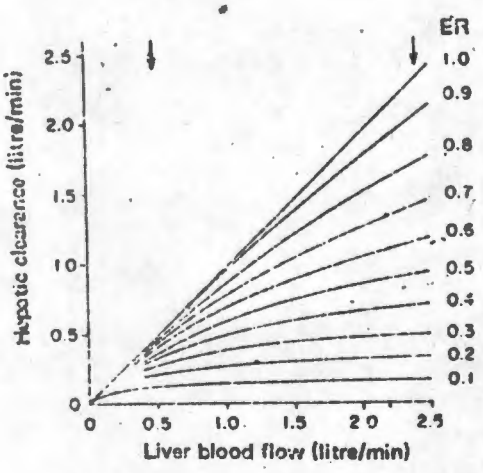


Fig. 6. The relationship between liver blood flow and hepatic clearance for drugs with varying extraction ratios (ER). The arrows indicate the range over which liver blood flow can vary and extraction ratios refer to a normal flow of 1,5 l/min (after Wilkinson and Shand: Clin, Pharm. Ther. 18, 377-390, 1975 (25)).

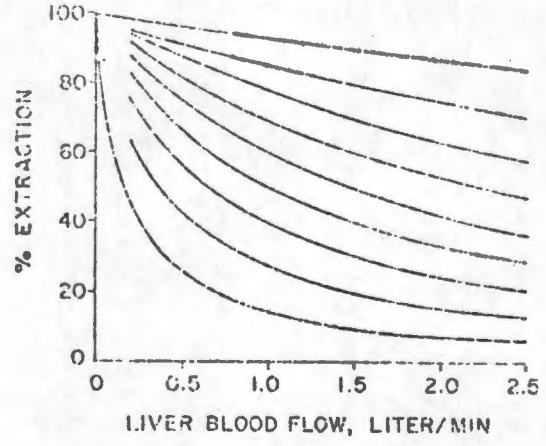


Fig. 7. The relationship between liver blood flow and hepatic extraction ratio for drugs with varying extraction ratios. The individual curves reflect a 10% stepwise change in extraction at a normal flow of 1,5 l/min and therefore each is complementary to the equivalent curve in Fig. 6.

The reason that very low intrinsic clearance (very low E) drugs have actual clearances that are independent of flow (as shown in Fig. 6) is that with these drugs an increase in blood

flow results in a nearly equal decrease in E so that the product of flow and E (i.e. actual clearance) remains relatively unchanged. With very high intrinsic clearance (very high E) drugs an increase in flow produces a minimal decrease in E and thus the actual clearance (i.e. flow x E) is definitely increased. With intermediate intrinsic clearance drugs, the effects of flow on E and thus on actual clearance are also intermediate.

1.5.7. The effect of changes in intrinsic clearance on hepatic drug clearance

As discussed in section 1.5.1., Cl_{int} is *inter alia* dependent on hepatic enzyme activity. Thus changes in hepatic metabolism can result in changes in Cl_{int} which in turn can affect actual drug clearance.

Changes in Cl_{int} will have the most marked effect on actual clearance in the case of drugs which have small Cl_{int} values. For example, an increase in Cl_{int} from 167 ml/min to 357 ml/min will produce an increase in E from 0,1 to 0,2 at a liver blood flow of 1,5 litres/min in humans ($E = \frac{Cl_{int}}{Cl_{int} + Q}$) and thus double the actual clearance (flow x E)(24).

On the other hand changes in Cl_{int} will not have a noticeable effect on actual clearance in the case of drugs with high Cl_{int} values. Thus an increase in Cl_{int} from 6 litres/min to 13,5 litres/min will increase E from 0,8 to 0,9 and thereby increase clearance by only 12,5%.

1.5.8. The effect of changes in drug binding on hepatic drug clearance

There is one other variable that must be considered when assessing drug clearance. Binding to plasma proteins and/or other blood constituents is an important determinant of drug disposition. If the availability of the drug to the metabolic site is limited to the unbound fraction of the drug, then binding to plasma proteins or other blood constituents could retard metabolism. This is certainly so for drugs with low Cl_{int} and low extraction ratios where the extraction ratio of a drug is slightly less than or equal to the unbound fraction

of drug (f_B): clearance is then termed <u>restrictive</u>. If the E is very much less than f_B then binding is not restrictive (i.e. restricted to the free fraction of drug). Since the extraction ratio of some drugs is in fact greater than the free fraction of drug, elimination in these cases is obviously not restricted to that free fraction which is delivered to the liver: this elimination is termed <u>non-restrictive</u>.

It would appear in the latter case that the removal of bound drug in the liver leads to dissociation (or stripping) of bound drug and some of this fraction is subsequently extracted. The blood then helps transport the drug to its site of elimination.

The effects of binding on hepatic clearance have been incorporated into the "venous-equilibration" model.

$$Cl_H = Q\,E = Q\left[\frac{f_B\ Cl_{int}}{Q + f_B\ Cl_{int}}\right]$$

where f_B = fraction of unbound drug in the blood.

Cl_{int} = intrinsic clearance of free drug. This is a measure of the inherent ability of the liver to clear drug from liver water under conditions of first-order metabolism (19).

$Cl_{int}\ f_B = Cl_{int}$ (i.e. intrinsic clearance of total drug, free and bound).

1.5.9. The effect of changes in flow on $t_{\frac{1}{2}}$ and bioavailability

The area under the curve in the systemic circulation after oral administration is independent of flow, and $Cl_{int} = \frac{D_o}{AUC_o}$ (equation 15) - see section 1.5.5.

As discussed previously (section 1.5.6.), drugs with small Cl_{int} values (small E) have systemic clearances that are independent of flow. Thus half-life and the shape of the areas under the curve after oral and intravenous administration are unchanged over a wide range of flow rates.

However, the systemic clearance of a highly extracted drug is definitely flow dependent (section 1.5.6.), as is its half-life. As flow increases, half-life decreases due to an increased hepatic clearance. Thus the area-under-

the-curve in the systemic circulation after intravenous administration will be decreased, $Cl_H = \frac{D_{iv}}{AUC_s}$ (equation 5).

However, as flow increases, the extraction ratio decreases to a certain extent so that bioavailability after oral administration is increased and peak concentrations are higher. This increase in bioavailability is offset by the decrease in half-life so that AUC_o after oral administration remains unchanged (as expected from equation 15) although the shape of the curve will vary with different flow rates.

1.5.10. The effect of changes in intrinsic clearance on $t_{\frac{1}{2}}$ and bioavailability

If the Cl_{int} of a drug with a low Cl_{int} (low E, e.g. 0,1) is increased twofold (say by enzyme induction) there will be an approximately twofold increase in E ($E = \frac{Cl_{int}}{Q+Cl_{int}}$, equation 10).

The area-under-the-curve in the systemic circulation after oral administration will therefore be halved ($Cl_{int} = \frac{D_o}{AUC_o}$, equation 15) in order for the Cl_{int} to double. Even though the change in extraction ratio (0,1 to 0,2) is large, the effect on bioavailability ($f = 1 - E$, equation 7) is not very marked, i.e. it decreases only from 0,9 to 0,8. Thus the 50% decrease in AUC_o (50% increase in Cl_{int}) must be due to the increased drug clearance and thus reduced half-life.

If, on the other hand, Cl_{int} is large (E = 0,9), then doubling the Cl_{int} will increase E by only a small fraction (say 0,9 to 0,95). However, although this first-pass extraction after oral administration is increased only a little, the bioavailability (1 - E) is greatly decreased (from 0,1 to 0,05). The reduction in the area-under-the-curve (due to the increase in Cl_{int}) is thus caused by the decreased fraction of drug reaching the circulation. The actual clearance and thus $t_{\frac{1}{2}}$ will not be affected by the change in Cl_{int}.

1.5.11. Validation of the "Venous-Equilibration" Model

The validity of the theoretical conclusions reached by the "venous-equilibration" model has been established by Shand et al using an isolated rat liver perfusion model shown in Fig. 8 (17).

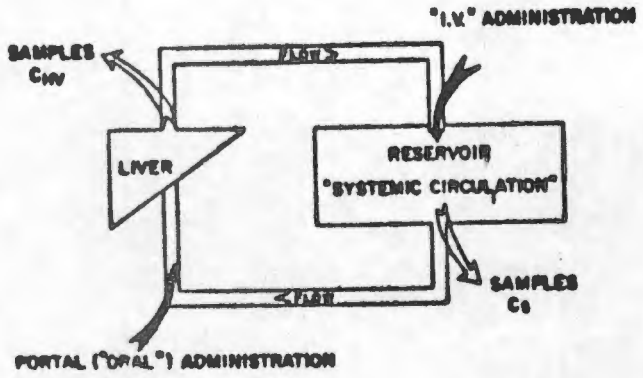


Fig. 8. Diagram of the perfusion circuit. Solid arrows represent the two sites of drug administration and open arrows the sampling sites from the reservoir and the hepatic vein (after Shand et al (17)).

Rat livers were perfused via the portal vein at a constant flow rate of 20 ml/min with 100 ml of 20% rat blood-80% KREBS buffer. The reservoir simulated the 'systemic circulation' and drug administered directly into the reservoir represented intravenous dosing. Drug administered into the portal vein simulated oral dosing since the drug had to pass through the liver before it could reach the reservoir ('systemic circulation'). 5 mg of propranolol were injected over 30 seconds either into the reservoir or into the portal vein and samples were taken at various times over 60 minutes from the reservoir and directly from the venous effluent (Fig. 8).

In another set of experiments the effect of changing the perfusate flow from 20 ml/min to 10 ml/min was examined. Using this model Shand et al were able to show the following:

(i) AUC_{HV} (AUC in the hepatic vein after intravenous dosing) and AUC_o (AUC in the reservoir after oral dosing) were not significantly different. ($Cl_{int} = \frac{D_{iv}}{AUC_{HV}} = \frac{D_o}{AUC_o}$) (section 1.5.5.)

(ii) AUC_{HV} and AUC_o were independent of flow changes, even though the shape of the curve (and the $t_{\frac{1}{2}}$) were different at the two flow rates (see section 1.5.9.).

(iii) The extraction ratio calculated as $E = \frac{Cl_{int}}{Cl_{int} + Q}$ (equation 10) agreed well with the value obtained by measuring the extraction ratio directly. (Equation 3.)

$$\frac{AUC_{PV} - AUC_{HV}}{AUC_{PV}}$$

These principles also held for lidocaine and diphenylhydantoin, drugs which have extraction ratios that are greater and smaller than propranolol respectively (17).

In humans it has been shown that oral drug clearance (and hence intrinsic clearance) is not affected by flow (18, 43) (see equation 15, section 1.5.5.).

Rane et al used an in vitro measurement of the kinetic constants V_{max} and k_m to obtain Cl_{int} and hence extraction ratio. They compared this value with the extraction ratio determined directly in the isolated liver perfusion. Allowing for liver weight, blood flow and drug binding they found a good correlation between the extraction ratios determined in vitro and those obtained from the perfusion model (30).

There is evidence to support the predicted effects of changes in liver blood flow, intrinsic clearance and drug binding on the clearance of propranolol (a high clearance, high Cl_{int} drug). (No data are available in the literature for pindolol.)

(i) Liver blood flow

Branch et al examined the effect of varying flow on the elimination of propranolol by the perfused rat liver (19). The effect of flow was greatest when Cl_{int} was greater than liver blood flow. Using equation 13, they showed that a plot of $\frac{1}{E}$ versus Q yielded a straight line with an intercept of 1 and a slope of $\frac{1}{Cl_{int}}$.

Nies et al have shown in the monkey that hepatic blood flow was the major determinant of propranolol clearance as would be expected for a drug with a high Cl_{int} (60). Racemate propranolol compared to its dextro isomer (devoid of beta-blocking activity) resulted in a lower clearance of the drug

due to a reduction in hepatoportal flow rate induced by beta-adrenergic blockade.

In human subjects a highly significant positive correlation was observed between hepatic blood flow and the clearance of dextro-propranolol (18, 82).

The clearance of propranolol was reduced in liver disease in proportion to the decrease in blood flow (107).

Phenobarbitone increased the clearance of propranolol in the monkey by causing an increased liver blood flow; on the other hand, it increased the clearance of antipyrine (a low Cl_{int} compound) by accelerating hepatic drug metabolism (125).

(ii) Intrinsic clearance

Vestal et al have studied the effect of chlorpromazine on propranolol metabolism (109). Once again the kinetic data were exactly as anticipated for a high clearance drug like propranolol. Chlorpromazine produced a reduction in intrinsic (oral) clearance of propranolol, indicating that propranolol metabolism was inhibited. However, this had an insignificant effect on systemic clearance but affected the bioavailability which increased significantly ($p<0,005$) from 25% to 32%.

Another study showed that a decrease in Cl_{int} from 5,27 litres/min to 2,62 litres/min had no significant effect on the systemic clearance of propranolol but did increase the bioavailability (41).

(iii) Drug binding

Shand et al have shown that in man, dog and rat the clearance of propranolol is not dependent on drug binding, i.e. clearance is non-restrictive (18, 43, 51). This is what is predicted since propranolol has a high extraction ratio which is much greater than the free fraction of drug. However, if drug binding becomes excessively high, as in the monkey, then clearance becomes restrictive (43).

1.5.12. Conclusions and Summary of Findings derived from the "Venous-Equilibration" Model

The "venous-equilibration" model permits the resolution of clearance into three measurable biological parameters: flow rate, intrinsic clearance (extraction ratio) and drug binding. From the practical standpoint, this allows the changes in clearance caused by liver disease, drug interactions and genetic factors to be analysed and predicted.

In clinical terms the intrinsic clearance can be measured conveniently and without recourse to invasive procedures by dividing the dose by the area-under-the-curve in the systemic circulation following a single oral administration of the drug (equation 15). The extraction ratio is easily calculated from the equation $\frac{Cl_{int}}{Q + Cl_{int}}$ (equation 10).

The "venous-equilibration" model thus allows for the classification of a drug in terms of its intrinsic clearance and extraction ratio from a single, simple measurement.

(i) Drugs which have a high extraction ratio (i.e. a high Cl_{int} value which greatly exceeds liver blood flow) will have hepatic clearances which are sensitive to changes in hepatic blood flow but not sensitive to changes in intrinsic clearance (flow-limited drugs). With such drugs, the elimination half-life will thus also be sensitive to changes in flow but not sensitive to changes in intrinsic clearance. There will be a significant first-pass effect after oral administration (in view of the high E). Although changes in Cl_{int} will have relatively little effect on extraction ratio, the bioavailability (1 - E) will be markedly affected by such changes. The extraction ratio of such drugs exceeds the free fraction of drug in the blood and thus elimination is non-restrictive.

(ii) Drugs which have a low extraction ratio (i.e. a low Cl_{int} value which is substantially less than liver blood flow) will have hepatic clearances which are not sensitive to changes in hepatic blood flow but are sensitive to changes in intrinsic clearance (capacity-limited drugs). With such drugs, elimination half-life will be independent of changes in blood flow

but highly sensitive to the liver's ability to metabolise the drug. There will be a small first-pass effect after oral administration (because of the low extraction ratio). Although changes in Cl_{int} will have major effects on extraction ratio, the bioavailability will be relatively unaffected by such changes. The extraction ratio of such drugs is limited to the free fraction of drug in the blood and elimination is thus restrictive.

(iii) Drugs which have intermediate extraction ratios (0,3 - 0,7) will show a partial sensitivity to all three biological variables - hepatic blood flow, hepatic metabolic activity and binding to blood constituents. Such drugs will show a mixed profile of the features of groups (i) and (ii), depending on the actual value of the extraction ratio.

1.6. Literary Review of the Pharmacokinetics of Propranolol

The pharmacokinetics of propranolol have been extensively reviewed (55, 69, 132).

1.6.1. Absorption

Propranolol is absorbed to the extent of more than 90% in man (12, 132). Peak plasma levels are obtained about 2 hours after administration; these vary from at least 7-fold to 20-fold with levels of between 30 ng/ml and 200 ng/ml (31, 120). Levels of between 50 and 100 ng/ml are required for β-blockade (55). Peak levels vary only 2-fold after intravenous administration (31). The attainment of individual peak plasma levels can be affected by the rate of gastric emptying (115).

1.6.2. Distribution

Propranolol has a large apparent volume of distribution in man and mean values of between 150 litres and 295 litres have been reported (18, 31, 68, 80). These volumes exceed the physiological body space and they indicate that propranolol is concentrated in extravascular sites. Propranolol is one of the most lipid soluble β-blockers and has a

distribution ratio in octanol/H_2O of 5,39.

In man, propranolol accumulates in the liver and in other organs including the heart, brain and lungs (50, 136). Accumulation in the lungs is marked and is sufficient to substantially depress the arterial concentration of the drug after intravenous injection (136).

Similar findings have been made in the rat, dog and monkey where propranolol was found in the lungs, brain, liver and kidney (52, 83, 106). Propranolol accumulated preferentially in the lungs followed by the brain. Accumulation in the heart was 5-fold less than in the lung (52) and the accumulation in the liver was also less than in these tissues (52, 83). Dollery and Junod have established the presence of two binding sites in the rat lung, one a high affinity, saturable and temperature-sensitive site, and the other without these properties (57). The binding was inhibited by desmethylimipramine, nortriptyline and chlorpromazine and was enhanced by lidocaine (83).

Hepatic uptake and binding have been extensively studied and these are discussed in section 1.6.6.

Propranolol is highly bound to plasma proteins in humans (83% to 96%) (43, 50, 135) and binding is extensive in rats (90%), dogs (96%) and monkeys (98,5%) (43). The binding of propranolol to human plasma cannot be accounted for by albumin alone and other proteins, e.g. $\alpha_1$acid glycoprotein, are implicated (43, 135). At low concentrations of propranolol plasma binding is co-operative (135).

Red blood cells also bind significant quantities of the drug (43). Blood/plasma ratios of approximately 0,85 have been found in monkey, dog and rat, and in man values between 0,85 and 1,5 have been reported (75, 76).

1.6.3. Metabolism

Propranolol is extensively metabolised in the liver in man, rat, dog and monkey. In humans only 25 µg of a 10 mg dose given intravenously were excreted as the parent drug (31). *In vitro* work has shown that propranolol is metabolised by the microsomal enzyme system requiring NADPH and oxygen. This

requirement is characteristic of mixed function oxidases. The metabolic pathways are qualitatively the same in man and animal species although there are quantitative differences.

In 1976 Bond characterised the metabolites as being about 30% acidic and 70% amphoteric (with only minute amounts of basic compounds), and he isolated two of the main metabolites, 4-hydroxy propranolol and propranolol glucuronide (110). Since then at least 19 metabolites have been identified (38, 114).

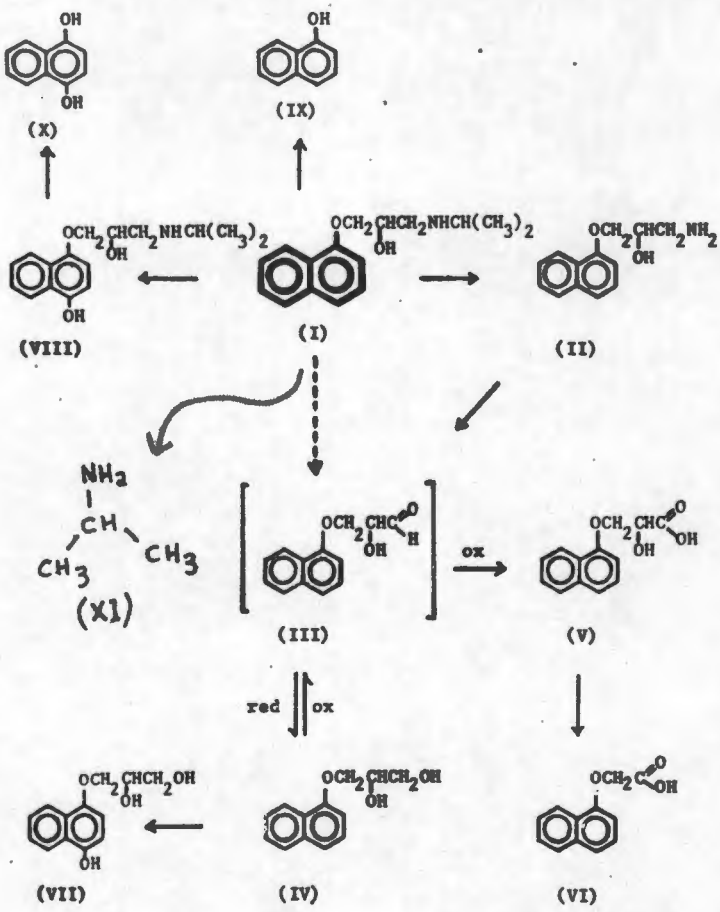


Fig. 9. Schematic representation of propranolol metabolism in man and dog. I, propranolol; II, desisopropyl propranolol; III, proposed aldehyde; IV, glycol; V, naphthoxylactic acid; VI, naphthoxyacetic acid; VII, OH-glycol; VIII, 4-hydroxy propranolol; IX, naphthol; X, dihydroxynaphthalene; XI, isopropylamine; (after Walle and Gaffney: (67)).

The most well documented pathways of metabolism are shown in Fig. 9. The four primary pathways involve O-dealkylation, side-chain oxidation, glucuronic acid conjugation and ring oxidation.

Naphthoxylactic acid accounts for 20% of a single oral dose but 40% of a single intravenous dose in man and is an important metabolite in the rat, dog and monkey. Propranolol glucuronide accounts for 2,5% to 25% of a given dose in man. The metabolites II, IV, VII, VIII, IX and X (Fig. 9) are usually excreted as glucuronides and ethereal sulphates (132).

Propranolol has fairly varied pharmacological effects and it is possible that several of the metabolites contribute to these properties (67). 4-hydroxy propranolol is the major metabolite and it is also an active one (111). Hayes and Cooper (52) and others (87) detected 4-hydroxy propranolol 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.

The isopropylamine metabolite (XI) has sympathomimetic activity and it is speculated that the glycol metabolite (IV) may contribute to propranolol's central nervous effects (9, 114).

1.6.4. Elimination

Propranolol is almost entirely eliminated by various metabolic systems in the liver (37, 51). The systemic clearance after intravenous administration (which is thus equal to the hepatic clearance) was about 1 litre/min with values varying from 0,6 to 1,5 litres/minute (31, 18, 58, 80). The plasma elimination half-life was between 2 - 3 hours but values of 4 hours have been reported (18, 58).

The intrinsic clearance (oral clearance) in man varied between 1,16 litres/min and 6,06 litres/min with an average of 2,71 litres/min (18, 31, 50). This is more than the average liver blood flow in man (approximately 1,5 litres/min) and indicates that propranolol is a flow-limited drug with a high extraction ratio (18, 82, 107).

In rats the Cl_{int} has been estimated as 10 ml/g/min (30, 41) from *in vitro* studies of metabolism although a lower value of 25 ml/min (uncorrected for liver weight) has been reported, using an isolated liver perfusion model (17). The liver blood flow in the rat is only 1,2 ml/g/min and this again

emphasises that propranolol is a flow-limited drug in this species.

Shand et al have shown that drug concentrations declined bi-exponentially after intravenous dosage in man. The early α-phase had a half-life of 10 minutes and the later β-phase one of 2,3 hours (31) (see Fig. 10). A biphasic curve has also been observed in the rat (15). Thus propranolol conformed to a two-compartment open model.

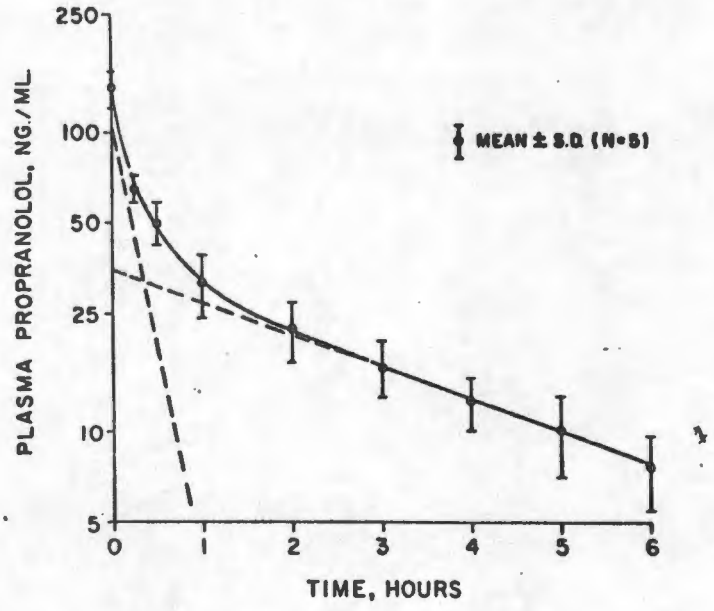


Fig. 10. Plasma propranolol levels after a 10 mg intravenous infusion at a rate of 1,03 mg per minute. Each point represents the mean and standard deviation of the levels determined in 5 subjects (after Shand et al (31)).

1.6.5. First-pass extraction

Since propranolol is a drug with a high Cl_{int} value, one would expect it to have a high extraction ratio, a large first-pass effect and a low bioavailability after oral administration. There is abundant evidence in the literature to support this. In man, Shand and Rangno have shown that after an oral dose of 30 mg or less, only traces of unchanged propranolol could be detected in the circulation and extraction was nearly complete (46). In a patient with portacaval anastamosis, where portal blood bypasses the liver, a large proportion of the drug was systemically available. This proved that the low bioavailability mentioned above was due to the first-pass extraction (46).

At doses of 40 mg and above the first-pass effect dropped to 0,72, indicating that this effect was saturable. Wood et al calculated an average bioavailability of 22% after a single dose but 34% after chronic dosage. Similar first-pass extractions of 0,75 (59) and 0,74 (82) were found after propranolol administration for several days. First-pass extractions, however, varied from 40% to 80% in studies by Kornhauser (18) and Shand (31).

Thus the first-pass extraction of propranolol is considerable and also extremely variable in man. This variation may be exaggerated by patient non-compliance, poorly controlled studies and insensitive assay procedures (70).

Animal studies have given similar extraction ratios. In studies with mongrel dogs, the extraction ratio was measured directly using arterial and portal venous blood concentrations and was found to be 0,90 (106). Breckenridge et al, using similar methods to measure extraction, found values that varied from 0,69 to 0,92 (4, 65).

In an in vivo experiment with rats, little or no drug appeared in the systemic circulation after intraportal administration of doses less than 0,8 mg/kg. As saturation occurred, however, extraction decreased from 97,5% (with a dose of 0,625 mg/kg) to 50% (with a dose of 5 mg/kg) (15). This was also shown using liver perfusions, where drug only appeared in the effluent perfusate when concentrations had been increased to 20 µg/ml.

The extraction ratio in monkeys averaged 0,49, which is slightly lower than in other animal species (60).

1.6.6. Hepatic Uptake of Propranolol

In the previous section the saturability of the hepatic extraction of propranolol was mentioned. This phenomenon has been extensively studied and it is apparent that the kinetics of propranolol after oral administration are complex.

Shand and Rangno found that with doses greater than 40 mg in man hepatic removal became saturated, after which there was an approximately 60% increase in bioavailability (46). Furthermore, it has been proposed that there is a second

extraction process (probably reflecting metabolism) which itself becomes saturated by the increased bioavailability (50,70).

Evans and Shand postulated that this first avid extraction process depended on the physical binding of the unchanged drug to sites in the liver (50).

After perfusing isolated rat livers with propranolol, Shand and his colleagues homogenised these livers and assayed the amount of unchanged drug therein. There was a liver/effluent concentration ratio of 170 : 1 at low concentrations of propranolol (10 µg/ml), and at high concentrations (160 µg/ml) the ratio decreased to 9,3 : 1 (15). Anderson calculated ratios of 12 : 1 to 36 : 1 after a dosage of about 11 mg (101).

Thus the extraction of propranolol during liver perfusions was dose-dependent. Drug concentration declined mono-exponentially following low doses (1 mg) and bi-exponentially following higher doses (5 mg and above) (14).

The accumulation of unchanged drug was not affected by enzyme inhibitors. This accumulation of drug reflected binding and uptake into the liver and is represented by the α-phase. The β-phase, on the other hand, was sensitive to enzyme inhibition and apparently represented drug metabolism. The α and β phases therefore resulted from different biological mechanisms (14, 101).

Several groups of workers have demonstrated that hepatic uptake of propranolol involves two binding sites, one with a high affinity and low capacity and the other with low affinity and a higher capacity (76, 83, 101). Anderson et al found that the high affinity site had a dissociation constant of 3 µg/ml and a capacity of 500 µg/g liver. The low affinity site had a dissociation constant of 160 µg/ml and a capacity of 6500 µg/g liver (101).

Autoradiography showed considerable concentrations of propranolol in the periportal zones of the liver, indicating that propranolol was efficiently cleared from the perfusate. Within these periportal zones certain areas were more heavily labelled with isotope than others, suggesting that a

particular type of cell (as yet unidentified) might be involved in propranolol uptake (101).

Some workers have attributed the high affinity binding to mitochondria (83, 101). Propranolol inhibited oxidative phosphorylation and increased the permeability of the inner mitochondrial membrane to sodium and potassium ions (127, 112).

Other investigators have minimised mitochondrial binding and claimed that the high affinity areas were confined to the microsomal fraction. Schneck et al found during in vitro studies that there was a correlation between high affinity binding and metabolism in the rat. Binding of propranolol actually occurred to cytochrome P450 which might itself have represented a high affinity site (73, 83).

It is probable that binding sites are found in both the mitochondrial and microsomal fractions (101). Binding to a cytosol protein has been excluded (83). Although the exact nature of the binding sites is unclear, it would appear that hepatic uptake is primarily a phenomenon of physical binding to several sites of different affinities, different capacities and different intrahepatic locations.

1.7. Literary Review of the Pharmacokinetics of Pindolol

The pharmacokinetics of pindolol in relation to other β-blockers have been recently reviewed (37).

1.7.1. Absorption

Pindolol absorption in man is fast and essentially complete (92%) in subjects fasted overnight (3, 21, 37). The absorption half-life is 25 minutes in man (12) and peak plasma levels (20 ng/ml to 80 ng/ml) were reached after about 80 minutes (21). Levels varied about 4-fold after oral administration. Food intake three hours before oral dosage delayed peak concentrations by 30 minutes (49). A plasma level of 10 ng/ml is required for β-blockade. Unlike propranolol plasma levels rise in proportion to the dose and there are no non-linear kinetics (5, 64).

1.7.2. Distribution

The mean apparent volume of distribution in man after intravenous and oral administration averaged 136 litres and 142 litres respectively (21). Pindolol is one of the most water soluble β-blockers and has a distribution ratio in octanol/H_2O of only 0,12 (1, 12). Its volume of distribution, although one of the lowest amongst the β-blockers, still significantly exceeds the physiological body space; this indicates that the drug is concentrated in various tissues.

There is medium binding to plasma albumin (57%) (21) and to other plasma proteins (50%) (56) and the average binding to serum is about 40% in humans, rats and dogs (100, 105).

Workers from the Sandoz Laboratories have claimed that binding of pindolol to red blood cells is an important determinant of its distribution, although they minimised the pharmacokinetic significance of this (100). The fraction of pindolol in erythrocytes is 0,52 in humans, 0,51 in rats and 0,59 in dogs (100). This fraction was decreased *in vivo* in rats when the anthranilic acid metabolite of pindolol displaced pindolol from the red blood cells. Uptake of pindolol by red blood cells has also been observed in other studies in humans (39, 56).

1.7.3. Metabolism

In man large doses of pindolol are required to obtain sufficient quantities of metabolites for the elucidation of their structure. The drug is more extensively metabolised in animal species and this has allowed some delineation of the metabolic pathways.

Four different pathways are recognised in rat, rabbit, dog and monkey: (i) side-chain conjugation with glucuronic acid, (ii) hydroxylation or oxidation with conjugation of the indole ring, (iii) oxidative ring scission, and (iv) side-chain oxidation and deamination (8).

Kiechel *et al* have identified nine metabolites from the urine of monkeys, rats, cats and rabbits. A diagram of the metabolic pathways is shown in Fig. 11 (8). The metabolites isolated from rat urine are circled in the diagram.

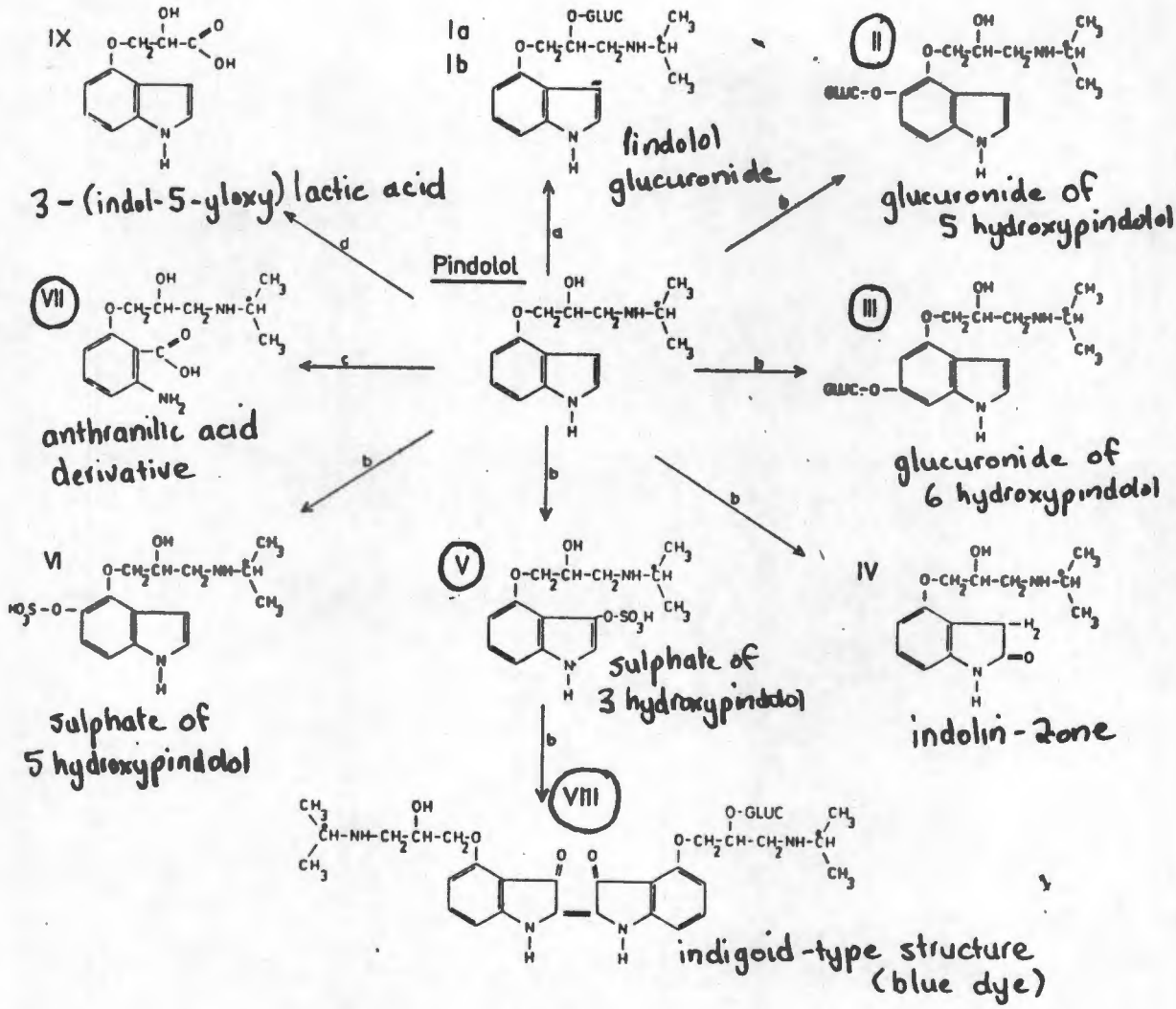


Fig. 11. Metabolic pathways (a - d) of pindolol in rat, monkey, rabbit and dog (after Kiechel et al (8)).

Metabolite VIII is a blue dye which causes a green coloration in the urine of rats.

To date no active metabolites of pindolol have been identified in man in whom over 90% of the metabolites are glucuronides and sulphates (11).

In rats, however, certain of the metabolites are active and appear to be important in mediating the effects of the drug on blood pressure and heart rate (13).

1.7.4. Elimination

The relative water solubility of pindolol facilitates its excretion by the kidneys. In man 40% of an administered dose is excreted unchanged by the kidneys (3, 11, 21, 56).

This marked extrahepatic elimination makes the estimation of hepatic intrinsic clearance difficult.

In dogs only 4% to 7% of a 25 mg oral dose was recovered unchanged in the urine so that extrahepatic elimination is less in this species (40) as it is also in the rat (13).

The total clearance of pindolol in man averaged 0,48 litres/min (21, 55, 56). The plasma elimination half-life varied between 3 and 4 hours (12, 21). The half-life is only 30 minutes in the rat (26).

Initial studies by Gugler suggested that pindolol obeyed a one-compartment open model. However the biphasic nature of the semi-logarithmic plots of concentration-versus-time - compatible with a two-compartment open model - has since been demonstrated by other workers (11, 39, 56) (see Fig. 12).

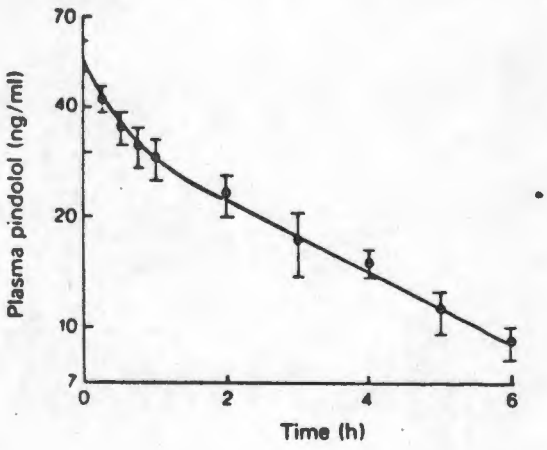


Fig. 12. Mean and s.e. mean of time-concentration curves obtained in four subjects following a 3 mg intravenous bolus of pindolol at zero time (after Jennings et al (39)).

1.7.5. First-pass extraction

There is considerable evidence that pindolol and practolol have the lowest first-pass extractions among the β-blockers in man.

Gugler et al calculated bioavailability after oral compared to intravenous administration to be 92% (21). This figure has been criticised by Jennings and colleagues on the grounds that different subjects were used for the oral and

intravenous administrations. In their study they obtained a bioavailability value of 53% which they regarded as being more in accord with the drug's rate of systemic clearance (39).

However, first-pass extractions of only 13% and 20% have been reported in the majority of cases (3, 56, 84). Pindolol is a drug with a low Cl_{int}, a low first-pass extraction and a large bioavailability.

The first-pass extraction was greater in animals. Two beagle dogs were given 25 mg of pindolol intravenously and then orally after a washout period of three weeks. Bioavailability was 54,2% and 55,2% respectively, indicating a first-pass extraction of only 45% (40). Metabolism (and thus extraction) is more extensive in rats than man (8, 13).

1.8. Factors affecting First-Pass Extraction

From equation 7, bioavailability (f) is equal to (1 - E). Thus factors which affect the extraction ratio inversely affect the bioavailability.

Propranolol

Propranolol has a high Cl_{int}, a large extraction ratio and therefore a small bioavailability. Even minor changes in extraction ratio will have marked effects on bioavailability e.g. a change in E from 0,9 to 0,8 will double bioavailability from 0,1 to 0,2 (see page 17). Thus changes in Cl_{int} (i.e. changes in metabolism), although producing small effects on first-pass extraction, can be practically important because of their large influences on bioavailability.

Changes in liver blood flow will cause slight (inverse) changes in the extraction ratio of a high Cl_{int} drug like propranolol, but these again will have pronounced effects on bioavailability.

Pindolol

In humans pindolol has a small Cl_{int} value, a small first-pass extraction and a high bioavailability. Even if the extraction ratio of this drug is substantially altered by changes in Cl_{int} or flow rate (e.g. if E doubles from 0,1 to 0,2), the bioavailability decreases by only 10% (see page 17).

This will not be of major practical importance.

In the various animal species studied the extraction of pindolol is more extensive than in man. One would expect this higher extraction ratio to be affected by the same factors which influence propranolol.

A multitude of constitutional and environmental factors, the presence of disease, and the coadministration of other agents can alter the pharmacokinetics of a drug. The following gives a brief outline of the factors which may affect the first-pass extraction of propranolol and (where data are available) pindolol.

1.8.1. Genetic factors

The metabolism of drugs is clearly under genetic control: monozygotic twins showed much greater similarity in their metabolism of drugs than did dizygotic twins (2, 79). Genetic factors certainly contribute to the large inter-individual (up to 20-fold) variation (120) found among normal subjects after propranolol administration.

1.8.2. Food

The influence of food on bioavailability has been studied by various workers with conflicting results. Some workers have found no correlation between food and bioavailability (70, 75).

Melander *et al* found an increased bioavailability of propranolol when the drug was administered after a meal (81). In one report the severity of side-effects in a patient especially after meals was attributed to the increased bioavailability of the drug (122).

The mechanism by which food decreases the first-pass extraction is not known. McLean found that an increased hepatic blood flow during drug absorption decreased first-pass extraction (123). Drug metabolism may also be affected by food. The content of the diet (e.g. charcoal-broiled beef, high protein, high carbohydrate) can affect metabolism in different ways (53, 102). The fact that Walle *et al* (68), unlike other workers, found only a one- to three-fold variation in plasma propranolol after oral administration was

explained by the rigid dietary control imposed on his subjects (132). The metabolism of drugs is reduced in chronically malnourished patients (88).

1.8.3. Age

The range of bioavailability of propranolol obtained in children is similar to that observed in adults (117).

Hepatic microsomal metabolism, intrinsic clearance and first-pass extraction are reduced in the elderly (5) who show higher plasma levels of propranolol than do young adults after single (130) and continued oral doses (90).

1.8.4. Drug Interactions

Halofenate caused a lowering of propranolol levels in four subjects on chronic therapy (129), probably by increasing the intrinsic clearance and first-pass extraction of propranolol.

Chlorpromazine resulted in a reduced intrinsic clearance of propranolol (4,3 litres/minute to 2,90 litres/minute) and an increased bioavailability (25% to 32%) (109). It appears from studies in dogs that chlorpromazine altered the metabolic pathway of propranolol (38).

Several tricyclic antidepressants inhibited propranolol metabolism *in vitro* in the rat (28). Drugs such as amitriptyline, nortriptyline and chlorpromazine displaced propranolol from its binding sites in the rat liver (101). Coadministration of these drugs would therefore tend to reduce the first-pass extraction of propranolol.

Ethanol and phenobarbital stimulated the *in vitro* metabolism of propranolol in rats (6, 16) and it is possible that they might also do so in man. Phenobarbital increased the capacity of the high affinity uptake site in rat liver (16) and would thus be expected to increase the first-pass extraction of propranolol.

Smoking increased propranolol metabolism (138) and this may result in a lower bioavailability of the drug.

1.8.5. Liver disease

Chronic liver disease can affect the various determinants of drug disposition simultaneously. Thus, liver disease results in a reduced hepatic metabolism (reduced protein synthesis), a decrease in overall hepatic blood flow and the shunting of blood around or within the liver. The effect of liver disease on drug elimination has been comprehensively discussed in recent review articles (22, 34, 126).

The extraction of propranolol is reduced in patients with cirrhosis in proportion to the severity of the disease (107). An increased systemic availability (54%) was found in these patients compared to controls (38%) (118) and a reduction in extraction ratio to as low as 0,41 has been reported (116).

While a reduction of Cl_{int} occurred in chronic cirrhosis (116, 118, 97), portasystemic shunts also contributed to the reduction in extraction ratio and first-pass effect.

The extraction ratio of propranolol was reduced in isolated perfused rat livers made cirrhotic by chronic carbon tetrachloride inhalation (133). The unexpected finding of a reduced (rather than increased) extraction ratio with decreasing blood flow in these livers was attributed to a shunting of the blood from normally functioning hepatocytes to non-functioning tissue (intact hepatocyte theory) (133).

1.8.6. Hypothermia

Hypothermia can substantially alter the pharmacokinetics of propranolol both *in vivo* (108, 93) and *in vitro* (66) by decreasing metabolism. This would suggest that temperature changes may affect first-pass metabolism.

1.8.7. Renal disease

A single oral dose of propranolol, although readily absorbed in patients with renal disease, underwent a reduced first-pass extraction (58). However, after multiple dosing, there was an increased extraction ratio (33), possibly owing to enzyme induction.

With pindolol there was a reduced bioavailability in patients with renal disease but this resulted from a

diminished gastrointestinal absorption (56, 84).

1.8.8. Thyroid disease (Hyperthyroidism)

The data in hyperthyroid subjects have been conflicting. Feely et al found an increased metabolism and extraction ratio of propranolol in hyperthyroid patients (91) while others found no change of pharmacokinetics in such patients (92).

1.8.9. Cardiac disease

The clearance of lignocaine (a very high clearance drug like propranolol) was reduced in patients with cardiac failure and cardiogenic shock (following myocardial infarction) as a result of reduced hepatic blood flow and impaired metabolism (36, 74).

It is probable that the extraction of propranolol may be similarly affected in these conditions.

1.8.10. Coeliac disease

There is an increased rate of absorption of propranolol in patients with coeliac disease (9, 10). The increased plasma levels in these patients has been attributed to saturation of the first-pass extraction (10), although others have suggested that these elevated levels result from a redistribution of the drug from the red blood cells into the plasma.

1.8.11. Inflammatory diseases

$\alpha_1$acid glycoprotein binds cationic drugs such as propranolol. The increase in $\alpha_1$acid glycoprotein in certain inflammatory diseases (Crohn's disease and rheumatoid arthritis) resulted in an enhanced binding of propranolol to this glycoprotein (9, 103, 105).

Schneider et al observed that patients with these diseases had a reduced oral clearance (intrinsic clearance) of propranolol with plasma levels up to seven times higher than normal (9, 104). They made the interesting suggestion that the binding of propranolol to glycoprotein somehow protected the drug from first-pass metabolism by the liver (104).

CHAPTER 2

2. METHODS

2.1. General Principles of the Isolated Liver Perfusion Model

The isolated rat liver perfusion has been used extensively in metabolic studies as well as studies investigating drug disposition and metabolism. Basically the technique involves cannulation of the portal vein allowing inflow of perfusion medium followed by removal of the liver from the carcass and its perfusion in a cabinet where physiological temperature, pH, oxygen and carbon dioxide tension are maintained.

Studies which use isolated microsomal systems to measure drug metabolism cannot meaningfully supply information on parameters such as hepatic extraction and drug clearance which depend on liver blood flow and tissue distribution.

However an isolated liver perfusion is ideal for studying hepatic drug clearance because:

(i) flow can be accurately calculated;
(ii) extraction can be measured directly;
(iii) oral and intravenous administrations can be simulated; and
(iv) the simple two-component system (liver and perfusate) enables hepatic extraction and clearance to be studied without contributions from other organs of elimination.

The duration of perfusion has varied with the requirements of the investigator. The liver is viable for at least 2 - 3 hours (61, 62) and livers have even been perfused for up to 6 hours (35).

Normal liver function is maintained during the perfusion. Kerly et al have shown that bile was secreted at nearly the same rate during perfusion as by anaesthetised rats (59). The water content of the liver remained within the range observed in intact animals. There was no appreciable haemolysis during perfusion and the sodium ion concentration of the medium did not change (59). Although there was a slight

increase in potassium ion concentrations after 1½ hours with a greater increase after 2½ hours, this change was similar from one liver to the next (59).

Rowland has found a good correlation between the drug clearance calculated using an isolated liver perfusion and the clearance calculated *in vivo* in the rat (42, 54).

The isolated liver perfusion model is an *in vitro* model. Caution is required when extrapolating results in absolute terms to the *in vivo* situation. Nevertheless, if the perfusions are carried out with care and if the conditions (e.g. temperature (66, 108) and perfusate volume (44, 78)) remain constant from one perfusion to the next, the technique can be reliably used to compare the extraction of two drugs.

2.2. Practical Applications of the Isolated Liver Perfusion Model

All perfusions were carried out in the Department of Medicine, University of Cape Town Medical School. Before the experiments reported in this study were performed, I had spent several months developing and perfecting the surgical techniques and the appropriate methods (see also section 2.3.).

2.2.1. Introduction

The perfusion model used was based on the method of Miller *et al* (35) with modifications to reduce the time of the operation by Kerly *et al* (59) and Hems *et al* (63). This model has been used by workers in the Department of Medicine (61, 62).

The model differs from that used by Shand and his colleagues (17, 32, 19). These workers pumped the perfusate through the liver at a *constant flow*. In my perfusions the liver was always at a *constant pressure* comparable to that in the intact animal and the liver determined its own flow depending on its size and the cannulation of the portal vein. This is thought to represent a more physiological situation. It is possible that under conditions of high pressure perfusion the entire liver may not be perfused. Furthermore, forced pumping may damage the liver.

My liver perfusion experiments lasted for only 90 minutes. This time was well within the time limits of viability reported by other workers.

2.2.2. Apparatus

The glass-fronted insulated perfusion cabinet was built in the Department of Medicine. A labelled diagram of the apparatus is shown in Fig. 12.

The heating element (h) ensured that the cabinet was kept at a temperature of 38°C.

The cabinet was humidified by means of steam from boiling water in a flask which was fed into the cabinet. The air was circulated by a fan.

The glass reservoir (r) had two openings for the inflow and outflow tubing as well as an opening for the oxygenation tubing and a wider opening for sample collection.

The oxygenation chamber or glass lung (o) consisted of five involuted chambers. The lung is constructed so that flow is retarded which, together with the involuted nature of the glass walls, ensured that the perfusion medium spread out as a thin film and received adequate oxygenation. 95% O_2. 5CO_2, after being bubbled through water, entered the glass lung as shown in the diagram.

The liver (l) was placed on a piece of wire gauze on a glass plate (g) which fitted into the reservoir as shown in the figure. The portal vein cannula (c) fitted onto the end of the glass lung.

Filters (f), which collected any small clots, were placed at the points shown in the figure.

Flexible vinyl tubing was used for connecting the apparatus.

The perfusion medium was pumped to the glass oxygenator by a Sarns Model 5500 pump (p) and thereafter flowed through the liver at a rate determined by a) the liver size, b) the bore of the portal vein cannula and c) the pressure head. The latter (d) was kept constant at 16 cm of water by adjusting the clamp (b) in the overflow bypass (e) so that the

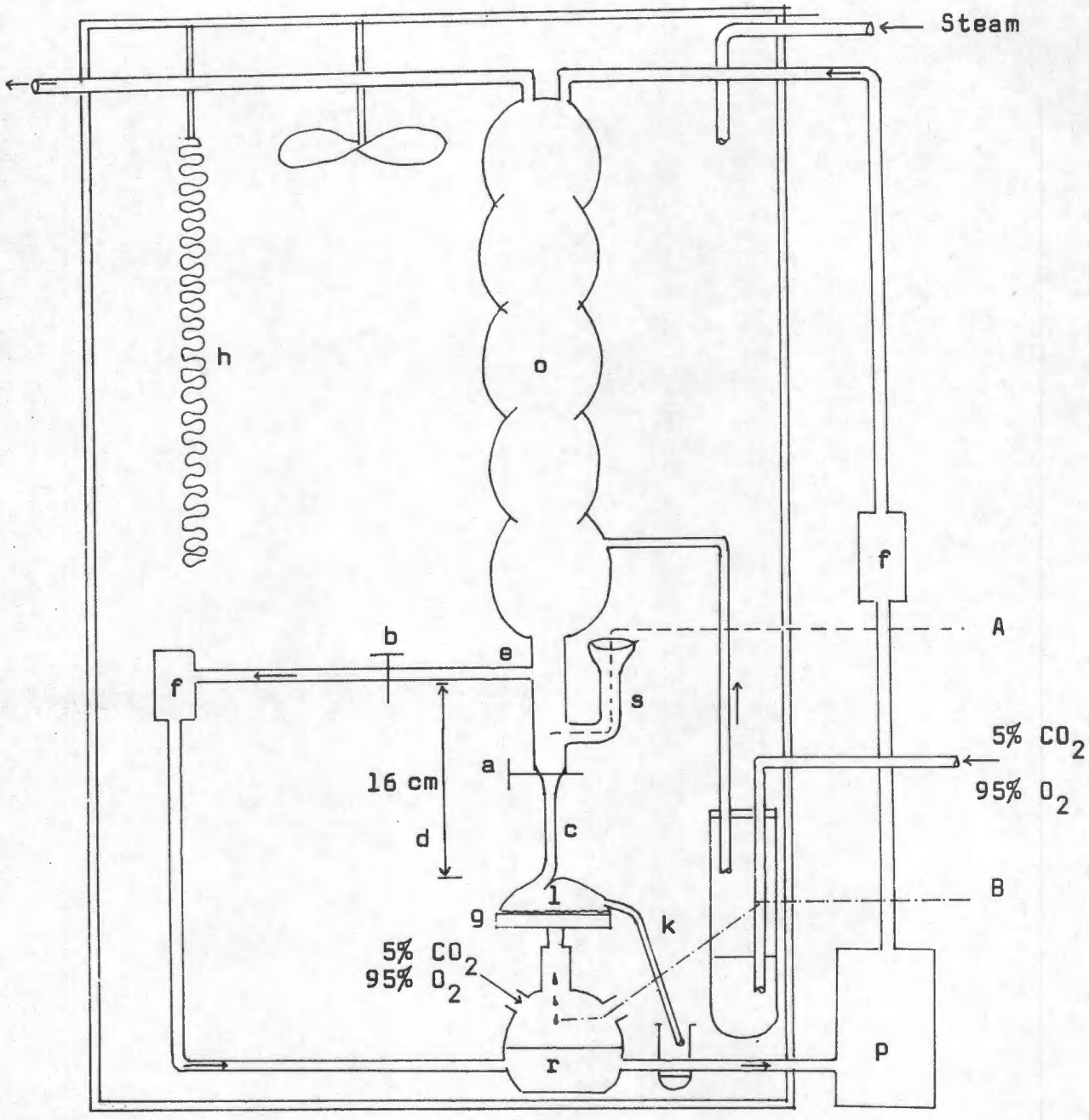


Fig. 12. The Perfusion System

A = portal vein sample
B = hepatic vein sample
l = liver
r = reservoir
p = pump
o = thin film glass oxygenator
f = filter
a = tap
b = clamp
k = bile duct cannula
s = pressure side-arm
d = pressure head
g = glass plate
c = portal vein cannula
e = overflow bypass
h = heating element

perfusate did not build up in the glass lung over the 16 cm mark. The overflow bypass drained back into the reservoir.

The outflow of perfusate from the hepatic vein fell directly into the reservoir which was continuously mixed by a magnetic stirrer.

The perfusion medium was 20% rat blood 80% KREBS buffer. Heparinised blood was obtained by cardiac puncture from Long-Evans rats under diethyl ether anaesthesia. KREBS buffer consisted of 118,3 mM NaCl; 4,69 mM KCl; 2,52 mM $CaCl_2$; 1,18 mM $MgSO_4.7H_2O$; 25 mM $NaHCO_3$ and 1,18 mM KH_2PO_4 according to Shand et al (16). The buffer was freshly made up every morning.

100 ml of blood-KREBS perfusion medium was circulated and oxygenated in the cabinet for 20 minutes before the liver was introduced into the system.

2.2.3. Surgical procedures

This part of the procedure was carried out with the extremely able assistance of Mr M. Parker of the Department of Medicine, University of Cape Town.

Male Long-Evans rats of approximately 300 g were operated on under diethyl ether anaesthesia. When the depth of anaesthesia was satisfactory, the rat was secured on an operating platform.

An incision was made down the length of the bloodless abdominal midline and two lateral incisions made to expose the abdominal cavity. The cavity was washed with warm saline throughout the procedure to prevent the tissues from drying out.

The stomach was carefully freed from the liver and the oesophagus cut in close proximity to the diaphragm. The liver lobes were covered with a swab moistened with warm saline.

The common bile duct was exposed and cannulated with fine portex tubing, ensuring that the side branches of the duct were not obstructed.

The abdominal contents were then moved over to the right. This exposed the portal vein.

Two ligatures were placed around the portal vein about

two centimetres apart and left untied. The two collateral veins were tied off tightly.

The portal vein cannula was connected up to a bottle of plasmalyte B so that the warm liquid dripped slowly from it. From then on the experiment proceeded as quickly as possible.

The lower ligature around the portal vein was secured. An incision was made just proximal to the top ligature and the vein was cannulated. This cannula was secured after ensuring that all the liver lobes were perfused. This could be observed because the lobes lightened in colour as the plasmalyte B washed through the liver.

The hepatic veins were cut flush with the liver and then the perfusion rate of plasmalyte B was increased slightly. The liver was rapidly dissected out, taking care to keep handling of the liver to a minimum.

Any adhering fat was trimmed from the liver and the organ was connected to the perfusion system via the portal vein cannula.

The liver lobes were carefully spread out and the liver arranged so that the portal vein cannula was not twisted and the blood-KREBS could issue freely from the hepatic vein.

With practice, the time taken from the incision in the portal vein to the establishment of the liver in the cabinet was usually under two minutes. Shock and anoxia were thus kept to a minimum.

The liver was allowed to equilibrate for 30 minutes before the actual experiment began.

2.2.4. Experimental Steps

After equilibrating the liver for 30 minutes, 5 mg of the drug dissolved in 3 ml of 20% blood - 80% KREBS were injected as a single bolus into the reservoir. This simulated an intravenous administration of drug. See Fig. 8, section 1.4.11.

Samples of about 300 µl were taken from just above the liver (the portal vein samples) by extending a piece of tubing down the pressure side-arm at point A in Fig. 12. Immediately

afterwards samples were collected from the effluent drops before they reached the reservoir (hepatic vein samples), point B in Fig. 12. Samples were taken 1, 3, 5, 10, 20, 30, 40 and 60 minutes after injection of the drug.

The kinetics of drug elimination are not appreciably affected if the sampling volumes are kept as small as they were in my experiments (78).

Perfusate flow was calculated by collecting and timing the drops from the hepatic vein when samples were collected. The volume of the sample was obtained by weighing and this volume was used to calculate the flow rate in ml/min.

Perfusate flow varied from 1,83 - 5,05 ml/gram wet liver tissue/minute. (21 ml/min - 60 ml/min.) Because of the less viscous nature of my perfusate this flow range is greater than the range of about 1 - 3 ml/g/min obtained with 100% whole blood. Perfusions were only acceptable if the flow rate was reasonably constant throughout the experiment.

Bile flow rate ranged from 15 seconds per drop at the beginning to 90 seconds per drop at the end of the perfusion although the range varied from one perfusion to the next. If the bile did not flow spontaneously and in regular droplets, the perfusion was discarded, although in most cases a sluggish bile flow was corrected by rearranging a twisted bile cannula.

The pH was kept constant between 7,4 and 7,6 - if necessary, by the addition of 4,2% sodium bicarbonate.

The packed cell volume was measured at both the beginning and at the end of the perfusion to check that there was no appreciable haemolysis.

A liver perfusion was only accepted for inclusion in this study if it satisfied all of the following criteria of viability:

(i) adequate bile production;
(ii) adequate perfusate flow;
(iii) the absence of visible infarcts and/or liver swelling; and
(iv) a stable portal pressure.

It is relevant to note that during the perfusions with pindolol a blue pigment appeared in the bile resulting in a dark green coloration. This was due to a metabolite of pindolol (metabolite VIII, Fig. 10, section 1.6.3) and it was a clear demonstration of the presence of an actively metabolising liver system. This was additional evidence of the viability of the perfusion model.

When the perfusion was complete the liver was weighed.

Samples were assayed for drug on the same day, or refrigerated overnight for analysis the next day.

2.3. Additional notes on Overflow Bypass

One apparently limiting factor of my perfusion model was that there was an overflow bypass (e, Fig. 12).

This bypass was designed to regulate the effects of flow on the pressure head (d, Fig. 12). Since the liver blood flows in my experiments varied between 21 ml/min and 60 ml/min the pressure tended to build up above the 16 cm mark, more particularly where the rate of liver blood flow was slow. In order to maintain the constant pressure head of 16 cm of water, it was necessary to drain off the excess blood volumes into the overflow by regulation of clamp b (Fig. 12). This blood flowed directly back to the reservoir and thereby escaped the liver. Such an arrangement gave a good approximation to the *in vivo* situation where a proportion of an intravenous dose does not pass through the liver in the first cycles.

The incorporation of the bypass tube into the perfusion would not have affected the pharmacokinetics. The extraction ratio which was obtained was an average figure calculated over a period of 60 minutes when all of the injected drug had been eliminated.

In any case, where flow was a factor influencing a pharmacokinetic parameter, that parameter was always analysed both in the overall propranolol and pindolol groups and in selected subgroups in which flow was controlled. It will be noted (see Results section) that the statistical significance

of the findings obtained in the complete groups was unchanged in the flow-matched subgroups. This confirmed that the varying amounts of perfusate entering the bypass had little, if any, impact on the interpretation of the data.

Furthermore, all the portal vein samples for drug assay were taken from a point <u>below</u> the overflow bypass (point A, Fig. 12) and this ensured that these samples were derived from blood that was definitely routed through the liver.

2.4. Additional notes on Perfusion Method selected

In the preliminary stages of this study I tried two variations of the experimental method:

(i) I injected the total dose into the reservoir and allowed the entire volume to perfuse through the liver only once with clamp b (Fig. 12) completely closed. I collected the entire emergent effluent and assayed this volume for total drug, thus obtaining a measure of the total amount of drug extracted.

This method was unsuitable because there was no control over pressure and flow, and rate of flow has a major effect on extraction ratio. There were also practical difficulties in ensuring that the total volume was in fact collected since small amounts remained entrapped in various parts of the perfusion system. At the end of the perfusion, air bubbles formed and passed through the liver.

Furthermore, this method would have limited the pharmacokinetic parameters that could have been measured.

(ii) Shand and colleagues (17), in their perfusion studies, conducted serial perfusions. They first injected the total dose into the reservoir; this simulated intravenous administration. This was the method which I adopted in my study. They followed this (after one hour) with an injection of the total dose into the portal vein; this simulated oral administration of drug.

I, too, attempted the latter mode of administration but had practical difficulties with the injection of drug into the portal vein. It was difficult to control the rate of infusion of the drug and, if the rate of injection was too fast, the

drug escaped into the overflow before going through the liver.

2.5. General Introduction to Analytical Methods

2.5.1. Propranolol

The fluorometric method of Shand *et al* (31) was used in this study to determine propranolol concentrations. This is the most widely applied method which has been extensively employed since 1970 up till the present day (121, 122, 124) not only by Shand and co-workers (14, 16, 17, 18, 19, 43, 50, 51, 109), but by numerous other investigators (4, 58, 70, 82, 83, 90, 108).

(i) Sensitivity

When the fluorometric method was compared to the high-performance liquid chromatographic method in human plasma assays of propranolol (41), there was an excellent correspondence between the respective determinations only if the subject's own blank plasma was used. The existence of a high blank reading was confirmed by some (89) but not other workers (31, 72). However, in the present study blank and standards were prepared in the same blood-KREBS as was used in the perfusion experiment that day, thus excluding the possibility of interference by blank plasma.

The limit of detection of propranolol has been set by various workers in a range varying from 5 ng/ml to 30 ng/ml (2, 72, 68).

(ii) Specificity

The specificity of the fluorescent method was originally established by Evans *et al* (16) and Shand *et al* (31) who found no interference by metabolites.

There are few quantitative data on metabolite concentrations in the rat. A major metabolite in man and rat is 4-hydroxy propranolol but this compound was poorly extracted by the method as it is converted to an unextracted conjugated derivative during the extraction procedure (59). Moreover it has minimal fluorescence in the 0,1 N HCl in which the samples were read (71). Naphthoxylactic acid is a major metabolite

in the rat but this was not extracted in the method employed (52).

Recently more metabolites have been investigated. Of the propranolol metabolites only N-desisopropyl propranolol and propranolol glycol have shown both significant fluorescence at the propranolol fluorescence wavelengths (71, 72) and extraction to any extent. However, very small amounts of these metabolites relative to propranolol were detected in human serum by gas-liquid chromatography. In the rat N-desisopropyl propranolol is only an intermediate metabolite in the conversion of propranolol to the poorly extracted propranolol glycol and to the unextracted naphthoxylactic acid (128).

In fact, a good correspondence was obtained from propranolol values derived from gas-liquid chromatography and those obtained by the fluorometric assay (71), (see Fig. 13). Kraml et al have performed several thousand fluorometric assays of propranolol and experienced only a 1% interference. They were satisfied that this method was specific both with whole blood and plasma (43, 72).

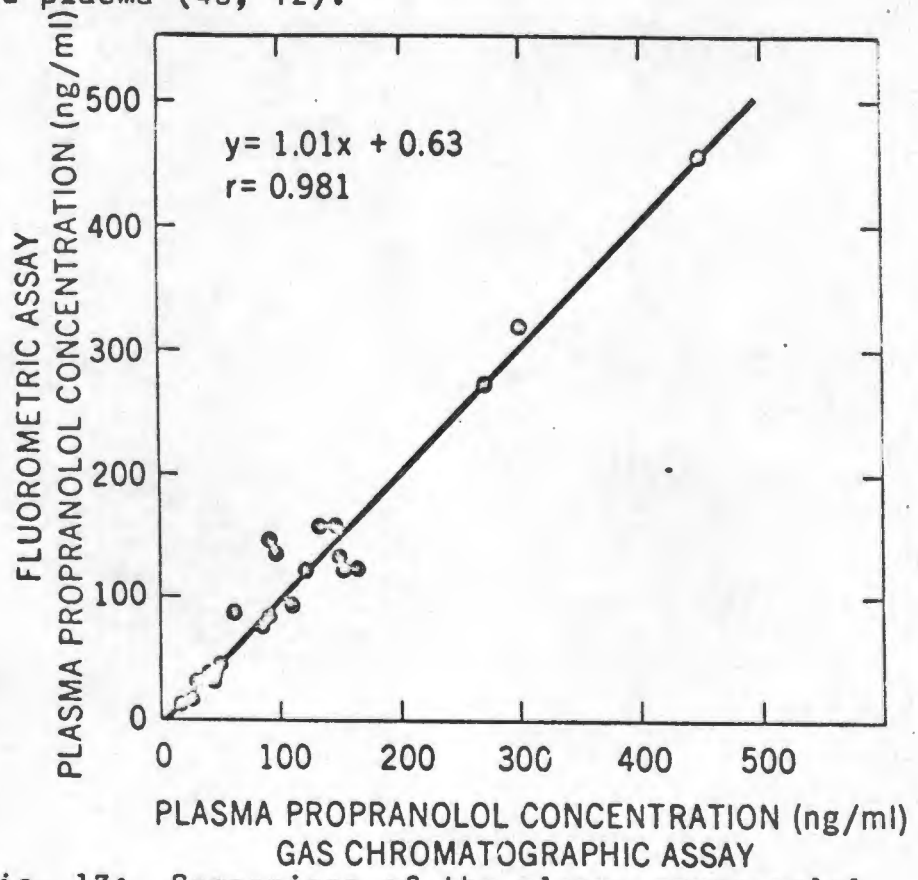


Fig. 13: Comparison of the plasma propranolol levels in 22 hypertensive patients as measured by the gas chromatographic assay versus those determined fluorometrically. The line pictured was fitted to the data using linear regression analysis (after Pritchard et al (71)).

2.5.2. Pindolol

The fluorometric method of Pacha (26) was used in this study to determine pindolol concentrations. This method has had extensive application in both clinical and experimental studies (3, 11, 13, 21, 39, 40, 56, 64, 84, 113).

(i) Sensitivity

The lower limit of detection has been claimed to be from 5 ng - 20 ng/ml (26, 39).

(ii) Specificity

Kiechel (7) has shown with thin-layer chromatography that all the polar metabolites of pindolol remain in the NaOH-phase during extraction and that only unchanged pindolol enters the HCl-phase, which is the one used for fluorometric measurements. He did find traces of an unidentified metabolite in the HCl-phase which consisted of small amounts of the side-chain of pindolol. However the fluorescing agent reacts specifically and exclusively with the indole nucleus so that this metabolite would not interfere with the assay.

It can thus be concluded that metabolites of pindolol do not interfere with the fluorometric determination of the parent compound.

2.6. Analytical Methods

2.6.1. Measurement of Total Drug Blood Concentrations

Equations used to calculate the extraction ratios, intrinsic clearances and hepatic clearances contain the term hepatic blood flow. In order to maintain consistency, drug concentrations in the blood rather than in plasma are measured (27, 94).

In addition, the extraction ratios for both propranolol and pindolol in the rat are greater than the free plasma fraction of the drug (43, 100). In these cases the blood actually transports the drug to its site of elimination and the relevant kinetic parameters are more meaningfully calculated from whole blood (or in my case, 20% whole blood - 80% KREBS) concentrations (24, 33, 50, 54, 98, 132).

2.6.2. Fluorometric Determination of Propranolol

Propranolol was assayed according to the method of Shand et al (31) with minor modifications in the volumes used. This method is efficient in whole blood as well as plasma (72, 43).

In order to test the reliability of the assay in 20% rat blood-80% KREBS buffer, a standard graph was done with each point in quadruplicate as shown in Graph 1. This shows a high degree of consistency in the assay measurements. The standard calibration curve was linear to at least 2000 ng in 500 µl of 20% blood-80% KREBS buffer. The lower limit of reliable detection was approximately 100 ng in 500 µl of 20% blood-80% KREBS.

Perfusion sample volumes ranged from 50 µl to 500 µl depending on the expected concentrations of drug (the higher the concentration, the lower the sample volume). All samples were made up to 500 µl with 20% rat blood-80% KREBS before extraction. 500 µl of blood-KREBS were used as a blank and three 500 µl aliquots were spiked with 250, 500 and 1000 ng of drug (dissolved in water) respectively. The 20% rat blood-80% KREBS was the excess of that used in the perfusion earlier in the day. The blank and standards were extracted simultaneously with the unknown samples.

The samples were basified with 1 ml of 1N NaOH and shaken with 10 ml of 1,5% isoamylalcohol, 98,5% heptane for 15 minutes on an automatic shaker. 8 ml of the heptane layer were removed and shaken with 4 ml of 0,1 N HCl for 15 minutes. This acid phase was then read in a Perkin-Elmer MPF - 44A Spectrofluorimeter at an excitation wavelength of 295 nm (slit 10 nm) and an emission wavelength of 360 nm (slit 8 nm).

Concentrations of drug were corrected for the different volumes extracted and expressed in µg/ml.

2.6.3. Fluorometric Determination of Pindolol

Pindolol was assayed according to the method of Pacha (26) with minor modifications in the volumes used. This method is reliable in whole blood as well as plasma (56, 84).

In order to check the reliability of the assay in 20% rat blood-80% KREBS buffer, a standard graph was done with each

point in quadruplicate as shown in Graph 2. This shows a high degree of consistency in the assay measurements. The standard calibration curve was linear to at least 2000 ng/ 500 µl 20% rat blood-80% KREBS buffer. The lower limit of reliable detection was approximately 100 ng/500 µl.

Perfusion sample volumes ranged from 50 µl to 500 µl depending on the expected concentrations of drug (the higher the concentration, the lower the sample volume). All samples were made up to 500 µl with 20% rat blood-80% KREBS before extraction. 500 µl of blood-KREBS were used as a blank and three 500 µl aliquots were spiked with 250, 500 and 1000 ng of drug (dissolved in water) respectively. The 20% rat blood-80% KREBS was the excess of that used in the perfusion earlier in the day. The blank and standards were extracted simultaneously with the unknown samples.

The samples were basified with 1 ml 1 N NaOH and shaken with 10 ml of diethyl ether for 15 minutes on an automatic shaker. 8 ml of the diethyl ether layer were removed and shaken with 4 ml of 0,1 N HCl for 15 minutes. 3 ml of the acid extract was then heated in a water bath at 50°C for 30 minutes with 200 µl of a 0,5% solution of phthaldialdehyde in MeOH. After cooling, the fluorescence of the solutions was stabilised by the addition of 500 µl of a 1% solution of ascorbic acid in water. The fluorescence of the solutions was measured in a Perkin-Elmer MPF - 44A Spectrofluorimeter at an excitation wavelength of 390 nm (slit 10 nm) and an emission wavelength of 440 nm (slit 8 nm).

Concentrations of drug were corrected for the different volumes extracted and expressed in µg/ml.

All the extractions were done in glass tubes with ground glass stoppers because rubber stoppers were found to alter the distribution of drug between red blood cells and plasma (29). Periodically the tubes were cleaned in chromic acid in addition to the normal washing procedure. The tubes were well rinsed with twice-distilled water and all the solutions were made up in twice-distilled water.

CHAPTER 3

3. RESULTS

3.1. Liver Blood Flow

As can be seen from Tables 1a and b, there was little variation in hepatic blood flow during the perfusions and an average value was taken for the purpose of the calculations.

3.2. Drug-Concentration-versus-Time Curves

Drug concentrations in 20% rat blood - 80% KREBS buffer (µg/ml) were plotted against time on semi-logarithmic graph paper (Graphs 3a - 9a for propranolol and 10a - 16a for pindolol). The best straight lines were fitted by eye and, where there was doubt, I took a consensus from two independent observers as to the most appropriate positioning of the line.

Points derived from the above straight lines were transposed on to linear graph paper to obtain drug-concentration-versus-time curves for the portal vein (Graphs 3b - 9b for propranolol and 10b - 16b for pindolol) and for the hepatic vein (Graphs 3c - 9c for propranolol and 10c - 16c for pindolol).

Areas under the drug-concentration-versus-time curves were calculated from the linear graph paper by counting the squares underneath the curves as shown in Graphs 3b and 3c (for propranolol) and 10b and 10c (for pindolol).

3.2.1. Sensitivity of the Assays

I found that the assays for both propranolol and pindolol were sensitive and reproducible to a lower limit of 100 ng/ml 20% blood-80% KREBS. Between 100 and 50 ng/ml readings were not always reliable and below 50 ng/ml they were generally inconsistent and unreliable.

However, the majority of concentrations obtained in my experiments were above the 100 ng/ml limit: those that fell below occurred at the tail-end of the drug-concentration-versus time curve (at 40 and 60 minutes) where an inspection of the

Graphs (3b,c - 16b,c) shows that their contribution to the overall area-under-the-curve values was negligible; in fact, some of the concentrations at 40 and 60 minutes were undetectable by the assay.

3.2.2. Specificity of the Assays

The possibility of drug metabolites interfering with the fluorescence of unchanged drug was considered. If interfering metabolites had been present they would have accumulated with time (and particularly so in the emergent hepatic vein perfusate) and falsely inflated the readings at the terminal end of the perfusions. Inspection of Graphs 3a - 16a shows that this did not occur; all readings had declined to near zero by 60 minutes.

3.2.3. Semi-logarithmic plots of Propranolol

Semi-logarithmic plots of blood-KREBS propranolol concentrations-versus-time in the portal vein produced a biphasic curve with an α and β phase reflecting the distribution and elimination processes respectively (Graphs 3a - 9a).

3.2.4. Semi-logarithmic plots of Pindolol

Semi-logarithmic plots of blood-KREBS pindolol concentrations-versus-time in the portal vein appeared to yield a single straight line (Graphs 10a - 16a). Although these blood-KREBS levels may have declined bi-exponentially, the initial phase must have been so rapid that my data were insufficient to define that portion of the curve. The results were therefore analysed as if the model behaved as a one-compartment open system.

3.2.5. Exclusion of the possibility of drug adherence to glass and apparatus material

Inspection of Graphs 1 and 2 shows that the plot of fluorescence intensity against concentration of the standards was a straight line which passed through zero. This indicates that there was no irreversible adherence of either propranolol or pindolol to glass during the extraction. It was also found that silanisation of the glass had no effect on the fluorescence readings obtained.

Graphs 3a - 16a clearly demonstrate that when the slope

of the portal vein concentration-time line for both propranolol and pindolol was extrapolated back to the y-axis to determine the theoretical concentration at t = 0, the derived figure was very close to the expected concentration of both drugs at instantaneous distribution throughout the perfusate, i.e. 5 mg of drug in 103 ml of blood-KREBS = 48,5 µg/ml. This again establishes the validity of the assay and excludes the possibility of drug adherence to the perfusion apparatus.

3.3. Abbreviations of Parameters Calculated

On the basis of the calculated areas-under-the-concentration-time curves a number of parameters were derived and compared for propranolol and pindolol.

The terms used are represented by the following abbreviations:

E	=	hepatic extraction ratio (first-pass extraction)
AUC_{PV}	=	area under the drug concentration-versus-time curve for the portal vein
AUC_{HV}	=	area under the drug concentration-versus-time curve for the hepatic vein
Cl_{int}	=	hepatic intrinsic clearance of total drug
Cl_H	=	hepatic clearance of total drug
D	=	total dose of drug administered
Q	=	hepatic flow rate
$t_{\frac{1}{2}}$	=	elimination half-life
V_d	=	apparent volume of distribution
c_o	=	concentration at time zero

Statistical Abbreviations[1]

t	=	t distribution (Student's t test)
r	=	correlation co-efficient (product moment)
p	=	probability
NS	=	not significant
df	=	degrees of freedom

3.4. Hepatic extraction ratio (E)

This ratio is equal to the first-pass extraction and reflects the fraction of the dose of drug removed during its first passage through the liver.

Three different formulae (see below) were used to calculate E and the three values obtained were averaged.

(i) $$E = \frac{AUC_{PV} - AUC_{HV}}{AUC_{PV}}$$ (equation 3)

(ii) $$E = \frac{Cl_{int}}{Q + Cl_{int}}$$ (equation 10)

where Cl_{int}[2] $= \frac{D_{iv}}{AUC_{HV}}$ (equation 13)

(iii) $$E = \frac{D_{iv}}{Q.\ AUC_{PV}}$$ (equation 5)

(In the perfusion model, after intravenous administration, the AUC_{PV} corresponds to the AUC_s in the intact animal.)

Tables 2a and 2b show the extraction ratios for propranolol and pindolol respectively. There is no significant difference in mean flow rate (t = 0,70; NS) between the two groups.

---

(1) The formulae for the statistical tests employed are listed in the Appendix.

(2) Calculation of Cl_{int} values obtained are set out in section 3.5.

However, the mean extraction ratio for propranolol was 0,71 and that for pindolol was 0,50. This difference is highly statistically significant ($t = 5,42$; $p<0,001$) and it indicates that the first-pass effect is greater in the case of propranolol.

3.4.1. Flow and Extraction Ratio

The individual perfusions in both the pindolol and propranolol groups are ranked from A to G in order of increasing flow, as shown in Tables 2a and 2b.

With pindolol (Table 2b) there is a highly significant negative correlation between flow and extraction ratio ($r = -0,88$; $t = 4,15$; $p<0,01$). There was a similar trend with propranolol for increasing flow to be accompanied by a reduced extraction ratio (Table 2a) but this correlation did not attain significance ($r = -0,74$; $t = 2,50$; $p<0,10$).

An inspection of Table 2b shows that for pindolol increasing flow is accompanied by a progressive decrease in extraction ratio except in the case of perfusions D and F where the respective intrinsic clearances exceed the mean intrinsic clearance. With propranolol (Table 2a) there is also a progressive decrease in E with increasing flow except in perfusions D and E where the respective intrinsic clearances are well below the mean intrinsic clearance.

Although there was no significant difference in the mean flow rates between the propranolol and pindolol groups, there was a difference in the range of flows (35 ml/min - 60 ml/min for propranolol; 21 ml/min - 60 ml/min for pindolol - Tables 2a and 2b). For this reason it was decided to exclude the possibly distorting effects of flow on extraction ratio by taking a subgroup of perfusions from both groups with identical, or nearly identical, flow rates, as shown in Table 2c. The results again clearly show a marked difference in E between propranolol (0,69) and pindolol (0,45). ($t = 5,66$; $p<0,01$.)

The proportionately more marked influence of flow rate on the extraction ratio of pindolol is shown in Table 2c.

The 71% and 67% increase in flow rate for the propranolol

and pindolol groups respectively was accompanied by a 12% decrease in E for propranolol and a 28% decrease in E for pindolol.

3.5. Hepatic Intrinsic Clearance (Total Drug) (Cl_{int})

This parameter reflects the maximum inherent ability or capacity of the liver to extract drug from blood assuming the drug is not limited in its access to hepatic cells by distributional factors (such as flow and binding to blood constituents).

Two different formulae (see below) were used to calculate Cl_{int} and these two values were averaged.

(i) $$Cl_{int} = \frac{D_{iv}}{AUC_{HV}}$$ (equation 13)

(ii) $$Cl_{int} = \frac{QE}{1 - E}$$ (equation 11)

where $$E = \frac{AUC_{PV} - AUC_{HV}}{AUC_{PV}}$$ (equation 3)

The total hepatic intrinsic clearances were expressed in ml/gram of wet liver tissue/minute.

Tables 3a and 3b show the intrinsic clearances for propranolol and pindolol respectively.

The mean intrinsic clearance for propranolol was 10,44 ml/g/min and that for pindolol was 3,42 ml/g/min. This difference is very highly significant. ($t = 11,56$; $p<0,001$)

3.5.1. Flow and Intrinsic Clearance

Tables 3a and 3b show that there is no correlation between hepatic flow and total hepatic intrinsic clearance in either the propranolol or the pindolol groups. ($r = 0,23$; $t = 0,54$; NS for propranolol; $r = 0,48$; $t = 1,24$; NS for pindolol)

3.6. Interrelationships between Extraction Ratio, Intrinsic Clearance and Hepatic Flow

From equation 13,

$$\frac{1}{E} = 1 + \frac{Q}{Cl_{int}}$$

Graph 18 shows a plot of $\frac{1}{E}$ versus Q (where Q is expressed in ml/gram of liver/minute), for both propranolol and pindolol. E was calculated from equation 10 ($E = \frac{Cl_{int}}{Cl_{int} + Q}$). The slope of the line was fitted by linear regression analysis and was found to have an intercept of 1,07 in the case of propranolol and 1,05 in the case of pindolol.

The slope of the propranolol line ($\frac{1}{Cl_{int}}$) was 0,088 and that of the pindolol line was 0,306. Hence the Cl_{int} ($\frac{1}{slope}$) is greater for propranolol (11,36 ml/g/min) than for pindolol (3,27 ml/g/min).

This graph also shows that E decreases with flow to a proportionately more marked extent for pindolol than propranolol.

Equation 13 (above) can be rearranged to give

$$E = \frac{Cl_{int}}{Q + Cl_{int}} \qquad \text{(equation 10)}$$

Table 4 shows the interrelationships between the individual flow rates (expressed in ml/g/min), intrinsic clearances and extraction ratios. The values for intrinsic clearance are those derived from the formula $\frac{D_{iv}}{AUC_{HV}}$ (section 3.3.) and thus were not directly derived from equation 13. Similarly, the values for E were an average of formulae (i) and (iii) (section 3.4.) and were therefore not derived from equation 13.

When flow is greater than the intrinsic clearance, then the extraction ratio is greater than 0,5 (equation 11). As is evident in Table 4 all the intrinsic clearances for propranolol exceeded the hepatic flow and all the extraction ratios exceeded 0,5.

In the case of pindolol, where the intrinsic clearances

exceeded the hepatic flow, E was greater than 0,5 (perfusions A and B); where the intrinsic clearances were less than flow, E was less than 0,5 (perfusions E and G); and where the intrinsic clearances were approximately equal to hepatic flow, E was approximately equal to 0,5 (perfusions C, D and F).

3.7. Hepatic clearance (Total Drug) (Cl_H)

This parameter is an index of the actual perfusate volume cleared of drug by the liver in unit time.

Two different formulae (see below) were used to calculate Cl_H and these two values were averaged and expressed as ml/gram of wet liver tissue/minute.

(i) $$Cl_H = Q\,E \qquad \text{(equation 4)}$$

(ii) $$Cl_H = \frac{D_{iv}}{AUC_{PV}} \qquad \text{(equation 5)}$$

(In the perfusion model AUC_{PV} corresponds to AUC_s in the intact animal.)

Table 5a and 5b show the hepatic clearances for propranolol and pindolol respectively.

The mean hepatic clearance for propranolol was 2,47 ml/g/min and that for pindolol was 1,56 ml/g/min. This difference is highly significant. ($t = 4{,}81$; $p<0{,}001$)

3.7.1. Flow and Hepatic Clearance

There was a significant correlation between flow and hepatic clearance in the pindolol group ($r = 0{,}87$; $t = 3{,}91$; $p<0{,}05$ - Table 5b.) With propranolol there was a very highly significant correlation between flow and hepatic clearance ($r = 0{,}97$; $t = 9{,}73$; $p<0{,}001$ - Table 5a.)

An inspection of Table 5a and 5b shows that increasing flow is accompanied by a progressive increase in hepatic clearance in all cases except those in which the intrinsic clearances are less than flow in the case of pindolol (perfusions E and G) and in which the intrinsic clearances are below the mean in the case of propranolol (perfusions D and E).

Table 6 represents two subgroups of perfusions from the propranolol and pindolol groups respectively where rate of flow is controlled. It is evident that the mean difference in hepatic clearance between the drugs is still highly statistically significant ($t = 3,84$; $p<0,01$) even when the possibly distorting effects of different flow rates are excluded.

The proportionately more marked influence of flow rate on the hepatic clearance of propranolol is shown in Table 6. An increase in flow of 71% for propranolol (35 ml/min to 60 ml/min) produced an increase in Cl_H of 49% whereas a 67% flow increase for pindolol (36 ml/min to 60 ml/min) produced an increase of only 15%.

3.8. Elimination Half-life ($t_{\frac{1}{2}}$)

The elimination half-life ($t_{\frac{1}{2}\beta}$) was calculated from the elimination (β) phase in both the portal vein and the hepatic vein logarithmic drug concentration-time plots. It represents the time taken for any given blood-KREBS drug concentration in the β phase to fall by 50 per cent.

Table 7 shows the $t_{\frac{1}{2}\beta}$ for the propranolol and pindolol groups in the portal and hepatic veins respectively.

The $t_{\frac{1}{2}\alpha}$ of propranolol was obtained from the slope (α) of the line calculated from the method of residuals as shown in Graphs 3a - 9a. This method plotted the difference between the actual concentration at a given time and the concentrations obtained from the extrapolated portion of the β-slope at the same time.

The $t_{\frac{1}{2}\alpha}$ was measured by the time taken for a given concentration on the α slope to fall by 50 per cent. Table 7 shows these $t_{\frac{1}{2}\alpha}$ values. The $t_{\frac{1}{2}\alpha}$ of propranolol was very short (1,0 minutes) while the $t_{\frac{1}{2}\beta}$ was much longer (5,2 minutes, portal vein; 5,0 minutes, hepatic vein).

The $t_{\frac{1}{2}\beta}$ for pindolol was 4,1 minutes and 3,8 minutes in the portal and hepatic veins respectively.

3.8.1. Elimination Half-life and Flow

Table 7 shows the relationship between flow and half-life for both propranolol and pindolol in the portal vein. There

was no correlation between flow rate and $t_{\frac{1}{2}\beta}$ for the propranolol perfusions ($r = -0,47$; $t = 1,18$; NS). There was a highly significant negative correlation between flow rate and $t_{\frac{1}{2}\beta}$ for the pindolol perfusions ($r = -0,93$; $t = 5,64$; $p<0,01$).

A correlation was also obtained between flow rate and the half-life of the α-phase for propranolol. A highly significant negative correlation emerged between these two variables ($r = -0,93$; $t = 5,67$; $p<0,01$).

Table 7 shows the relationship between flow and half-life in the hepatic vein. There was no correlation between flow rate and $t_{\frac{1}{2}\beta}$ for the propranolol perfusions ($r = -0,63$; $t = 1,82$; NS). There was a highly significant negative correlation between flow rate and $t_{\frac{1}{2}\beta}$ for the pindolol perfusions ($r = -0,91$; $t = 5,09$; $p<0,01$).

3.9. Apparent Volume of Distribution (V_d)

This parameter reflects the apparent volume into which the drug appears to be distributed.

Two different formulae (see below) were used to calculate V_d and these two values were averaged and expressed as ml/gram of wet liver tissue.

(i) $$V_d = \frac{Cl_H \cdot t_{\frac{1}{2}}}{0,693}$$ (equation 6)

(ii) $$V_d = \frac{D_{iv}}{c_o}$$ (equation 2)

where c_o is the drug concentration at time zero, derived (in the case of propranolol) by extrapolation of the β-phase slope to the y-axis, as shown in Graphs 3a - 9a.

Table 8 shows the apparent volumes of distribution for propranolol and pindolol. The mean V_d of propranolol (21,23 ml/g) is highly significantly greater than pindolol (8,72 ml/g) ($t = 9,13$; $p<0,001$).

3.10. Instantaneous Extraction Ratios

This ratio was calculated from the following formula:

$$E = \frac{C_{PV} - C_{HV}}{C_{PV}}$$

where C_{PV} = drug concentration in the portal vein
and C_{HV} = drug concentration in the hepatic vein.
These concentrations were derived from the mean drug concentration-time-curves shown in Graphs 17a and b.

Table 10 shows the instantaneous extraction ratios for propranolol and pindolol at time points throughout the perfusion.

3.11. Capacity of the high affinity site of propranolol

The capacity of the high affinity site of propranolol was calculated from the amount of drug extracted after 5 minutes divided by the liver weight.

These values expressed in µg/g wet liver tissue are shown in Table 9. The mean capacity was 329 µg/g of liver.

3.12. Summary of Results

1. Graphs 17a and b are semi-logarithmic plots of the mean concentration values for propranolol and pindolol against time in the hepatic and portal veins. Graph 17a shows the biphasic curve of propranolol in the portal vein.

2. The mean extraction ratio for propranolol was highly significantly greater than that for pindolol ($p<0,001$). This difference occurred even when flow was controlled ($p<0,01$). While the correlation between flow and extraction ratio for propranolol did not attain significance ($p<0,10$), there was a trend in that increasing flow ratios were accompanied by decreasing extraction ratios. In the case of pindolol, there was a highly statistically significant negative correlation between flow and extraction ratio ($p<0,01$) (section 3.4.).

3. The mean hepatic intrinsic clearance of total drug in the blood-KREBS was very highly significantly greater for propranolol than for pindolol ($p<0,001$). This parameter was independent of flow for both propranolol and pindolol (section 3.5.).

4. The interrelationships between E, Cl_{int} and Q were confirmed in the perfusion model such that $\frac{1}{E} = 1 + \frac{Q}{Cl_{int}}$ (section 3.6.).

5. The hepatic clearance of total blood-KREBS propranolol was significantly greater than that of pindolol ($p<0,001$). This difference occurred even when flow was controlled ($p<0,01$). There was a positive correlation between flow and hepatic clearance and this effect was more marked in the case of propranolol (section 3.7.).

6. The elimination half-life ($t_{\frac{1}{2}\beta}$) of pindolol was shorter than that of propranolol. There was a highly significant negative correlation between flow and $t_{\frac{1}{2}\alpha}$ with propranolol ($p<0,01$) but no significant correlation emerged between flow and $t_{\frac{1}{2}\beta}$. In the case of pindolol, however, there was a highly significant negative correlation between $t_{\frac{1}{2}\beta}$ and flow ($p<0,01$).

7. The apparent volume of distribution for propranolol was highly significantly greater than that for pindolol ($p<0,001$) (section 3.9.).

Table 1a

Perfusate Flow Rates (Propranolol)

Perfusion A		B		C		D		E		F		G	
Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min
3	35	3	42	3	44	3	43	3	43	3	47	3	
5	36	5	41	5	44	5	44	5	45	5	48	5	60
10	38	10	42	10	46	10	47	10	47	10	49	10	63
20	35	20	45	20	45	20	46	20	48	20	49	20	63
30	36	30	44	30	44	30	47	30	46	30	47	30	61
40	36	40	40	40	43	40		40	45	40	46	40	58
60	31	60	43	50	43	60	43	60		60	45	60	57
Mean	35		42		44		45		46		47		60

Table 1b
Perfusate Flow Rates (Pindolol)

Perfusion A		B		C		D		E		F		G	
Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min	Time min	Flow ml/min
3	21	3		3	38	3	43	3	47	3		3	60
5	21	5	25	5	39	5	43	5	46	5	56	5	60
10	23	10	24	10	37	10	41	10	47	10	58	10	61
20	21	20	27	20	36	20	45	20	46	20	55	20	63
30	23	30	25	30		30	43	30	45	30	58	30	60
40	21	40	27	40	33	40	43	40	48	40	57	40	57
60	20	60	26	60	32	60	40	60	41	60		60	60
Mean	21		26		36		43		46		57		60

Table 2a

Extraction Ratios of Propranolol

Individual Perfusions	Q ml/min	Liver weight (grams)	Cl_{int} (1) ml/g/min	AUC_{PV} µg/ml.min	AUC_{HV} µg/ml.min	(i) $E \simeq \frac{AUC_{PV}-AUC_{HV}}{AUC_{PV}}$	(ii) $E = \frac{Cl_{int}}{Q + Cl_{int}}$	(iii) $E = \frac{D_{iv}}{Q.AUC_{PV}}$	E (Average)
A	35	12,06	10,10	209	44	0,79	0,76	0,68	0,74
B	42	12,18	11,12	190	41	0,78	0,74	0,63	0,72
C	44	13,20	11,32	160	35	0,78	0,76	0,71	0,75
D	45	13,28	8,77	169	45	0,73	0,71	0,66	0,70
E	46	11,47	8,72	178	54	0,70	0,67	0,61	0,66
F	47	12,69	11,52	164	37	0,77	0,74	0,65	0,72
G	60	11,87	11,51	149	42	0,72	0,66	0,56	0,65
Mean (± S.E.M.)	45,57 (± 2,83)	12,39 (±0,26)	10,44 (±0,47)						0,71 (2) (±0,01)

(1) Calculations of Cl_{int} are set out in section 3.3.

(2) Comparisons against pindolol are shown in Table 2b.

Table 2b

Extraction Ratios of Pindolol

Individual Perfusions	Q ml/min	Liver weight (grams)	Cl_{int} (1) ml/g/min	AUC_{PV} µg/ml.min	AUC_{HV} µg/ml.min	(i) $E = \frac{AUC_{PV}-AUC_{HV}}{AUC_{PV}}$	(ii) $E = \frac{Cl_{int}}{Q + Cl_{int}}$	(iii) $E = \frac{D_{iv}}{Q.AUC_{PV}}$	E (Average)
A	21	11,45	3,08	363	138	0,62	0,63	0,66	0,64
B	26	11,97	3,30	359	137	0,62	0,58	0,54	0,58
C	36	12,64	2,83	280	141	0,50	0,50	0,50	0,50
D	43	11,16	4,47	254	111	0,56	0,51	0,46	0,51
E	46	14,02	2,41	282	159	0,44	0,41	0,39	0,41
F	57	11,45	5,13	202	94	0,53	0,48	0,43	0,48
G	60	13,12	2,69	238	148	0,38	0,36	0,35	0,36
Mean (±S.E.M.)	41,28 (2) (±5,54)	12,26 (3) (±0,40)	3,42 (±0,38)						0,50 (4) (±0,04)

(1) Calculations of Cl_{int} are set out in section 3.3.
(2) t = 0,70; NS (when compared against mean flow rate in propranolol group, Table 1a)
(3) t = 0,27; NS (when compared against mean liver weight in propranolol group, Table 1a)
(4) t = 5,42; p<0,001; df = 12 (when compared against mean liver weight of propranolol, Table 1a)

Table 2c

Comparison of the Extraction Ratio of Propranolol and Pindolol with Flow Rate Controlled

	PROPRANOLOL			PINDOLOL	
	Q ml/min	E		Q ml/min	E
A	35	0,74	C	36	0,50
B	42	0,72	D	43	0,51
E	46	0,66	E	46	0,41
G	60	0,65	G	60	0,36
Mean (±S.E.M.)	45,75	0,69[1] (±0,02)		46,25	0,45[1] (±0,04)

(1) $t = 5,66$; $p<0,01$; $df = 12$

Table 3a

Total Hepatic Intrinsic Clearances of Propranolol

	Q ml/min	AUC_{HV} (1) µg/ml. min	(i) $Cl_{int} = \frac{D_{iv}}{AUC_{HV}}$ ml/min	(ii) $Cl_{int} = \frac{QE}{1-E}$ ml/min	Cl_{int} (Average) ml/min	$\frac{Cl_{int}}{\text{Liver-weight}}$ (2) ml/g/min
A	35	44	113,64	130,00	121,82	10,10
B	42	41	121,95	148,91	135,43	11,12
C	44	35	142,86	156,00	149,43	11,32
D	45	45	111,11	121,67	116,39	8,77
E	46	54	92,59	107,33	99,96	8,72
F	47	37	135,14	157,35	146,25	11,52
G	60	42	119,05	154,29	136,67	11,51
Mean	45,57					10,44 (3)
(±S.E.M.)	(±2,83)					(±0,47)

(1) There is no correlation between Q and AUC_{HV} ($r = -0,086$; $t = 0,081$; NS).
(2) For liver weights see Table 1a.
(3) Comparisons against pindolol are shown in Table 3b.

Table 3b
Total Hepatic Intrinsic Clearances of Pindolol

	Q ml/min	AUC_{HV} (1) µg/ml. min	(i) $Cl_{int} = \frac{D_{iv}}{AUC_{HV}}$ ml/min	(ii) $Cl_{int} = \frac{QE}{1-E}$ ml/min	Cl_{int} (Average) ml/min	$\frac{Cl_{int}}{\text{Liver weight}}$ (2) ml/g/min
A	21	138	36,23	34,26	35,25	3,08
B	26	137	36,50	42,42	39,46	3,30
C	36	141	35,46	36,00	35,73	2,83
D	43	111	45,05	54,72	49,89	4,47
E	46	159	31,45	36,14	33,80	2,41
F	57	94	53,19	64,28	58,74	5,13
G	60	148	33,78	36,77	35,28	2,69
Mean (±S.E.M.)		41,28 (±5,54)				3,42 (3) (±0,38)

(1) There is no correlation between Q and AUC_{HV} (r = -0,22; t = 0,53; NS).
(2) For liver weights see Table 1b.
(3) t = 11,56; p<0,001; df = 12 (against mean total hepatic intrinsic clearance of propranolol, Table 3a).

Table 4

Relationship between Blood Flow, Intrinsic Clearance and Extraction Ratio for Propranolol and Pindolol

	PROPRANOLOL				PINDOLOL		
	Q ml/g/min	Cl_{int} (1) ml/g/min	E (2)		Q ml/g/min	Cl_{int} (1) ml/g/min	E (2)
A	2,90	9,42	0,74	A	1,83	3,16	0,64
B	3,45	10,01	0,71	B	2,17	3,05	0,58
C	3,33	10,82	0,75	C	2,85	2,81	0,50
D	3,39	8,37	0,70	D	3,85	4,04	0,51
E	4,01	8,07	0,66	E	3,28	2,24	0,42
F	3,70	10,64	0,71	F	4,98	4,65	0,48
G	5,05	10,03	0,69	G	4,57	2,57	0,37

---

(1) derived from the formula $\frac{D_{iv}}{AUC_{HV}}$

(2) derived from the formulae, $E = \frac{AUC_{PV} - AUC_{HV}}{AUC_{PV}}$ and

$$E = \frac{D_{iv}}{Q.AUC_{PV}}$$

Table 5a

Total Hepatic Drug Clearances of Propranolol

	Q ml/min	Q ml/g/min	AUC_{PV} µg/ml.min	Cl_{int} ml/g/min	E	(i) $Cl_H = Q\ E$ ml/min	(ii) $Cl_H = \frac{D}{AUC_{PV}}$ ml/min	Average Cl_H ml/min	$\frac{Cl_H}{\text{Liver weight}}$ (1) ml/g/min
A	35	2,90	209	10,10	0,74	25,90	23,92	24,91	2,06
B	42	3,45	190	11,12	0,72	30,24	26,32	28,28	2,32
C	44	3,33	160	11,32	0,75	33,00	31,25	32,13	2,43
D	45	3,39	169	8,77	0,70	31,50	29,59	30,55	2,30
E	46	4,01	178	8,72	0,66	30,36	28,09	29,23	2,55
F	47	3,70	164	11,52	0,72	33,84	30,49	32,17	2,54
G	60	5,05	149	11,51	0,65	39,00	33,55	36,28	3,06
Mean (±S.E.M.)	45,57 (±2,83)	3,69		10,44 (±0,47)	0,71 (±0,01)				2,47 (2) (±0,12)

(1) For liver weights see Table 1a.
(2) Comparisons against pindolol are shown in Table 5b.

Table 5b

Total Hepatic Drug Clearances of Pindolol

	Q ml/min	Q ml/g/min	AUC_{PV} µg/ml.min	Cl_{int} ml/g/min	E	(i) $Cl_H = Q\,E$ ml/min	(ii) $Cl_H = \frac{D}{AUC_{PV}}$ ml/min	Average Cl_H ml/min	$\frac{Cl_H}{\text{Liver weight}}$ (1) ml/g/min
A	21	1,83	363	3,08	0,64	13,44	13,77	13,61	1,19
B	26	2,17	359	3,30	0,58	16,12	13,92	15,02	1,25
C	36	2,85	280	2,83	0,50	18,00	17,86	17,93	1,42
D	43	3,85	254	4,47	0,51	21,93	19,69	20,81	1,86
E	46	3,28	282	2,41	0,41	18,86	17,73	18,30	1,31
F	57	4,98	202	5,13	0,48	27,36	24,75	26,06	2,28
G	60	4,57	238	2,69	0,36	21,60	21,01	21,31	1,63
Mean (±S.E.M.)	41,28 (±5,54)	3,36		3,42 (±0,38)	0,50 (±0,04)				1,56 (2) (±0,15)

(1) For liver weights, see Table 1b.
(2) $t = 4,81$; $p < 0,001$; $df = 12$ (when compared against mean total hepatic drug clearance of propranolol, Table 5a).

Table 6

Effect of Flow on Hepatic Clearances of Propranolol and Pindolol

	PROPRANOLOL				PINDOLOL		
	Q ml/min	Cl_H ml/min	Cl_H ml/g/min		Q ml/min	Cl_H ml/min	Cl_H ml/g/min
A	35	24,91	2,06	C	36	17,93	1,42
B	42	28,28	2,32	D	43	20,81	1,86
E	46	29,23	2,55	E	46	18,30	1,31
G	60	36,28	3,06	G	60	21,31	1,63
Mean	45,75	29,68(1)	2,50(2)			19,59(1)	1,56(2)
(±S.E.M.)		(±2,38)	(±0,21)			(±0,86)	(±0,12)

(1) $t = 3,97$; $p<0,01$; $df = 6$
(2) $t = 3,84$; $p<0,01$; $df = 6$

Table 7
Elimination Half-life for Propranolol and Pindolol

	PROPRANOLOL					PINDOLOL		
	Q ml/min	Portal vein $t_{\frac{1}{2}\beta}$ min	Hepatic vein $t_{\frac{1}{2}\beta}$ min	$t_{\frac{1}{2}\alpha}$ min		Q ml/min	Portal vein $t_{\frac{1}{2}\beta}$ min	Hepatic vein $t_{\frac{1}{2}\beta}$ min
A	35	6,0	6,0	1,2	A	21	5,2	5,2
B	42	5,6	5,6	1,1	B	26	5,4	4,8
C	44	4,6	4,4	1,0	C	36	4,1	3,6
D	45	5,7	5,0	1,0	D	43	3,8	3,9
E	46	6,0	5,5	1,0	E	46	4,2	4,0
F	47	4,0	3,9	0,9	F	57	3,0	3,1
G	60	4,8	4,4	0,8	G	60	3,2	1,9
Mean		5,2	5,0	1,0			4,1	3,8

Table 8

Apparent Volumes of Distribution of Propranolol and Pindolol

	PROPRANOLOL						PINDOLOL				
	Cl_H ml/min	$t_{\frac{1}{2}}$ min	(i) $V_d = \frac{Cl_H \times t_{\frac{1}{2}}}{0,693}$ ml	(ii) $V_d = \frac{D_{iv}}{c_o}$ (1) ml	Average V_d / Liver weight ml/g		Cl_H ml/min	$t_{\frac{1}{2}}$ min	(i) $V_d = \frac{Cl_H \times t_{\frac{1}{2}}}{0,693}$ ml	(ii) $V_d = \frac{D_{iv}}{c_o}$ (1) ml	Average V_d / Liver weigh ml/g
A	24,91	6,0	215,67	250,00	19,32	A	13,61	5,2	102,12	103,09	9,00
B	28,28	5,6	228,53	264,55	20,28	B	15,02	5,4	117,04	103,09	9,19
C	32,13	4,6	213,27	287,36	18,94	C	17,93	4,1	106,08	103,09	8,31
D	30,55	5,7	251,28	352,11	22,74	D	20,81	3,8	114,11	103,09	9,77
E	29,23	6,0	253,07	340,14	25,89	E	18,30	4,2	110,91	103,09	7,63
F	32,17	4,0	185,69	226,24	16,23	F	26,06	3,0	112,81	103,09	9,43
G	36,28	4,8	251,29	347,22	25,19	G	21,31	3,2	98,40	103,09	7,70
Mean (±S.E.M.)					21,23 (2) (±1,33)						8,72 (2) (±0,32)

(1) For c_o values see Graphs 3a - 16a

(2) $t = 9,13$; $p<0,001$; $df = 12$

Table 9
Capacity of the High Affinity Site of Propranolol

Individual Perfusions	Capacity (µg/g)
A	317
B	323
C	315
D	316
E	361
F	316
G	356
Mean	329

Table 10

Instantaneous Extraction Ratios of Propranolol and Pindolol

Time minutes	PROPRANOLOL $E^{(1)} = \frac{C_{PV} - C_{HV}}{C_{PV}}$	PINDOLOL $E^{(1)} = \frac{C_{PV} - C_{HV}}{C_{PV}}$
1	0,99(2)	0,75
3	0,94(2)	0,48
5	0,69(2)	0,42
10	0,26	0,46
20	0,35	0,53
30	0,43	0,59
40	0,52	0,64

(1) Based on the mean concentration values shown in Graphs 17a and 17b.

(2) These values are the instantaneous extraction ratios during the α-phase.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

The primary aim of this study was to compare the first-pass extraction of the two beta-blockers, pindolol and propranolol, in view of reports from various sources that propranolol undergoes a larger first-pass effect.

The present project was set up to evaluate the pharmacokinetic parameters of the two drugs within the context of an isolated rat liver perfusion model. The advantage of the latter approach was that these drugs could be tested and compared under identical experimental circumstances. As far as is known, this was the first attempt to make direct comparisons between propranolol and pindolol in the rat under standardised conditions.

Extraction Ratio

The results show that the mean extraction ratio (first-pass effect) for propranolol (0,71) was highly significantly greater than for pindolol (0,50) ($p<0,001$). The figure of 0,71 for propranolol was very close to the mean extraction ratio value of 0,68 (at a flow rate of 20 ml/min) obtained from various rat liver perfusion studies (15, 17).

Very little information exists on the extraction ratio of pindolol in the rat, and the only value available (obtained from Sandoz Laboratories - unpublished data) was one of 0,5 which is the same figure as that derived from my experiments.

Most of the work on pindolol relates to man where the extraction ratio (first-pass effect) was 0,13 (4), 0,10 (21), 0,23 (56) and 0,47 (39). Except for the latter, all the other figures are considerably lower than that calculated by me in the rat. Values for propranolol do not appear to vary substantially between humans and animals (4, 15, 41, 46) with the exception of the monkey where the extraction ratio was found to be 0,49 (60). It would therefore seem that in the case of pindolol the extraction ratio is larger in the rat than in man. Metabolism in experimental animals is often

greater than in humans (137).

Hepatic Intrinsic Clearance

The marked difference in extraction ratio between propranolol and pindolol was reflected by a very great difference in their respective hepatic intrinsic clearances (10,44 ml/g/min for propranolol; 3,42 ml/g/min for pindolol; $p<0,001$). Values of 10 ml/g/min (30) and 24 ml/min (uncorrected for liver weight) (17) have been reported for propranolol in the rat.

Intrinsic hepatic clearance is a measure of the innate capacity of the liver to eliminate and metabolise a drug and it is independent of any limitations of blood flow. This in fact was confirmed in my experimental model where the correlations between flow rates and intrinsic clearances of both propranolol ($r = 0,23$) and pindolol ($r = 0,48$) were non-significant.

Propranolol can thus be characterised as a high Cl_{int} drug. Its Cl_{int} value always exceeds that of the liver blood flow (perfusate flow) and its extraction ratio is greater than 0,7. Pindolol has an intermediate Cl_{int} (extraction ratio is between 0,3 and 0,7) which is sometimes greater than, and at other times equal to, or less than, hepatic blood flow. It should be recalled that when blood flow is equal to Cl_{int} then extraction ratio is 0,50 ($E = \frac{Cl_{int}}{Q + Cl_{int}}$). A low Cl_{int} drug has an extraction ratio that is less than 0,3.

My results support the "venous-equilibration" model in that the extraction ratio calculated directly as $\frac{AUC_{PV}-AUC_{HV}}{AUC_{PV}}$ (equation 3) and $\frac{D_{iv}}{Q.AUC_{PV}}$ (equation 5) agreed well with the extraction ratio calculated from the equation $\frac{Cl_{int}}{Q + Cl_{int}}$ derived from the "venous-equilibration" model. (See Tables 2a and b.) For example, in perfusion C of pindolol when Cl_{int} was equal to the perfusate flow, the extraction ratio was 0,5 even when calculated according to equations 3 and 5.

The AUC_{HV} and Cl_{int} were independent of flow rate, as predicted by the model.

Influence of Flow Rate on Extraction Ratio and Hepatic Clearance

According to the theoretical predictions of the "venous-equilibration" model, high Cl_{int} (high extraction ratio) drugs are classified as being flow-limited, i.e. their hepatic clearances are sensitive to changes in flow (page 13). Low Cl_{int} (low extraction ratio) drugs, on the other hand, are capacity-limited, i.e. their hepatic clearances are sensitive not to flow but to the metabolic (enzymatic) capacity of the liver. Intermediate Cl_{int} (intermediate extraction ratio) drugs have hepatic clearances that are determined partly by flow and partly by hepatic metabolic function.

In terms of the above classification, propranolol with its high Cl_{int} and high extraction ratio can be categorised as a flow-limited drug, while pindolol can be best regarded as a part flow-limited/part capacity-limited drug on account of its intermediate Cl_{int} and extraction ratio status.

Results obtained from my experiments confirm these predictions. Reference to Graph 18 shows the relationship between flow rate and extraction ratio. A plot of $\frac{1}{E}$ against flow gave a straight line with an intercept of 1 and a slope of $\frac{1}{Cl_{int}}$ as predicted by the "venous-equilibration" model. This plot illustrates that increasing flow is accompanied by more marked changes in extraction ratio with pindolol (the slope of the line is greater) than with propranolol. This is also reflected in the highly significant negative correlation between flow and extraction ratio in the case of pindolol and the non-significant correlation in the propranolol group.

Increasing liver blood flow therefore resulted in a decreasing extraction ratio but the decrease was proportionately much greater with pindolol than with propranolol. Hepatic clearance is the product of extraction ratio and blood flow. Thus with pindolol (an intermediate Cl_{int}, intermediate extraction ratio), the increase in blood flow was paralleled by an appreciable decrease in extraction ratio, such that the product (i.e. hepatic clearance) was not predominantly affected by changes in flow (the latter being partly offset by opposite changes in extraction ratio). The situation with propranolol

(a high Cl_{int}, high extraction ratio drug) was different. Here increasing blood flow was accompanied by only a marginal decrease in extraction ratio; thus the product of the two variables (i.e. hepatic clearance) rose steeply. The hepatic clearance of propranolol was thus predominantly affected by changes in flow rate.

My results clearly highlight these differences. An increase in flow of 71% resulted in a 49% increase in the hepatic clearance of propranolol whereas a similar increase in flow (67%) caused only a 15% increase in hepatic clearance of pindolol. As expected, the correlation between flow and hepatic clearance was larger for the propranolol perfusions ($r = 0,97$; $p<0,001$) than for pindolol ($r = 0,87$; $p<0,05$). The fact that a significant correlation emerged in the latter confirmed that pindolol was an intermediate Cl_{int} drug, i.e. one whose hepatic clearance was <u>partly</u> sensitive to flow. It is unlikely that a low Cl_{int} drug would have yielded a significant correlation between flow and hepatic clearance.

As propranolol has a higher extraction ratio than pindolol, one would expect that its hepatic clearance at any flow rate would be greater than that of pindolol. This was shown in my experiments where the mean Cl_H of the propranolol perfusions (2,47 ml/g/min) was significantly greater than that for the pindolol perfusions (1,56 ml/g/min) ($p<0,001$).

It might be argued that since flow is an important determinant of extraction ratio in the case of pindolol, the lower extraction ratio for pindolol in my study was an artefact. Although the mean flow rates between the pindolol and propranolol groups did not differ significantly, the range of flow rates showed a slight variation. In order to exclude any distorting effects of flow on extraction ratio, subgroups of perfusions were taken from the propranolol and pindolol groups, the flow rates in the subgroups being almost identically matched. A statistical analysis of these subgroups revealed no change in the significance of the findings previously demonstrated in the total groups. The mean extraction ratio for propranolol was still highly significantly greater than that for pindolol ($p<0,01$) (Table 2c).

The same procedure was followed for the mean hepatic clearance: propranolol again yielded significantly higher values than pindolol ($p<0,01$) (Table 6).

Compartment Models

A notable difference in the pharmacokinetics of the drugs was the biphasic portal venous concentration-versus-time curve for propranolol in comparison to the monophasic curve obtained in the case of pindolol. This indicated that propranolol obeyed a two-compartment open model whereas pindolol appeared to conform to a one-compartment model.

The biphasic curve of propranolol represents: (a) a rapid α or distribution phase which primarily reflects the movement of drug from the central to the peripheral compartment; and (b) a slower β or elimination phase which is a hybrid of redistribution (peripheral to central compartment) and elimination. (See Fig. 3, page 5.)

The apparent volume of distribution (V_d) is an index of the volume into which the drug appears to be distributed. Propranolol had a mean V_d of 21,23 ml/g of liver tissue (262 ml); this figure far exceeds the volume of the perfusate (103 ml). This indicated that the drug was extensively distributed beyond the central compartment. Pindolol, on the other hand, had a mean V_d of 8,72 ml/g of liver tissue (106 ml). This figure, being nearly equal to the volume of perfusate, suggested that pindolol was virtually confined to the intravascular (central) compartment.

I measured drug concentrations in total blood-KREBS perfusate: thus the central compartment included red blood cells and plasma. In my perfusion model the extravascular accumulation of propranolol could only have occurred in the liver. Such an assumption, however, would not be tenable unless the binding of propranolol to glass or to other constituents of the apparatus had been discounted as a source of error. Particular care was taken to exclude such a possibility and this aspect is more fully discussed in an earlier section (page 52).

Other workers have also observed a biphasic curve after rat liver perfusions with propranolol at doses of 5 mg and

above (14, 101). However, they also found that at low doses drug concentrations declined monoexponentially. This indicated that the initial uptake of drug depended on a saturable process which at low doses resulted in complete extraction and at higher doses was responsible for the α-phase of the biexponential curve. Almost complete extraction of propranolol at low doses has been observed in man and dogs (4, 46, 65).

This initial rapid and avid uptake of propranolol has been shown to be due to physical binding of unchanged drug to liver tissue. The α-phase was found to be independent of factors which inhibit metabolism such as temperature, oxygen and enzyme inhibitors, whereas the β-phase was sensitive to these factors (14, 101). When livers were perfused in the absence of oxygen, there was virtually no β-phase although uptake in the α-phase was not affected (101). Thus it would appear that the uptake and binding processes of propranolol (α-phase) are qualitatively different from those involved in metabolism (β-phase).

The binding of propranolol has been studied quantitatively in the rat. Homogenisation experiments have yielded estimates of over 170 : 1 for liver/perfusate drug concentrations at low concentrations whereas at higher concentrations (160 µg/ml) this ratio decreased to 9,3 : 1 (15). At concentrations of 90 µg/ml unchanged propranolol was found in the liver in amounts 12 to 36 times higher than in the perfusate (101). Autoradiography has demonstrated that propranolol localises in the periportal zones and possibly also in certain cell types (101).

It is hypothesised that propranolol binds to several sites each with different affinities within the liver. Equilibrium dialysis and Scatchard plots have demonstrated high affinity-low capacity sites and low affinity-high capacity sites (101). The α-phase reflects binding to the high affinity site which, as previously mentioned, is saturable. Therefore, after saturation at higher doses, further binding takes place in the lower affinity-higher capacity areas.

Table 9 shows my calculations of the total amount of propranolol extracted from the perfusate at 5 minutes, i.e.

at the end of the α-phase. The average value was 329 μg/g of liver which may be regarded as an indication of the capacity of the so-called high affinity site. The only other such estimation in the literature was one of 500 μg/g of liver (101).

Distribution and Elimination Half-Life

There was a very highly significant negative correlation between flow and the α-phase half-life ($t_{\frac{1}{2}\alpha}$) of propranolol ($p<0,01$). This has also been shown by other workers (19, 101). The elimination half-life ($t_{\frac{1}{2}\beta}$) of propranolol (5,1 minutes) was considerably longer than the $t_{\frac{1}{2}\alpha}$ (1 minute) but there was no correlation between $t_{\frac{1}{2}\beta}$ and flow rate. Pindolol, on the other hand, showed a highly significant negative correlation between flow and $t_{\frac{1}{2}\beta}$ ($p<0,01$). Furthermore, the $t_{\frac{1}{2}\beta}$ of pindolol was shorter than that of propranolol.

Table 10 shows the instantaneous extraction ratios for propranolol and pindolol at various times throughout the perfusion. Within the α-phase of propranolol there was an exceedingly high extraction ratio, especially at 1 minute (0,99) and 3 minutes (0,94). During the β-phase there was a marked decline in extraction ratio; in fact, the average extraction ratio of propranolol (0,39) in this phase was lower than the average extraction ratio for pindolol (0,52).

It thus appears that propranolol is an exceptionally high extraction ratio drug in the α-phase because of the rapid (1 minute) and avid high affinity liver binding. Thereafter, in the β-phase it converts to a low extraction ratio drug, lower even than pindolol.

This observation explains my finding of a high negative correlation between flow and $t_{\frac{1}{2}\alpha}$. Half-life is inversely related to hepatic clearance which, in the case of a very high extraction ratio drug (e.g. propranolol in the α-phase), is strongly determined by flow. Increasing flow rates would therefore lead to increasing hepatic clearances and to decreasing half-lives.

In the β-phase of propranolol, when the drug has converted to a lower extraction ratio status, hepatic clearance is not so sensitive to flow but rather to metabolic factors. As I have demonstrated, there is no significant correlation

between flow and $t_{\frac{1}{2}\beta}$.

Pindolol has a fairly consistent extraction ratio throughout the perfusion (0,50 - 0,60) and, as described earlier, falls into the intermediate category where hepatic clearance is partly sensitive to flow changes. It is thus not unexpected to obtain a negative correlation between flow and $t_{\frac{1}{2}\beta}$.

The $t_{\frac{1}{2}\beta}$ of pindolol is shorter than that of propranolol. No direct statistical analysis was done between the $t_{\frac{1}{2}\beta}$ of propranolol and pindolol because the two drugs conformed to different compartmental models. In the case of the two-compartment, biexponential model the β-phase is a composite of redistribution as well as elimination processes whereas the one-compartment, monoexponential system does not involve redistribution to any extent.

The longer $t_{\frac{1}{2}\beta}$ of propranolol compared to pindolol may be explained in terms of both the redistribution and the elimination of propranolol.

Previous work by Shand et al showed that, (when the liver was washed with drug-free perfusate) after perfusion with 4 mg propranolol, 34% of propranolol was displaced from the rat liver into the perfusate (14). Since the dosage used in my experiments was even higher (5 mg), it was probable that there was appreciable redistribution of the drug back into the central compartment. The extent of this first-order redistribution would have accordingly prolonged the $t_{\frac{1}{2}\beta}$. In the case of pindolol, such a redistribution effect (if any) is likely to have been negligible owing to its one-compartment disposition.

Secondly, the longer half-life of propranolol might be accounted for by its reduced elimination in the β-phase. It has already been shown from the instantaneous extraction ratios that the extraction ratio of propranolol during the β-phase is lower than that of pindolol. However, this finding may also have arisen from redistribution - with higher effluent (hepatic vein) concentrations resulting in lower extraction ratios ($E = \frac{AUC_{PV} - AUC_{HV}}{AUC_{PV}}$).

In the absence of quantitative data on the total amount of non-metabolised drug in the liver after the perfusion, it is not possible to ascertain whether there are actual metabolic differences between the two drugs. It must also be noted that elimination is an overall process which includes such processes as tissue binding and biliary excretion.

The larger mean extraction ratio of propranolol (0,71) compared to pindolol (0,50) represents the first-pass effect. It is obvious that this overall greater first-pass effect is due to the very high extraction ratio values obtained for propranolol in the α-phase and that these represent initial rapid and avid uptake and binding. The same reasoning would apply to the much higher hepatic intrinsic clearance derived for propranolol. It must be recalled that hepatic intrinsic clearance is an index not only of liver weight (I have corrected for this) and liver metabolism but also of the partitioning of drug into the liver. This concept would explain the seemingly paradoxical situation where propranolol has a higher extraction ratio and a higher intrinsic clearance than pindolol and yet has a longer elimination half-life. It is, in fact, this specific property of physical binding to liver tissue that endows propranolol with its high clearance status.

Hepatic uptake of the kind described for propranolol may be a feature of drugs with substantial first-pass effects, such as chlorpromazine, imipramine and nortriptyline. It is interesting that the latter drugs displace propranolol from rat liver binding sites (101) and that imipramine binds to rat tissue subcellular fractions.

The location of liver binding sites is still unclear. Phenobarbitone can increase the capacity of the high affinity site in the rat liver, indicating that this site may be an inducible protein or lipoprotein (16). Propranolol is an amine with a pk_a of 9,45 so that most of its molecules, being protonated cations at physiological pH, can bind to extracellular and intracellular anions.

The binding process may be dependent on the lipid solubility of propranolol. Propranolol binds to mitochondrial membranes, microsomal sites and to the membrane constituents

cardiolipin and phosphatidyl ethanolamine (101).

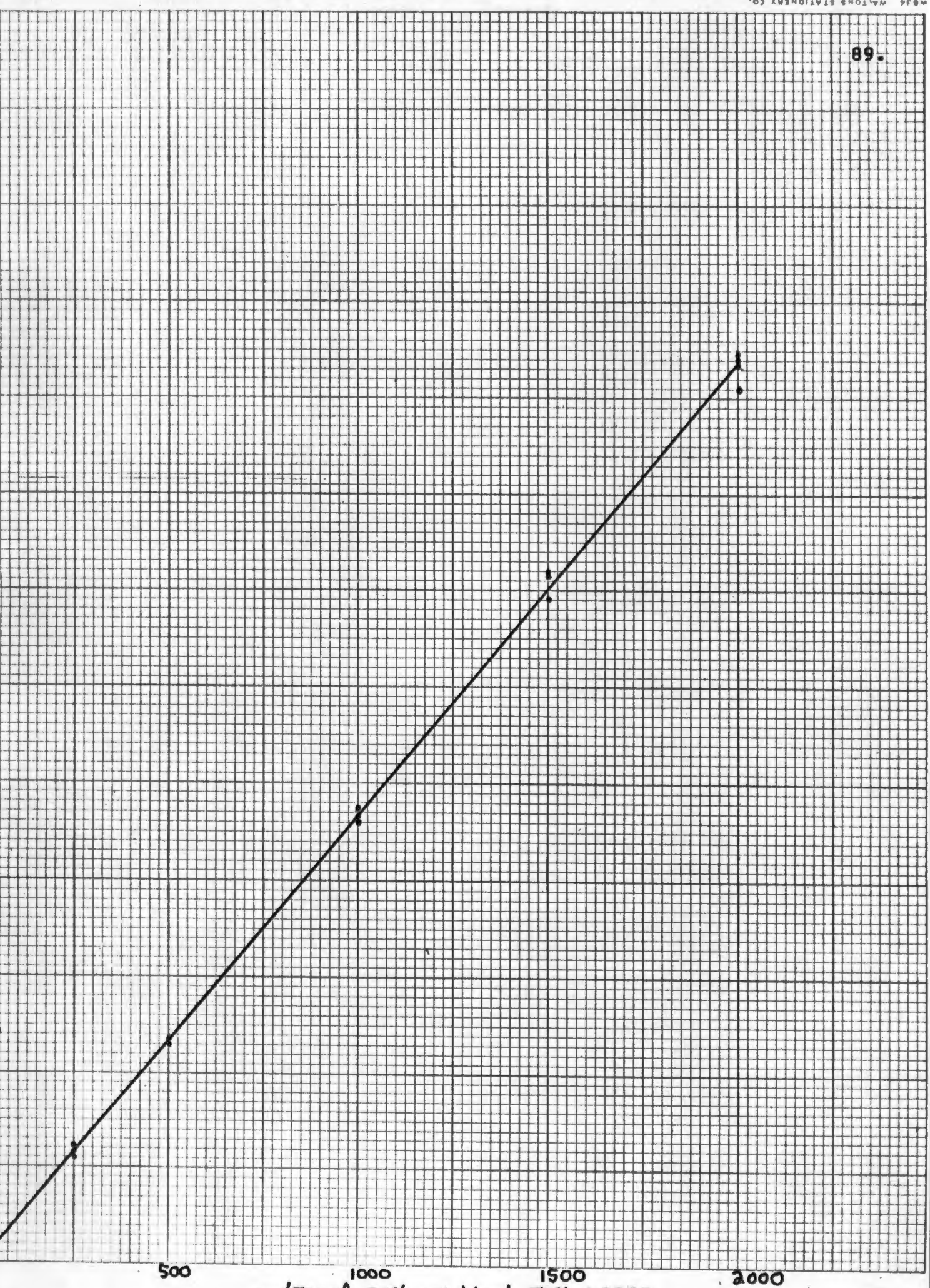
It is interesting to note that propranolol and alprenolol have the highest lipid solubilities within the β-blocker class with octanol/water distribution ratios of 5,39 and 3,27 respectively. These drugs also have the highest first-pass extractions in man (70% and 90% respectively). Practolol has a very low lipid solubility (octanol/water 0,009) and it is virtually devoid of a first-pass effect. Pindolol has a low octanol/water distribution ratio (0,12) and a small first-pass extraction (13%).

It seems possible that the great difference in the lipid solubility (and perhaps tissue binding) between propranolol and pindolol may contribute substantially to the larger first-pass effect of the former.

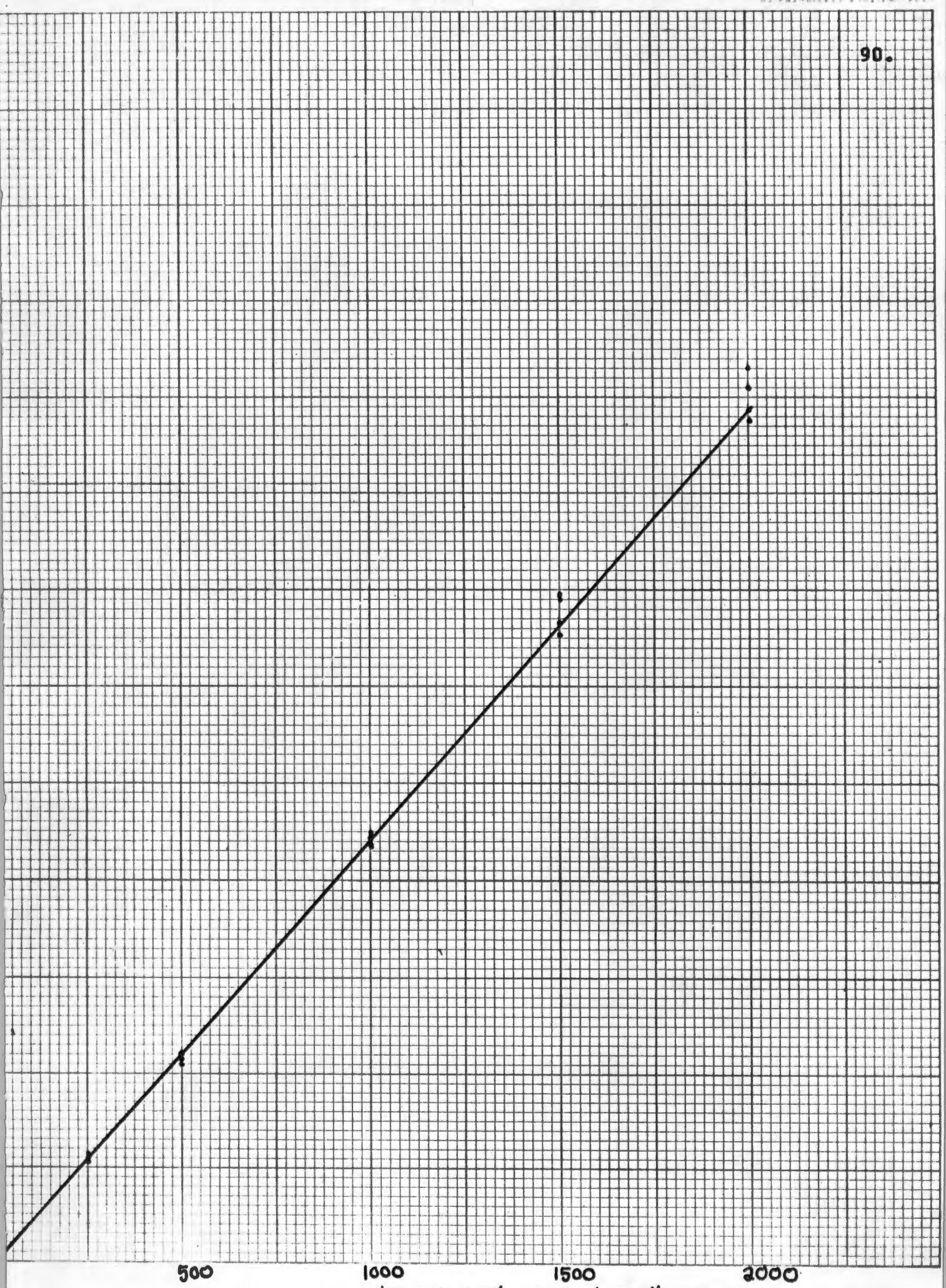
This project has confirmed the validity of the rat liver perfusion model and has demonstrated its application in predicting the disposition of drugs. Although the results from an isolated <u>in vitro</u> rat model cannot necessarily be extrapolated to man, the data derived therefrom may provide meaningful insights into the pharmacokinetics of a drug, particularly its first-pass extraction (bioavailability) and possible binding characteristics.

The model has shown that hepatic intrinsic clearance is an important pharmacokinetic parameter. Its calculation is clinically simple, convenient and non-invasive. Furthermore, it can be derived clinically after administration of a single oral dose (from the formula $\frac{D_o}{AUC_o}$) and from this one can gain a reliable estimate of the first-pass effect (= $\frac{Cl_{int}}{Q + Cl_{int}}$) and the bioavailability (= 1 - E) of the drug (assuming a liver blood flow of 1,5 l/min and complete absorption). Hepatic intrinsic clearance provides an index of the drug's capacity to be eliminated by <u>all</u> channels. It thus offers a useful basis by which to assess changes in drug clearance due to disease processes and/or interaction with other drugs.

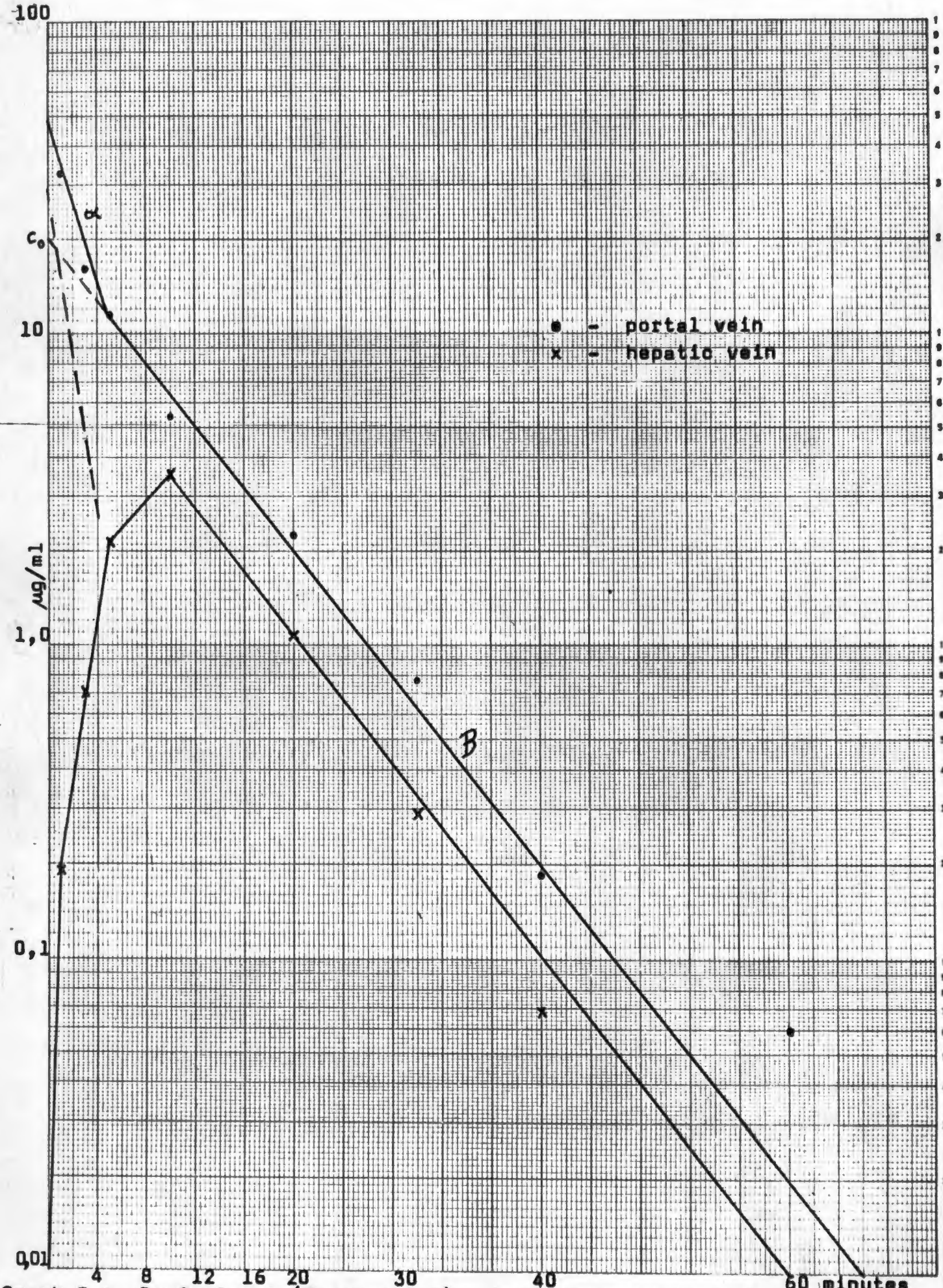
The perfusion-limited rat model as exemplified in this research might contribute usefully to a study of the pharmacokinetics of a new drug in the preclinical phases as well as to comparative evaluations against existing compounds.



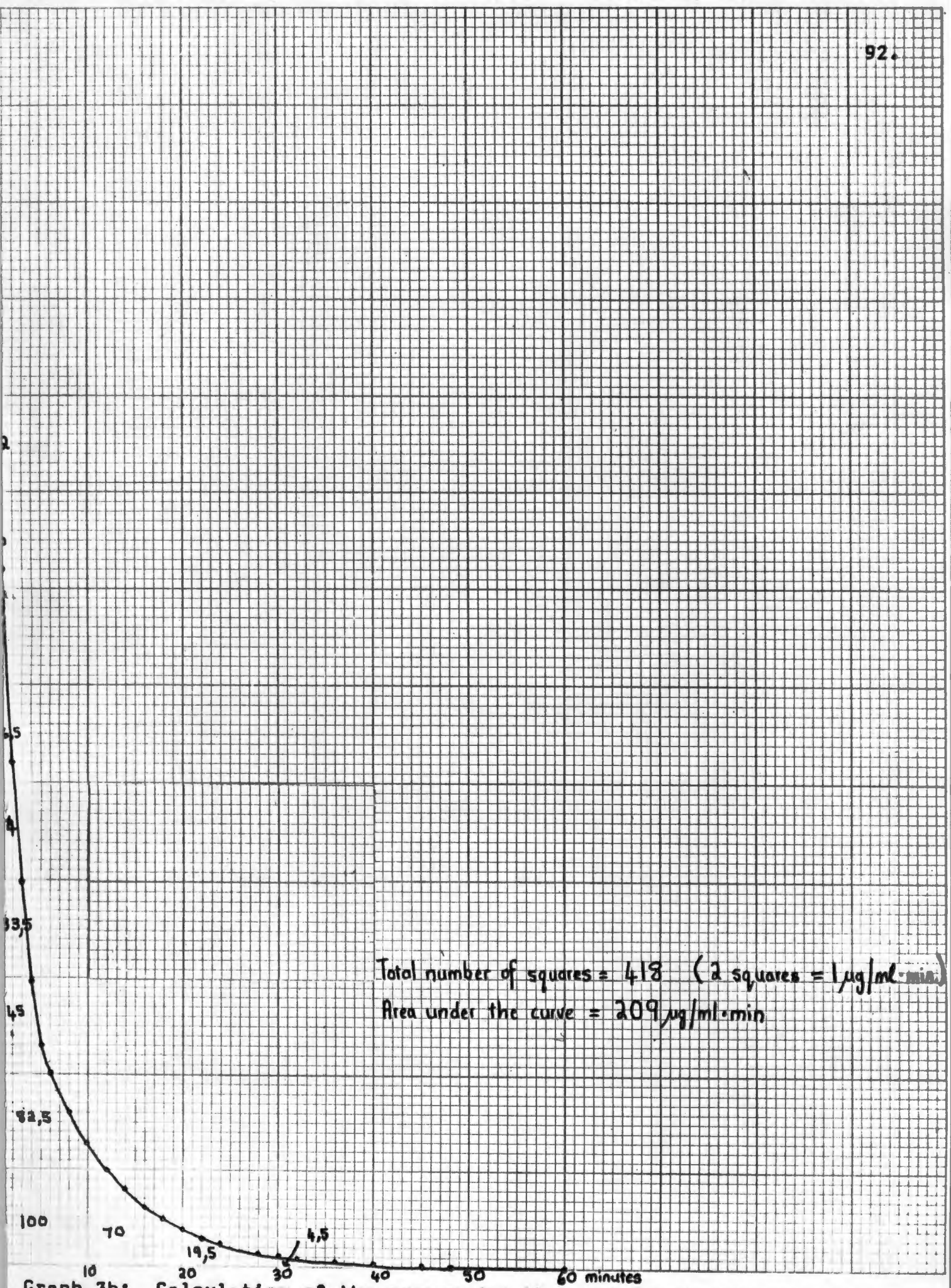
Graph 1: Fluorometric Determination of Propranolol. A plot of fluorescence intensity versus concentration with each point in quadruplicate.



Graph 2: Fluorometric Determination of Pindolol. A plot of fluorescence intensity versus concentration with each point in quadruplicate.



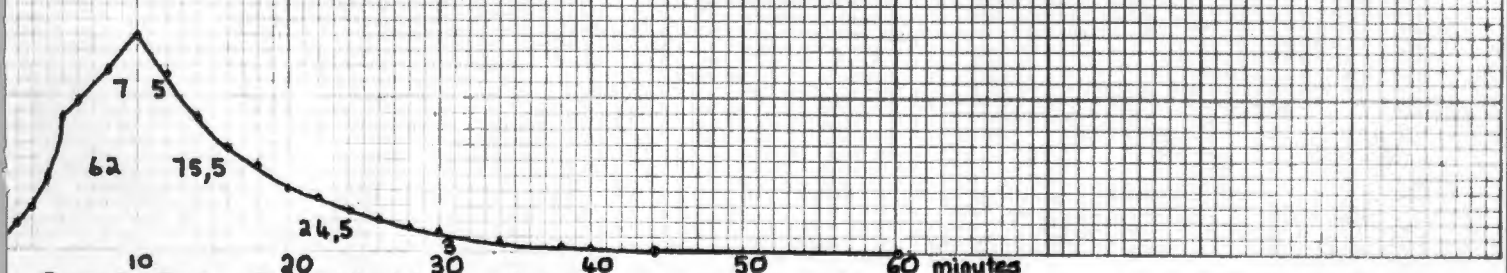
Graph 3a: Perfusion A (Propranolol). Semi-logarithmic plot of concentration (g/ml) versus time (minutes). Flow rate: 35 ml/min or 2,90 ml/g/min.



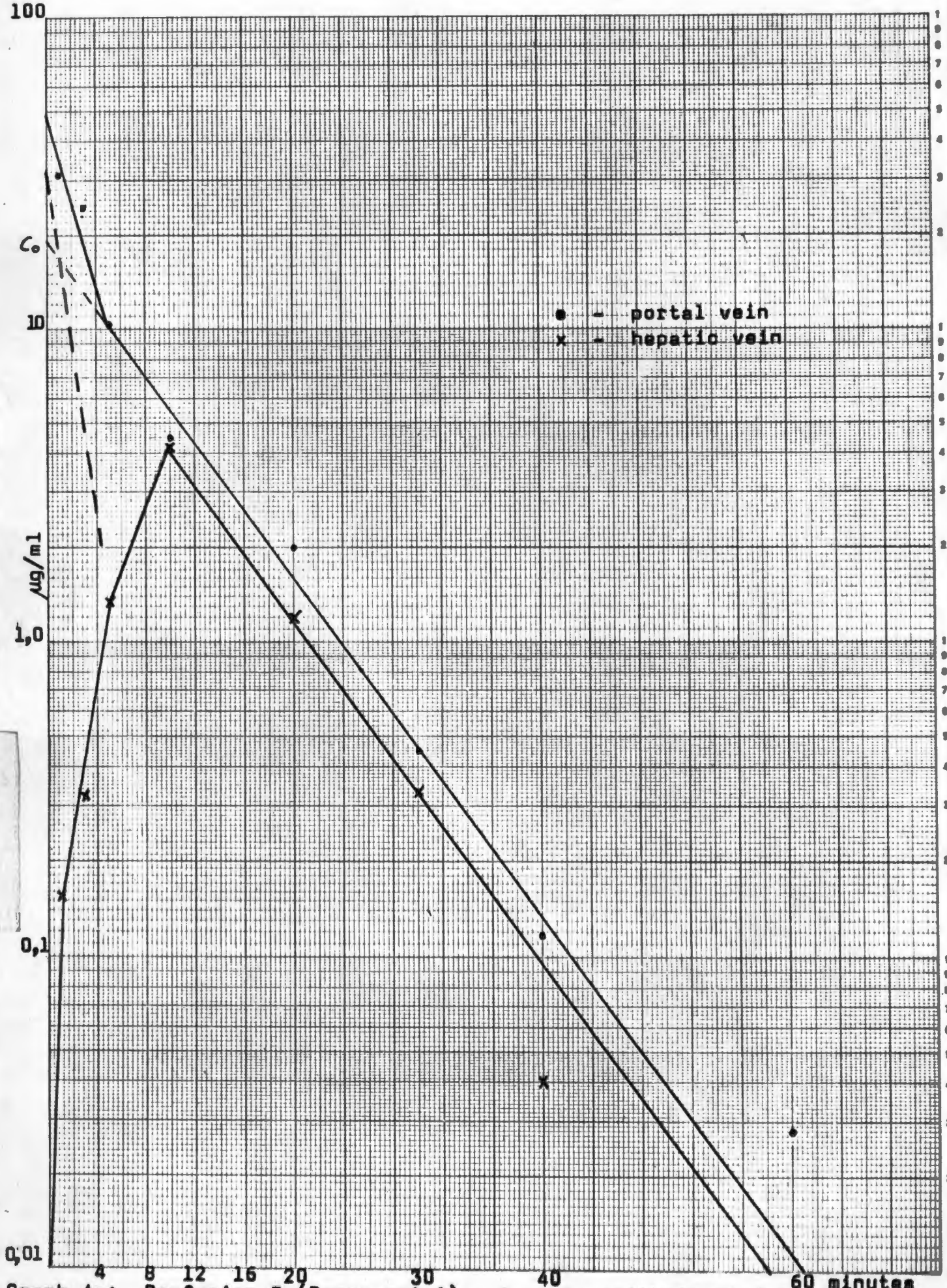
Graph 3b: Calculation of the area under the portal vein concentration versu time curve for perfusion A (Propranolol). Points were obtained from the lin fitted in Graph 3a.

Total number of squares = 177 (4 squares = 1 µg/ml·min.)

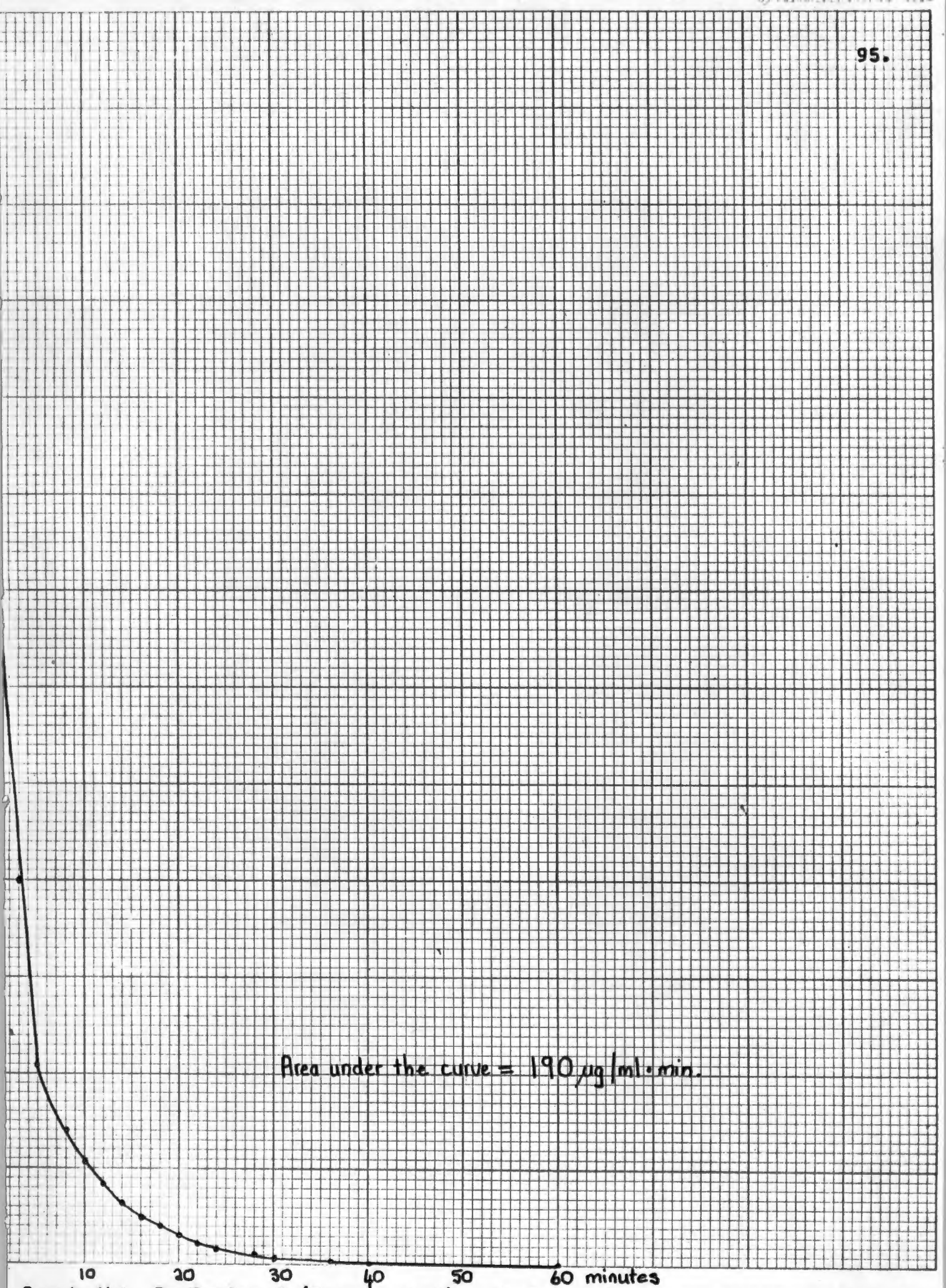
Area under the curve = 44 µg/ml·min.



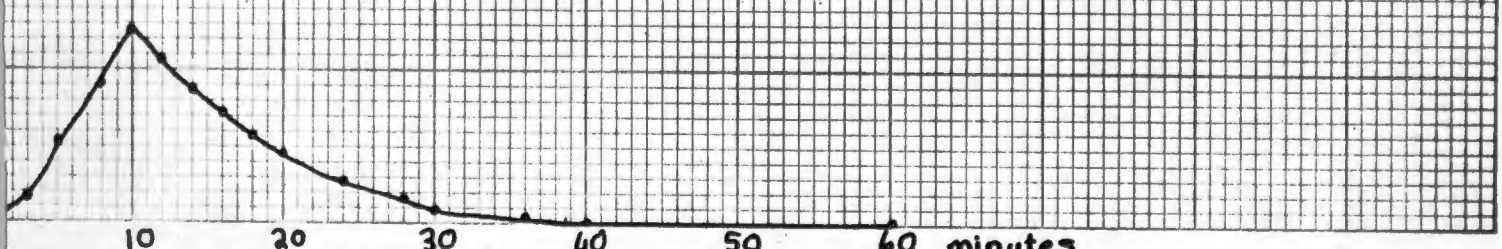
Graph 3c: Calculation of the area under the hepatic vein concentration versus time curve for perfusion A (Propranolol). Points were obtained from the line fitted in Graph 3a.



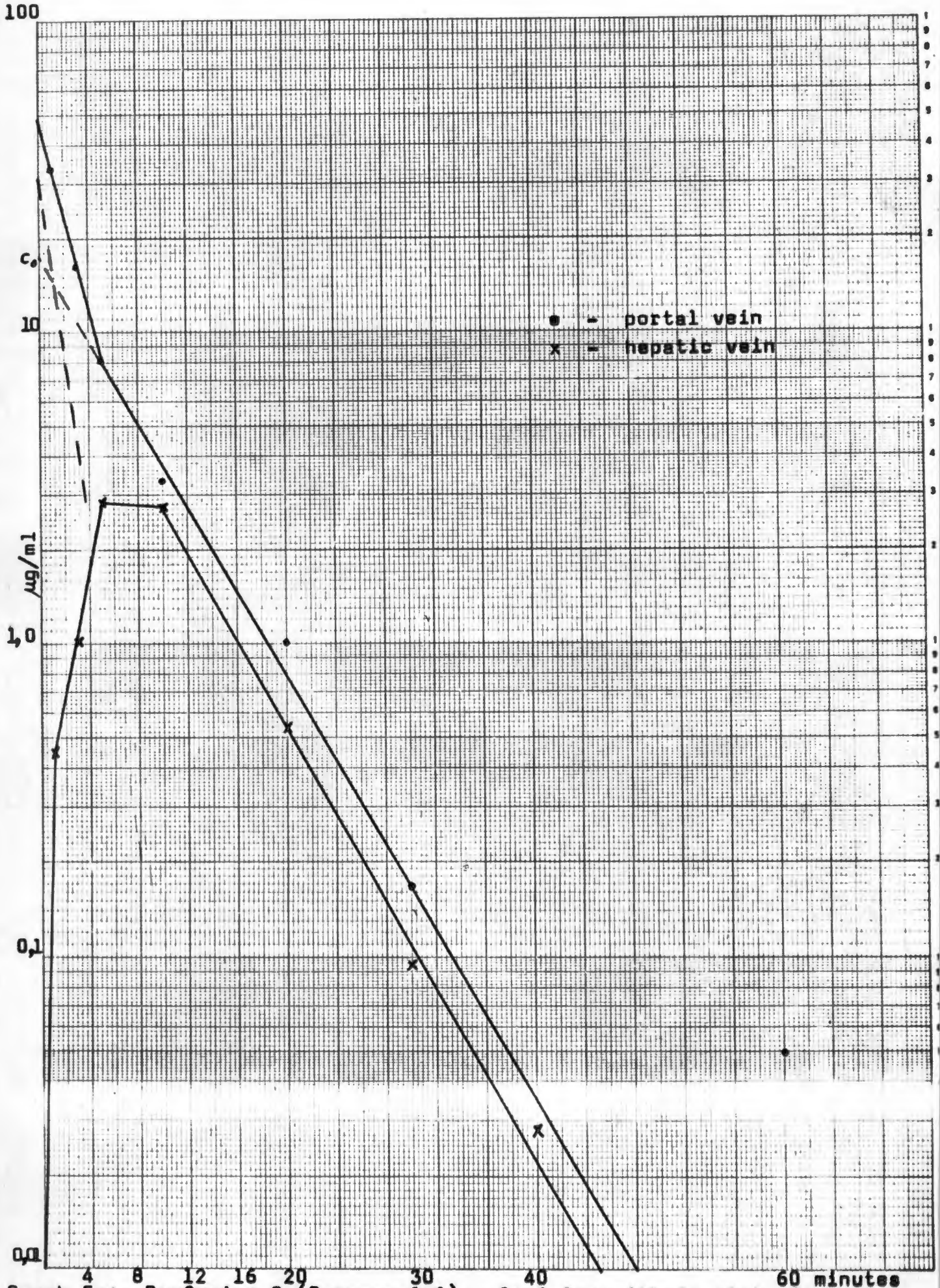
Graph 4a: Perfusion B (Propranolol). Semi-logarithmic plot of concentration versus time. Flow rate: 42 ml/min or 3,45 ml/g/min.



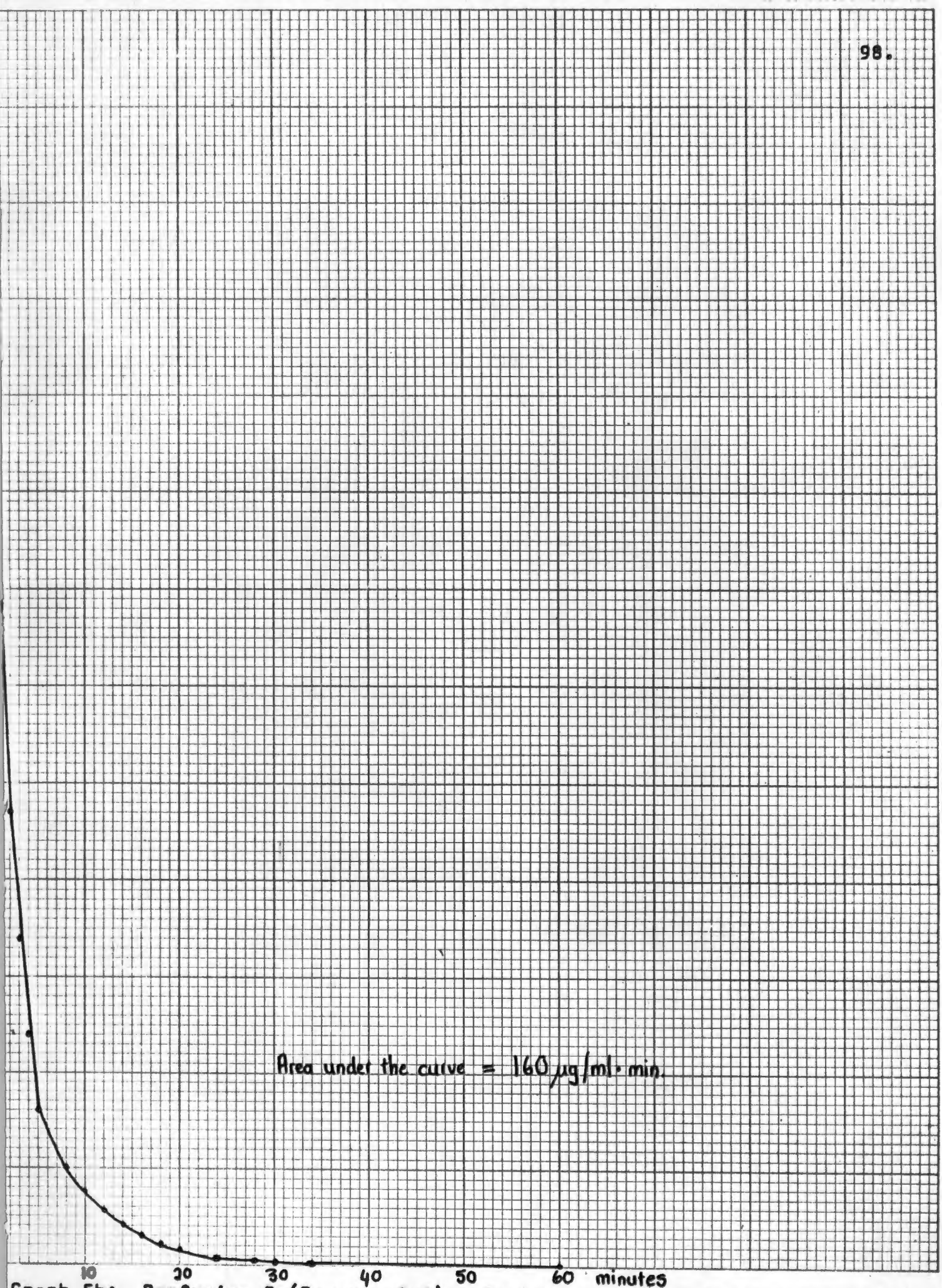
Graph 4b: Perfusion 8 (Propranolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 4a.



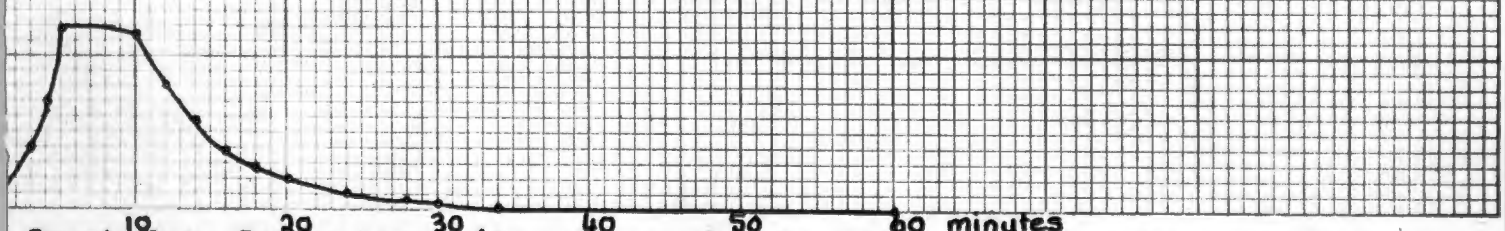
Graph 4c: Perfusion B (Propranolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 4a.



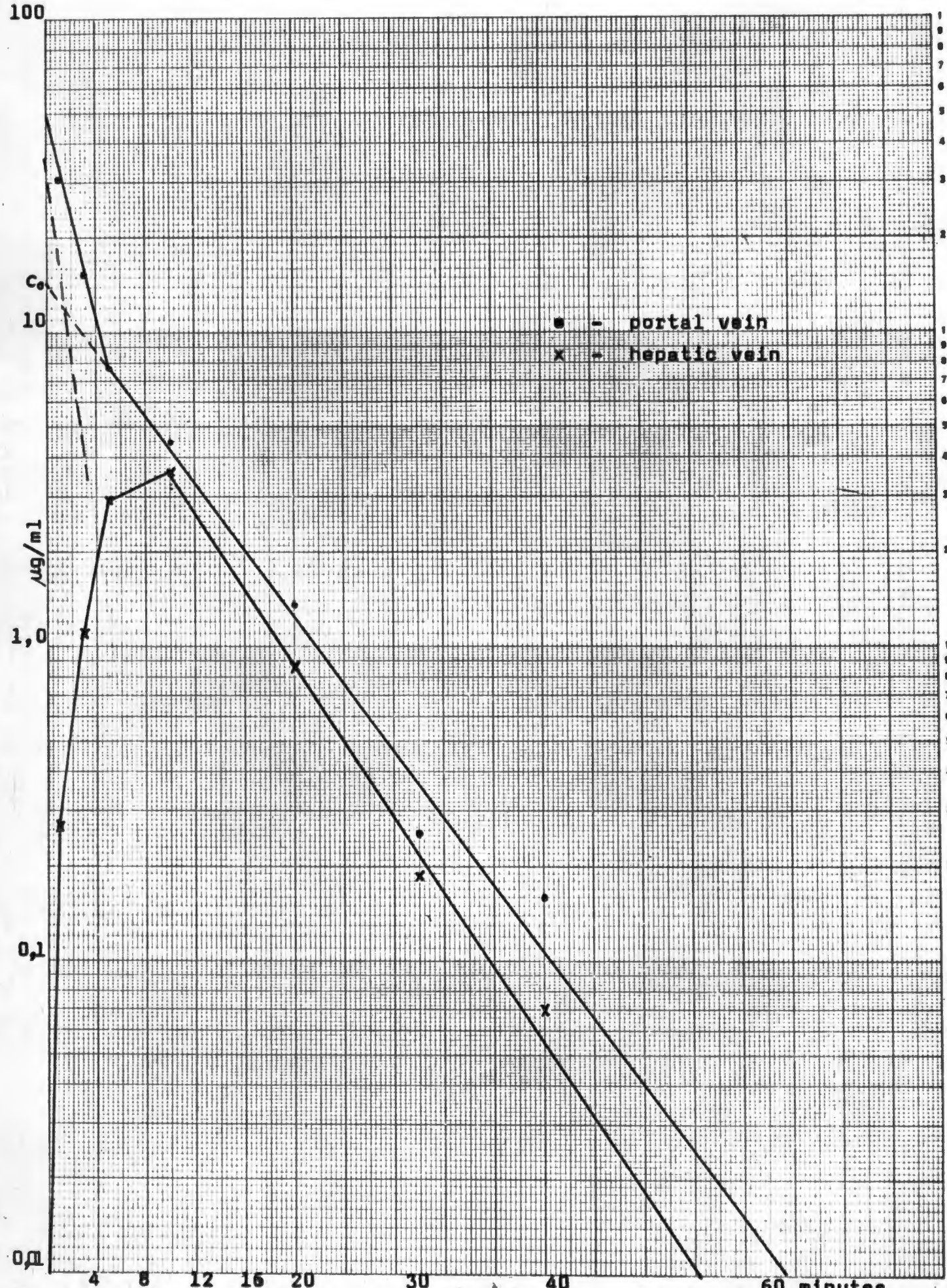
Graph 5a: Perfusion C (Propranolol). Semi-logarithmic plot of concentration versus time. Flow rate: 44 ml/min or 3,33 ml/g/min.



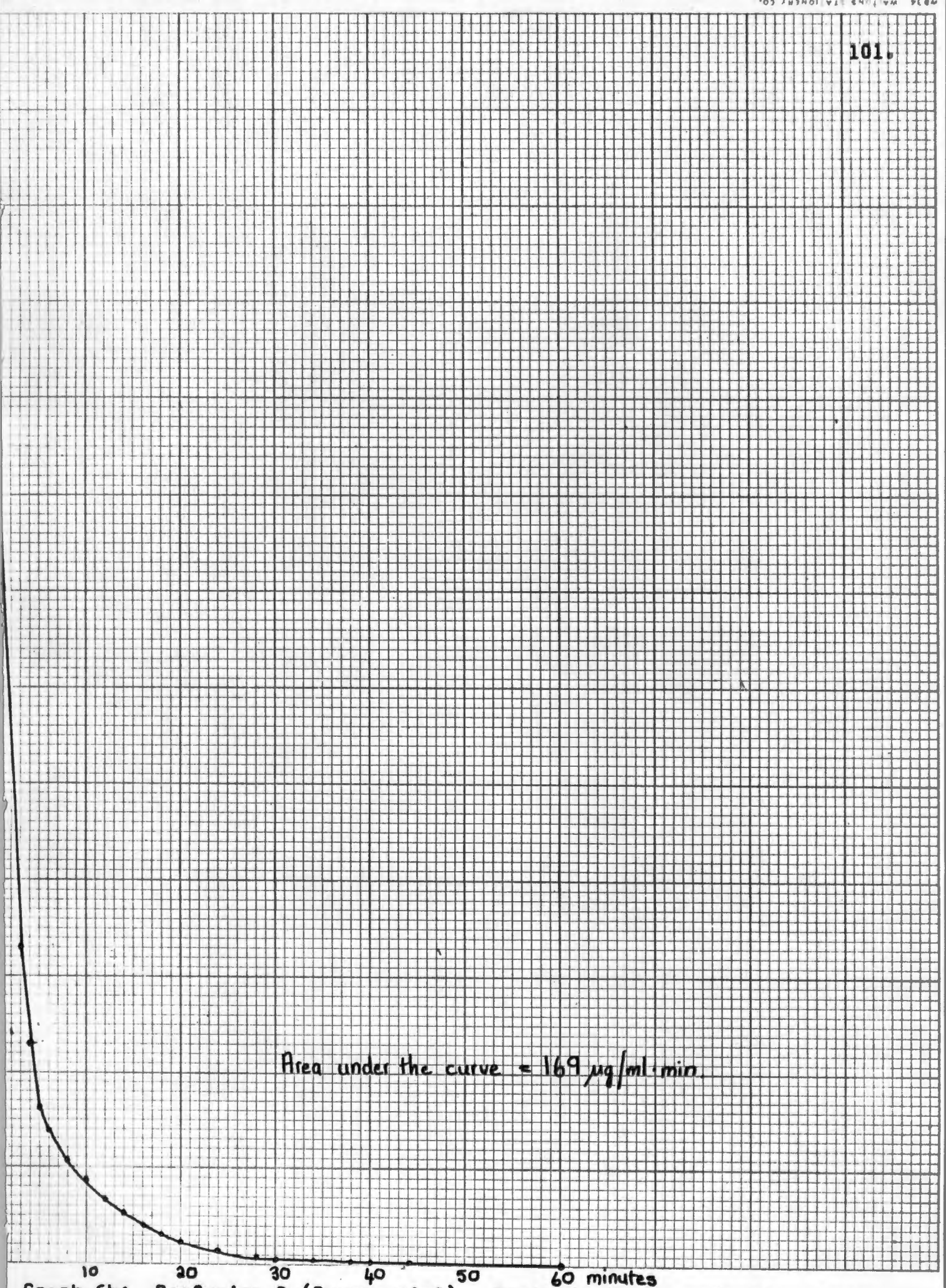
Graph 5b: Perfusion C (Propranolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 5a.



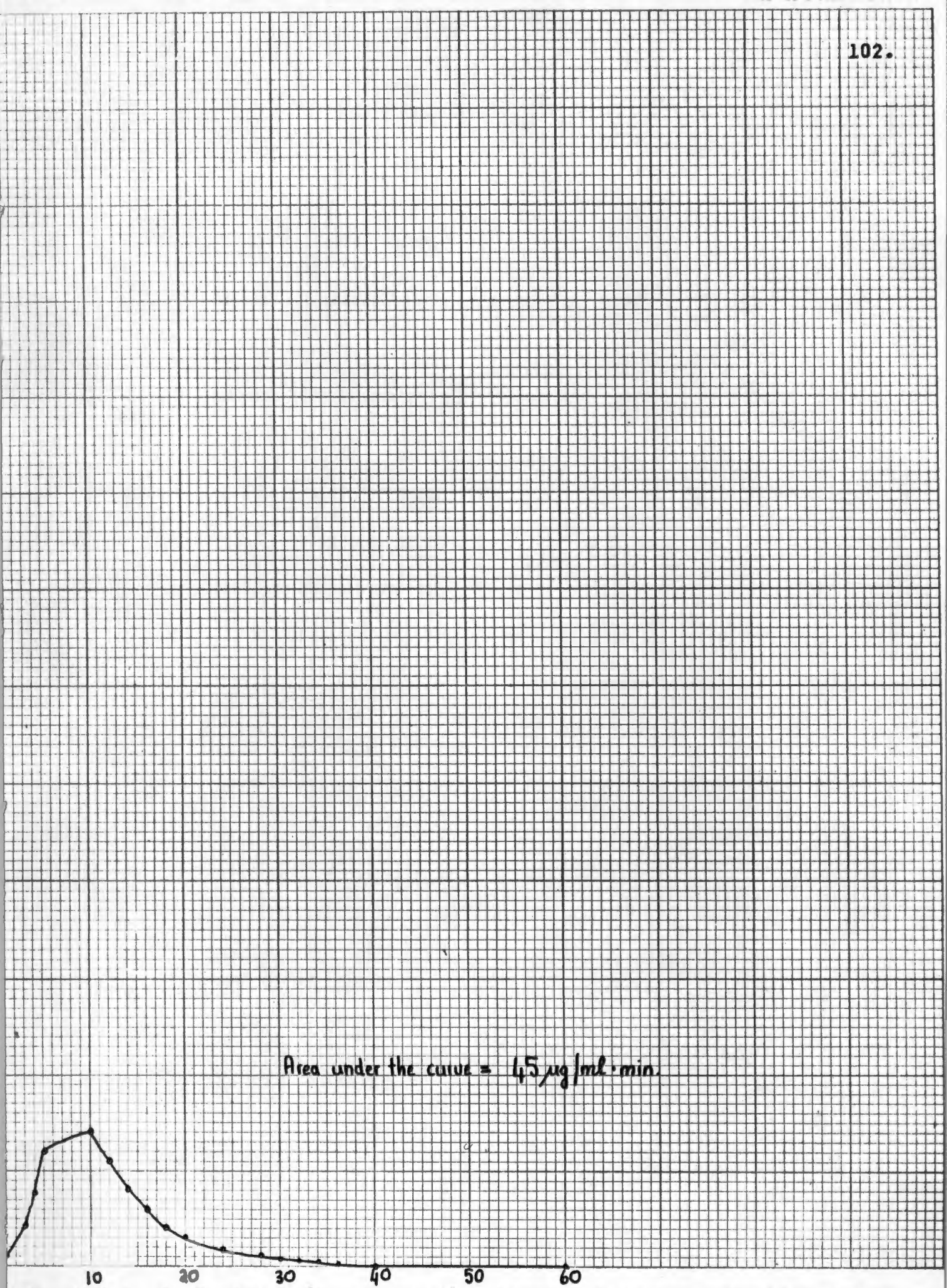
Graph 5c: Perfusion C (Propranolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 5a.



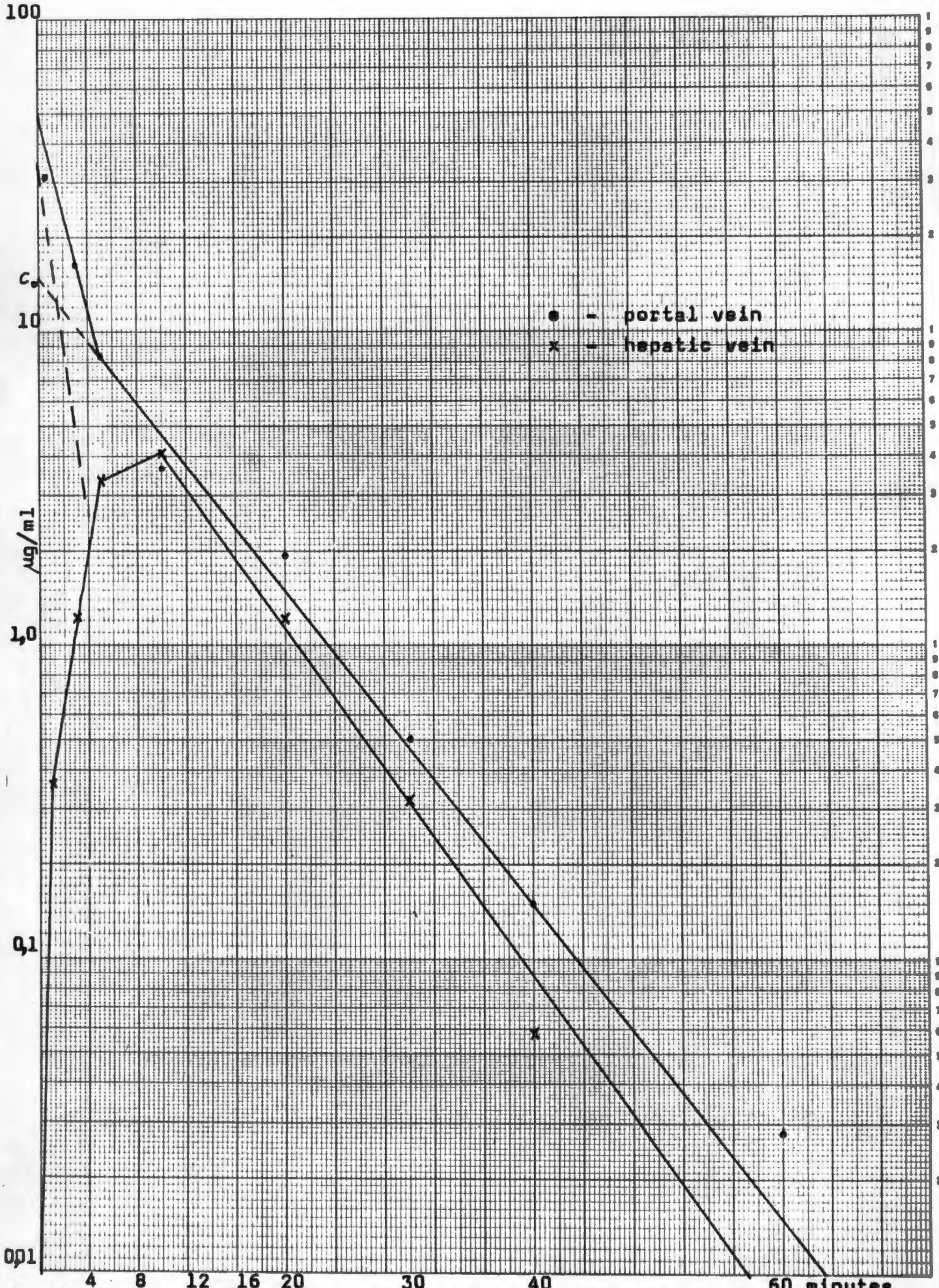
Graph 6a: Perfusion D (Propranolol). Semi-logarithmic plot of concentration versus time. Flow rate: 45 ml/min or 3,39 ml/g/min.



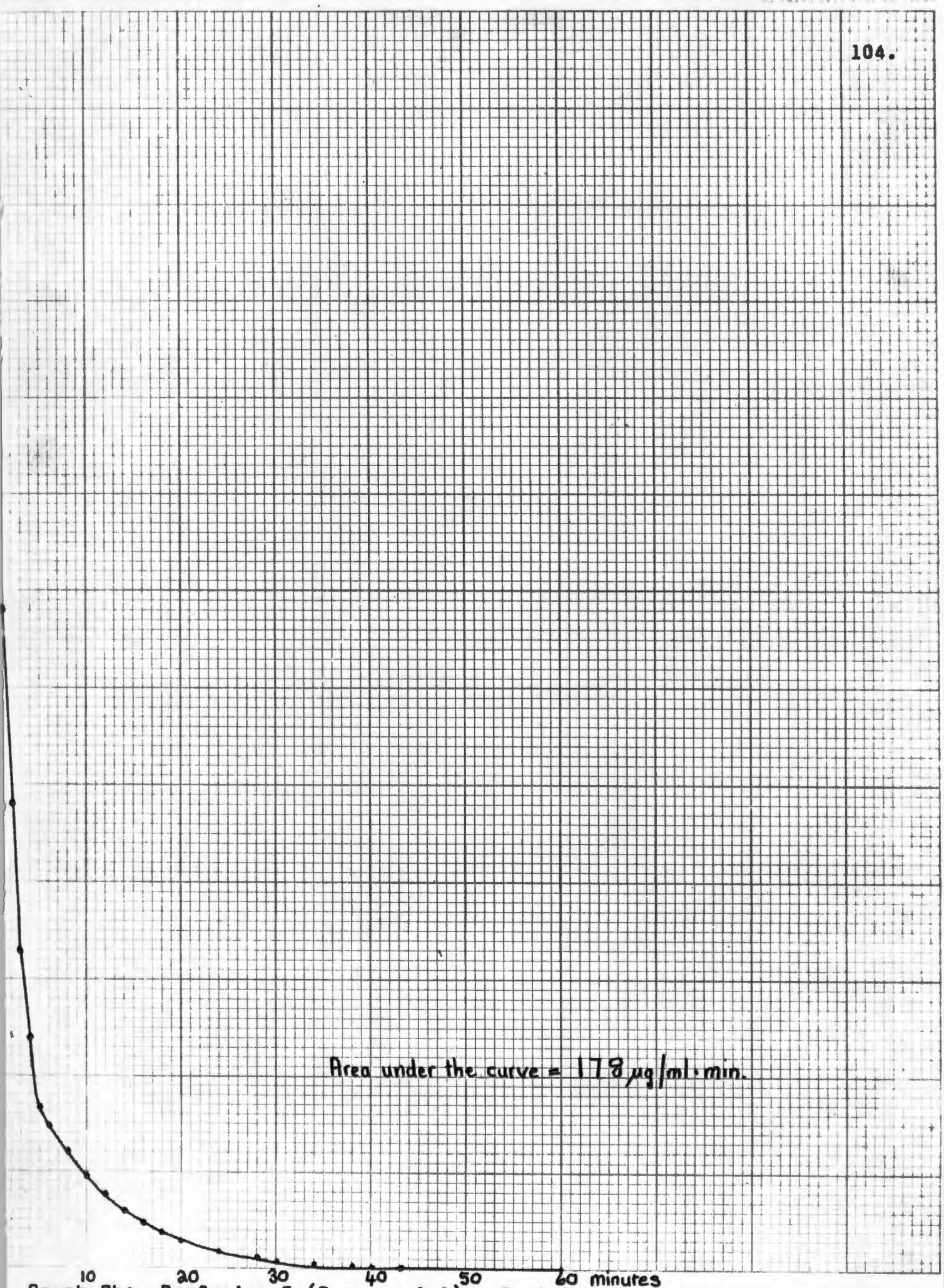
Graph 6b: Perfusion D (Propranolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 6a.



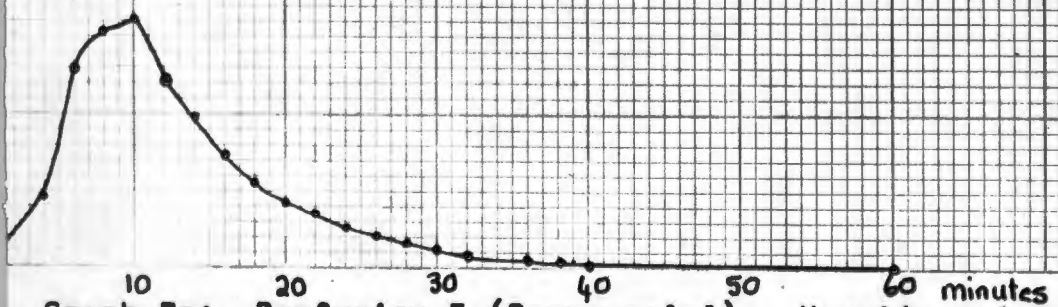
Graph 6c: Perfusion D (Propranolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 6a.



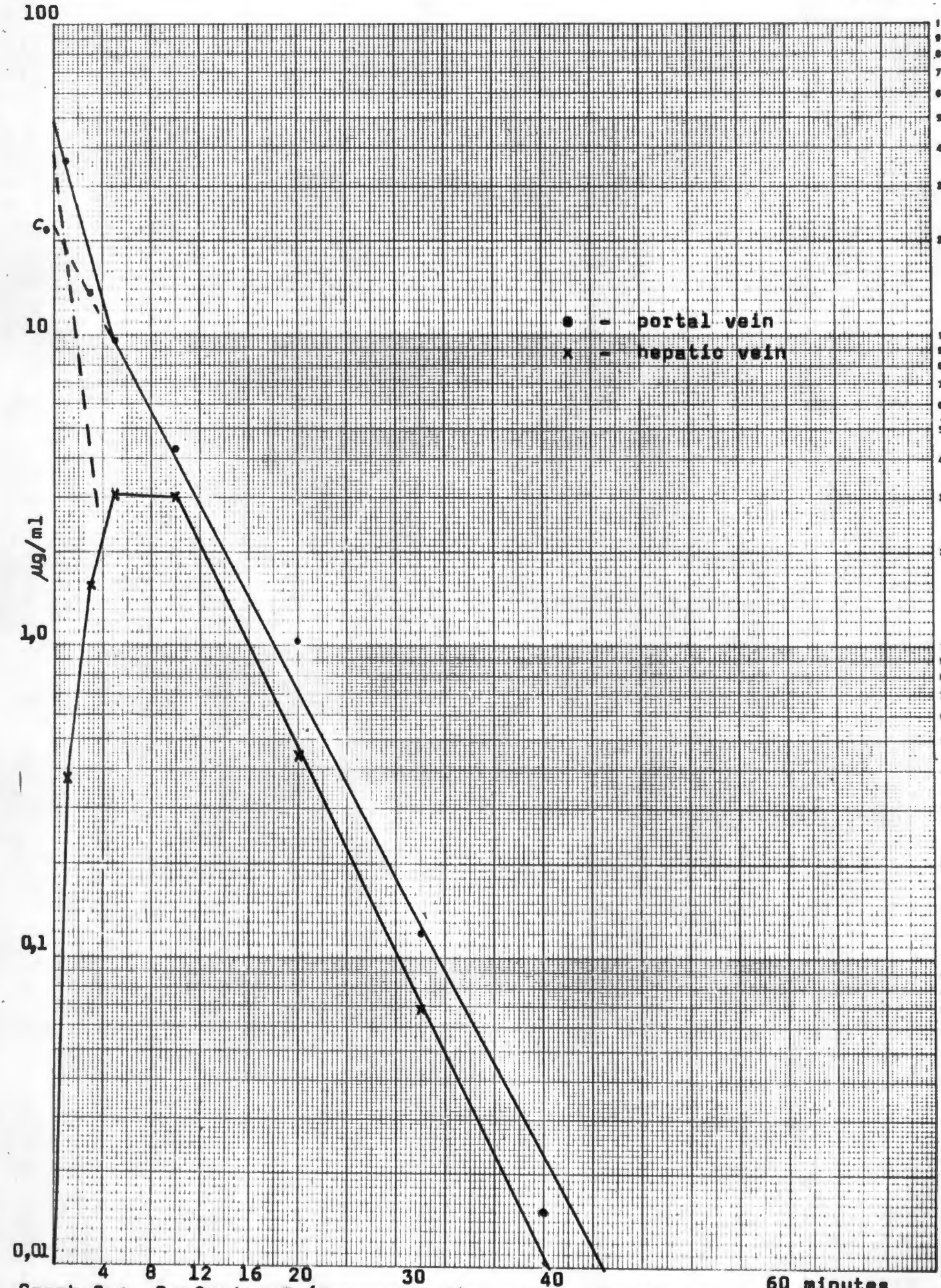
Graph 7a: Perfusion E (Propranolol). Semi-logarithmic plot of concentration versus time. Flow rate: 46 ml/min or 4,01 ml/g/min.



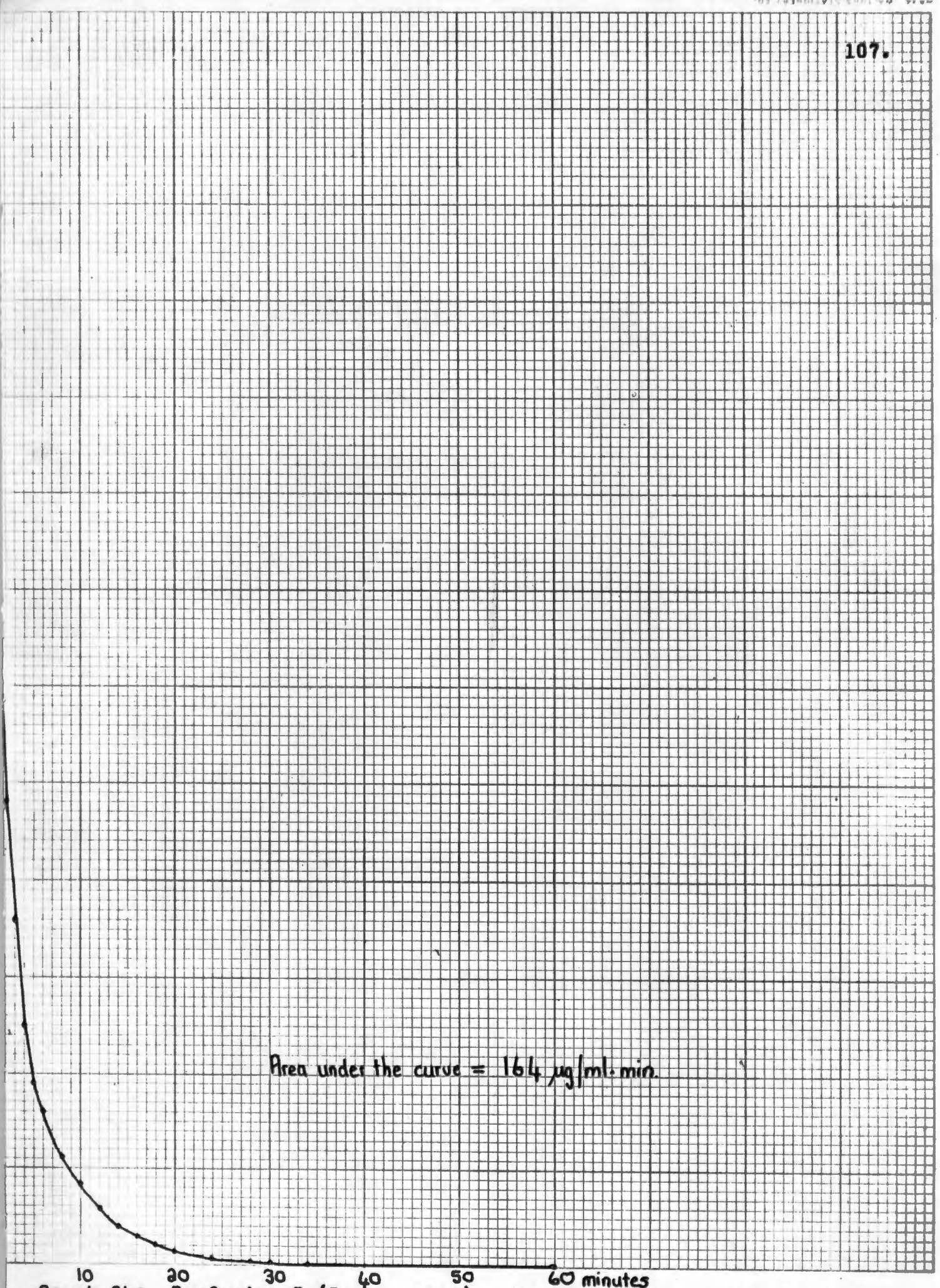
Graph 7b: Perfusion E (Propranolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 7a.



Graph 7c: Perfusion E (Propranolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 7a.

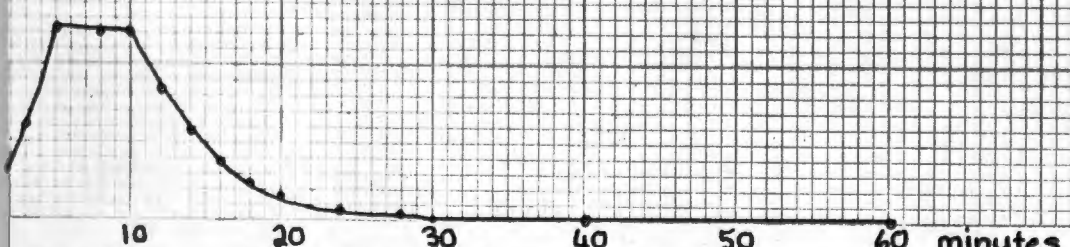


Graph 8a: Perfusion F (Propranolol). Semi-logarithmic plot of concentration versus time. Flow rate: 47 ml/min or 3,70 ml/g/min.

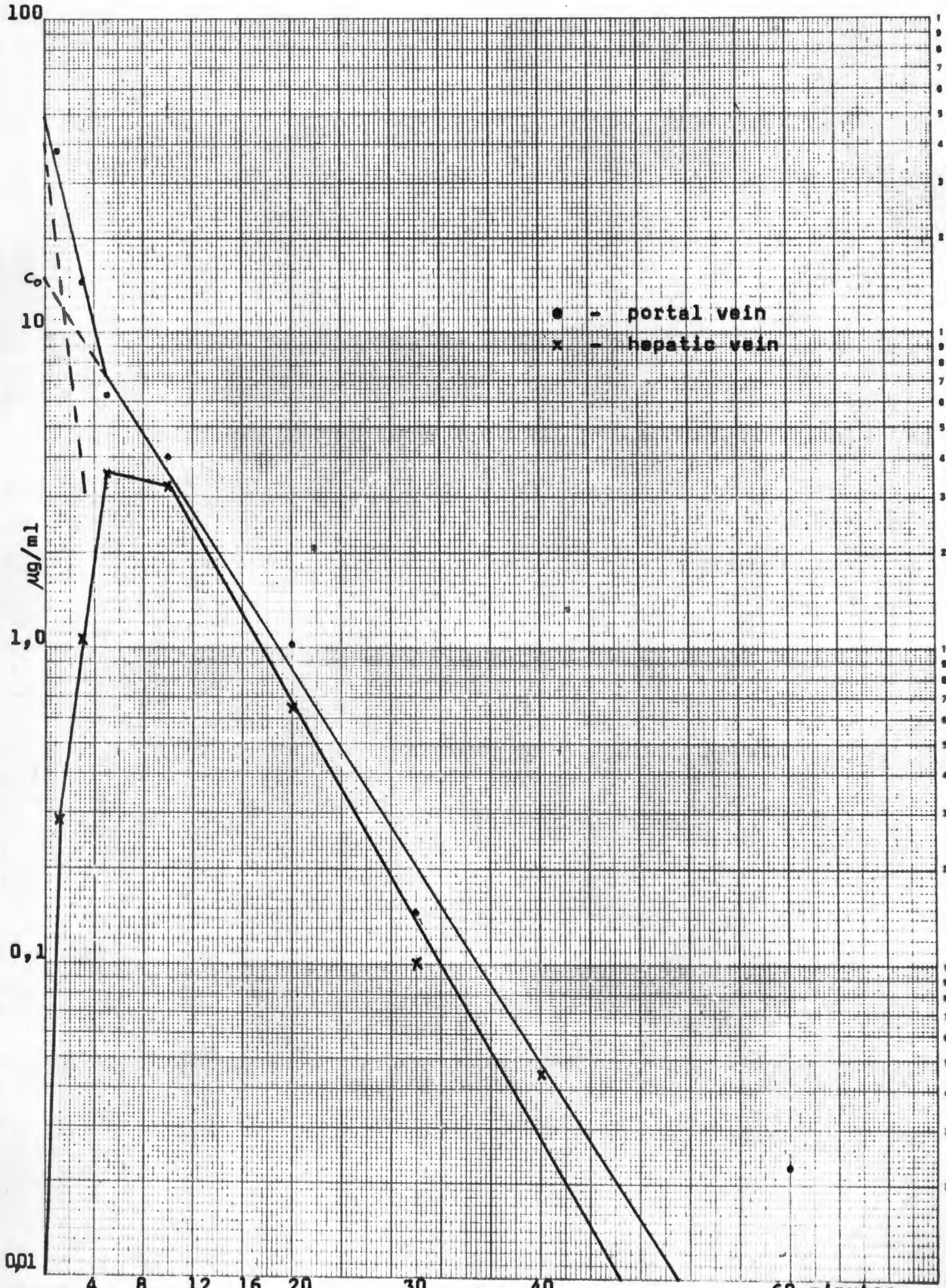


Graph 8b: Perfusion F (Propranolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 8a.

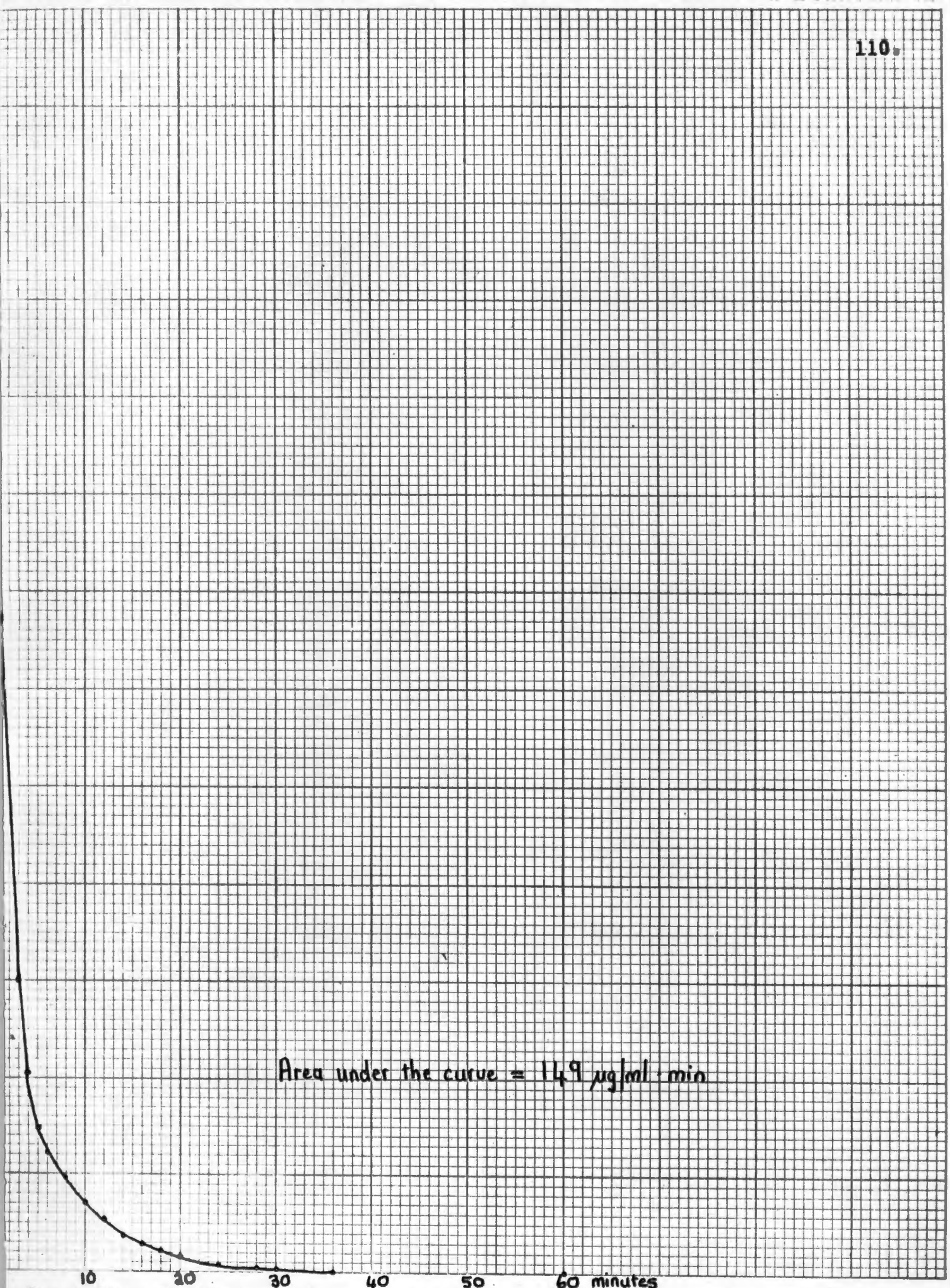
Area under the curve = 37 µg/ml·min.



Graph 8c: Perfusion F (Propranolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 8a.

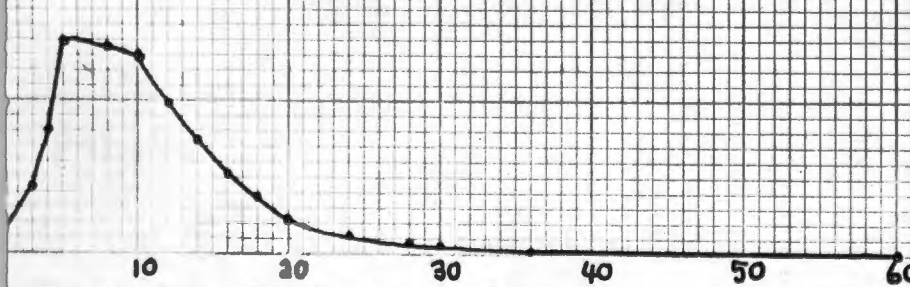


Graph 9a: Perfusion G (Propranolol). Semi-logarithmic plot of concentration versus time. Flow rate: 60 ml/min or 5,05 ml/g/min.

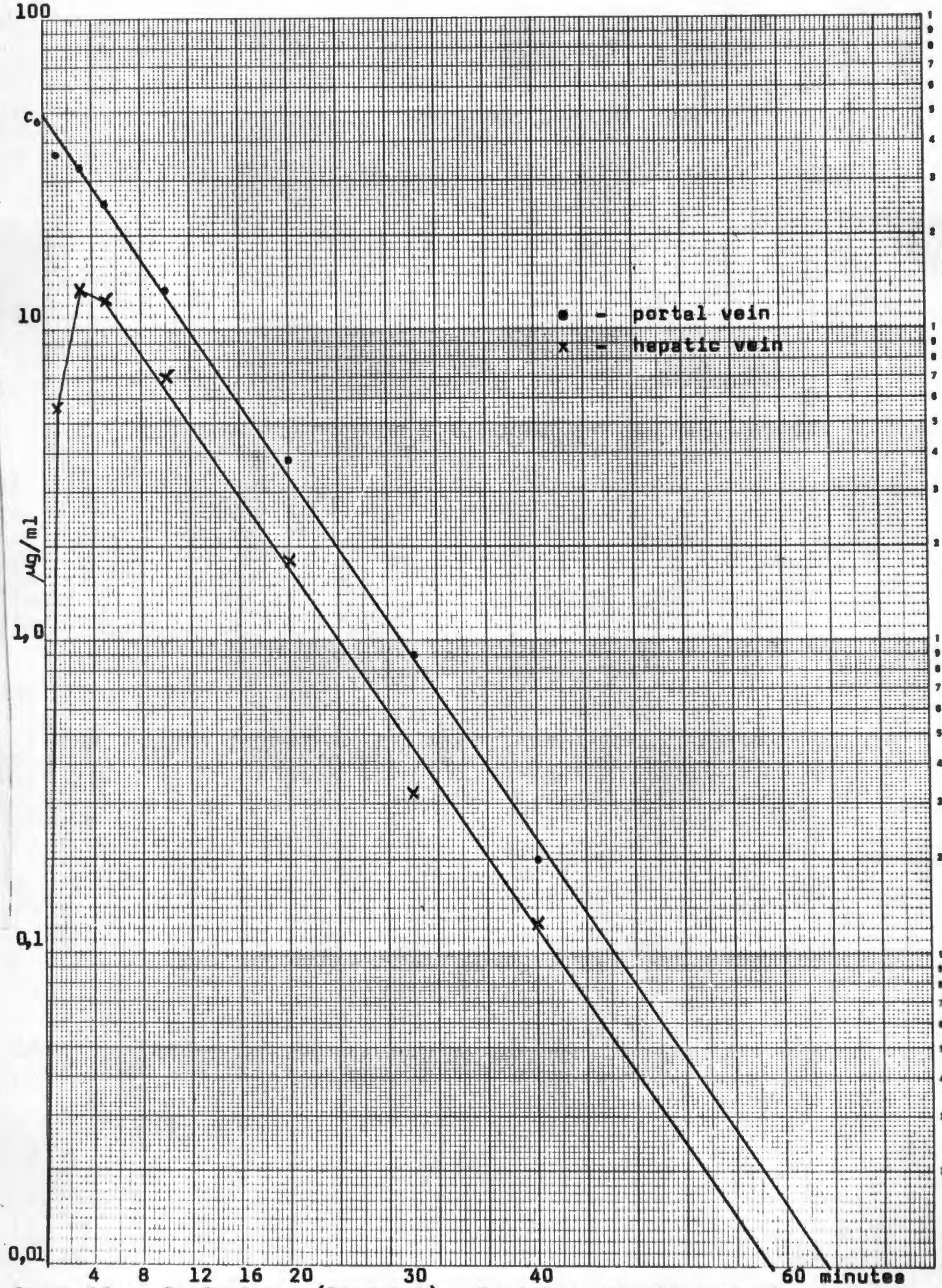


Graph 9b: Perfusion G (Propranolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 9a.

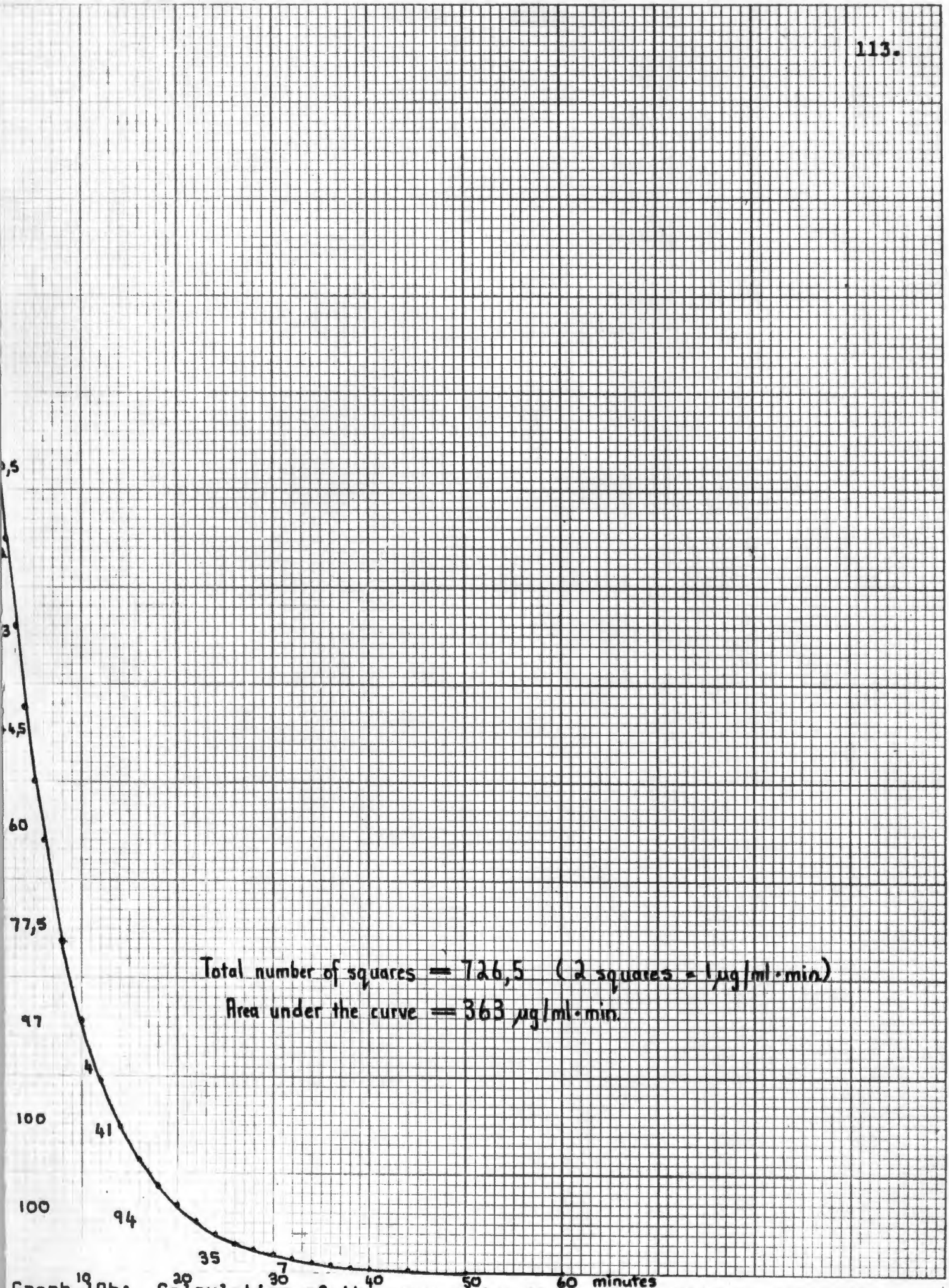
Area under the curve = 42 µg/ml·min.



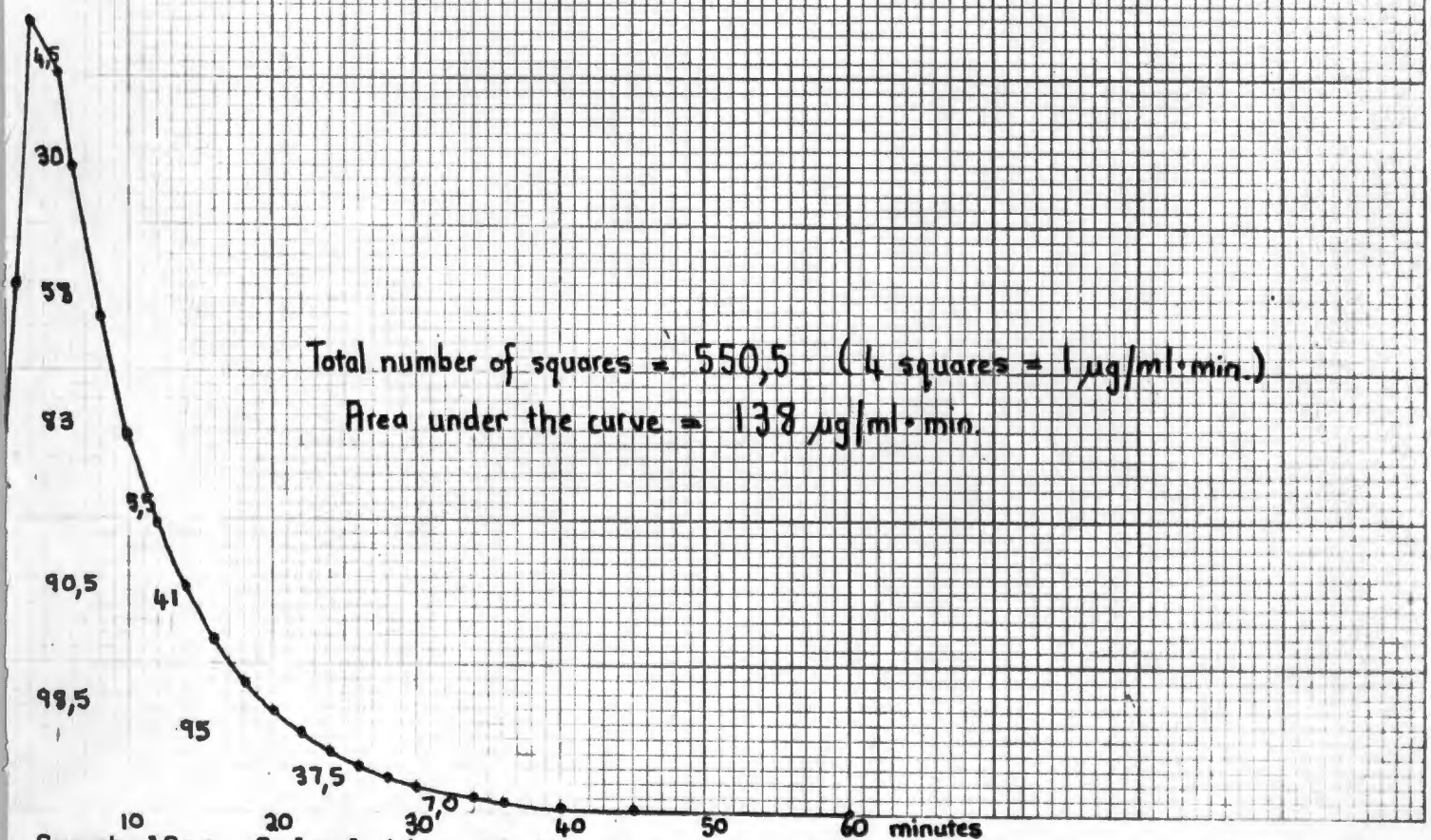
Graph 9c: Perfusion G (Propranolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 9a.



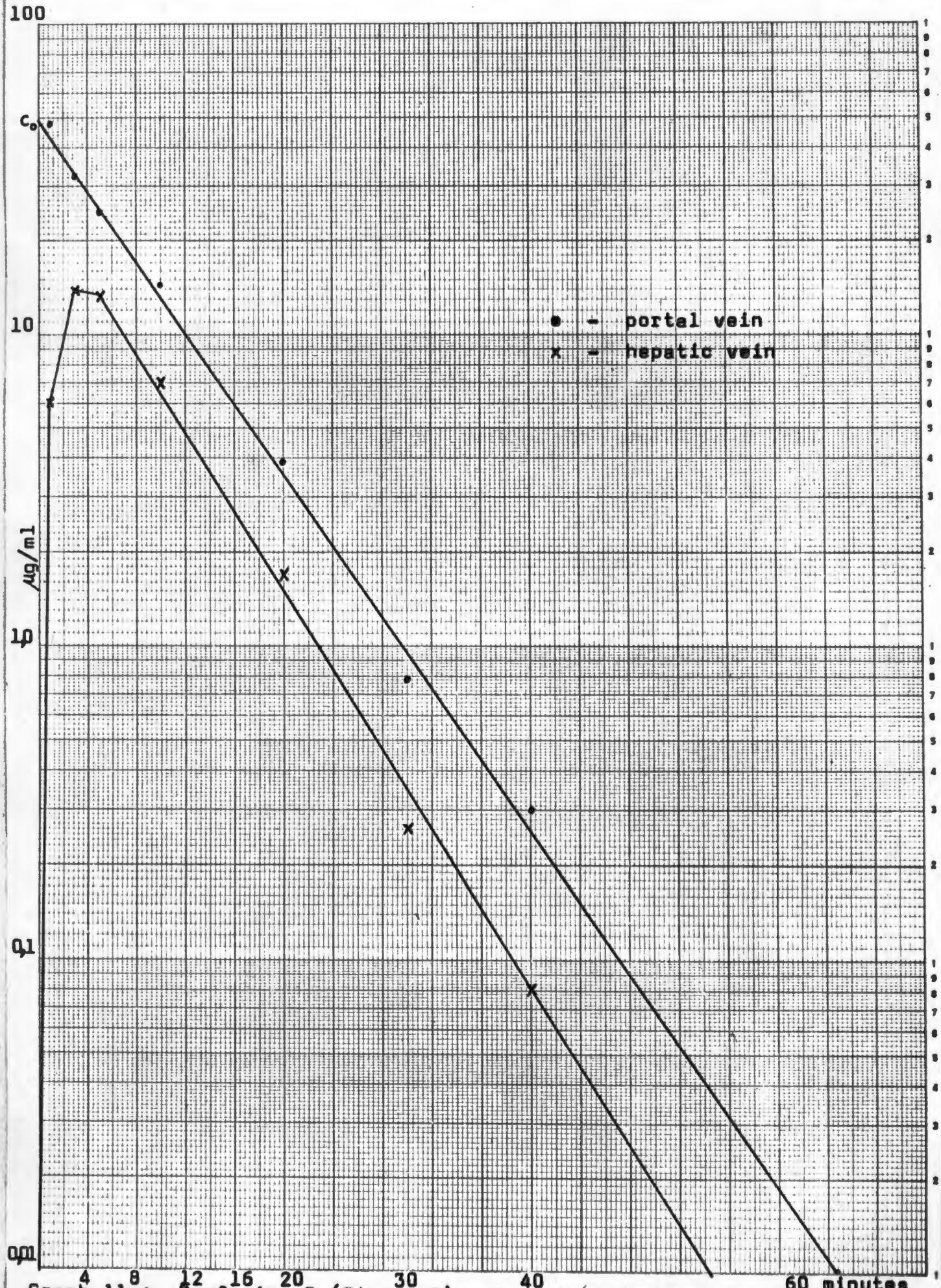
Graph 10a: Perfusion A (Pindolol). Semi-logarithmic plot of concentration (g/ml) versus time (minutes). Flow rate: 21 ml/min or 1,83 ml/g/min.



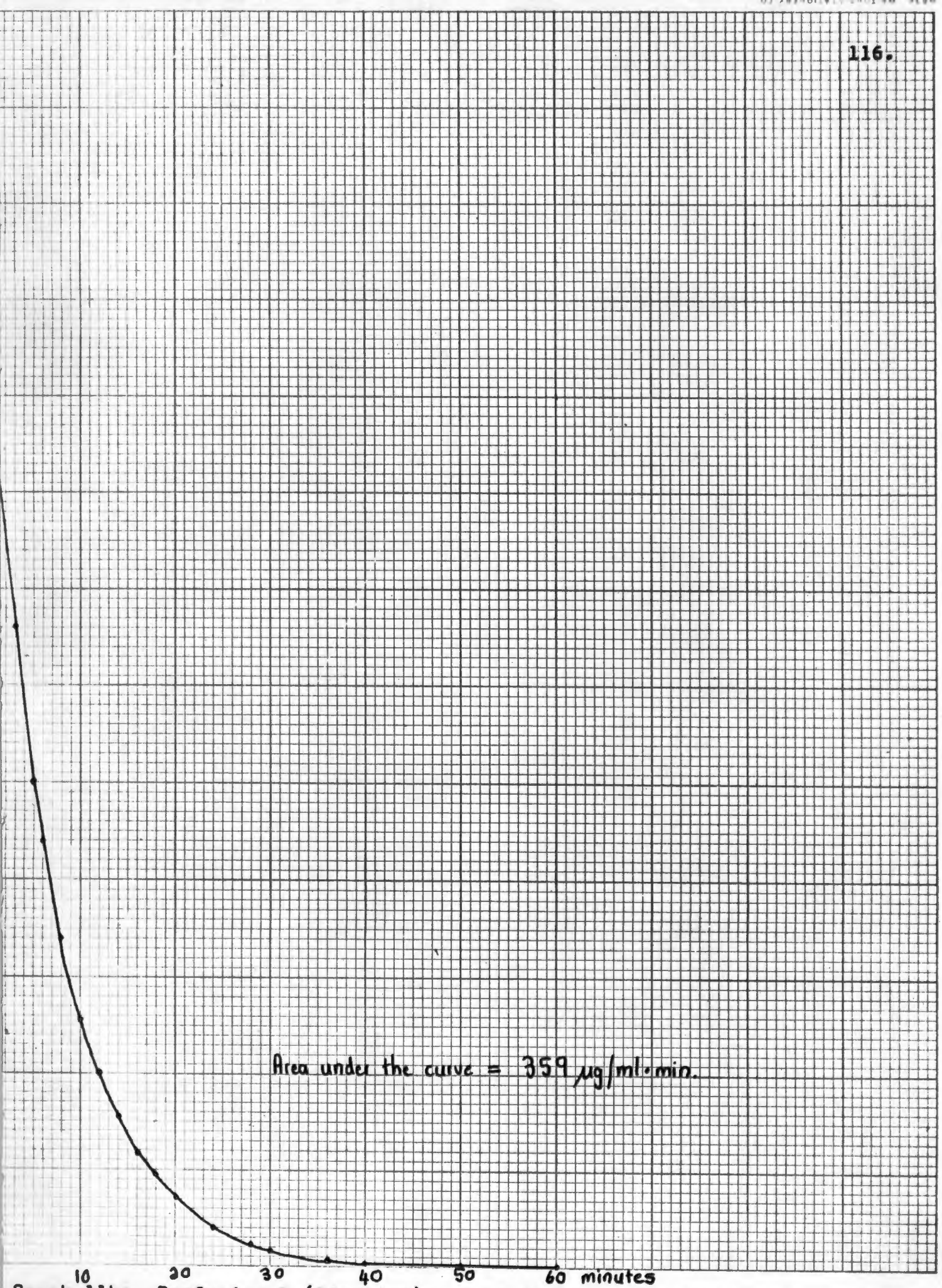
Graph 10b: Calculation of the area under the hepatic vein concentration versus time curve for perfusion A (Pindolol). Points were obtained from the line fitted in Graph 10a.



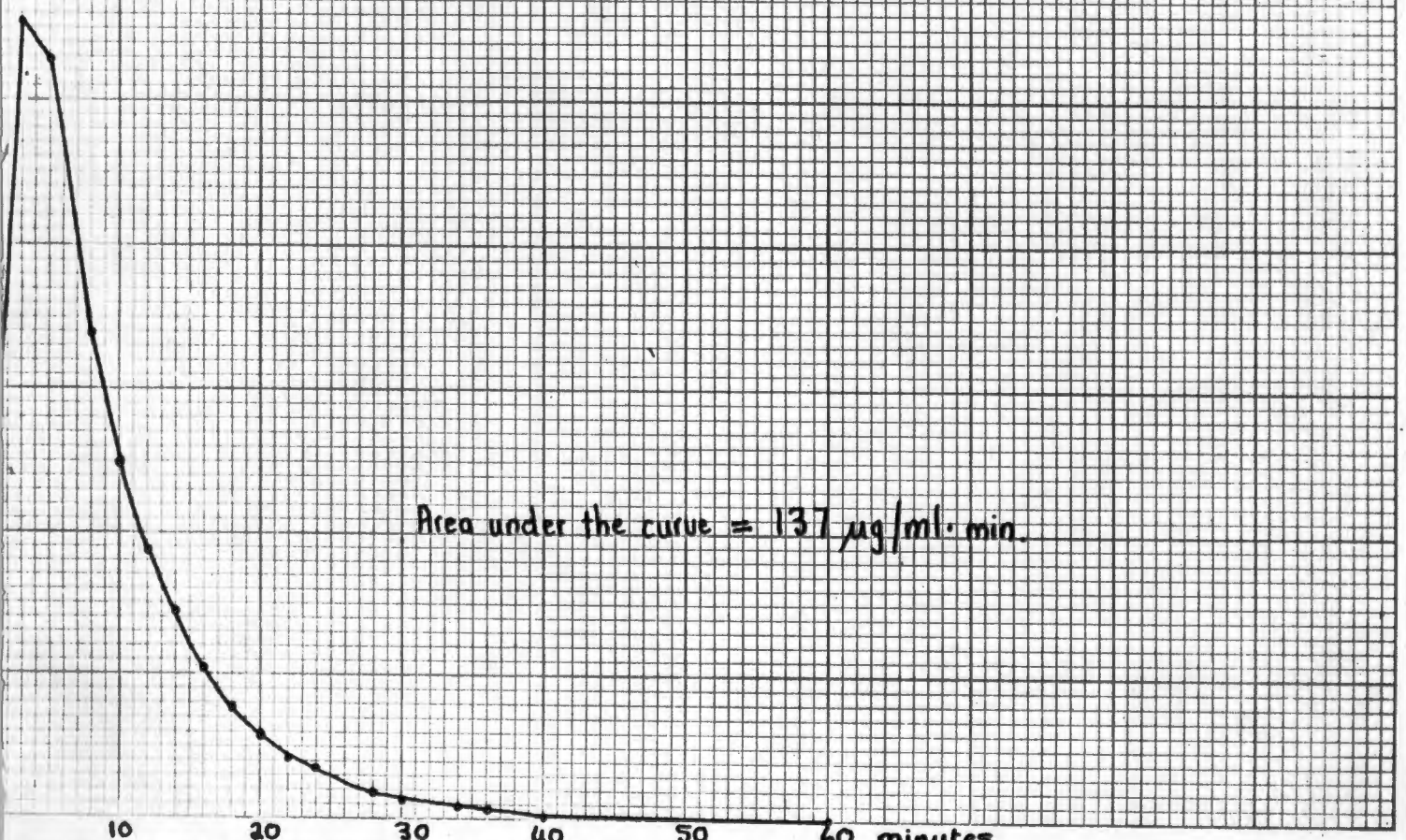
Graph 10c: Calculation of the area under the hepatic vein concentration versus time curve for perfusion A (Pindolol). Points were obtained from the line fitted in Graph 10a.



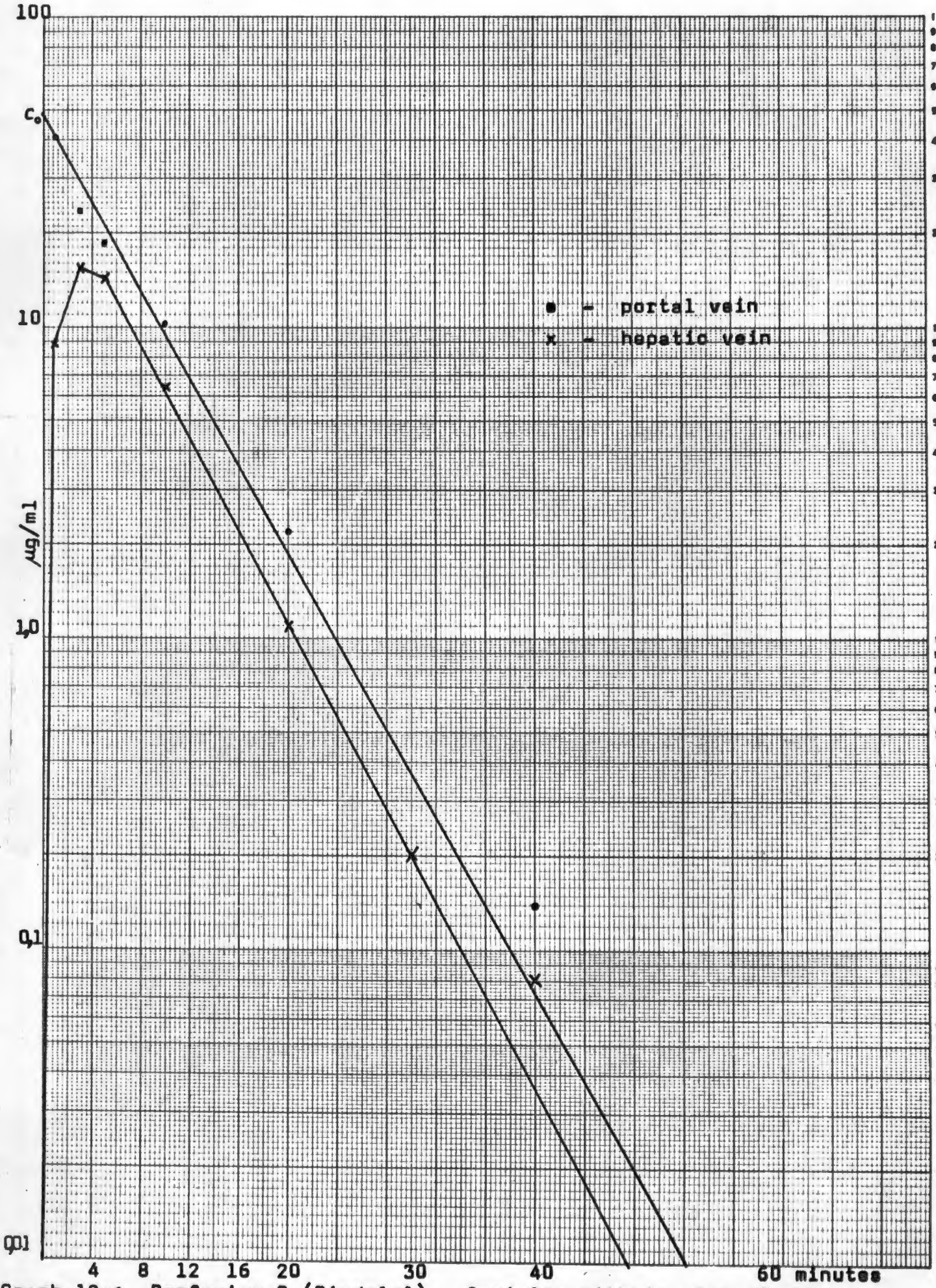
Graph 11a: Perfusion B (Pindolol). Semi-logarithmic plot of concentration versus time. Flow rate: 26 ml/min or 2,17 ml/g/min.



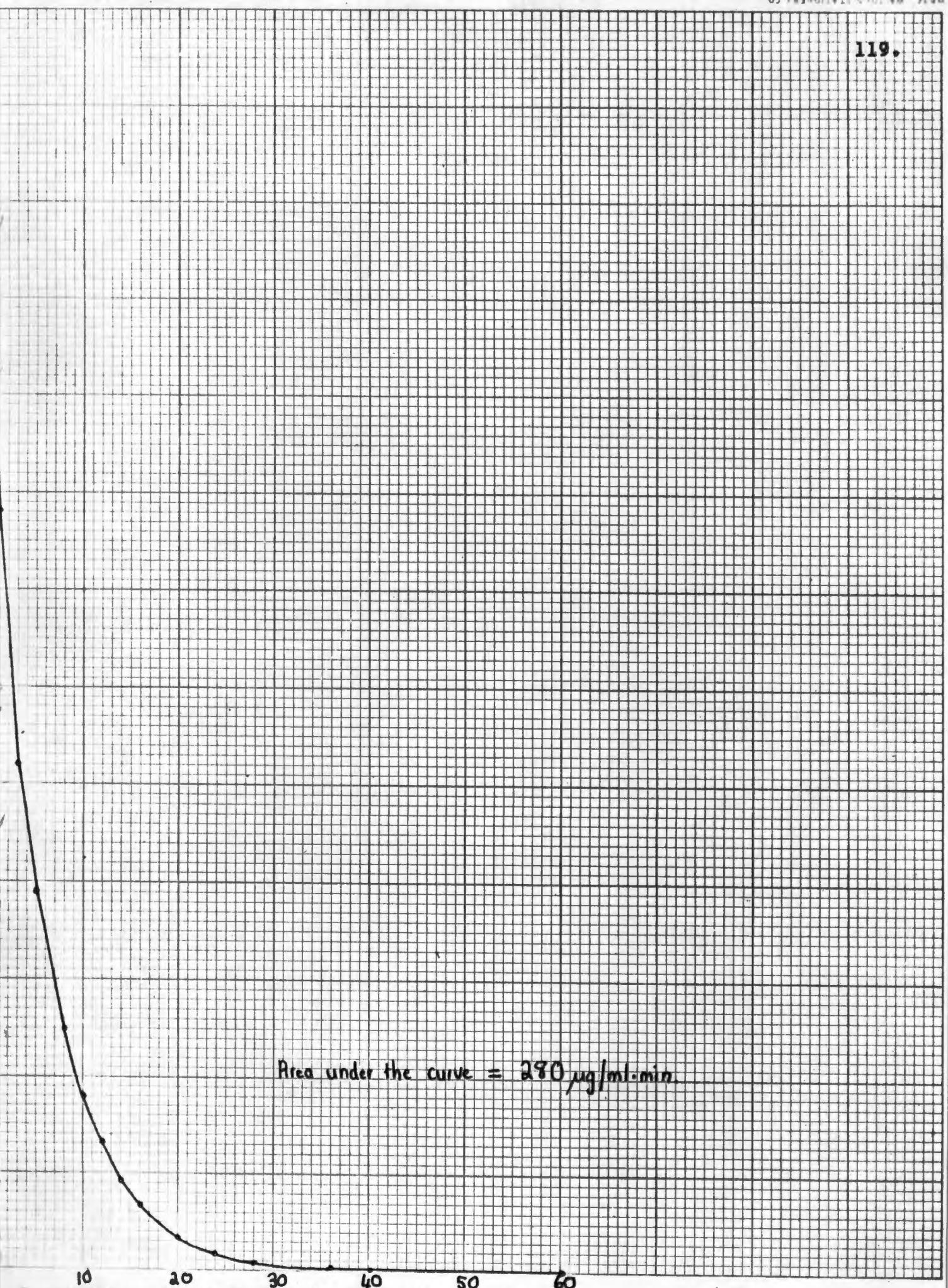
Graph 11b: Perfusion B (Pindolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 11a.



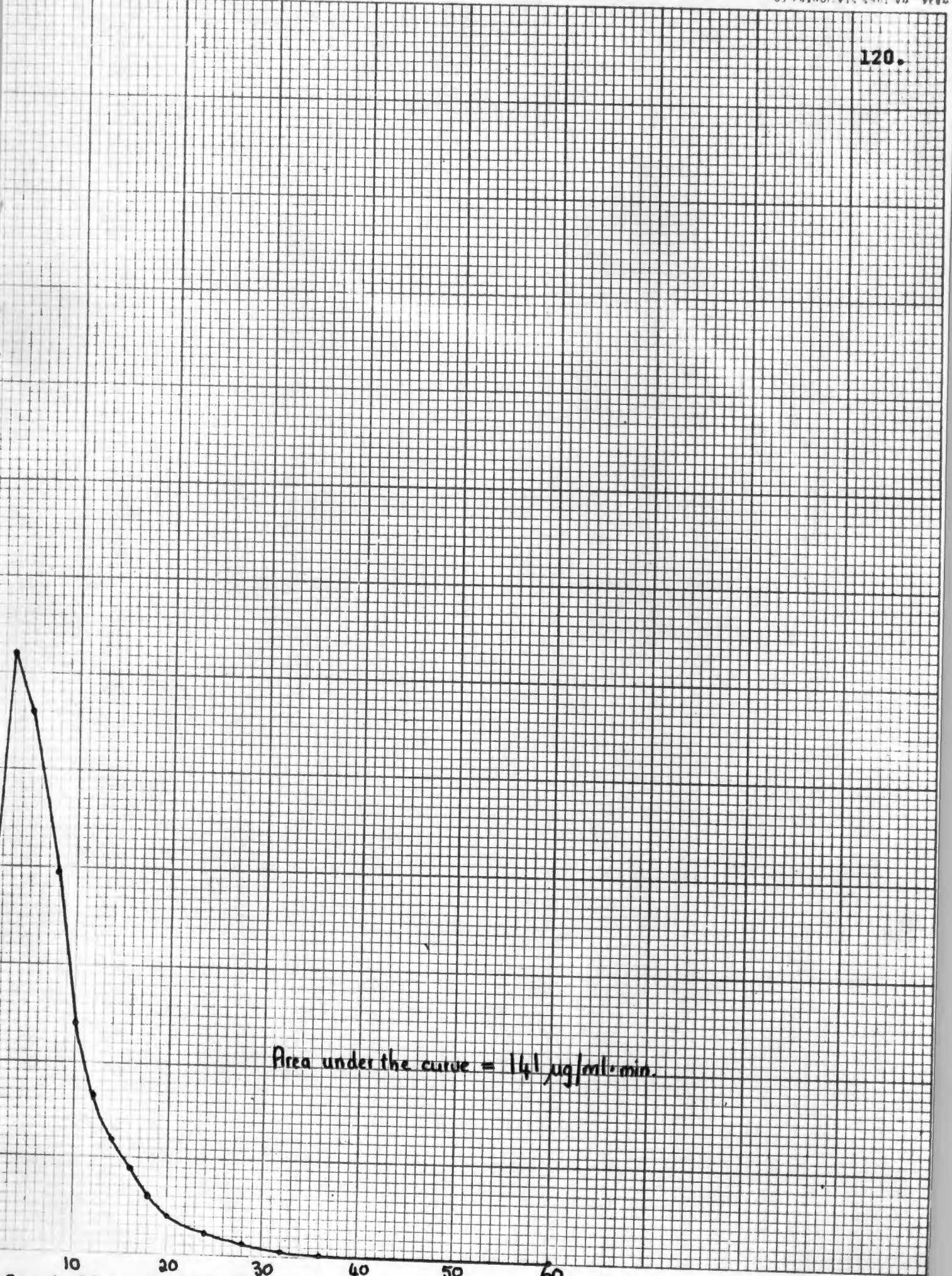
Graph 11c: Perfusion B (Pindolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 11a.



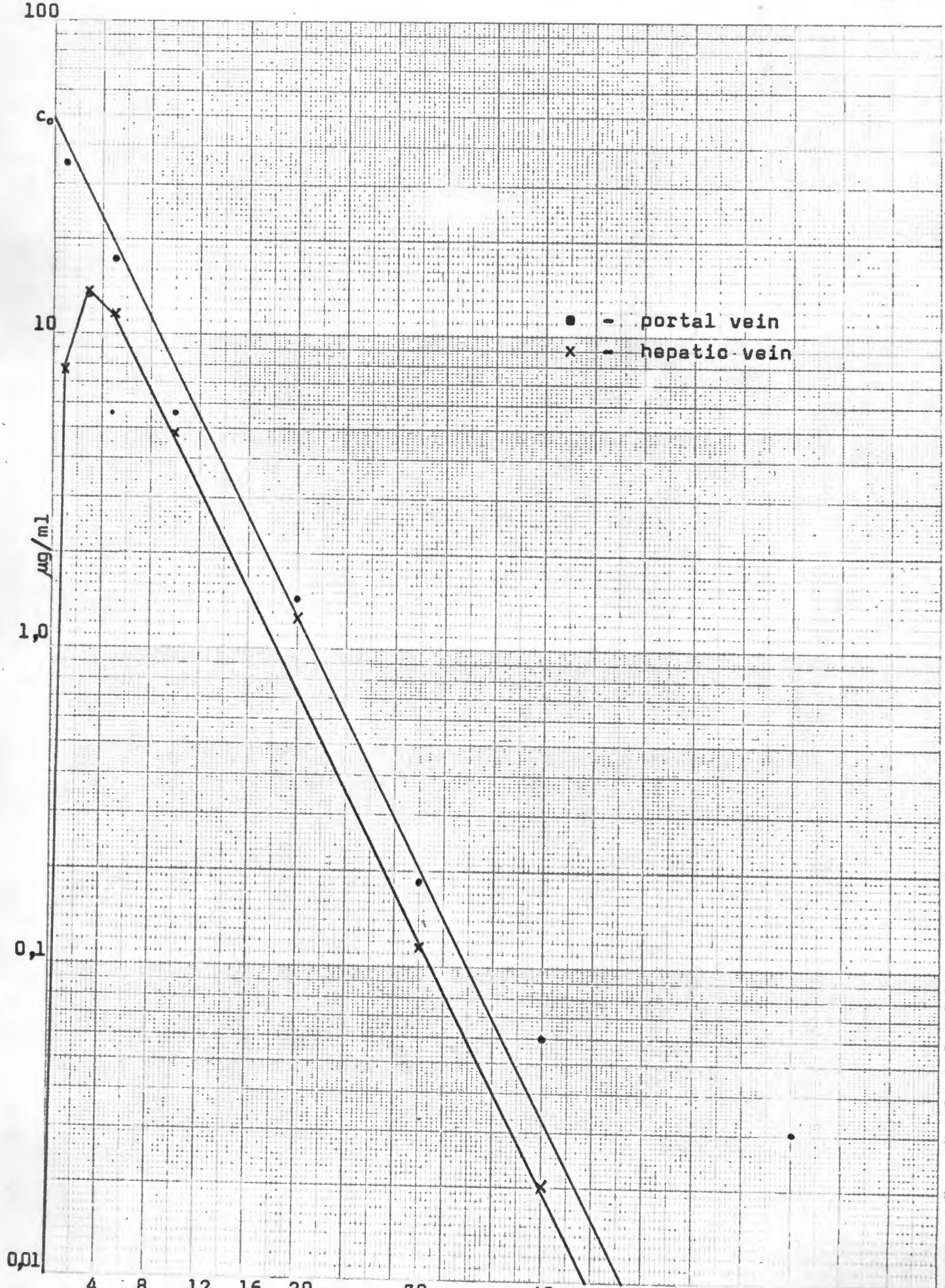
Graph 12a: Perfusion C (Pindolol). Semi-logarithmic plot of concentration versus time. Flow rate: 36 ml/min or 2,85 ml/g/min.



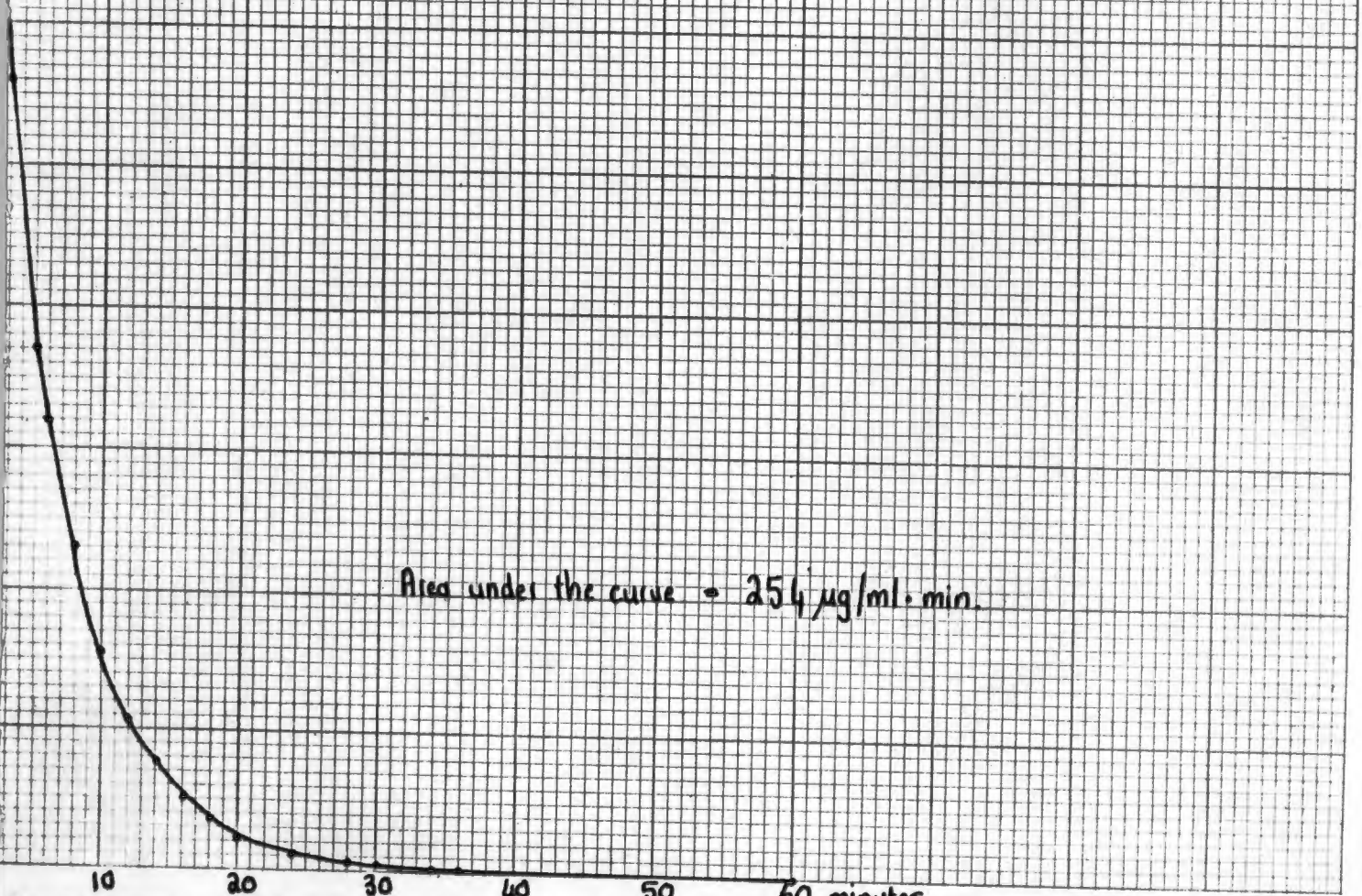
Graph 12b: Perfusion C (Pindolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 12a.



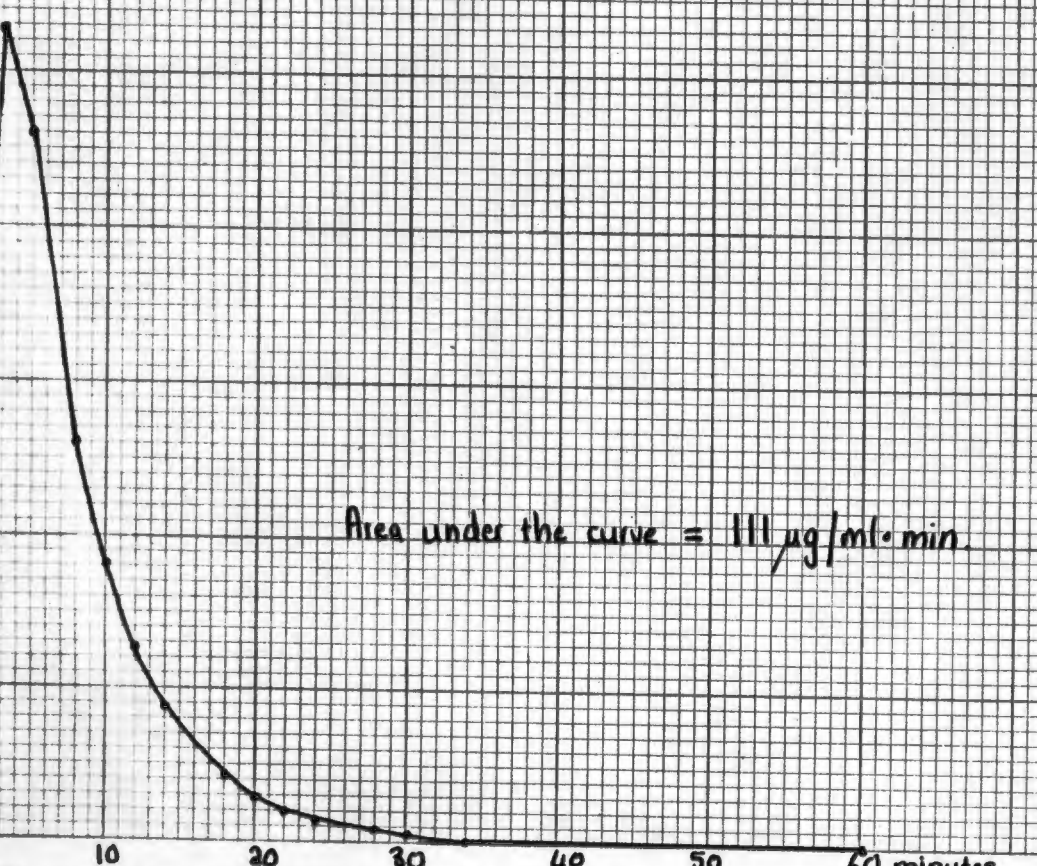
Graph 12c: Perfusion C (Pindolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 12a.



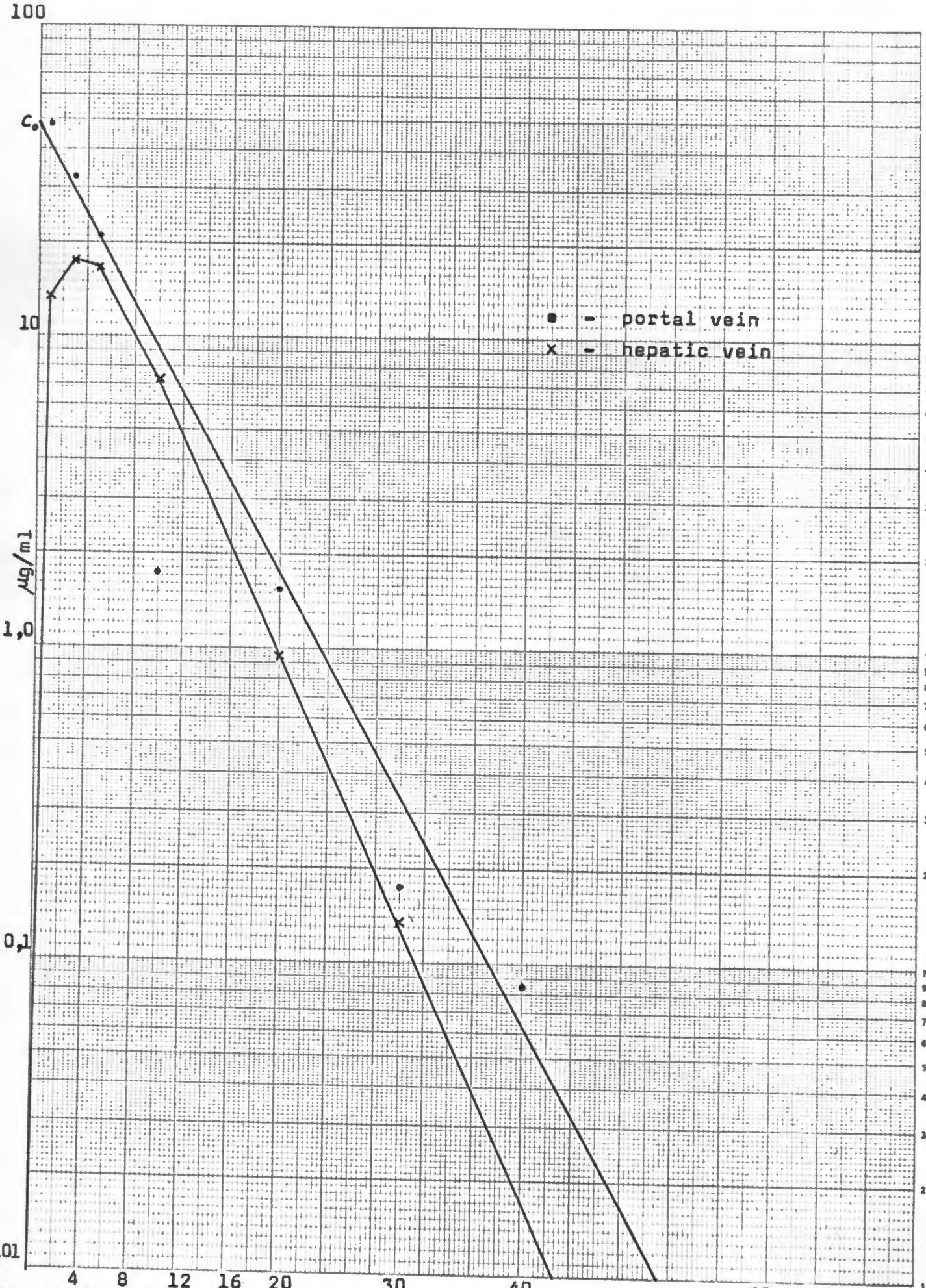
Graph 13a: Perfusion D (Pindolol). Semi-logarithmic plot of concentration versus time. Flow rate: 43 ml/min or 3,85 ml/g/min.



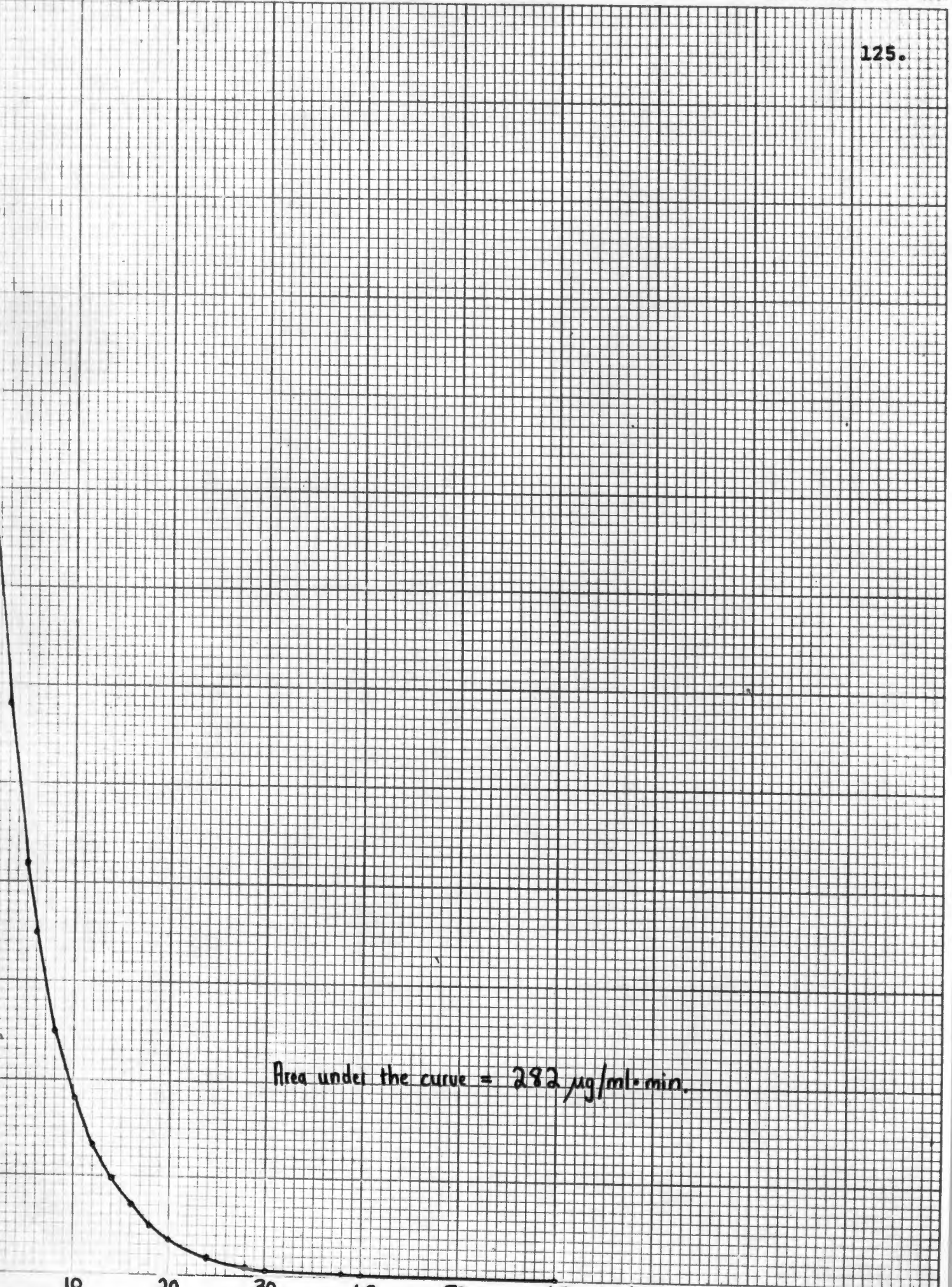
Graph 13b: Perfusion D (Pindolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 13a.



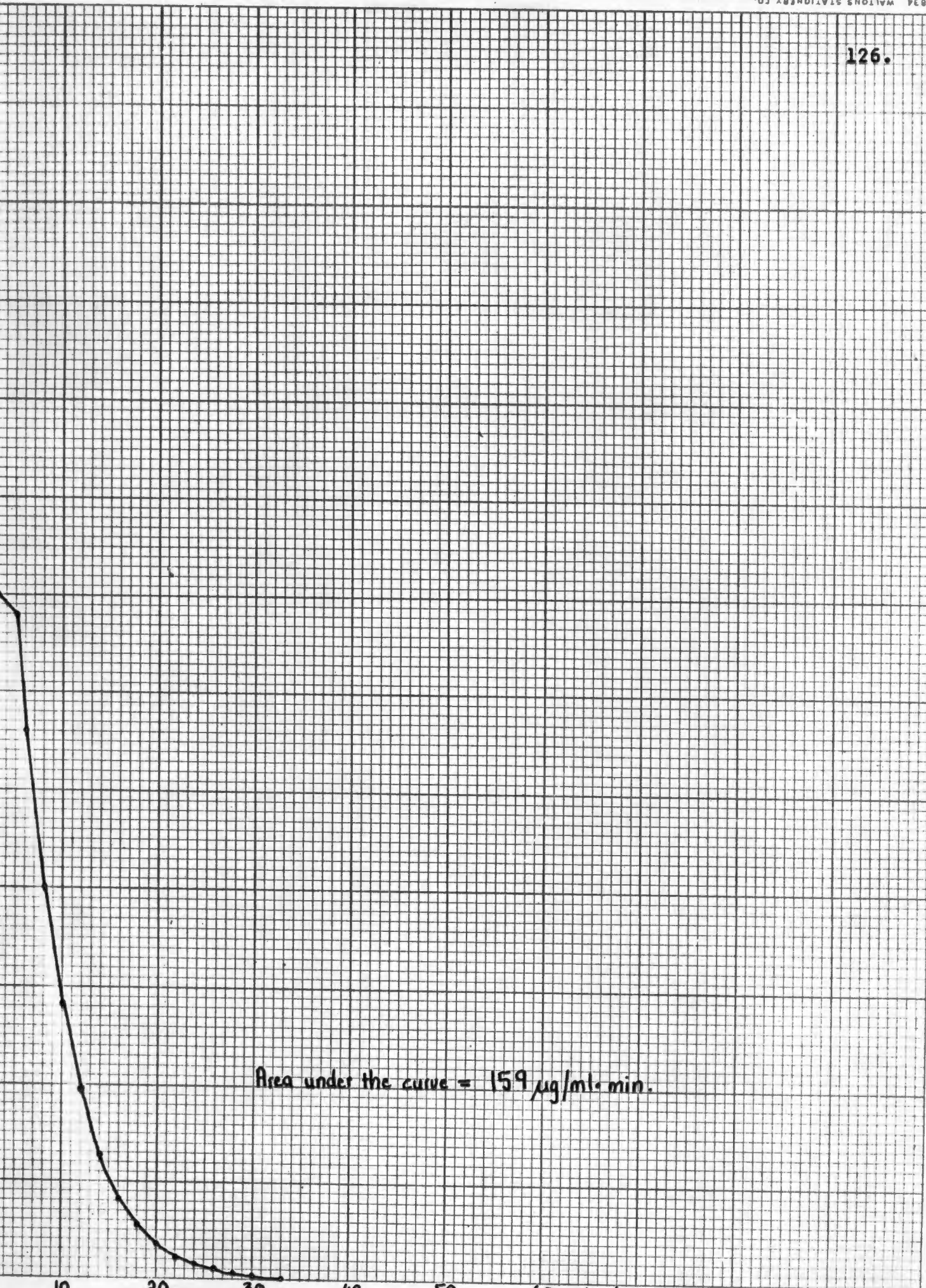
Graph 13c: Perfusion D (Pindolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 13a.



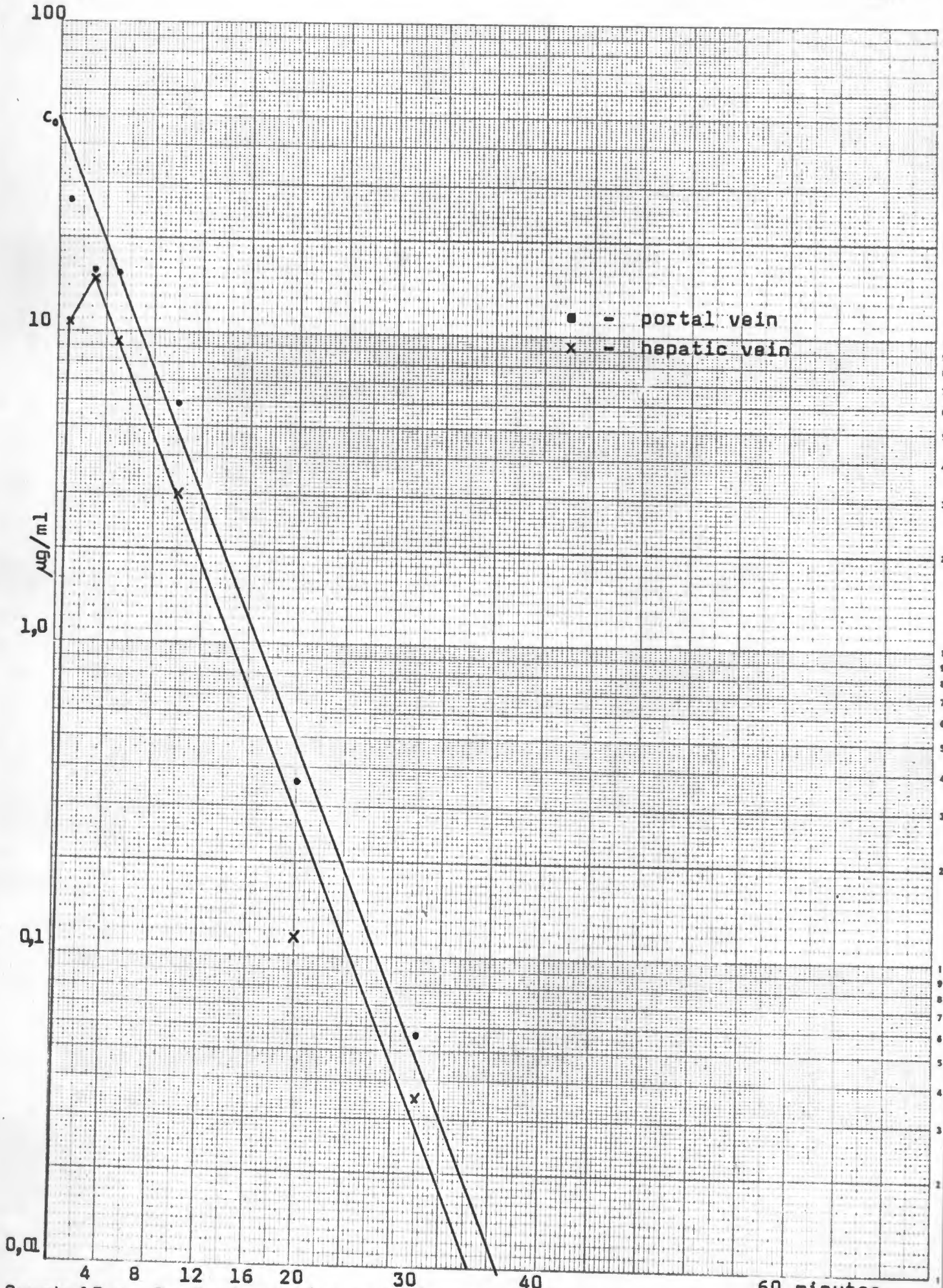
Graph 14a: Perfusion E (Pindolol). Semi-logarithmic plot of concentration versus time. Flow rate: 46 ml/min or 3,28 ml/g/min.



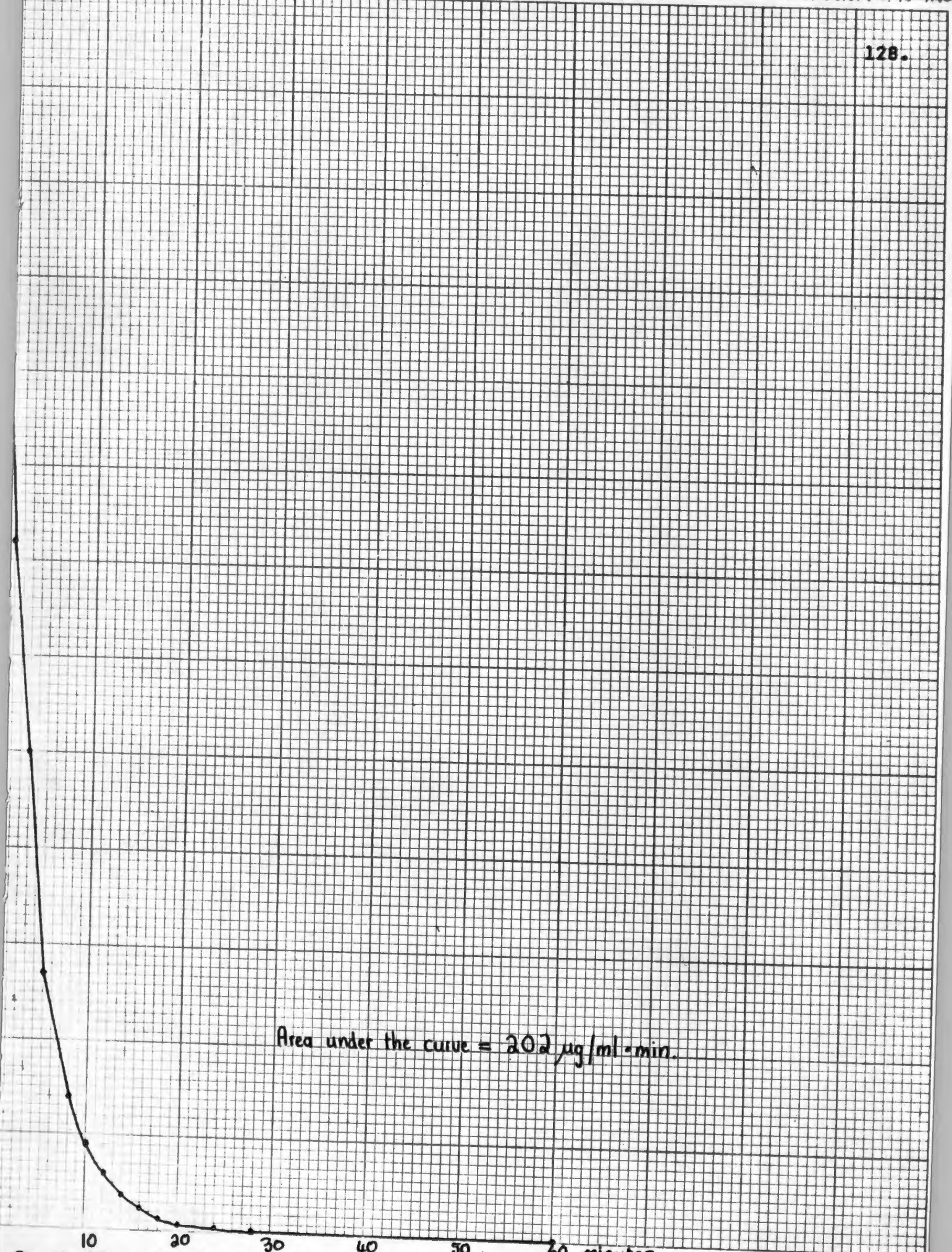
Graph 14b: Perfusion E (Pindolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 14a.



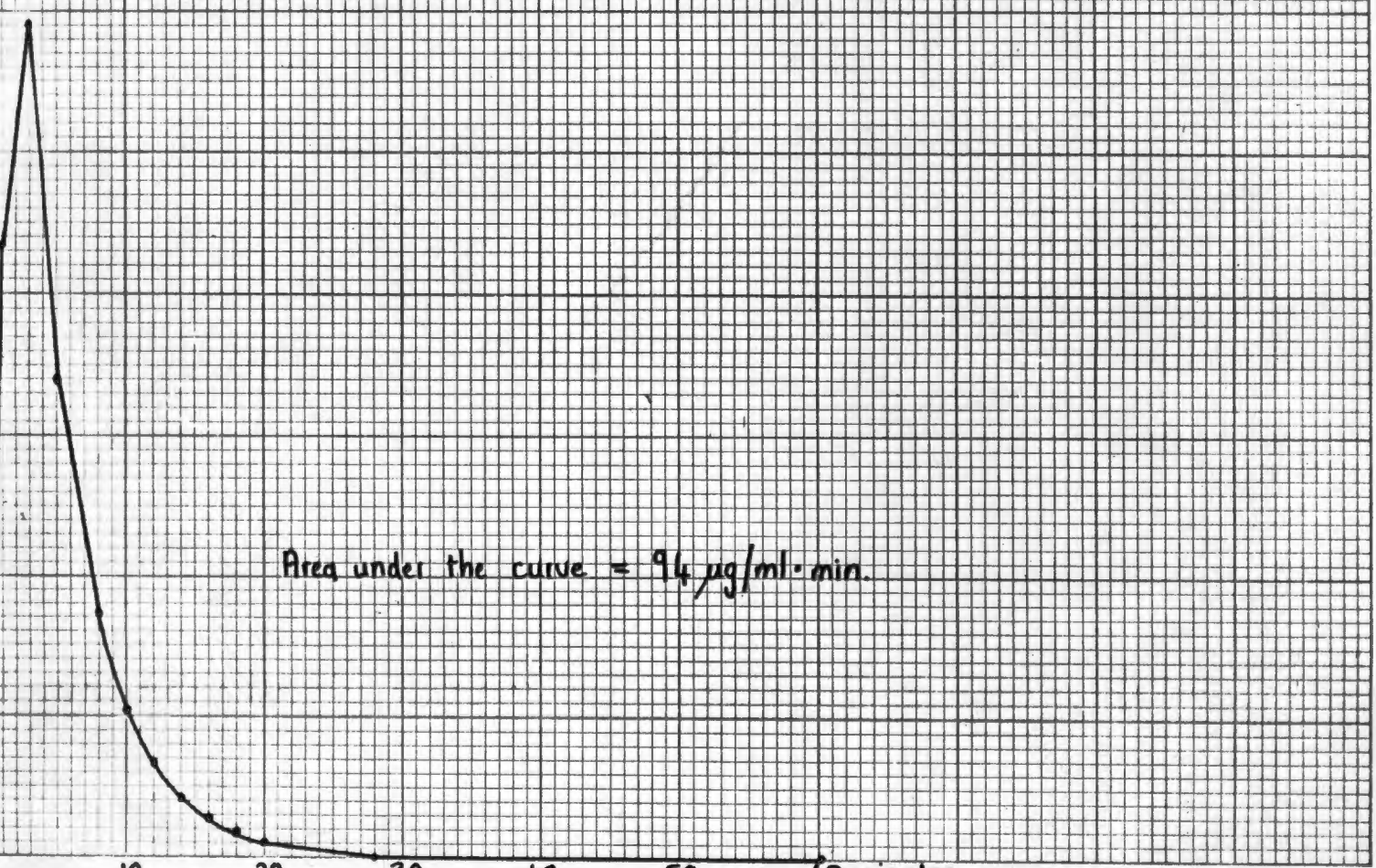
Graph 14c: Perfusion E (Pindolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 14a.



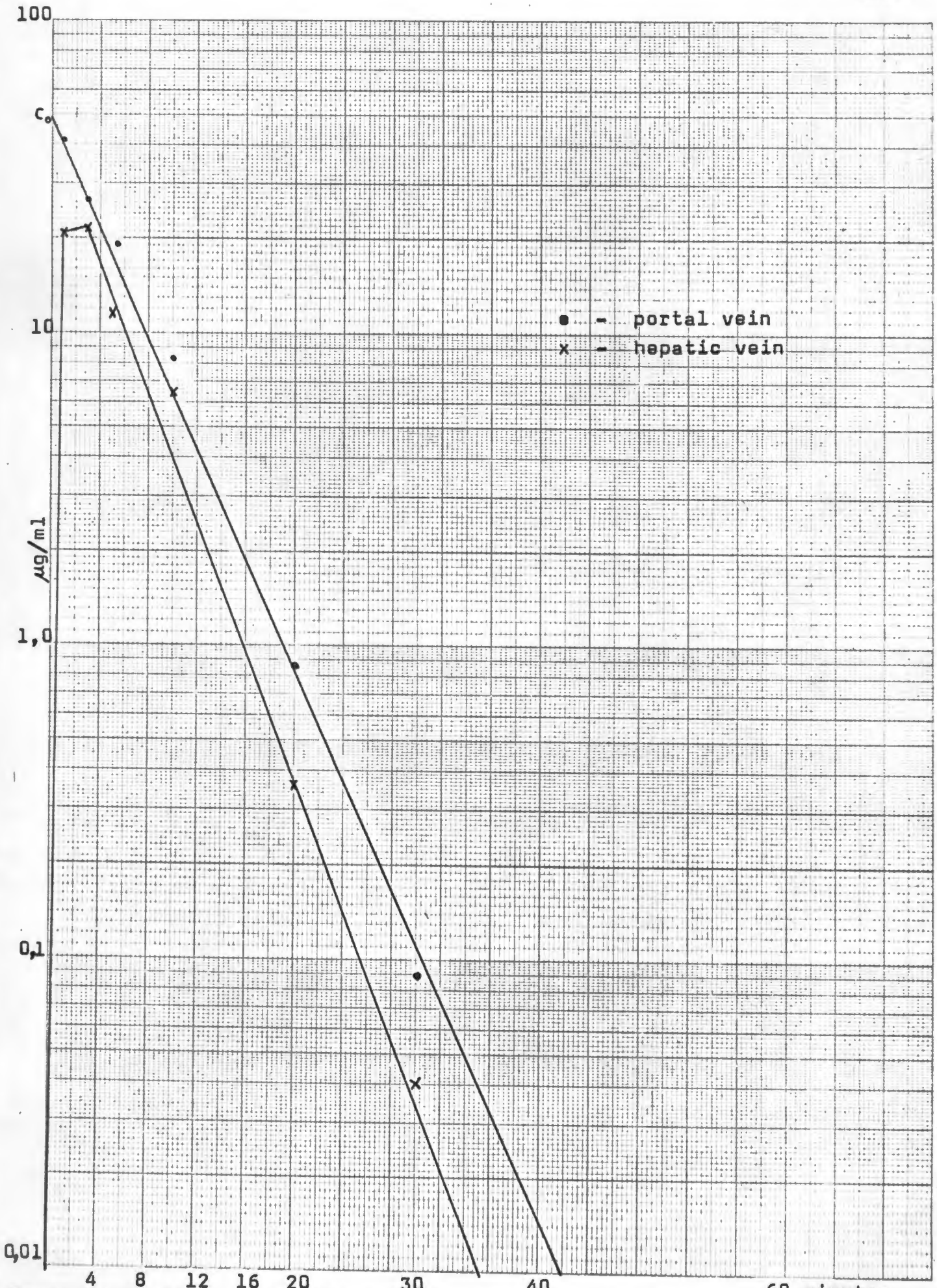
Graph 15a: Perfusion F (Pindolol). Semi-logarithmic plot of concentration versus time. Flow rate: 57 ml/min or 4,98 ml/g/min.



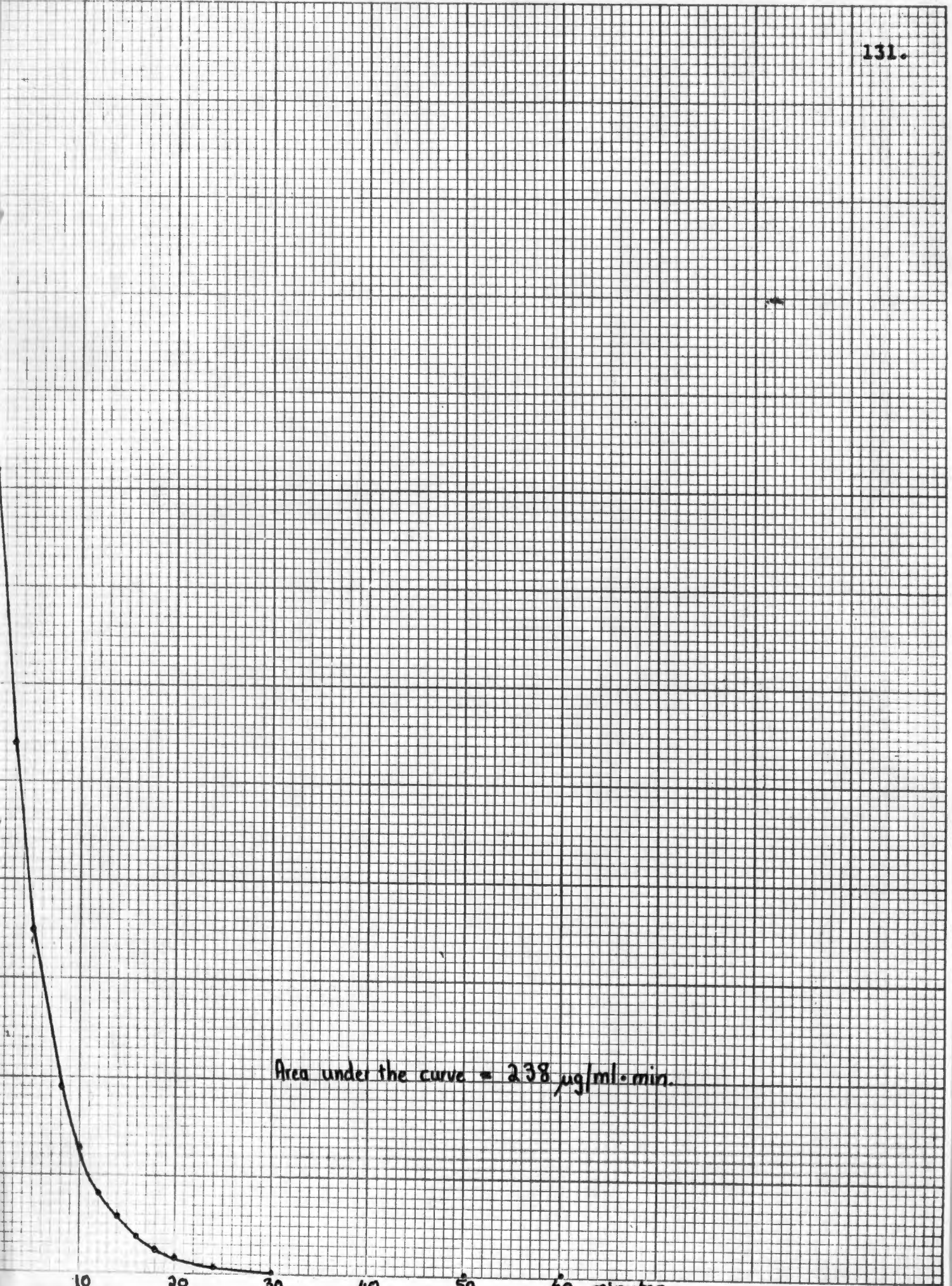
Graph 15b: Perfusion F (Pindolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 15a.



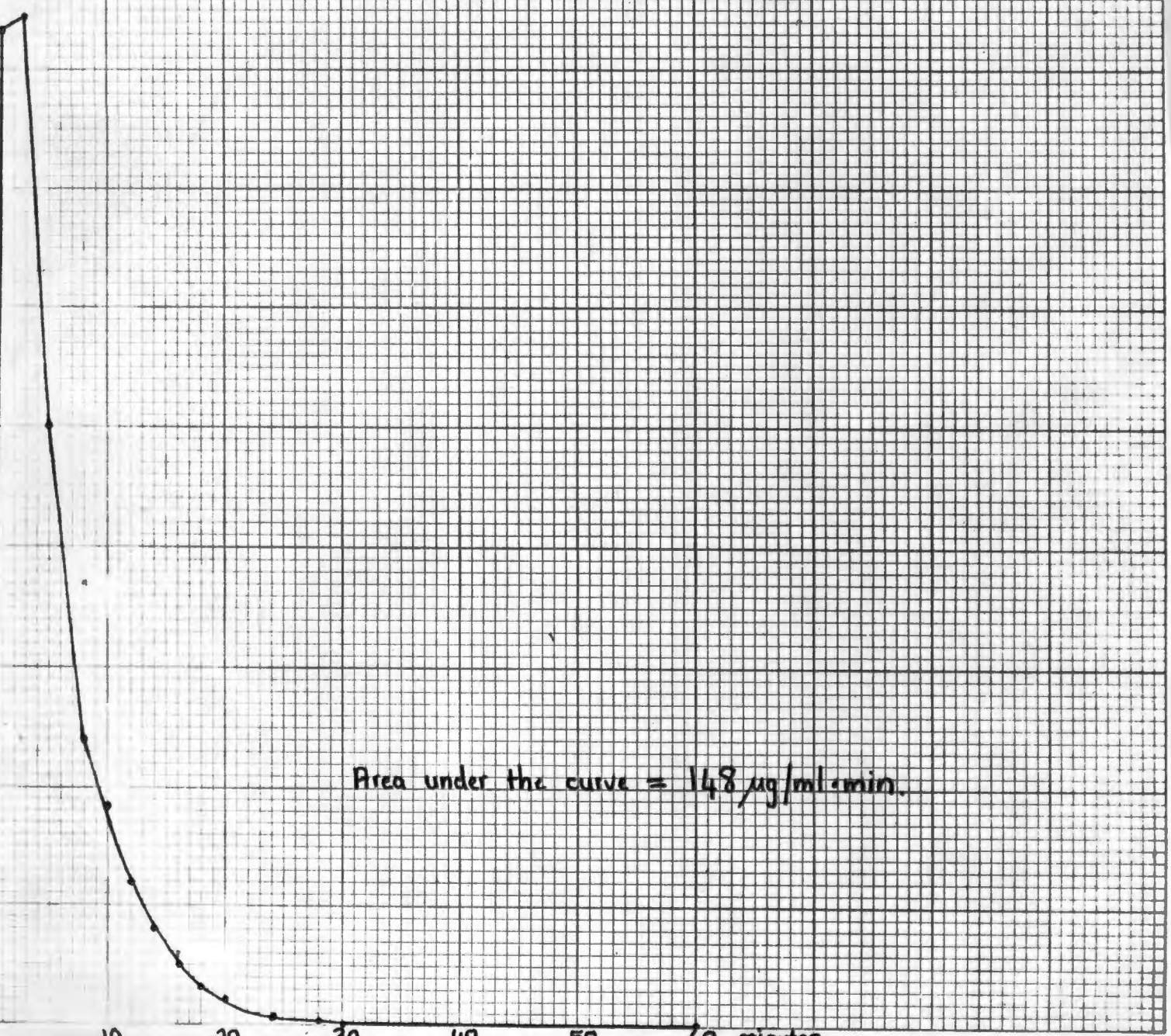
Graph 15c: Perfusion F (Pindolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 15a.



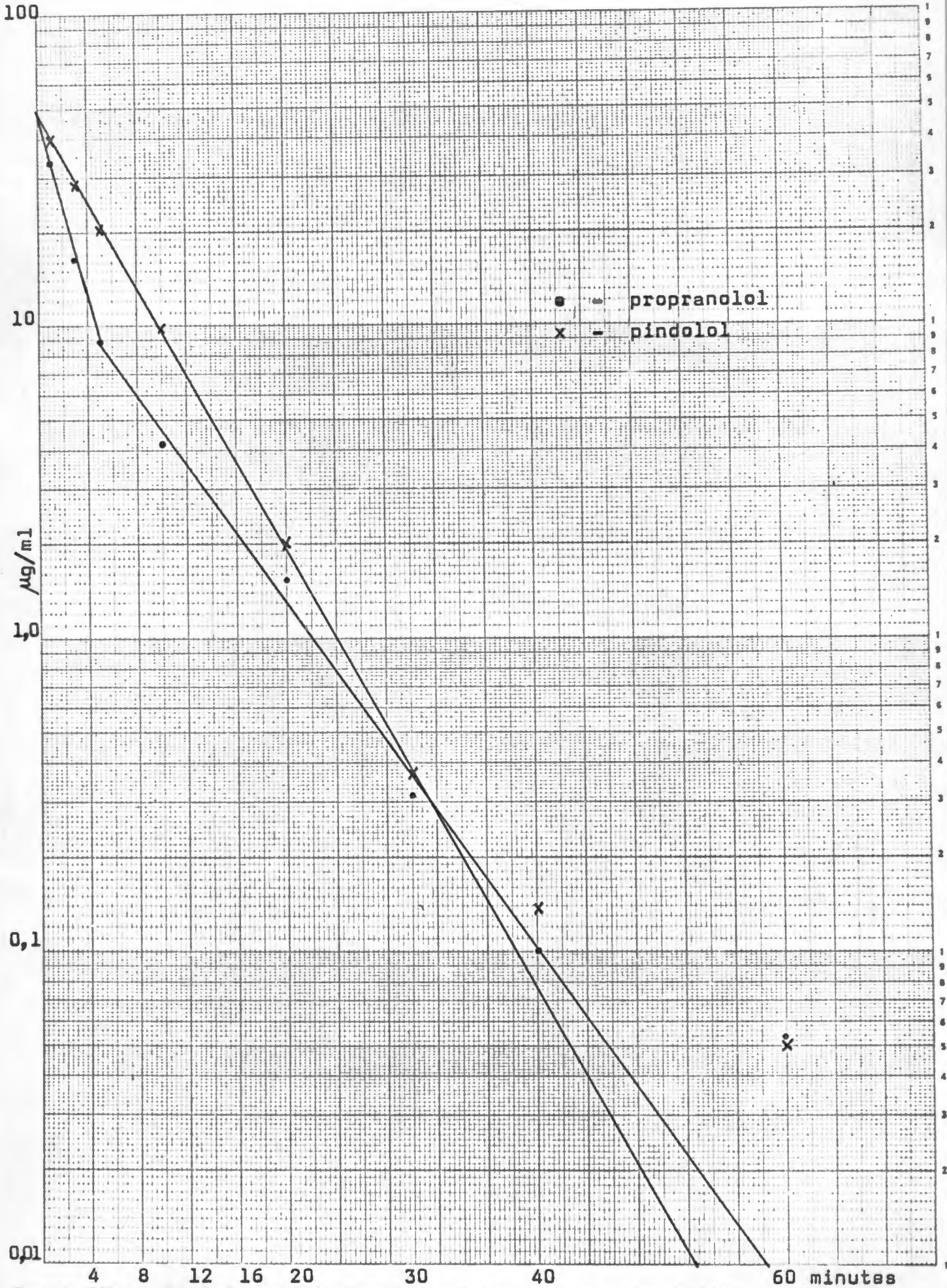
Graph 16a: Perfusion G (Pindolol). Semi-logarithmic plot of concentration versus time. Flow rate: 60 ml/min or 4,57 ml/g/min.



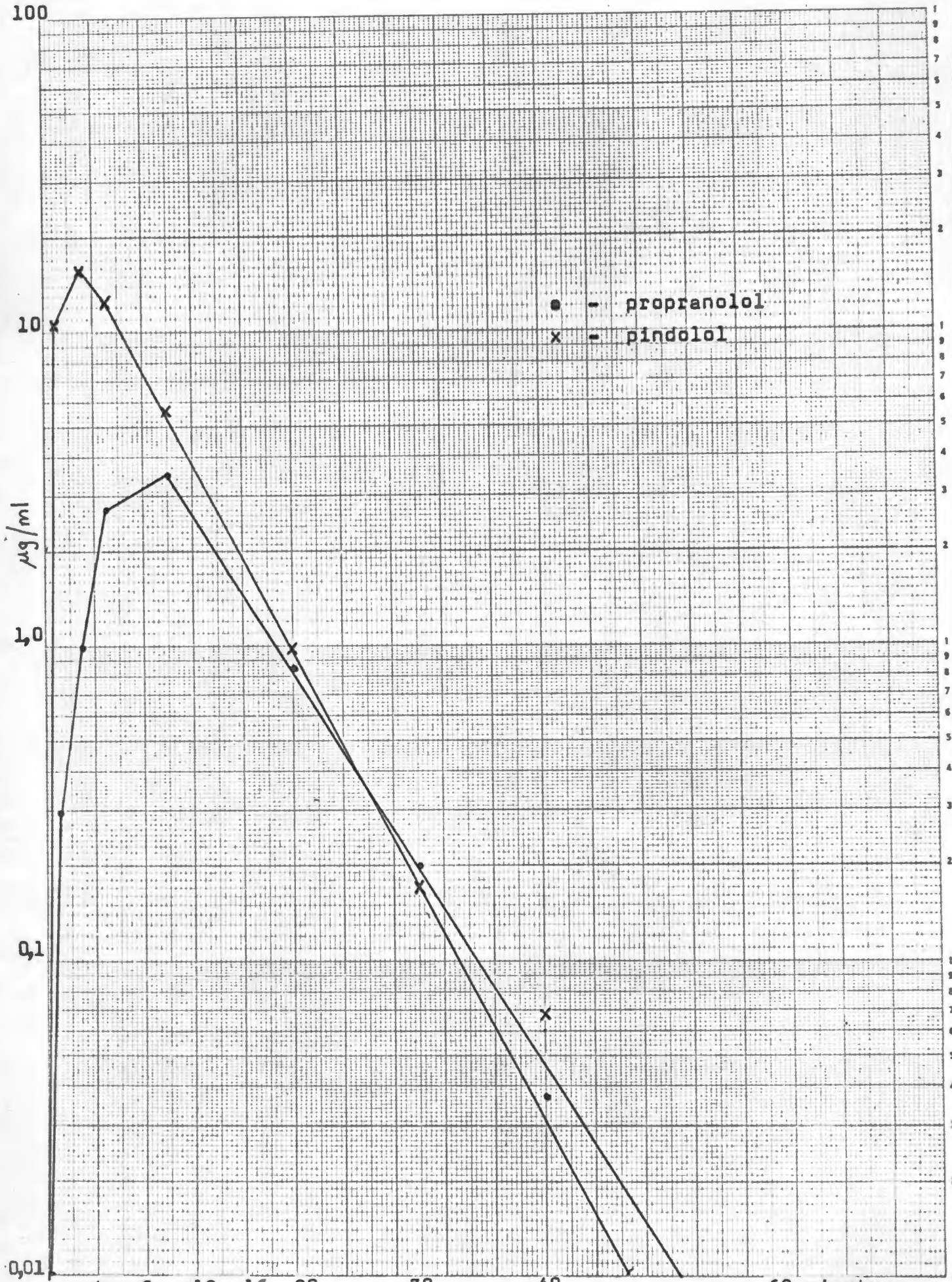
Graph 16b: Perfusion G (Pindolol). Portal vein concentration versus time curve. Points were obtained from the line fitted in Graph 16a.



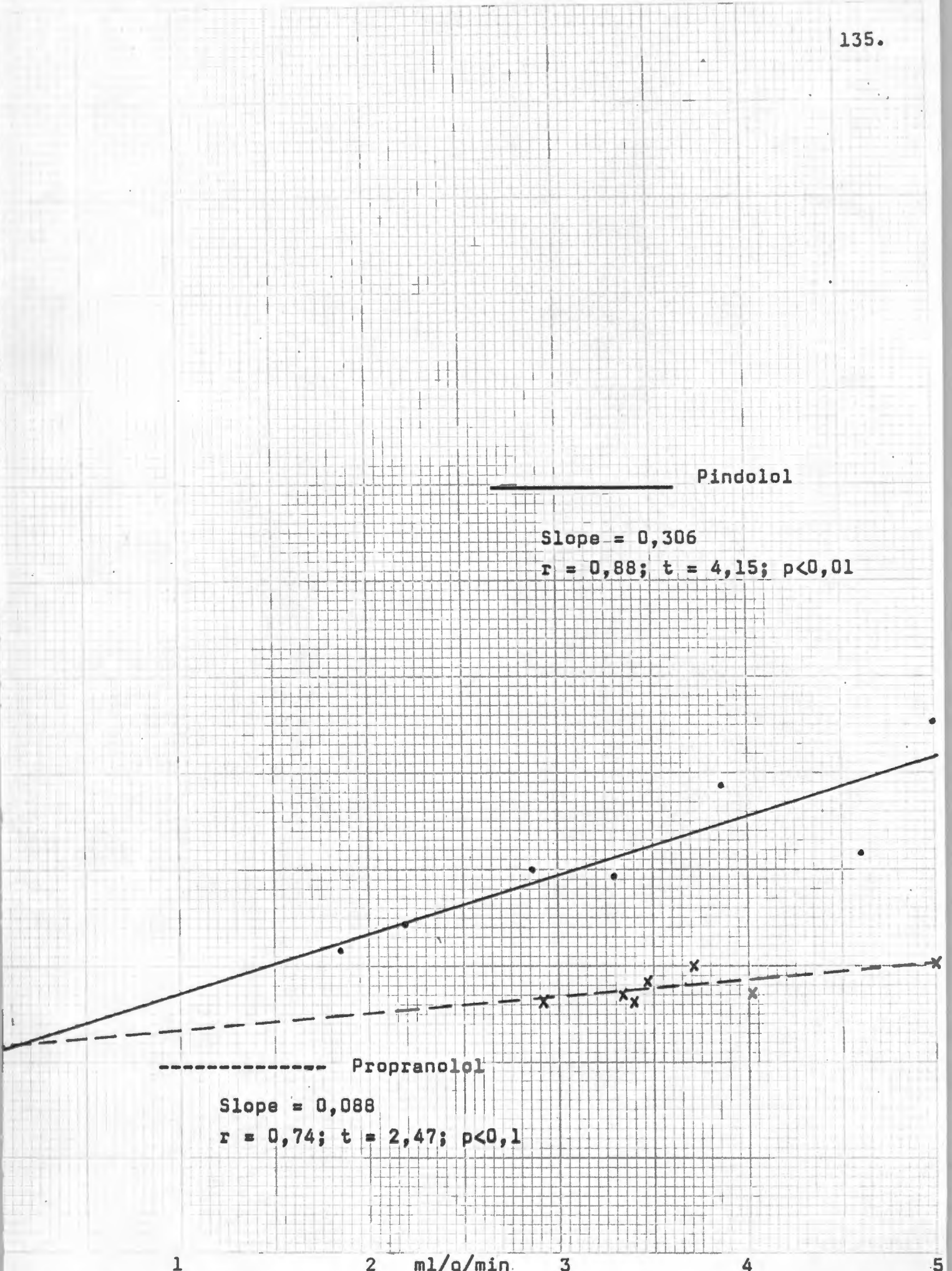
Graph 16c: Perfusion G (Pindolol). Hepatic vein concentration versus time curve. Points were obtained from the line fitted in Graph 16a.



Graph 17a: Semi-logarithmic plot of the mean concentration values for propranolol (— • —) and pindolol (— x —) in the portal vein versus time.



Graph 17b: Semi-logarithmic plot of the mean concentration values for propranolol (— • —) and pindolol (— x —) in the hepatic vein versus time.



Graph 18: The effects of flow on extraction ratio. A plot of $\frac{1}{E}$ versus flow (ml/g/min).

APPENDIX A

Statistical Methods

Mean $\bar{x} = \frac{\Sigma x}{N}$

where N = sample number

Standard Deviation SD $= \sqrt{\frac{(x - \bar{x})^2}{N - 1}}$

Standard Error of the Mean S.E.M. $= \frac{SD}{\sqrt{N}}$

Student's t test:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(SD_1)^2}{N_1} + \frac{(SD_2)^2}{N_2}}}$$

Degrees of freedom $= (N_1 - 1) + (N_2 - 1)$

Correlation Co-efficient (Product Moment):

$$\Sigma x^2 = \Sigma X^2 - \frac{(\Sigma X)^2}{N}$$

$$\Sigma y^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{N}$$

$$\Sigma xy = \Sigma XY - \frac{\Sigma X \Sigma Y}{N}$$

$$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 \ \Sigma y^2}}$$

$$t = \frac{r\sqrt{N - 2}}{\sqrt{1 - r^2}}$$

Degrees of freedom $= N - 1$

APPENDIX B

ABBREVIATIONS

AUC_{HV}	=	area under the hepatic vein drug concentration versus time curve
AUC_o	=	area under the drug concentration versus time curve in the systemic circulation after oral administration
AUC_{PV}	=	area under the portal vein drug concentration versus time curve
AUC_s	=	area under the drug concentration versus time curve in the systemic circulation after intravenous administration
Cl_H	=	hepatic clearance of total drug
Cl_{int}	=	hepatic intrinsic clearance of total drug
Cl_{int}'	=	hepatic intrinsic clearance of free drug
c_o	=	drug concentration at time zero
D_{iv}	=	total intravenous dose
D_o	=	total oral dose
E	=	hepatic extraction ratio
f	=	bioavailability
f	=	fraction of unbound drug in the blood
Q	=	hepatic flow rate
$t_{\frac{1}{2}}$	=	elimination half-life
V_d	=	apparent volume of distribution

REFERENCES

1. RIESS W., BRECHBÜHLER S., BRUNNER L., IMHOF P.R. and JACK D.B.: The metabolism of beta-blockers in relation to their pharmacokinetic and pharmacodynamic behaviour, Beta-Blockers - present status and future prospects. International Symposium, Juan-les-Pins, 27-29 (1974). Ed. W. Schweizer.

2. O'MALLEY K., CROOKS J., DUKE E. and STEVENSON I.H.: Effect of Age and Sex on Human Drug Metabolism. Br. Med. J. 3, 607-609 (1971).

3. MEIER J. and NÜESCH E.: Pindolol, a β-adrenoreceptor blocking agent with a negligible first-pass effect. Br. J. of Clin. Pharmacol. 4, 371-372 (1977).

4. GEORGE C.F., ORME M.L., BURANAPONG P., MACERLEAN D., BRECKENRIDGE A.M. and DOLLERY C.T.: Contribution of the Liver to Overall Elimination of Propranolol. J. of Pharmacokinetics and Biopharmaceutics 4, 17-27 (1976).

5. SHEPHARD A., SALEM S., RAJJAYABUN P. and STEVENSON I.: Induction of Drug Metabolism in the Elderly. Clin. Research 26, 295A (1978).

6. PRITCHARD J.F. and SCHNECK D.W.: Effects of Ethanol and Phenobarbital on the Metabolism of Propranolol by 9000g Rat Liver Supernatant. Biochem. Pharmacol. 26, 2453-2454 (1977).

7. KIECHEL J.R.: Specificity of the fluorometric estimation of LB46. Report for Sandoz Ltd. (1972).

8. KIECHEL J.R., NIKLAUS P., SCHREIER E. and WAGNER H.: Metabolites of Pindolol in Different Animal Species. Xenobiotica 5, 741-754 (1975).

9. SCHNEIDER R.E., BABB J., BISHOP H. and MITCHARD M.: Plasma levels of Propranolol in Treated Patients with Coeliac disease and Patients with Crohn's disease. Br. Med. J. 2, 794-795 (1976).

10. PARSONS R.L., KAYE C.M., RAYMOND K., TROUNCE J.R. and TURNER P.: Absorption of Propranolol and Practolol in Coeliac Disease. Gut 17, 139-143 (1976).

11. OHNHAUS E.E., NÜESCH E., MEIER J. and KALBERER F.: Pharmacokinetics of Unlabelled and ^{14}C - labelled Pindolol in Uraemia. Europ. J. of clin. Pharmacol. 7, 25-29 (1974).

12. JOHNSSON G. and REGARDH C.G.: Clinical Pharmacokinetics of β-Adrenoreceptor Blocking Drugs. Clinical Pharmacokinetics 1, 233-263 (1976).

13. BUCKINGHAM R.E., HAMILTON T.C. and ROBSON D.: Studies on the cardiovascular effects of pindolol in Doca/Saline Hypertensive Rats. Br. J. of Pharmac. 6, 461-469 (1977).

14. SHAND D.G., BRANCH R.A., EVANS G.H., NIES A.S. and WILKINSON G.R.: The Disposition of Propranolol VII, The Effects of Saturable Hepatic Tissue Uptake on Drug Clearance by the Perfused Rat Liver. Drug Met. and Disposition 1, 679-686 (1973).

15. SHAND D.G., RANGNO R.E. and EVANS G.H.: The Disposition of Propranolol II, Hepatic Elimination in the Rat. Pharmacology 8, 344-352 (1972).

16. EVANS G.H., WILKINSON G.R. and SHAND D.G.: J. of Pharm. and Exp. Ther. 186, 447-454 (1973).

17. SHAND D.G., KORNHAUSER D.M. and WILKINSON G.R.: Effects of Route of Administration and Blood Flow on Hepatic Drug Elimination. J. of Pharmacol. and Exp. Ther. 195, 424-432 (1975).

18. KORNHAUSER D.M., WOOD A.J., VESTAL R.E., WILKINSON G.R., BRANCH R.A. and SHAND D.G.: Biological determinants of Propranolol Disposition in Man. Clinical Pharmacol. and Ther. 23, 165-174 (1978).

19. BRANCH R.A., NIES A.S. and SHAND D.G.: The Disposition of Propranolol VIII, General Implications of the Effects of Liver Blood Flow on Elimination from the Perfused Rat Liver. Drug. Met. and Disposition 1, 687-690 (1973).

20. ROWLAND M., BENET L.Z. and GRAHAM G.G.: Clearance Concepts in Pharmacokinetics. J. of Pharmacokinetics and Biopharmaceutics 1, 123-136 (1973).

21. GUGLER R., HEROLD W. AND DENYLER H.J.: Pharmacokinetics of Pindolol in Man. Europ. J. of clin. Pharmacol. 7, 17-24 (1974).

22. BRANCH R.A. and SHAND D.G.: Propranolol Disposition in Chronic Liver Disease: A Physiological Approach. Clinical Pharmacokinetics 1, 264-279 (1975).

23. BLASCHKE T.F.: Protein Binding and Kinetics of Drugs in Liver Diseases. Clinical Pharmacokinetics 2, 32-44 (1977).

24. NIES A.S., SHAND D.G. and WILKINSON G.R.: Altered Hepatic Blood Flow and Drug Disposition. Clinical Pharmacokinetics 1, 135-156 (1976).

25. WILKINSON G.R. and SHAND D.G.: A physiological approach to hepatic drug clearance. Clin. Pharm. and Ther. 18, 377-390 (1975).

26. PACHA W.L.: A Method for the Fluorimetric Determination of 4 - (2-Hydroxy-3-isopropylaminopropoxy) - indole (LB 46), a β-Blocking Agent, in Plasma and Urine. Experientia 25, 802-803 (1969).

27. GIBALDI M., BOYES R.N. and FELDMAN S.: Influence of First-Pass Effect on Availability of Drugs on Oral Administration. J. of Pharm. Sci. 60, 1338-1340 (1971).

28. SHAND D.G. and OATES J.A.: Metabolism of Propranolol by Rat Liver Microsomes and its inhibition by Phenothiazines and Tricyclic Antidepressants. Biochem. Pharmacol. 20, 1720-1723 (1971).

29. COTHAM R.H. and SHAND D.G.: Spuriously low plasma propranolol concentrations resulting from blood collection methods. Clin. Pharmacol. and Ther. 18, 535-538 (1975).

30. RANE A., WILKINSON G.R. and SHAND D.G.: Prediction of Hepatic Extraction Ratio from *in vitro* Measurement of Intrinsic Clearance. J. of Pharmacol. and Exp. Ther. 200, 420-424 (1977).

31. SHAND D.G., NUCKOLLS E.M. and OATES J.A.: Plasma propranolol levels in adults with observations in four children. Clin. Pharmacol. and Ther. 11, 112-120 (1970).

32. SHAND D.G., COTHAM R.H. and WILKINSON G.R.: Perfusion-limited effects of plasma drug binding on hepatic drug extraction. Life Sciences 19, 125-130 (1976).

33. LOWENTHAL D.T. and MUTTERPERL R.: The Pharmacokinetics of Multiple Dose Propranolol in Chronic Renal Disease. Clin. Pharm. and Ther. 19, 111 (1976).

34. WILKINSON G.R. and SCHENKER S.: Effects of Liver Disease on Drug Disposition in Man. Biochem. Pharmacol. 25, 2675-2681 (1976).

35. MILLER L.L., BLY C.G., WATSON M.L. and BALE W.F.: Role of the Liver in Plasma Protein Synthesis. J. of Exp. Med. 94, 431-453 (1951).

36. SOTANIEMI E.A., HUHTI E.A., PALATSI I.J. and TAKKUNEN J.T.: Evaluation of drug metabolising capacity in patients with myocardial infarction. Clin. Pharm. and Ther. 17, 244-245 (1975).

37. MEIER J.: Pindolol: a pharmacokinetic comparison with other beta-adrenoreceptor blocking agents. Current Medical Research and Opinion 4, 31-38 (1977).

38. VU V.T. and ABRAMSON F.P.: Quantitative Analysis of Propranolol and Metabolites by a Gas Chromatograph Mass Spectrometer Computer Technique. Biomedical Mass Spectrometry 5, 686-691 (1978).

39. JENNINGS G.L., BOBIK A., FAGAN E.T. and KORNER P.I.: Pindolol Pharmacokinetics in relation to the Time Course of Inhibition of Exercise Tachycardia. Br. J. of clin. Pharmac. 7, 245-256 (1979).

40. MEIER J. and WAGNER O.: First Pass Effect of the beta-Adrenoreceptor Blocking Agent, Pindolol, in the Dog. Br. J. of Pharmacol. 59, 492P (1977).

41. WOOD A.J., CARR K., VESTAL R.E., BELCHER S., WILKINSON G.R. and SHAND D.G.: Direct Measurement of Propranolol Bioavailability during accumulation to Steady State. Br. J. of clin. Pharmac. 6, 345-350 (1978).

42. VON BAHR C., ANDERSON B., AZARNOFF D.L., SJOQVIST F. and ORRENIUS S.: Drug Metabolism in the Perfused Liver. Eur. J. of Pharmacol. 9, 99-105 (1970).

43. EVANS G.H., NIES A.S. and SHAND D.G.: Plasma Binding of Propranolol. J. of Pharmacol. and Exp. Ther. 186, 114-122 (1973).

44. NAGASHIMA R. and LEVY G.: Effect of Perfusion Rate and Distributional Factors on Drug Elimination Kinetics in a Perfused Organ System. J. of Pharm. Sci. 57, 1991-1993 (1968).

45. REMMER H.: The Röle of the Liver in Drug Metabolism. Am. J. of Med. 49, 617-629 (1970).

46. SHAND D.G. and RANGNO R.E.: The Disposition of Propranolol I: Elimination during Oral Absorption in Man. Pharmacology 7, 159-168 (1972).

47. AELLIG W.H.: β-Adrenoreceptor Blocking Activity and Duration of action of Pindolol and Propranolol in Healthy Volunteers. Br. J. of clin. Pharmac. 3, 251-257 (1976).

48. HILL R.C. and TURNER P.: Preliminary Investigations of a new beta-Adrenoreceptor Blocking Drug LB46 in Man. Br. J. of Pharmacol. 36, 368-372 (1969).

49. GUGLER R., HÖBEL W., BODEM G. and DENGLER H.: The effect of Pindolol on Exercise-induced Cardiac Acceleration in relation to Plasma Levels in Man. Clin. Pharm. and Ther. 17, 127-133 (1975).

50. EVANS G.H. and SHAND D.G.: The Disposition of Propranolol V, Drug Accumulation and Steady-State Concentrations during Chronic Oral Administration in Man. Clin. Pharm. and Ther. 14, 487-493 (1973).

51. EVANS G.H. and SHAND D.G.: The Disposition of Propranolol VI, Independent Variation in Steady-State Circulating Drug Concentrations and Half-Life as a result of Plasma Drug Binding in Man. Clin. Pharm. and Ther. 14, 494-500 (1973).

52. HAYES A. and COOPER R.G.: Studies on the Absorption, Distribution and Excretion of Propranolol in Rat, Dog and Monkey. J. of Pharm. and Exp. Ther. 176, 302-311 (1971).

53. CONNEY A.H., PANTUCK E.J., HSIAO K., GARLAND W.A., ANDERSON K.E., ALVARES A.P. and KAPPAS A.: Enhanced Phenacetin Metabolism in Human Subjects fed Charcoal-broiled beef. Clin. Pharm. and Ther. 20, 633-642 (1976).

54. ROWLAND M.: Application of Clearance Concepts to some Literature Data on Drug Metabolism in the Isolated Perfused Liver Preparation and *in vivo*. Eur. J. of Pharmacol. 17, 352-356 (1972).

55. SHAND D.G.: Pharmacokinetic Properties of the β-Adrenoreceptor Blocking Drugs. Drugs 7, 39-47 (1974).

56. LAVENE D., WEISS Y.A., SAFAR M.E., LORIA Y., AGORUS N., GEORGES D. and MILLIEZ P.C.: Pharmacokinetics and Hepatic Extraction Ratio of Pindolol in Hypertensive Patients with Normal and Impaired Renal Function. J. of Clin. Pharmacol. 17, 501-508 (1977).

57. DOLLERY C.T. and JUNOD A.F.: Concentration of (±) - Propranolol in Isolated, Perfused Lungs of Rat. Br. J. of Pharmacol. 57, 67-71 (1976).

58. LOWENTHAL D.T., BRIGGS W., GIBSON T.P., NELSON H. and CIRKSENA W.: Pharmacokinetics of Oral Propranolol in Chronic Renal Disease. Clin. Pharm. and Ther. 16, 761-769 (1974).

59. FISHER M.M. and KERLY M.: Amino acid Metabolism in the Perfused Rat Liver. J. of Physiology 174, 273-294 (1974).

60. NIES A.S., EVANS G.H. and SHAND D.G.: Hemodynamic Effects on the Flow-Dependent Hepatic Clearance of Propranolol. J. of Pharm. and Exp. Ther. 184, 716-720 (1973).

61. KELMAN L., SAUNDERS S.S., WICHT S., FRITH L., CORRIGALL A., KIRSCH R.E. and TERBLANCHE J.: The Effects of Amino acids on Albumin Synthesis by the Isolated Perfused Rat Liver. The Biochemical Journal 129, 805-809 (1972).

62. LLOYD E.A., CROZIER N., PAMPHLET G., WELLS M. and SAUNDERS S.J.: Some Observations on Liver Cell Proliferation in the Isolated Perfused Rat Liver. Br. J. of Exp. Path. 55, 251-259 (1974).

63. HEMS R., ROSS B.D., BERRY M.N. and KREBS H.A.: Gluconeogenesis in the Perfused Rat Liver. Biochemical Journal 101, 284-292 (1966).

64. GUGLER R. and BODEM G.: Single and Multiple Dose Pharmacokinetics of Pindolol. Europ. J. of clin. Pharmacol. 13, 13-16 (1978).

65. BRECKENRIDGE A., BURANAPONG P., DOLLERY C.T., GEORGE C.F., MACERLEAN D. and ORME M.: Hepatic Clearance of Propranolol in Dogs. Br. J. of Pharmacol. 48, 336P-337P (1973).

66. COHEN G.H. and DAVIES D.S.: Uptake of Propranolol by the isolated Perfused Rat Liver. Br. J. of Pharmacol. 50, 472P (1974).

67. WALLE T. and GAFFNEY T.E.: Propranolol Metabolism in Man and Dog: Mass Spectrometric Identification of Six New Metabolites. J. of Pharm. and Exp. Ther. 182, 83-92 (1972).

68. CHIDSEY C.A., MORSELLI P., BIANCHETTI G., MORGANTI A., LEONETTI G. and ZANCHETTI A.: Studies of the Absorption and Removal of Propranolol in Hypertensive Patients During Therapy. Circulation 52, 313-318 (1975).

69. NIES A.S. and SHAND D.G.: Clinical Pharmacology of Propranolol. Circulation 52, 6-15 (1975).

70. WALLE T., CONRADI E.C., WALLE K., FAGAN T. and GAFFNEY T.: The predictable relationship between plasma levels and dose during chronic propranolol therapy. Clin. Pharm. and Ther. 24, 668-677 (1978).

71. PRITCHARD J.F., SCHNECK D.W., RACZ W.J. and HAYES A.H.: The Contribution of Propranolol Metabolites to the Fluorometric Assay of Propranolol in Human Plasma. Clinical Biochemistry 11, 121-125 (1978).

72. KRAML M. and ROBINSON W.: Fluorimetry of Propranolol and its Glucuronide: Applicability, Specificity and Limitations. Clinical Chemistry 24, 169-170 (1978).

73. McEWEN J., SHAND D.G. and WILKINSON G.R.: Br. J. of Pharm. 52, 140P-141P (1974).

74. PRESCOTT L.F., ADJEPON-YAMOAH K. and TALBOT R.G.: Impaired lignocaine metabolism in patients with myocardial infarction and cardiac failure. Br. Med. J. 1, 939-941 (1976).

75. VERVLOET E., PLUYM B. and MERKAS F.: Blood/Plasma concentration ratio of propranolol. Clin. Pharm. and Ther. 23, 133 (1978).

76. SAWCHUCK R.J., RABAYO J. and MILLER K.W.: The Distribution of Propranolol between Blood and Plasma in Hypertensive Patients. Br. J. of clin. Pharm. 1, 440-442 (1974).

77. PERRIER D. and GIBALDI M.: Clearance and Biological Half-life as Indices of Intrinsic Hepatic Metabolism. J. of Pharm. and Exp. Ther. 191, 17-24 (1974).

78. TIMMER C.J. and WIJNAND H.P.: Influence of Sampling on Drug Kinetics in Liver Perfusion. J. of Pharmacokin. and Biopharmaceutics 5, 335-358 (1977).

79. VESSELL E.S.: Factors causing interindividual variations of drug concentrations in blood. Clin. Pharm. and Ther. 16, 135-148 (1974).

80. SHAND D.G.: Pharmacokinetics of Propranolol: a Review. Postgrad. Med. J. 52, 22-25 (1976).

81. SCHNECK D.W., PRITCHARD J.F. and HAYES A.H.: Enhancement of the bioavailability of Propranolol and Metoprolol by Food. Clin. Pharm. and Ther. 22, 108-112 (1977).

82. WEISS Y.A., SAFAR M.E., LEHNER J.P., LEVENSON J.A., SIMON A. and ALEXANDRE J.M.: (+)- Propranolol Clearance, an Estimation of Hepatic Blood Flow in Man. Br. J. of clin. Pharmac. 5, 457-460 (1978).

83. SCHNECK D.W., PRITCHARD J.F. and HAYES A.H.: Studies on the Uptake and Binding of Propranolol by Rat Tissues. J. of Pharm. and Exp. Ther. 203, 621-629 (1977).

84. CHAU N.P., WEISS Y.A., SAFAR M.E., LAVENE D.E., GEORGES D.R. and MILLIEZ P.L.: Pindolol Availability in Hypertensive Patients with Normal and Impaired Renal Function. Clin. Pharm. and Ther. 22, 505-510 (1977).

85. GILLETTE J.R.: Factors affecting Drug Metabolism. Annals of N.Y. Acad. of Sciences 179, 43-66 (1971).

86. GIBALDI M.: Pharmacokinetic Aspects of Drug Metabolism. Annals of N.Y. Acad. of Sciences 179, 19-31 (1971).

87. DOLLERY C.T., DAVIES S. and CONOLLY M.E.: Differences in the metabolism of Drugs depending on their Routes of Administration. Annals of N.Y. Acad. of Sciences 179, 108-112 (1971).

88. RUMACK B.H., HOLTZMAN J. and CHASE P.: Hepatic Drug Metabolism and Protein Malnutrition. J. of Pharm. and Exp. Ther. 186, 441-446 (1973).

89. DISALLE E., BAKER K.M., BAREGGI S.R., WATKINS W.D., CHIDSEY C.A., FRIGERIO A. and MORSELLI P.L.: A sensitive Gas-Chromatographic method for the Determination of Propranolol in Human Plasma. J. of Chromatography 84, 347-353 (1973).

90. CASTLEDEN C.M. and GEORGE C.F.: The Effect of Ageing on the Hepatic Clearance of Propranolol. Br. J. of clin. Pharmac. 7, 49-54 (1979).

91. FEELY J., CROOKS J. and STEVENSON I.H.: Alterations in plasma propranolol steady-state concentrations in thyroid disease. Clin. Pharm. and Ther. 23, 112 (1978).

92. BELL J.M., RUSSELL C.J. and NELSON J.K.: Studies on the effect of thyroid dysfunction on the elimination of beta-adrenoreceptor blocking drugs. Br. J. of clin. Pharm. 4, 79-82 (1977).

93. McALLISTER R.G., TAN G.T. and TODD E.P.: Effect of hypothermia on the metabolism of propranolol, quinidine and verapamil. Clin. Pharm. and Ther. 23, 121 (1978).

94. GIBALDI M. and PERRIER D.: 'Pharmacokinetics' Pages 232-241. Marcel Dekker Inc. (1975).

95. ROWLAND M.: Influence of Route of Administration on Drug Availability. J. of Pharm. Sci. 61, 70-74 (1972).

96. LEVY J.Y.: β-Adrenergic Receptor Blocking Drugs in Spontaneous Hypertension. Am. J. of Med. 61, 779-789 (1976).

97. BRANCH R.A., JAMES J.A. and READ A.E.: The influence of chronic liver disease on the elimination of d-propranolol, antipyrine and indocyanine green. Gut 15, 837-838 (1974).

98. WILKINSON G.R.: Pharmacokinetics of Drug Disposition: Hemodynamic Considerations. Ann. Review of Pharmacol. 15, 11-27 (1975).

99. KEIDING S., JOHANSEN S., WINKLER K., TONNENSEN K. and TYGSTRUP N.: Michaelis-Menten kinetics of galactose elimination by the isolated perfused pig liver. Am. J. of Physiol. 230, 1302-1313 (1976).

100. LEMAIRE M. and MEIER J.: LB46, Binding Studies with Blood Cells and Plasma Proteins. Report for Sandoz Ltd. (1978).

101. ANDERSON J.H., ANDERSON R.C. and IBEN L.S.: Hepatic Uptake of Propranolol. J. of Pharm. and Exp. Ther. 206, 172-180 (1978).

102. KAPPAS A., ANDERSON K.E., CONNEY A.H. and ALVARES A.P.: Influence of Dietary Protein and Carbohydrate on Antipyrine and Theophylline Metabolism in Man. Clin. Pharm. and Ther. 20, 643-653 (1976).

103. BORGA O., ODAR-CEDERLOF I., PIAFSKY K.M. and SJOQVIST F.: Plasma protein binding of propranolol in disease states. Br. J. of clin. Pharm. 4, 627P (1974).

104. SCHNEIDER R.E., BISHOP H., HAWKINS C.F. and KITS G.: Drug binding to α_1-glycoprotein. Lancet 1, 554 (1979).

105. PIAFSKY K.M., BORGA O., ODAR-CEDERLOF I., JOHANSSEN C. and SJOQVIST F.: Increased plasma Protein Binding of Propranolol and Chlorpromazine mediated by Disease-induced Elevations of Plasma Acid Glycoprotein. New Engl. J. of Med. 299, 1435-1439 (1978).

106. SHAND D.G., EVANS G.H. and NIES A.S.: The Almost Complete Hepatic Extraction of Propranolol During Intravenous Administration in the Dog. Life Sciences 10, 1417-1421 (1971).

107. BRANCH R.A., JAMES J. and READ A.E.: A study of factors influencing Drug Disposition in Chronic Liver Disease, using the Model Drug (+) -Propranolol. Br. J. of clin. Pharmac. 3, 243-249 (1976).

108. McALLISTER R.G., BOURNE D.W., TAN T.G., ERICKSON J.L., WACHTEL C. and TODD E.P.: Effects of Hypothermia on Propranolol Kinetics. Clin. Pharm. and Ther. 25, 1-7 (1979).

109. VESTAL R.E., KORNHAUSER D.M., HOLLIFIELD J.W. and SHAND D.G.: Inhibition of Propranolol Metabolism by Chlorpromazine. Clin. Pharm. and Ther. 25, 19-24 (1979).

110. BOND P.A.: Metabolism of Propranolol. Nature 213, 721 (1967).

111. FITZGERALD J.D. and O'DONNELL S.R.: Pharmacology of 4- Hydroxy Propranolol, a Metabolite of Propranolol. Br. J. of Pharmacol. 43, 222-235 (1971).

112. SAKURADA A., VOSS D.O., BRANDAO D. and CAMPELLO A.P.: Effects of Propranolol on Heart Muscle Mitochondria. Biochem. Pharmacol. 21, 535-540 (1972).

113. ANAVEKAR S.N., LOUIS W.J., MORGAN T.O., DOYLE A.E. and JOHNSTON C.I.: The relationship of plasma levels of pindolol in hypertensive patients to effects on blood pressure, plasma renin and plasma noradrenaline levels. Clin. and Exp. Pharmacol. and Physicl. 2, 203-212 (1975).

114. ISHIZAKI T., PRIVITERA P.J., WALLE T. and GAFFNEY T.E.: Cardiovascular action of a new metabolite of Propranolol, Isopropylamine. J. of Pharm. and Exp. Ther. 189, 626-632 (1974).

115. CASTLEDEN C.M., GEORGE C.F. and SHORT M.D.: Contribution of individual differences in gastric emptying to variability in plasma propranolol concentrations. Br. J. of clin. Pharmac. 5, 121-122 (1978).

116. PESSAYRE D., LEBREC D., DESCATOIRE V., PEIGNOUX M. and BENHAMOU J.: Mechanisms for reduced Drug Clearance in patients with Cirrhosis. Gastroenterology 74, 566-571 (1978).

117. WILSON J., ATWOOD G. and SHAND D.G.: Disposition of Propoxyphene and Propranolol in Children. Clin. Pharm. and Ther. 19, 264-270 (1976).

118. WOOD A.J., KORNHAUSER D.M., WILKINSON G.R., SHAND D.G. and BRANCH R.A.: The Influence of Cirrhosis on Steady-State Blood Concentrations of Unbound Propranolol after Oral Administration. Clin. Pharmacokin. 3, 478-487 (1978).

119. BRAUER R.W.: Liver Circulation and Function. Physiol. Rev. 43, 115-213 (1963).

120. SHAND D.G.: Clinical Review of Propranolol. New Engl. J. of Med. 293, 280-285 (1975).

121. RUBENFIELD S., SILVERMAN V.E., WELCH K.A., MALLETTE L.E. and KOHLER P.O.: Variable Plasma Levels in Thyrotoxicosis. New Engl. J. of Med. 300, 353-354 (1979).

122. SHEPPARD G.P.: High-dose Propranolol in Schizophrenia. Br. J. of Psychiat. 134, 470-476 (1979).

123. McLEAN A.J., McNAMARA P.J., DU SOUICH P., GIBALDI M. and LALKA D.: Food, Splanchnic Blood Flow and Bioavailability of Drugs subject to First-pass Metabolism. Clin. Pharm. and Ther. 24, 5-10 (1978).

124. WARBURTON S., OPIE L.H., KENNELLY B.M. and MULLER F.O.: Does Cimetidine alter the Cardiac Response to Exercise and Propranolol. S.A. Med. J. 55, 1125-1127 (1979).

125. BRANCH R.A., SHAND D.G., WILKINSON G.R. and NIES A.S.: Increased Clearance of Antipyrine and d-Propranolol after Phenobarbital Treatment in Monkey. J. of Clin. Investigation 53, 1101-1107 (1974).

126. ROBERTS R.K., DESMOND P.V. and SCHENKER S.: Drug Prescribing in Hepatobiliary Disease. Drugs 17, 198-212 (1979).

127. JARVISALO J.O. and SARIS N.L.: Biochem. Pharmacol. 24, 1309-1312 (1975).

128. TINDELL G.C., WALLE T. and GAFFNEY T.E.: Rat Liver Microsomal Metabolism of Propranolol: Identification of Seven Metabolites by Gas Chromatography-Mass Spectrometry. Life Sciences 11, 1029-1038 (1972).

129. HUFFMAN D.H., AZARNOFF D.L., SHOEMAN D.W. and DUJONE C.A.: The interaction between halofenate and propranolol. Clin. Pharm. and Ther. 19, 807-812 (1976).

130. CASTLEDEN C.M., KAYE C.M. and PARSONS R.L.: Br. J. of clin. Pharm. 2, 303-306 (1975).

131. PANG K.S. and ROWLAND M.: Hepatic Clearance of Drugs I. Theoretical Considerations of a "Well-Stirred" Model and a "Parallel Tube" Model. J. of Pharmacokin. and Biopharmaceutics 5, 625-653 (1977).

132. ROUTLEDGE P.A. and SHAND D.G.: Clinical Pharmacokinetics of Propranolol. Clin. Pharmacokinet. 4, 73-90 (1979).

133. WOOD A.J., VILLENEUVE J.P., BRANCH R.A., ROGERS L.W. and SHAND D.G.: Intact Hepatocyte Theory of Impaired Drug Metabolism in Experimental Cirrhosis in the Rat. Gastroenterology 76, 1358-1362 (1979).

134. CURRY S.H.: 'Drug Disposition and Pharmacokinetics' Chapter 7, Blackwell Scientific Publications (1977).

135. BARBER H.E., GABRIELLE M., HAWKESWORTH N.R., KITTERINGHAM N.R., PETRIE J.C., SWANN J.M. and PETERSEN J.: Protein binding of atenolol and propranolol to human serum albumin and human plasma. Proceedings of the British Pharmacol. Society C21, Page 19 (1978).

136. GEDDES D.M., NESBITT K. and TRAILL T.: First pass uptake of [^{14}C]-propranolol by lung in man. Proceedings of the British Pharmacol. Society C22, Page 20 (1978).

137. GILLETTE J.R.: in 'Proceedings of International Symposium of Drug Metabolism, University of Surrey' Pages 147-168 (1976).

138. VESTAL R.E., WOOD A.J., BRANCH R.A. and SHAND D.G.: The effects of ageing and cigarette smoking on propranolol's disposition in man. Clin. Pharm. and Ther., submitted 1979.

139. KELMAN L.: The Role of Amino Acids in Albumin Synthesis and Catabolism, M.D. Thesis (1971).