Dienst Landbouwkundig Onderzoek
Rijks-Kwaliteitsinstituut voor land- en tuinbouwprodukten
Agricultural Research Department
State Institute for Quality Control of Agricultural Products

REPORT OF RESEARCH PROJECT
No. R 98-130 *(NWO-BC)*

THE PERMEABILITY OF POLYETHYLENE GLYCOL OLIGOMERS IN THE ISOLATED PERFUSED RAT LIVER

Dr. M.J.B. Mengelers

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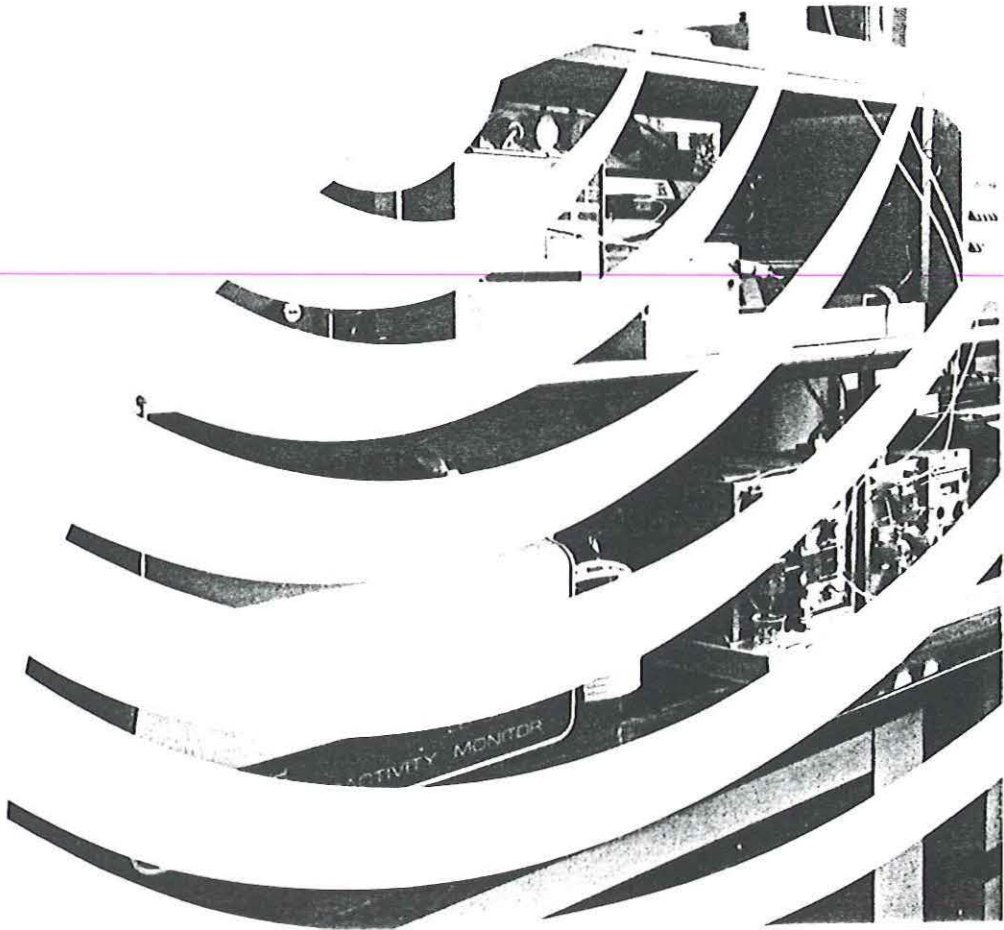

Report 96.51

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INTRODUCTION

Information about the visiting scientist

The visiting scientist is working as a senior scientist at the department of Food Safety and Health (formerly the department of Risk Assessment and Toxicology) of the State Institute for Quality Control of Agricultural Products (RIKILT-DLO) seated at Wageningen, The Netherlands. RIKILT-DLO is a research institute of the Agricultual Research Department of the Ministry of Agriculture, Nature Management and Fisheries. At this institute analytical and toxicological research is carried out in order to assess the quality and safety of food products and food production chains. At the department of Food Safety and Health, foods or compounds present in foods are studied which may influence the health of the consumer in a negative or positive manner. Compounds of interest can be divided into two major groups, namely contaminants like polychlorinated organic compounds, natural toxins and residues of veterinary drugs and health promoting agents like vitamines and (iso-)flavonoids. The main task of the undersigned is to study the kinetics of these compounds in order to assess possible harmful or benificial effects in humans or food-producing animals. For various reasons the applicant is interested in physiologically based pharmacokinetic (PB-PK) modeling.

Information about the host institute

The Pharmacokinetics Group within the Department of Pharmacy, University of Manchester, is one of the premier groups within the world engaged in all facets of pharmacokinetics - theory, experimental, modeling and data analysis. There are few such centres within Europe, and none specifically within The Netherlands.

This group, under the supervision of prof. dr. M. Rowland, is well known for its scientific contributions in physiologically based pharmacokinetics, which extend over 17 years. In addition to theoretical developments, much of the research has been carried out in isolated perfused organs and whole animal studies, invariably in the rat, for which it has all the necessary experimental facilities. Also available are computer systems and software for model simulation and data analysis.

Aims

General. Pharmacokinetic models are used to describe, in mathematical terms, the time course of drug and metabolite concentrations in body fluids and tissues. A novel approach is the application of physiologically based pharmacokinetic (PB-PK) models. These PB-PK models have been used to describe drug disposition in laboratory animals (mostly rats) and in humans (only recently). It is our purpose to apply these models for describing and predicting drug and metabolite concentrations in food-producing animals and to study the kinetic behaviour of food compounds in man. The general aim is to gain insight in and knowledge of PB-PK modeling by means of a sabbatical leave of six months. Due to this short period a specified project was designed to perform fundamental research on hepatic dispersion and disposition.

Specific. Processes occuring in the liver are very important for the description of the kinetic behaviour of drugs and compounds present in food. Therefore this research was focussed on dispersion and distribution processes occuring in the rat liver. Polyethylene glycols were used as test compounds because they are mixtures of different oligomers and offer a set of molecules with different molecular weights and sizes. The aim was to study the influence of molecular size on the permeability of different oligomers of polyethylene glycol (PEG) in the isolated perfused rat liver. Furthermore, the feasibility of performing these studies with non-radioactive labelled PEGs was studied.

Grant
The Netherlands Organisation for Scientific Research (NWO), in collaboration with the British Council, provided a grant for international travel and subsistence which covered a period of three months.

1. PHYSICO-CHEMICAL PROPERTIES OF PEG OLIGOMERS

1.1 General properties and nomenclature

Polyethylene glycols (PEGs) represent a family of inert, synthetic, linear polymers employed as viscosity enhancing agents, solubilizing agents and lubricants in a wide variety of commercial settings, including the pharmaceutical, cosmetic and food industries. PEGs are also referred to as macrogols. Some authorities use the general formula $H.(CH_2.O.CH_2)_m.OH$ to describe PEGs [1]. In using this formula the number asssigned to *m* for a specified PEG is 1 more than that of *n* in the general formula $CH_2OH.[CH_2.O.CH_2]_n.CH_2OH$ which is used to describe the macrogols in this report. The PEGs are mixtures of condensation polymers of ethylene oxide and water. Those with an average molecular weight (MW) of 200 to 700 Dalton (Da) are liquid; those with an average MW of more than 1000 Da vary in consistence from soft unctuous to hard wax-like solids. The average molecular weight is indicated by a number in the name; thus PEG400 has an average molecular weight of about 400 Da. The different oligomers are sometimes indicated by the letter 'E' followed by a number indicating the number (*n*) of $CH_2.O.CH_2$ moieties. This means that E6 has a molecular weight of 326 Da. Molecular structures and weights of tested PEG oligomers are given in annex 1.

1.2 Solubility and hydrophilicity of PEG400 and PEG600 oligomers

The liquid and semi-solid PEGs are hygroscopic and miscible with water, alcohol, acetone, chloroform and with other glycols. They are practically insoluble in ether and aliphatic hydrocarbons but soluble in aromatic hydrocarbons.

PEG400 is a mixture of oligomers with an average molecular weight of no less than 380 and no more than 420 Da. It is a colourless, viscous liquid with a freezing point of about 3°C and a specific gravity of 1.13 g/ml. PEG600 is a mixture of oligomers with an average molecular weight of 570-630 Da. It is a colourless, viscous liquid at room temperture with a freezing point of about 20°C and a specific gravity of 1.13 g/ml. A 5% w/v solution in water has a pH of 4.5 to 7.

Various partition coefficients have been described in the literature [2-5]. It is difficult to compare these results due to different organic solvents used in the lipid-water systems. The partition coefficient (P) in an octanol-water system varied from 0.01 to 0.0001 [4,5]. Furthermore, there is no complete agreement on the relationship between molecular weight and partition coefficient of the different oligomers. Theoretically, as the chain length increases with a constant molecular fragment (namely C_2H_40) the lipophilicity would increase with a constant factor if no other processes (interactions) would be involved. However, only one paper described an increase of P with increasing molecular weight. Unfortunately none of the above-mentioned studies has been carried out according to the 'Shake-Flask' method as described in the OECD test guideline [6].

2. DETERMINATION OF PEG OLIGOMERS WITH HPLC

2.1 Introduction

The HPLC analysis of low-molecular weight PEGs has mostly been carried out with reversed-phase HPLC [5,7,8]. Stationary phases used were C18, C8 or styrene divinylbenzene analytical columns. Mobile phases usually consisted of mixtures of water and methanol. Also size exclusion (gel filtration) columns have been used for the determination of PEGs in human urine [9].

All methods applied isocratic elution of the oligomers resulting in relatively long run times (20-30 minutes) for single PEGs. If detection limits were low, or high concentrations were used, more oligomers could be detected and run times were even longer.

Due to the absence of UV-absorption of PEGs most methods applied refractive index detection.

The purpose of this study was to develop a rapid HPLC method with refractive index detection for the determination of a large number of oligomers ($n > 12$) in media used for rat liver perfusions. Mixing PEG400 and PEG600 allowed us to study oligomers with a range in molecular weight from 326 (=E6) to 854 (=E18), in some cases even from 282 to 898 Da. Therefore, the possibility of applying gradient elution was studied.

2.2 Materials and methods

Chemicals

For identification purposes, stock solutions of two purified oligomers, namely E6 (MW = 326) and E9 (MW = 458), were diluted to standard solutions of 1 mg/ml distilled water. A fresh stock solution of 10 mg PEG400 and 10 mg PEG600 per ml distilled water was prepared monthly. For linearity testing this stock solution was diluted to standard solutions containing 0.1, 0.3, 0.5, 0.8, 1, 3, 5, and 8 mg PEG[400+600]/ml. A stock solution of radioactive, ^{14}C-labelled PEG400 of 2 mCi/ml was diluted to a standard solution of 5 μCi/ml.

Sample pre-treatment

Samples obtained from the liver perfusions were centrifuged and filtered simultaneously in specially designed micro test tubes (Eppendorf).

Separation

Two HPLC columns were used, namely a Techsphere C8 column (25 cm x 3.0 mm I.D.) and a Spherisorb C8 column (25 cm x 4.6 mm I.D.). Unless stated otherwise, the temperature of the column oven was 30°C. Mobile phases consisted of water/methanol or water/acetonitril mixtures. Injection volume was always 50 μl. System 1 consisted of the Techsphere C8 column and a mobile phase of water/methanol = 70/30. System 2 consisted of the Spherisorb C8 column and an

eluent of water/methanol = 65/35.

Detection
Detection was carried out with an R401 differential refractometer (Waters Associates) or with an on-line radioactive detector (Radiomatic 500TR). The usual temperature of the refractive index (RI) detector was set at 35°C, unless stated otherwise.

2.3 Results

Separation
Identification. First of all, the oligomers of PEG400 were separated by means of isocratic elution. The different peaks were identified by using standard solutions of two purified oligomers, namely E6 and E9. An overlay plot of chromatograms of E6, E9 and PEG400 is given in annex 2. Standard E6 is very pure but standard E9 is less pure and slightly contaminated with at least 2 other oligomers, one of which is E6.
Structural relationships. An increase in the MW of the oligomer causes an increase of the retention time, independent of the system used (stationary and mobile phase). Therefore, complete separation of all peaks is feasible although the total run time increases exponentially. The elution of 11 oligomers on system 1 takes about 40 minutes. The relationship between retention time and MW is depicted in annex 3. Correcting the retention time (t_R) of each oligomer for the dead volume (t_0) gives the capacity factor 'k' of each oligomer $\{k = (t_R-t_0)/t_0\}$. Using the capacity factor instead of the retention time improves the correlation with the MW of the oligomers, as can be seen in annex 4. The correlation coefficient (r) for the log-linear relation between k and MW of 11 oligomers on HPLC system 1 is 0.9996. The correlation on a different HPLC system with more oligomers (n = 15) is even better: r = 0.9999. These results are depicted in annex 5. The hydrophobic character of a (series of) compound(s) can be correlated with its liquid chromatographic behaviour. For example, the capacity factor of a series of compounds can be related to the n-octanol/water partition coefficient and HPLC can be used to determine the partition coefficient [10-12]. Officially, this relationship is limited to compounds with log P between 0 and 6 [10]. Therefore, the relationship between capacity factor and molecular weight of PEG oligomers may not be based on hydrophobicity but on another phenomenon. Nevertheless, it is a very good relationship which deserves closer attention.
Gradient elution. In order to improve the run time of approximately 45 minutes during isocratic elution the feasability of a gradient elution was investigated. However, despite of a lot of attempts and changes of the chromatographic system, variations in the composition of the eluent all lead to an unacceptable drift of the base line of the refractive index detector. On-line mixing of i.e. water and methanol caused to much base line drift although their difference in refractive index is as low as 0.004. The use of an on-line low or high pressure mixing chamber slightly

improved the situation but results were still unacceptable. This problem is related to the use of a refractive index detector (will be discussed in the next section).

Detection

One of the aims of this research was to investigate the feasibility of performing *in vivo* studies with non-radioactive labelled PEGs by applying an HPLC method not based on radioactive detection. Due to the absence of UV-absorption of PEGs the detection was carried out with a refractometer, as had been applied by many other investigators. The limit of detection of standard solutions is dependent on the applied HPLC system and the MW of the oligomer. Applying HPLC system 2 and a mixture of 0.1 mg PEG400 and 0.1 mg PEG600 per ml water, oligomers E6 to E17 could be quantified. The limit of detection for E6 was approximately 5 μg/ml and the limit of quantitation is about 10 μg/ml. The disadvantages of refractive index detection became apparent after many different adaptations of the original HPLC set-up. Summarized, these disadvantages are:

- sensitive to changes in temperature
- sensitive to changes in pressure (and therefore in flow rate)
- sensitive to variations in the composition of the eluent
- degassing of the mobile phase is crucial
- only restricted back-pressure is allowed
- lack of sensitivity (compared to other detection systems)

Therefore, the determination of PEG oligomers with HPLC and refractive index detection is only possible with isocratic elution and in order to obtain reproducible results many factors must be kept constant.

Linearity

Calibration curves were generated by measuring the peak areas of each oligomer (E5-E19) over a 30 tot 100-fold range of polymer concentrations. Mixtures of equal amounts of PEG400 and PEG600 were used. Calibration curves were linear for all oligomers over the range 0.1 - 3 mg/ml. The calibration curves are shown in annexes 6, 7 and 8. Calibration curves of E5-E12 deviated from linearity above 3 mg PEG[400+600]/ml, although calibration curves of E13-E19 remained linear untill 10 mg PEG[400+600]/ml. These curves are depicted in annexes 9, 10 and 11. Fortunately, as will be shown in the next chapter, outflow concentrations of PEG[400+600] into the hepatic vein of the isolated perfused rat livers were lower than 3 mg PEG[400+600]/ml.

2.4 Conclusions

Due to the properties of a refractometer used on-line in an HPLC system it was not possible to apply gradient elution of PEG oligomers. The isocratic elution of a large number of oligomers (n=15) is possible but many factors like temperature, pressure, eluent composition must be kept constant. The achieved run time for one sample is approximately 45 minutes.

Calibration curves, based upon peak area, are linear over a concentration range of 0.1 to 3 mg PEG[400+600]/ml. The limit of detection for E6 was approximately 5 μg/ml and the limit of quantitation is about 10 μg/ml. Oligomers E6 to E17 could be quantified in a mixture of 0.1 mg PEG400 and 0.1 mg PEG600 per ml water A very good log-linear relationship between molecular weight and capacity factor was found on two different HPLC systems. The investigated molecular weight range varied from 282 to 898 Da. It is not clear whether this correlation indicates that the hydrophobicity of these oligomers increases as molecular weight increases.

3. RAT LIVER PERFUSION. EXPERIMENT 1

3.1 Introduction

Polyethylene glycols have been extensively used to study intestinal permeability, i.e. intestinal paracellular absorption, but no information is available on the permeability of these compounds in the liver. PEGs are mixtures of different oligomers and offer a set of molecules with different molecular weights and sizes. Especially the influence of molecular size on the permeability of these hydrophilic compounds in the rat liver is of interest for physiologically based pharmacokinetic modeling and drug development.

In order to investigate the influence of molecular size on the dispersion profile and washout kinetics of different oligomers a perfusion of an isolated rat liver was performed.

3.2 Materials

All reagents were of analytical grade. The following stock solutions were used:

Tritiated water: 25 mCi/ml

^{14}C-labelled sucrose: 0.1 mCi/ml

PEG400: 2 mCi/ml

PEG[400+600]: 5 mg PEG400 and 5 mg PEG600 per ml Krebs-Ringer buffer solution enriched with glucose.

3.3 Experimental design

An experiment was designed in which the hepatic dispersion of PEG oligomers could be studied applying a bolus of ^{14}C-labelled PEG400 and the washout kinetics of the oligomers could be investigated after an infusion with a mixture of cold PEG400 and PEG600. The infusion was carried out with cold PEGs because it would be far to expensive to use ^{14}C-labelled PEG to obtain a steady state in a single-pass isolated perfused rat liver. Two radioactive labelled reference markers were used, namely tritiated water as a water space marker and ^{14}C-labelled sucrose as an extracellular marker.

In order to evaluate the influence of the infusion with cold PEGs, the markers as well as ^{14}C-labelled PEG400 were administered as a bolus before and during the infusion with cold PEG. The lay-out of the experimental design is schematically shown in annex 12.

A male Sprague-Dawley rat weighing 357 g was used in the first liver perfusion experiment. The isolated perfused *in situ* liver preparation was the same as that described previously [13-15]. In short, under intraperitoneal anesthesia the bile duct was cannulated. After rapid cannulation and ligation of the portal vein the liver was perfused at a flow rate of 15 ml/min with Krebs-Ringer bicarbonate buffer containing glucose. Liver effluent was collected via a cannula inserted into the superior vena cava through the right atrium without interruption of portal perfusion.

After a stabilization period of 15 minutes, 50 μl of a mixture of tritiated water (0.5 μCi) and ^{14}C-labelled sucrose (0.0027 μCi) was injected as a bolus into the portal vein. An automatic turntable was activated simultaneously to collect the effluent at 1-sec intervals for 1 min. Subsequently, samples were collected at 10-sec intervals for 1 min. During this minute the rate of outflow (liver effluent) was determined. The next 3 minutes were pooled into one final sample. Then 50 μl of PEG400 (100 μCi) was injected as a bolus into the portal vein. The total effluent was again collected for 5 min in the same manner as was done for the marker mixture. Thereafter, the liver was perfused with a mixture of 5 mg PEG400 and 5 mg PEG600 per ml Krebs-Ringer buffer solution. After 10 min of infusion the same bolus of marker mixture was injected and samples were collected as before. Subsequently, the same bolus of ^{14}C-labelled PEG400 was injected and samples were collected as before. Five minutes after the last sample collection the PEG infusion was terminated and the liver was perfused with Krebs-Ringer buffer solution. Again the automatic turntable was activated simultaneously to collect the effluent at 2-sec intervals for 2 min and then at 5-sec intervals for 3 min. The total perfusion lasted 55 minutes.

Viability of the liver was assessed by its gross appearance, flow recovery and bile production. The bile was collected approximately every 20 minutes. The wet liver weight was determined immediately after the experiment.

The concentrations of the radiolabelled markers in the effluent samples were measured by a liquid scintillation counter after addition of scintillant. Determination of the oligomers of ^{14}C-labelled PEG400 was carried out by HPLC with on-line radioactivity detection (HPLC system 1). The 'cold' oligomers were determined by HPLC with on-line refractive index detection (HPLC system 2).

3.4 Results

Visual observation of the liver throughout the experiment indicated no oedema. The wet liver weight was 14.1 g: 3.9 percent of total body weight. The outflow rates of liver and bile increased during the experiment, see table 1.

The observed concentrations of cold oligomers after the infusion fell within the linear range of the calibration curve. The detection limit enabled the determination of oligomers E5 up to E18 for 3 minutes after cessation of the infusion.

Table 1. Outflow rates (ml/min) of liver and bile effluent during the first liver perfusion.

Organ	Phase	Compound	Number of observations	Period (minutes)	Flow rate ± s.d.
Liver	before infusion	$^{3}H_2O$+^{14}C-Sucrose	6	1	13.63 ± 1.0
Liver	before infusion	^{14}C-PEG400	6	1	13.93 ± 0.8
Liver	during infusion	$^{3}H_2O$+^{14}C-Sucrose	6	1	14.75 ± 0.5
Liver	during infusion	^{14}C-PEG400	6	1	14.86 ± 0.8
Bile	before infusion	N.A.	1	20	0.006
Bile	before and during infusion	N.A.	1	25	0.008
Bile	during and after infusion	N.A.	1	20	0.011

N.A. = Not Applicable

The outflow versus time profiles for the radiolabelled markers are shown in annexes 13-15. Unintentionally, a low dose of ^{14}C-labelled sucrose was used. Nevertheless, it is obvious that ^{14}C-labelled sucrose reached its maximum earlier than tritiated water and then decayed more quickly (see annex 13). There was little difference between the first part (ascending part) of the outflow curves of tritiated water before and during the PEG infusion, resulting in approximately the same t_{max} (see annex 14). After maximum was reached the elution of the second bolus was slightly slower than the first bolus of tritiated water. Surprisingly, the elution profile of the second bolus of ^{14}C-labelled sucrose was superimposed on a descending slope (see annex 15). Apparently, some ^{14}C-labelled radioactive material was still being eluted. It is not clear whether this was due to the previous bolus of ^{14}C-labelled PEG400. Although different injection needles were used for the injection of markers and PEG400 some residual PEG400 might still have been present during the second bolus of markers. After maximum was reached the elution of the second bolus was slightly slower than the first bolus of ^{14}C-labelled sucrose. The elution profiles of the different ^{14}C-labelled oligomers of PEG400 were very similar. In annex 16 the outflow curves are shown of 4 oligomers, covering a range of molecular weights of 326 (= E6) to 722 Da (= E15). There were some differences in t_{max} of each oligomer. Fitting the data using a one-compartment axial dispersion model will reveal if there is a relationship between t_{max} and molecular weight of the oligomers. The elution profile of PEG400 oligomers after the second bolus injection, during PEG[400+600] infusion, was slightly different (see annex 17). After the maximum was reached the elution of all oligomers was slightly slower than after the first bolus of ^{14}C-labelled PEG400. In general, the elution profile of the PEG oligomers resembled the outflow curves of

tritiated water, although the PEG400 oligomers were retained somewhat longer than tritiated water. However, the axial dispersion model parameters were not established due to lack of time.
After 10 minutes of infusion steady-state was reached as was indicated by analysing the levels of cold oligomers in samples taken just before, during and after the second bolus injection of the markers. After cessation of the infusion the different oligomers were washed out at approximately the same rate (see annex 18). There was a tendency that as molecular weight increased the elution rate was increased. Detailed analysis of the available data will show if there were significant differences in the different elution rates.

3.5 Conclusions

After the infusion with non-radioactive labelled PEGs the concentrations of the 'cold' oligomers could be successfully determined with the developed HPLC method (applying refractive index detection).
The outflow curves of markers and PEG oligomers obtained in the second half of the experiment (during infusion with cold PEGs) were all slightly different from those in the first part of the experiment. Because liver and bile outflow rates increased during the experiment there is serious doubt on the viability of the liver during the experiment. It is not clear whether this was due to the PEG infusion or to the deterioration of the liver.
The dispersion of PEG oligomers is quite similar to water, i.e. the oligomers do enter the liver cells, in contrast to absorption processes occuring in intestinal cells. If the PEG400 oligomers were retained somewhat longer than tritiated water this would indicate a larger volume of distribution of the PEG400 oligomers. In general, there were no clear differences in dispersion and elution of the tested oligomers.
In order to draw unequivocal conclusions these kind of experiments have to be repeated several times. Although there was only little time left to perform another experiment it was decided to carry out another liver perfusion for two important reasons. First of all, for future experimentation it is vital to know whether the infusion with a relatively high dose of cold PEGs affected the viability of the liver. Secondly, it was necessary to know whether the occurrence of residual ^{14}C radioactivity, during the second bolus injection of the markers, could be avoided in future experiments. Therefore, another liver perfusion was carried out and as many samples as possible were analysed to rule out the above-mentioned uncertainties.

4. RAT LIVER PERFUSION. EXPERIMENT 2

4.1 Introduction

The first rat liver perfusion was repeated and based upon the results of that liver perfusion, some changes were made in the experimental design. Special attention was given to overcome residual radioactivity during the experiment (see items 1 and 2) and some minor adjustments were made to allow a better interpretation of the obtained data (see items 3, 4 and 5).

1. Precautions were taken to avoid cross-contamination of markers and PEG oligomers (rinsing of injection valve and connected tubing).
2. The period between the first bolus injection of ^{14}C-labelled PEG400 and the start of the infusion with cold oligomers was increased to 10 minutes.
3. The dose of ^{14}C-labelled sucrose was increased to 0.08 μCi.
4. The automatic turntables were started *after* the markers and PEG400 were injected and *after* the infusion was started (compared to the first experiment this means a shortening of the lag time with approximately 3 seconds).
5. Outflow rates of liver effluent were determined during 3 minutes over intervals of 30 seconds instead of during 1 minute over periods of 10 seconds.

4.2 Materials

The same reagents and stock solutions were used as in the first liver perfusion.

4.3 Experimental design

The hepatic dispersion of PEG oligomers was studied applying a bolus of ^{14}C-labelled PEG400 and after an infusion with a mixture of cold PEG400 and PEG600 the washout kinetics of the oligomers were investigated. Tritiated water and ^{14}C-labelled sucrose were used as radioactive labelled reference markers.

In order to evaluate the influence of the infusion with PEGs the markers as well as ^{14}C-labelled PEG400 were administered as a bolus before and during the infusion with cold PEG. Compared to the first experiment, the washout of the first bolus of ^{14}C-labelled PEG400 was prolonged with 5 minutes and the total duration of the second perfusion was 60 minutes. The lay-out of the experimental design is schematically shown in annex 12.

A male Sprague-Dawley rat weighing 298 g was used in the second liver perfusion experiment. The *in situ* liver preparation was the same as in the first experiment. After a stabilization period of 15 minutes, 50 μl of a mixture of tritiated water (0.5 μCi) and ^{14}C-labelled sucrose (0.08 μCi) was injected as a bolus into the portal vein. An automatic turntable was activated immediately after the mixture was injected to collect the effluent at 1-sec intervals for 1 min. Subsequently, samples were collected at 10-sec intervals for 1 min and then at 30-sec intervals for a further 3 minutes. During the last 3 minutes the rate of outflow (liver effluent) was determined. Then 50 μl of PEG400 (100 μCi) was injected as a bolus into the

portal vein. The total effluent was again collected for 5 min in the same manner as was done for the marker mixture. Thereafter, the liver was perfused with buffer solution for another 5 minutes before the liver was perfused with a mixture of 5 mg PEG400 and 5 mg PEG600 per ml Krebs-Ringer buffer solution. After 10 min of infusion the same bolus of marker mixture was injected and samples were collected as before. Subsequently, the same bolus of ^{14}C-labelled PEG400 was injected and samples were collected as before. Five minutes after the last sample collection the PEG infusion was terminated and the liver was perfused with Krebs-Ringer buffer solution. The automatic turntable was activated immediately after the buffer solution was switched to collect the effluent at 2-sec intervals for 2 min and then at 5-sec intervals for 3 min. Now, the total perfusion lasted 60 minutes.
Viability of the liver was assessed by its gross appearance, flow recovery and bile production. The bile was collected approximately every 20 minutes. The wet liver weight was determined immediately after the experiment.
The concentrations of the radiolabelled markers in the effluent samples were measured by a liquid scintillation counter after addition of scintillant. Determination of the oligomers of ^{14}C-labelled PEG400 was carried out by HPLC with on-line radioactivity detection (HPLC system 1). The 'cold' oligomers were determined by HPLC with on-line refractive index detection (HPLC system 2).

4.4 Results

Visual inspection of the liver during the experiment indicated no oedema. The wet liver weight was 13.7 g: 4.6 percent of total body weight. The outflow rates of liver and bile were constant during the experiment, see table 2.

Table 2. Outflow rates (ml/min) of liver and bile effluent during the second liver perfusion.

Organ	Phase	Compound	Number of observations	Period (minutes)	Flow rate ± s.d.
Liver	before infusion	$^{3}H_2O$+^{14}C-Sucrose	6	3	13.86 ± 0.3
Liver	before infusion	^{14}C-PEG400	6	3	13.83 ± 0.2
Liver	during infusion	$^{3}H_2O$+^{14}C-Sucrose	6	3	13.96 ± 0.2
Liver	during infusion	^{14}C-PEG400	6	3	13.92 ± 0.3
Bile	before infusion	N.A.	1	20	0.006
Bile	before and during infusion	N.A.	1	25	0.006
Bile	during and after infusion	N.A.	1	25	0.007

N.A. = Not Applicable

The outflow versus time profiles for the radiolabelled markers are shown in annexes 19-21. The adjustment of the dose of ^{14}C-labelled sucrose allowed a better interpretation of the obtained data. Clearly, ^{14}C-labelled sucrose reached its maximum earlier than tritiated water and subsequently decayed more quickly (see annex 19). Except for a difference in lag time there was no significant difference in the outflow of tritiated water before and during the infusion (see annex 20). The outflow of ^{14}C-labelled sucrose was also not affected by the infusion with a relatively high dose of cold PEG oligomers (see annex 21). As intended, the lag time was shortened for both markers with a few seconds compared to the first liver perfusion. Apparently, the problem with the residual radioactivity, encountered in the first experiment, was avoided by the precautionary measures. Unfortunately, there was no time left to analyse the samples of the elution profiles of the different ^{14}C-labelled oligomers of PEG400.

Steady-state was reached after 10 minutes of infusion as was indicated by analysing the levels of cold oligomers in samples taken just before, during and after the second bolus injection of the markers. After cessation of the infusion the different oligomers were washed out at approximately the same rate (see annex 22). Detailed analysis of the available data will show if there were significant differences in the different elution rates.

4.5 Conclusions

The outflow curves of the applied markers obtained in the second half of the experiment (during infusion with cold PEGs) were not significantly different from those in the first part of the experiment. Liver and bile outflow rates were constant during this experiment and there were no indications that deterioration of the liver had occurred during this experiment. Therefore, it can be concluded that the infusion with a relatively high dose of cold PEGs did not affect the viability of the liver. This is an important prerequisite for the design of future experiments.

No residual radioactivity was observed during the experiment after taking the necessary precautions to avoid cross-contamination of markers and PEG oligomers and increasing the washout period of the first bolus injection of ^{14}C-labelled PEG400.

In general, there were no striking differences in the washout kinetics of the tested oligomers after infusion with a mixture of PEG400 and PEG600.

5. GENERAL CONCLUSIONS AND DISCUSSION

Determination of PEG oligomers with HPLC

One of the aims of this research was to investigate the feasibility of performing *in vivo* studies with non-radioactive labelled PEGs by applying an HPLC method not based on radioactive detection. Due to the absence of UV-absorption of PEGs the detection was carried out with a refractometer. Fifteen oligomers (E6-E19) could be studied simultaneously in the *in situ* rat liver perfusions by the application of a mixture of PEG400 and PEG600. Therefore, an HPLC method was developed successfully to analyse 15 compounds in one run within an acceptable run time. Despite of a lot of attempts, gradient elution of PEG oligomers was not possible due to the properties of a refractometer when used on-line in an HPLC system. Calibration curves, based upon peak area, are linear over a concentration range of 0.1 to 3 mg PEG[400+600]/ml. The limit of detection for E6 was approximately 5 μg/ml and the limit of quantitation is about 10 μg/ml. Oligomers E6 to E17 could be quantified in a mixture of 0.1 mg PEG400 and 0.1 mg PEG600 per ml water. A very good log-linear relationship between molecular weight and capacity factor was found on two different HPLC systems. The investigated molecular weight range varied from 282 to 898 Da. It is not clear whether this correlation indicates that the hydrophobicity of these oligomers increases as molecular weight increases.

Rat liver perfusions

Another aim was to study the influence of molecular size on the permeability of different oligomers of PEG in the isolated perfused rat liver. It was concluded that the infusion with a relatively high dose of cold PEGs did not affect the viability of the liver. Therefore, *in situ* rat liver perfusions can be designed in which bolus injections of ^{14}C-labelled PEG can be combined with an infusion of cold PEG. The dispersion of PEG oligomers is quite similar to water, i.e. the oligomers enter the liver cells and distribute and elute in a similar manner as water. There were no clear differences in dispersion and elution between the different oligomers tested. Detailed kinetic and statistical analysis of the data will have to be performed to discriminate significant differences between oligomers and to reveal relationships between molecular weight and dispersion or disposition. However, the investigated range of molecular weights, from 282 to 810 Da, might be too small to detect any significant differences in dispersion and disposition.

Future research

This research has led to a better understanding of kinetic processes occuring in the rat liver, especially the influence of molecular size on the dispersion profile and washout kinetics of PEG oligomers. This kind of fundamental research leads to an improved knowledge of the variations in transit time of compounds on passage through a tissue, i.c. the liver. Extension and refinement of the physiologically

based pharmacokinetic models will strengthen the predictive power of these models, in particular with respect to identifying target site(s) of drugs. Future research, carried out at RIKILT-DLO, will concentrate on PB-PK models and its application for describing and predicting the kinetic behaviour of biologically active (non-) nutrients and drugs in man and food-producing animals. This research has lead to a better understanding of essential aspects of PB-PK modeling and I am thankful to those who made this possible: the Netherlands Organisation for Scientific Research (NWO), in collaboration with the British Council, and prof. dr. Rowland from the Manchester University in the UK.

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REFERENCES

1. Martindale, The Extra Pharmacopoeia, 28th edition, Eds. J.E.F. Reynolds and A.B. Prasad, London, The Pharmaceutical Press, 1982, pp 709-711.

2. Ukabam, SO and Cooper, BT. 1984. Small intestinal permeability to mannitol, lactulose, and polyethylene glycol 400 in celiac disease. *Digestive Diseases and Sciences*, **29**, 809-816.

3. Philipsen, EK, Batsberg, W, and Christensen, AB. 1988. Gastroinstestinal permeability to polyethylene glycol: an evaluation of urinary recovery of an oral load of polyethylene glycol as a parameter of intestinal permeability in man. *European Journal of Clinical Investigation*, **18**, 139-145.

4. Ma, TY, Hollander D, Krugliak P, and Katz, K. 1990. PEG 400, a hydrophilic molecular probe for measuring intestinal permeability. *Gastroenterology*, **98**, 39-46.

5. Ruddy, SB, and Hadzija, BW. 1994. High-performance liquid chromatographic method for the simultaneous determination of low-molecular-mass oligomers of polyethylene glycol in aqueous skin extracts. *Journal of Chromatography B*, **657**, 83-92.

6. OECD-OCDE. 1981. Partition Coefficient (n-octanol/water), Flask-shaking method. *OECD guideline for testing of chemicals*, no. 107.

7. Young, GO, Ruttenberg, D, and Wright, JP. 1990. Measurement of polyethylene glycol 400 in urine by direct-injection high-performance liquid chromatography. *Clinical Chemistry*, **36**, 1800-1802.

8. Oliva, A, Armas, H, and Farina JB. 1994. HPLC determination of polyethylene glycol 400 in urine: oligomeric profile in healthy and celiac disease subjects. *Clinical Chemistry*, **40**, 1571-1574.

9. Ryan CM, Yarmush ML, and Tompkins RG. 1992. Separation and quantitation of polyethylene glycols 400 and 3350 from human urine by high-performance liquid chromatography. *Journal of Pharmaceutical Sciences*, **81**, 350-352.

10. OECD-OCDE. 1989. Partition coefficient (n-octanol/water), High Performance Liquid Chromatography (HPLC) Method. *OECD guideline for testing of chemicals*, no. 117.

11. Hafkenscheid TL, and Tomlinson E. 1986. Estimation of physicochemical properties of organic solutes using HPLC retention parameters. *Advances in Chromatography*, **25**, 1-62.

12. Terada, H. 1986. Determination of log P_{oct} by high-performance liquid chromatography, and its application in the study of quantitative structure-activity relationships, *Quantitative Structure-Activity Relationships*, **5**, 81-88.

13. Hussein Z, and Rowland M. 1992. Distribution of antipyrine in the rat liver. *Journal of Pharmacy and Pharmacology*, **44**, 766-768.

14. Chou C-H, Evans AM, Fornasini G, and Rowland M. 1993. Relationship between lipophilicity and hepatic dispersion and distribution for a homologous series of barbiturates in the isolated perfused *in situ* rat liver. *Drug Metabolism and Disposition*, **21**, 933-938.

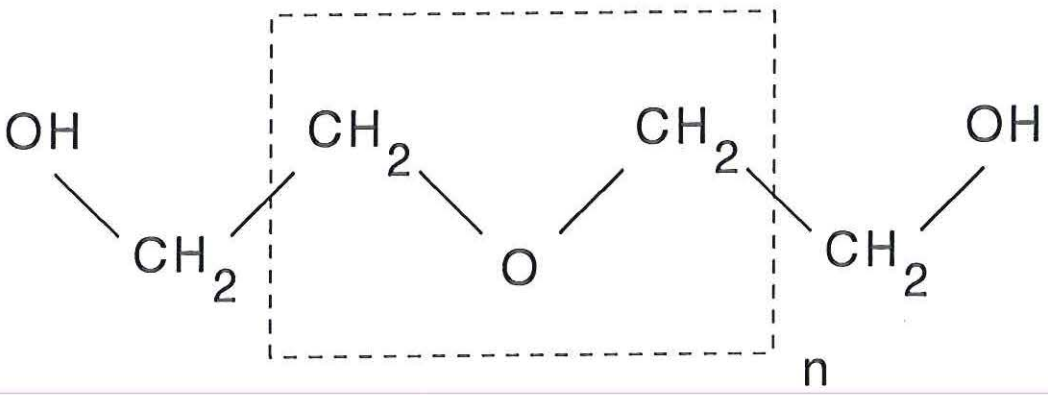
15. Chou C-H, McLachlan AJ, and Rowland M. 1995. Membrane permeability and lipophilicity in the isolated perfused rat liver: 5-ethyl barbituric acid and other compounds. *Journal of Pharmacology and Experimental Therapeutics*, **275**, 933-940.

ANNEXES

LIST OF ANNEXES

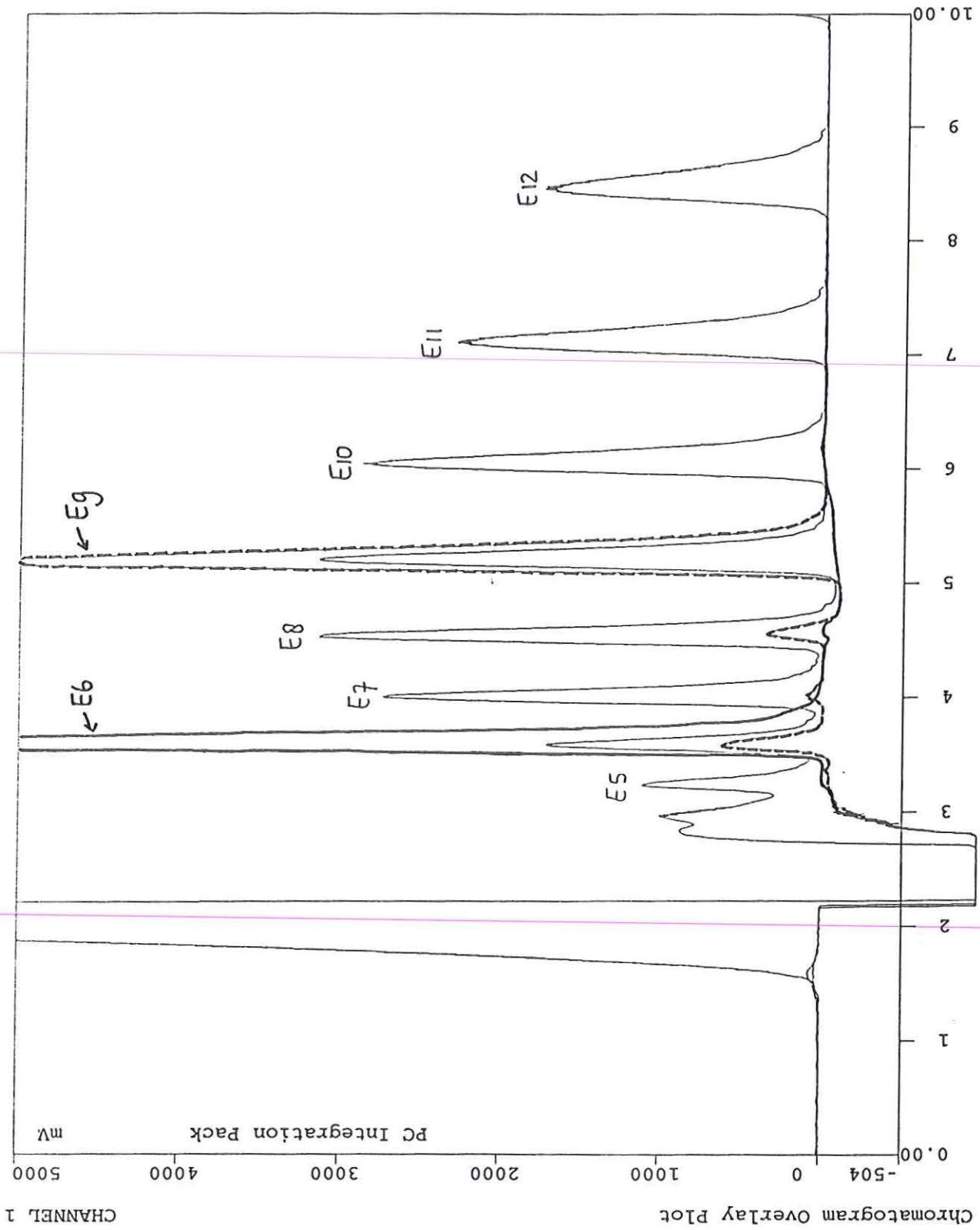
ANNEX 1. Molecular structures and weights of tested PEG oligomers.

Structure and molecular weight of tested PEG oligomers

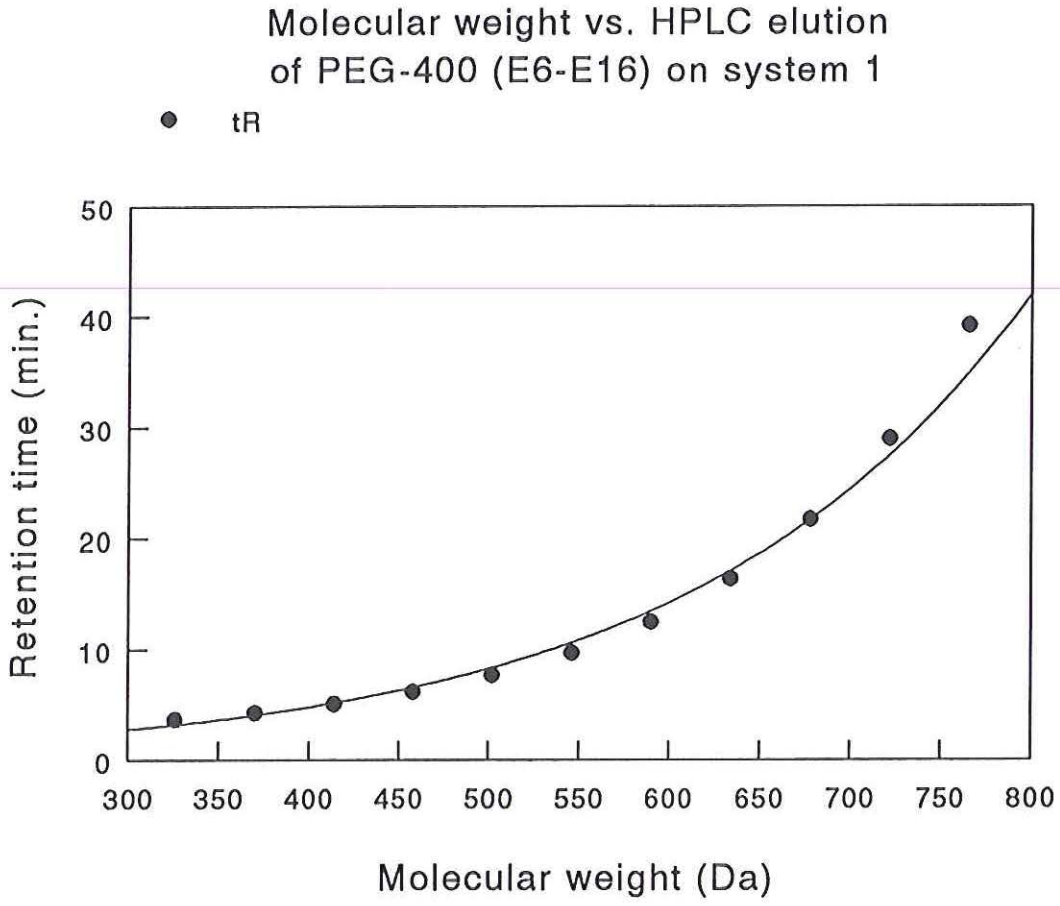


Compound	Oligomer	MW
PEG400	E5	282
	E6	326
	E7	370
	E8	414
	:	:
	E15	722
	E16	766
PEG600	E17	810
	E18	854
	E19	898

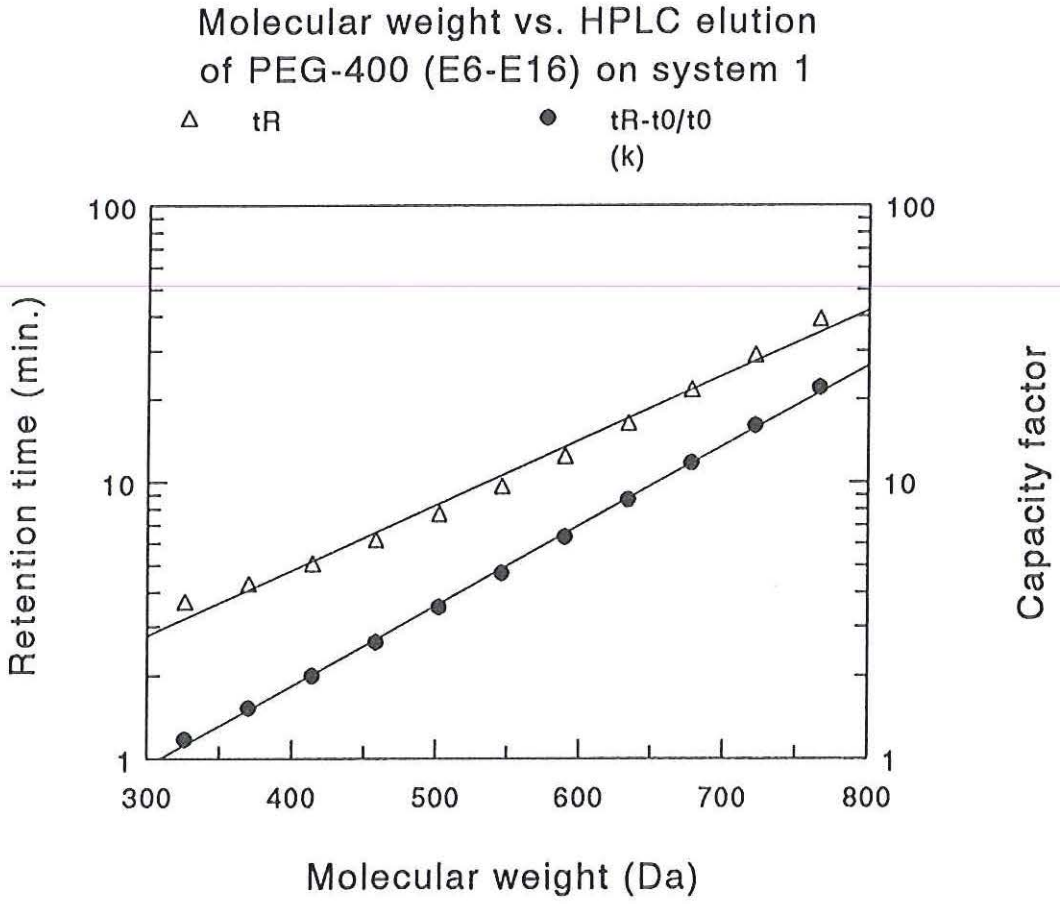
ANNEX 2. Overlay plot of chromatograms of standard solutions of PEG400 and purified E6 and E9.



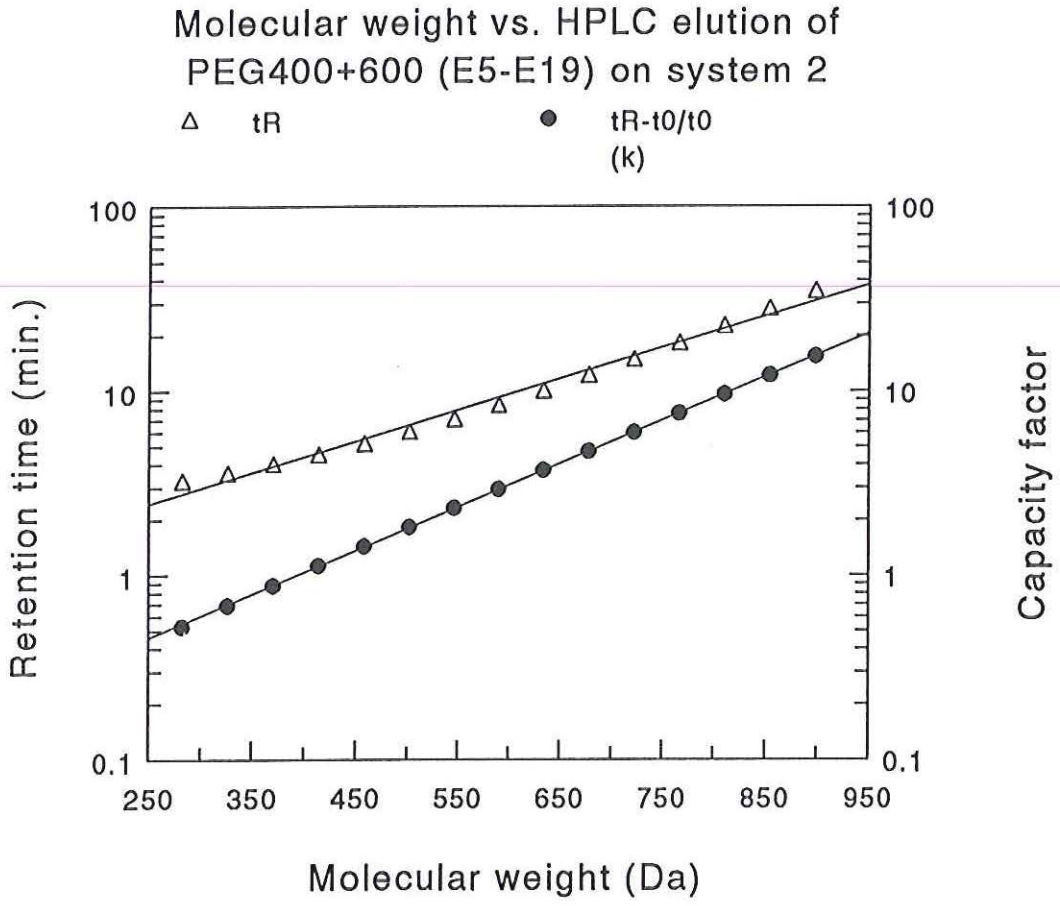
ANNEX 3. Relationship between molecular weight and retention time of oligomers of PEG400 (E6-E16) on HPLC system 1.



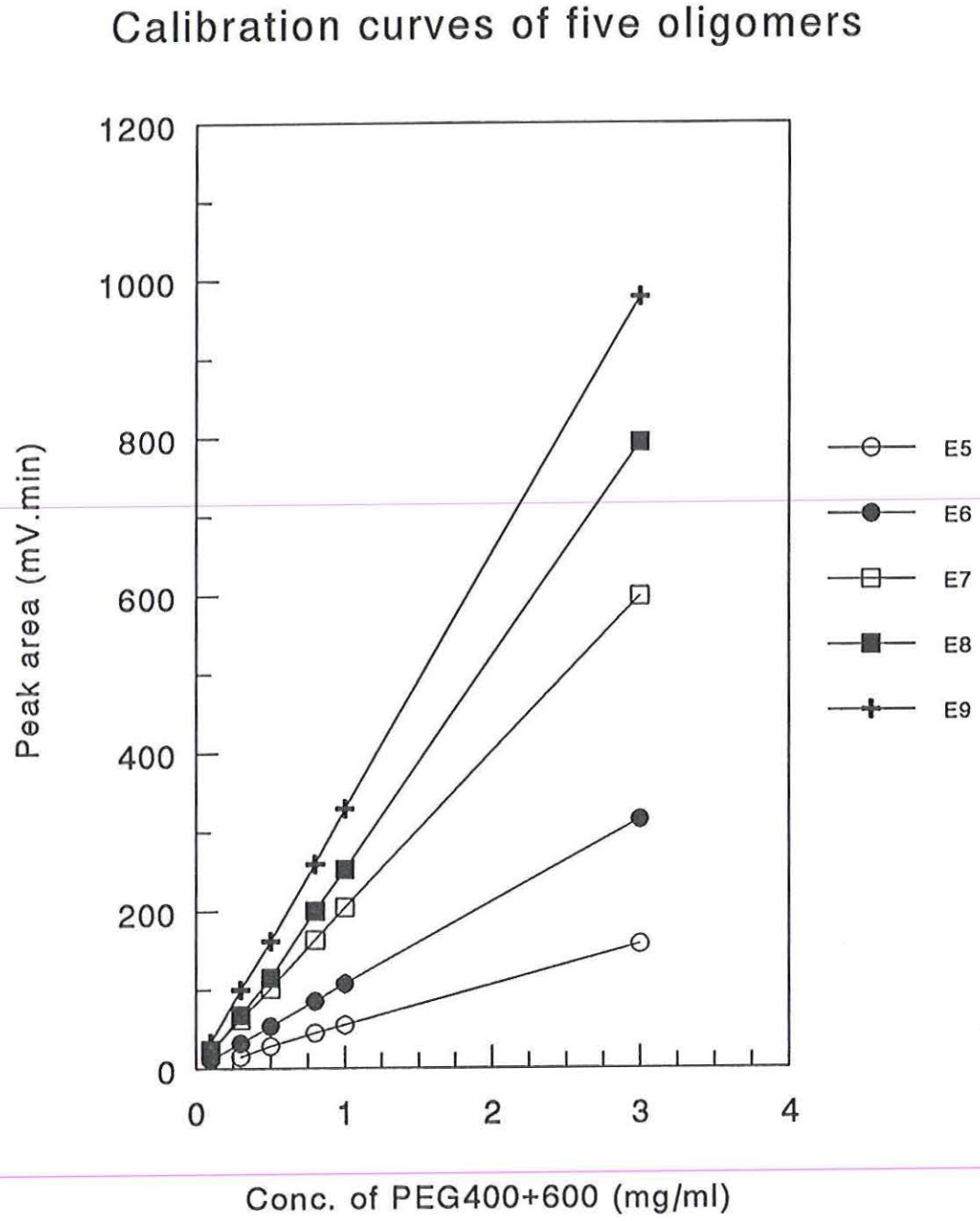
ANNEX 4. Log-linear relationship between molecular weight and capacity factors of oligomers of PEG400 (E6-E16) on HPLC system 1.



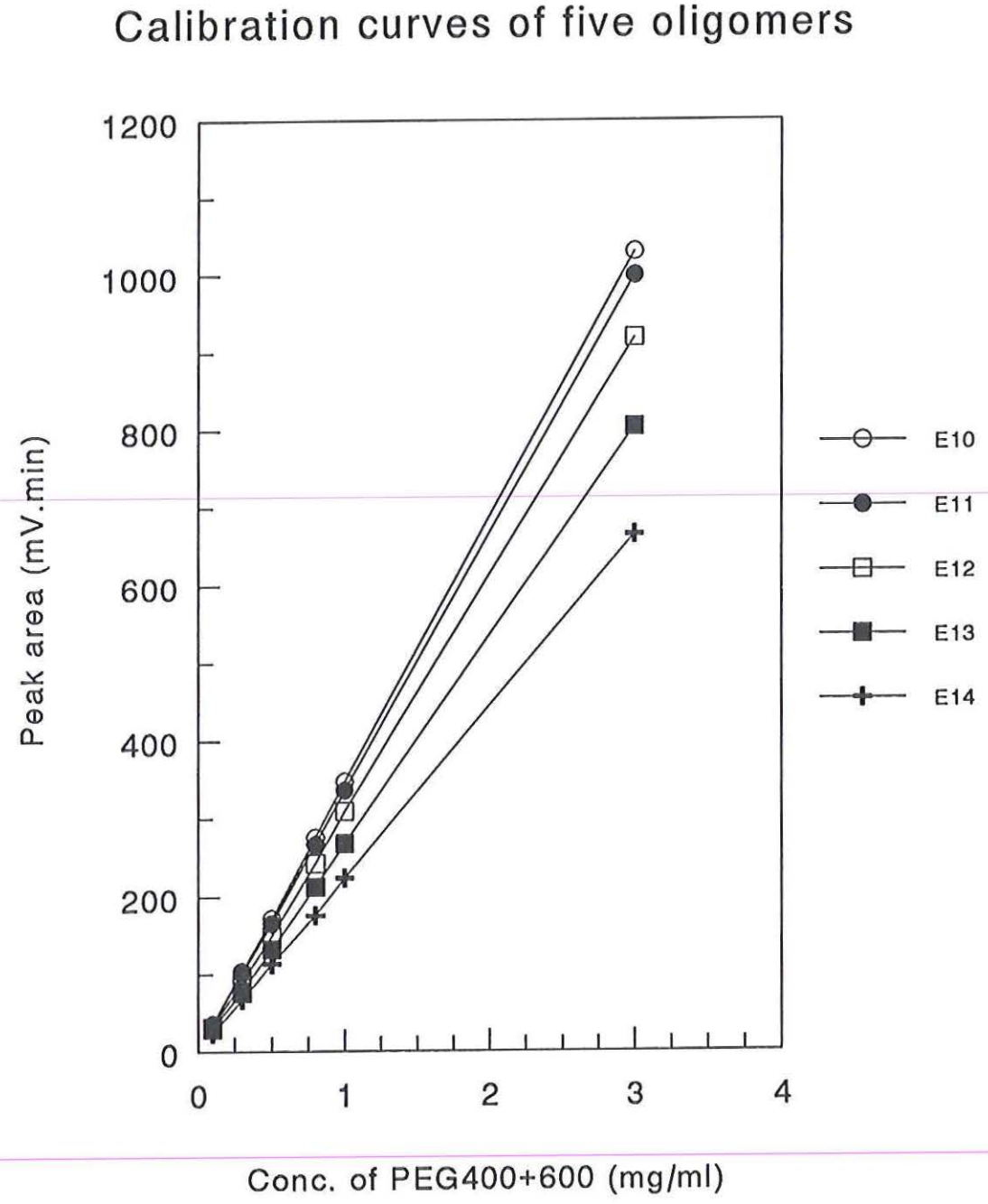
ANNEX 5. Log-linear relationship between molecular weight and capacity factors of oligomers of a mixture of PEG400 and PEG600 (E5-E19) on HPLC system 2.



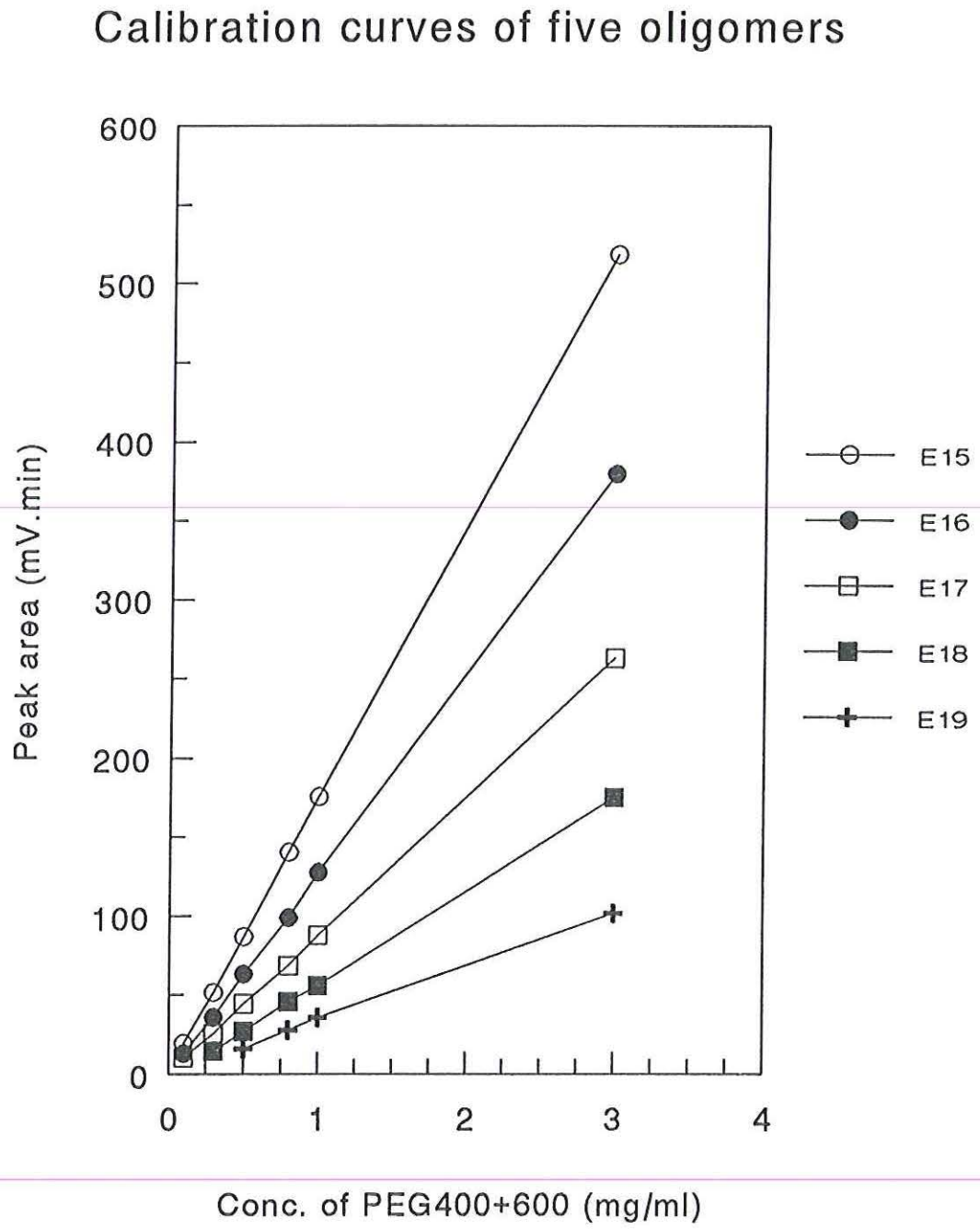
ANNEX 6. Calibration curves for oligomers E5-E9 of a mixture of PEG400 and PEG600 in the range of 0.1 - 3 mg PEG[400+600] mg/ml.



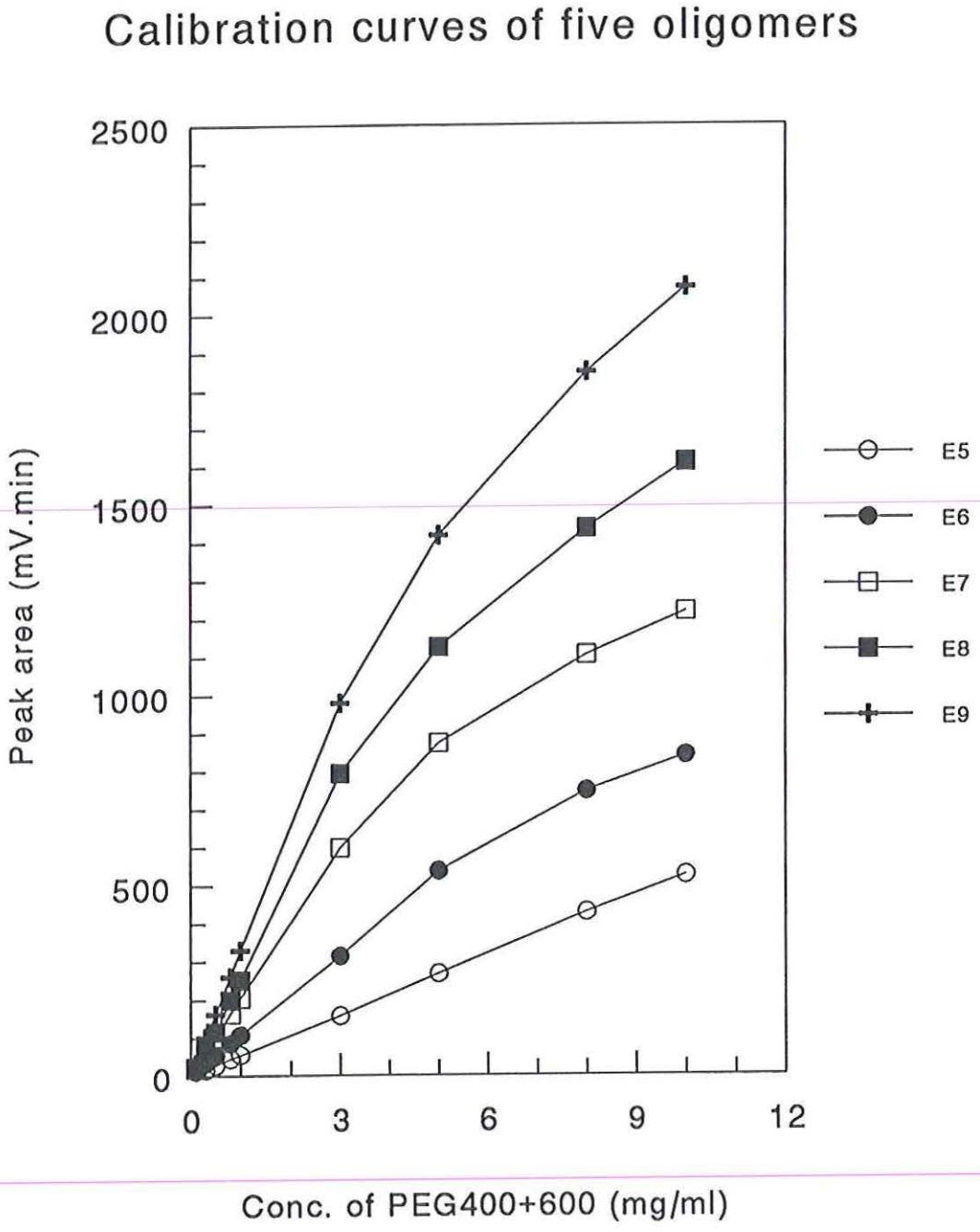
ANNEX 7. Calibration curves for oligomers E10-E14 of a mixture of PEG400 and PEG600 in the range of 0.1 - 3 mg PEG[400+600] mg/ml.



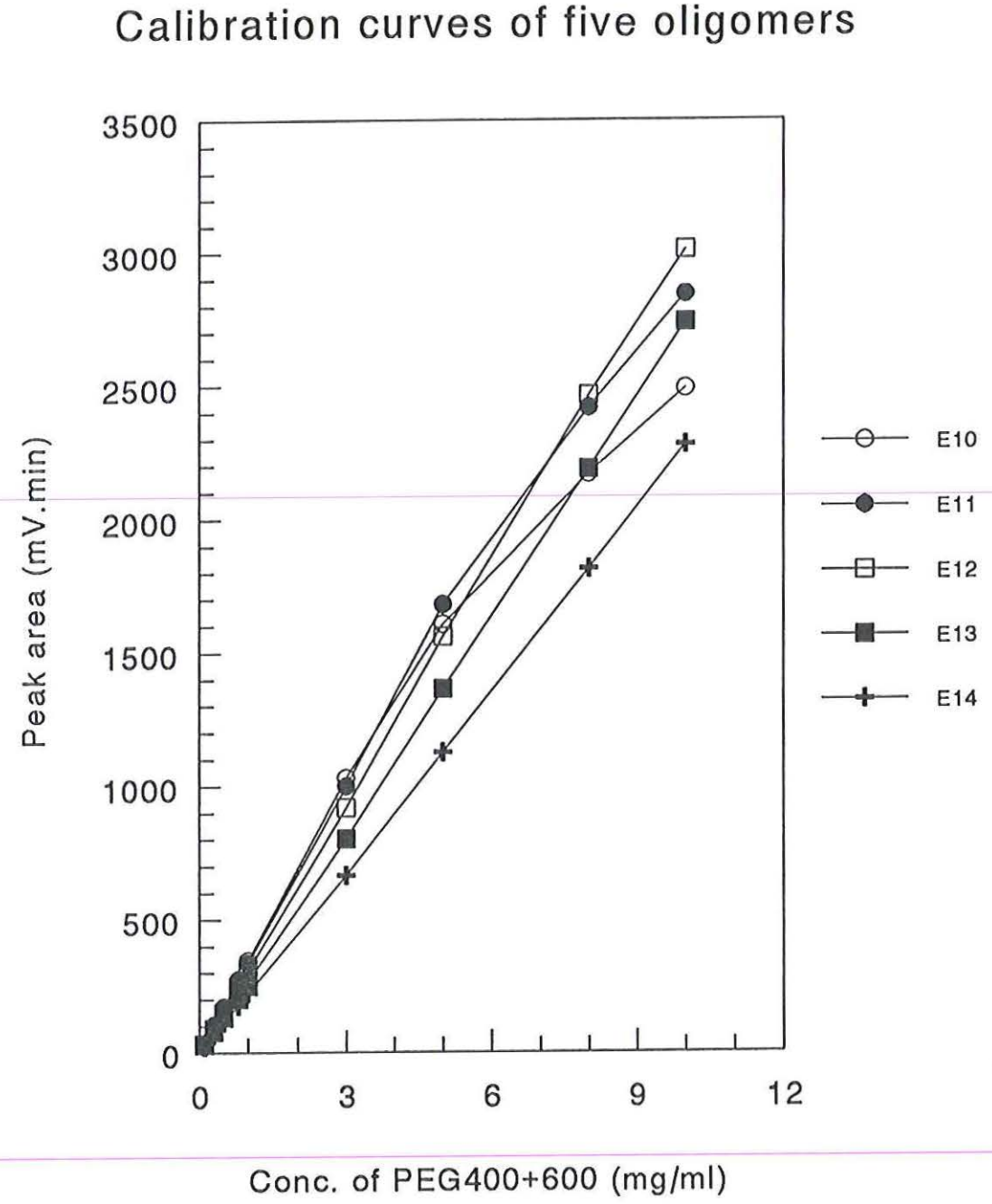
ANNEX 8. Calibration curves for oligomers E15-E19 of a mixture of PEG400 and PEG600 in the range of 0.1 - 3 mg PEG[400+600] mg/ml.



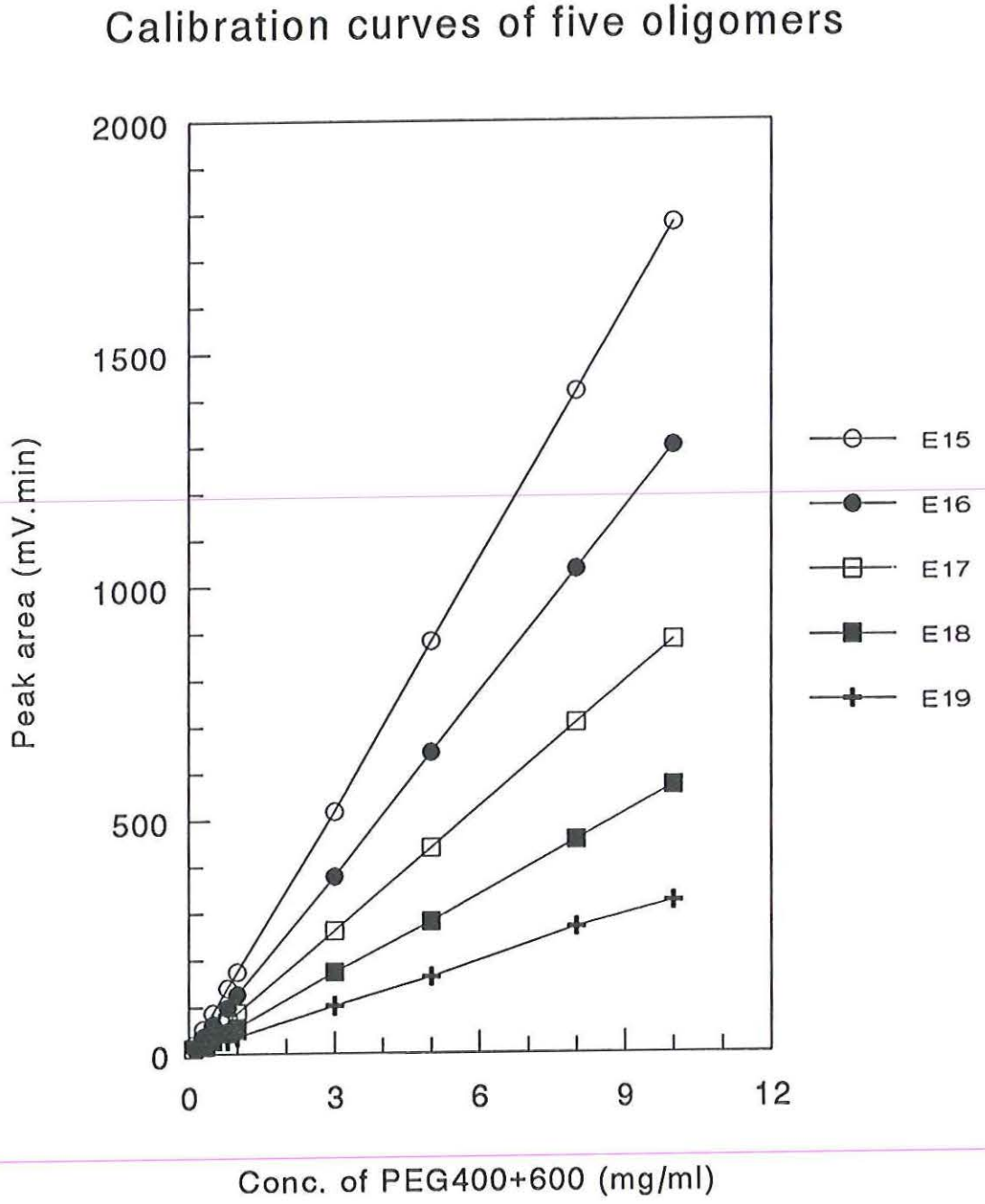
ANNEX 9. Calibration curves for oligomers E5-E9 of a mixture of PEG400 and PEG600 in the range of 0.1 - 10 mg PEG[400+600] mg/ml.



ANNEX 10. Calibration curves for oligomers E10-E14 of a mixture of PEG400 and PEG600 in the range of 0.1 - 10 mg PEG[400+600] mg/ml.



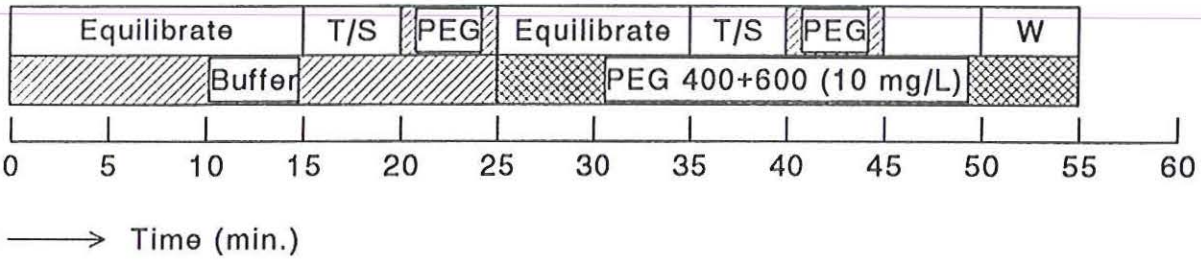
ANNEX 11. Calibration curves for oligomers E15-E19 of a mixture of PEG400 and PEG600 in the range of 0.1 - 10 mg PEG[400+600] mg/ml.



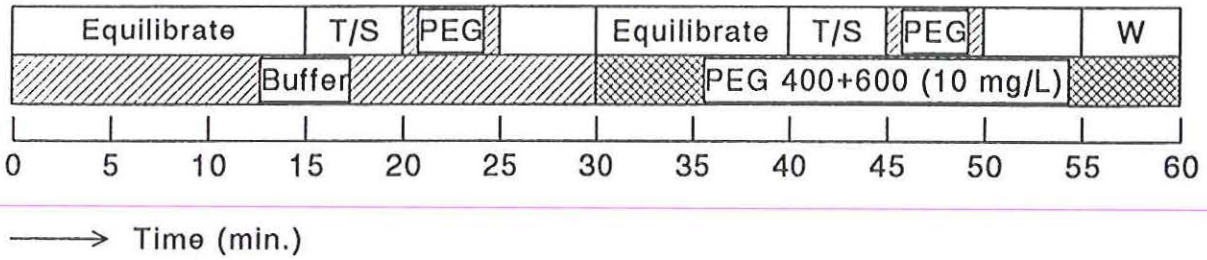
ANNEX 12. Lay-out of the in situ rat liver perfusions using a bolus of ^{14}C-labelled PEG400 combined with an infusion of a mixture of 'cold' PEG400 and PEG600.

Lay-out of in situ rat liver perfusion using a bolus of 14C-labelled PEG400 combined with an infusion of cold PEG400+600

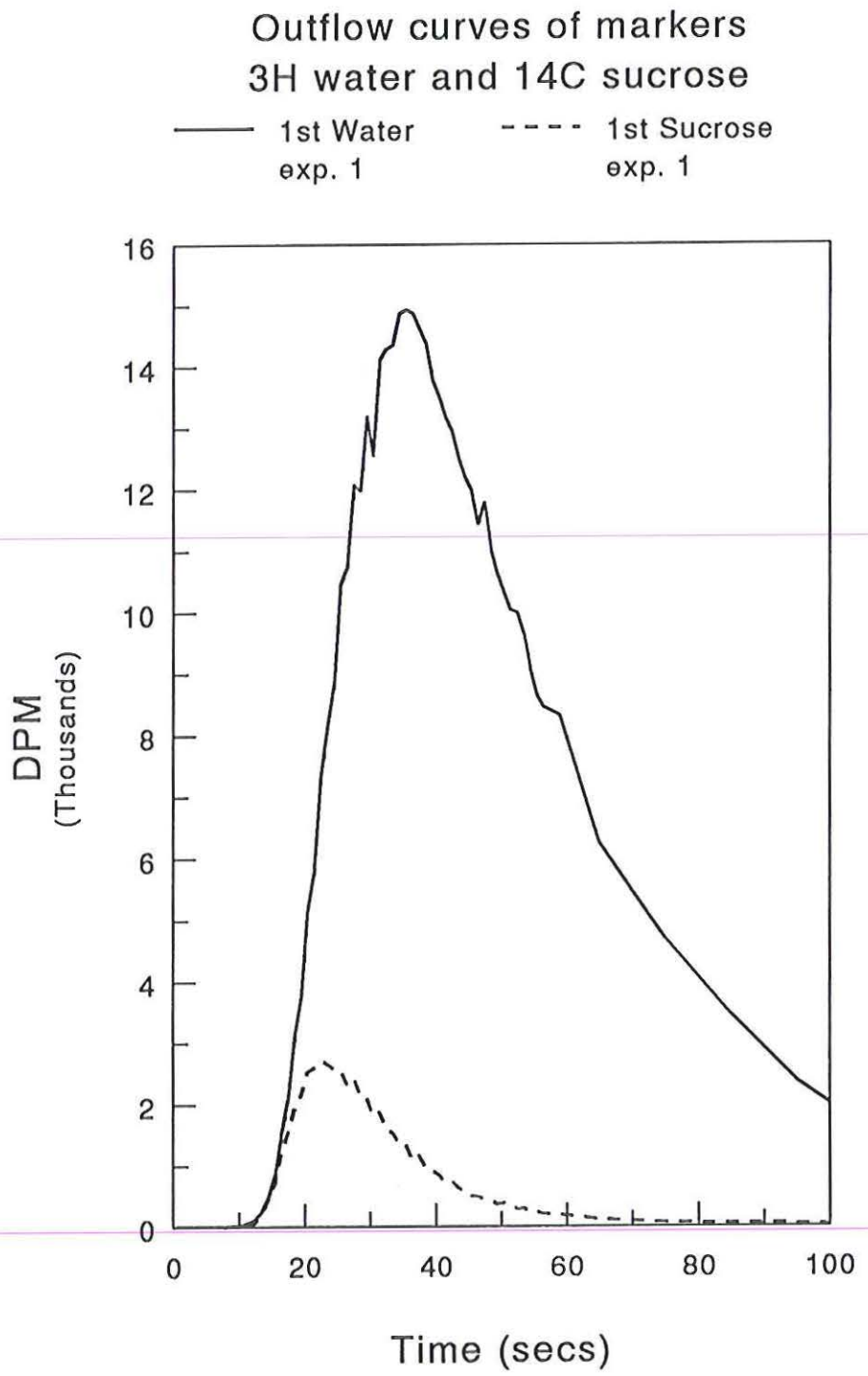
1st experiment:



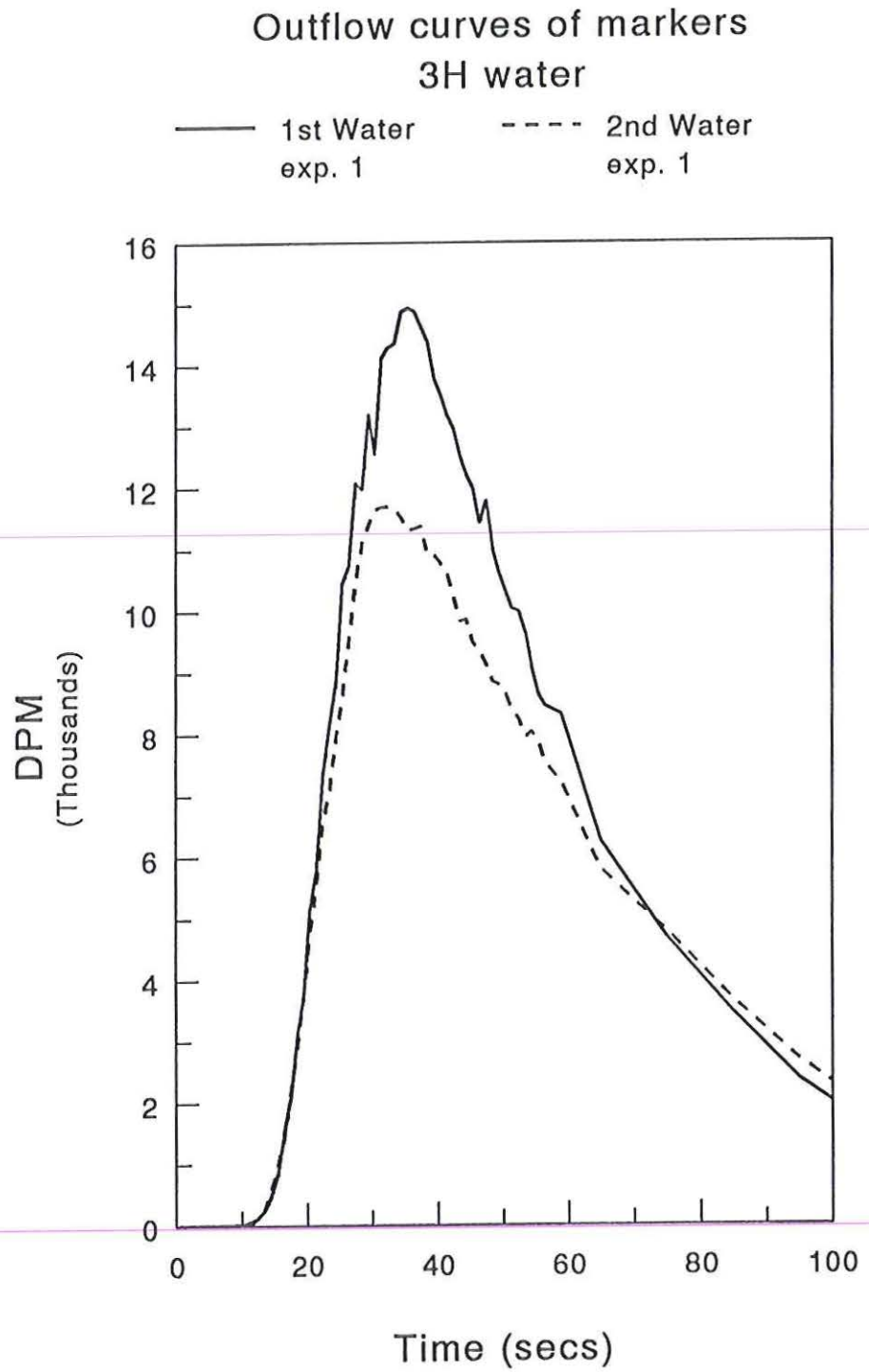
2nd experiment:



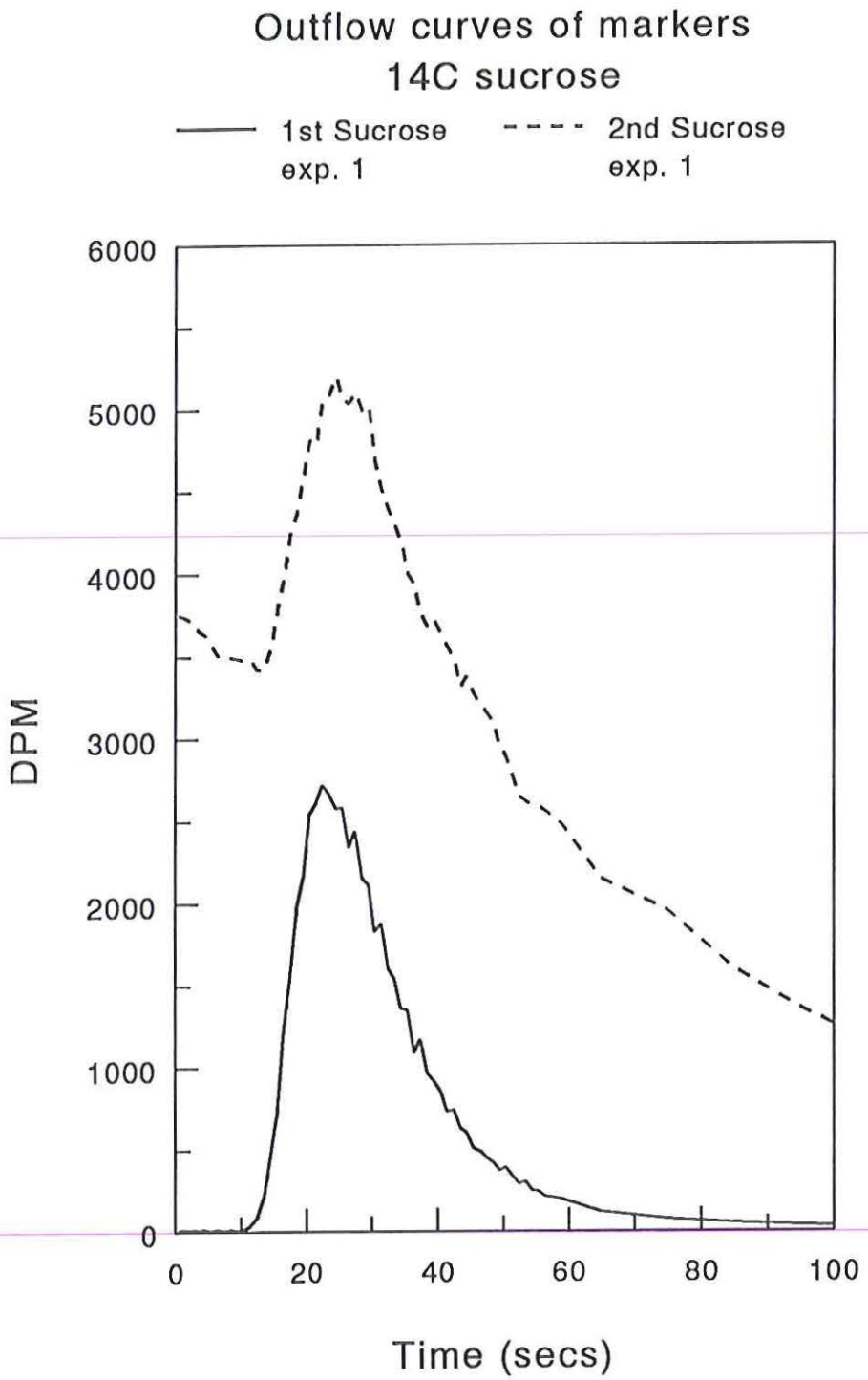
ANNEX 13. Outflow curves of the first bolus of tritiated water and ^{14}C-labelled sucrose in the first rat liver perfusion.



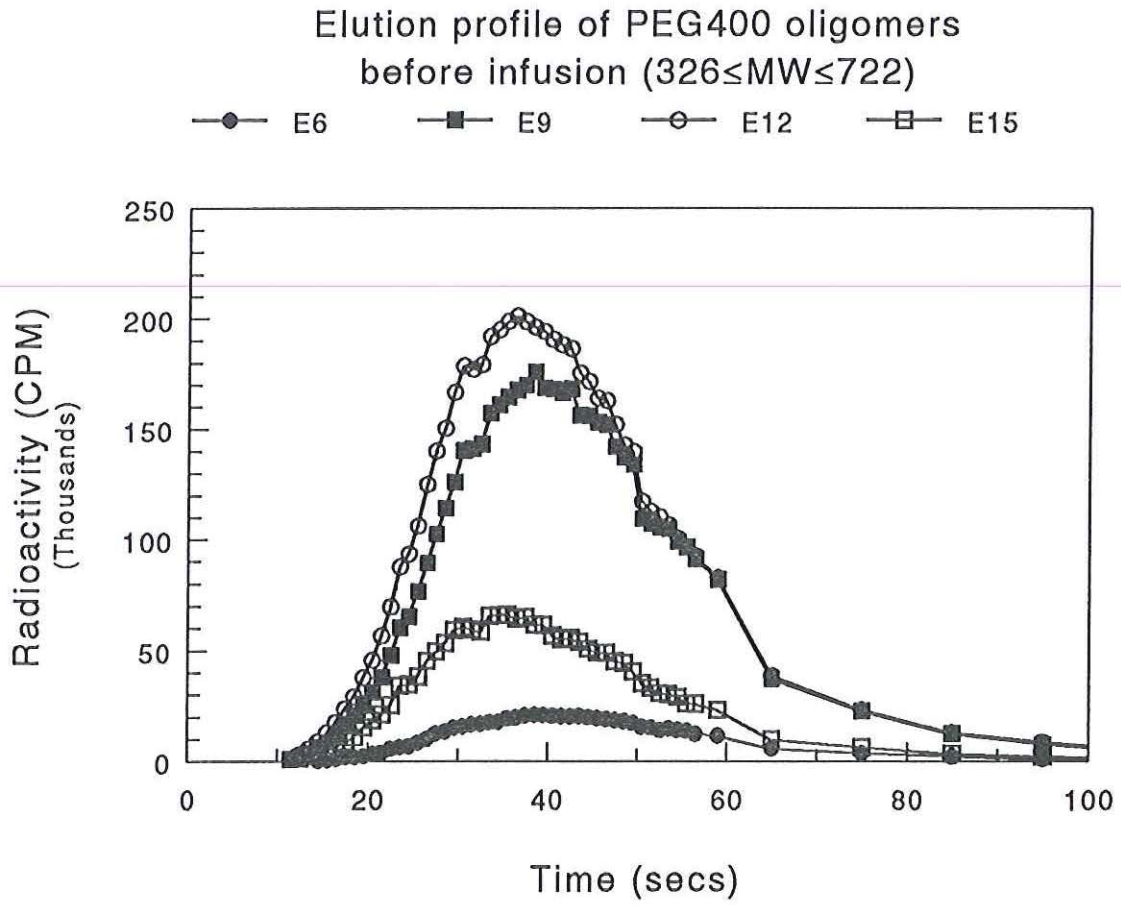
ANNEX 14. Outflow curves of the first and second bolus of tritiated water in the first rat liver perfusion.



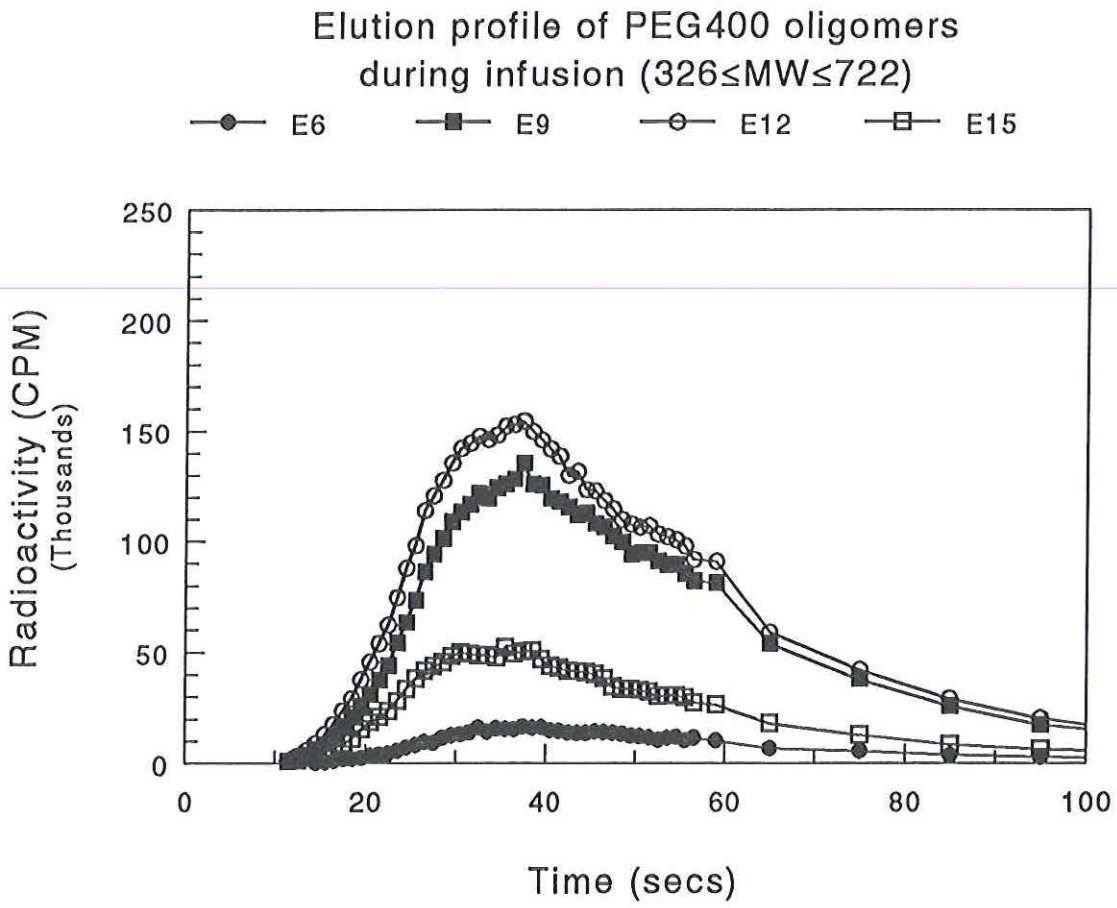
ANNEX 15. Outflow curves of the first and second bolus of ^{14}C-labelled sucrose in the first rat liver perfusion.



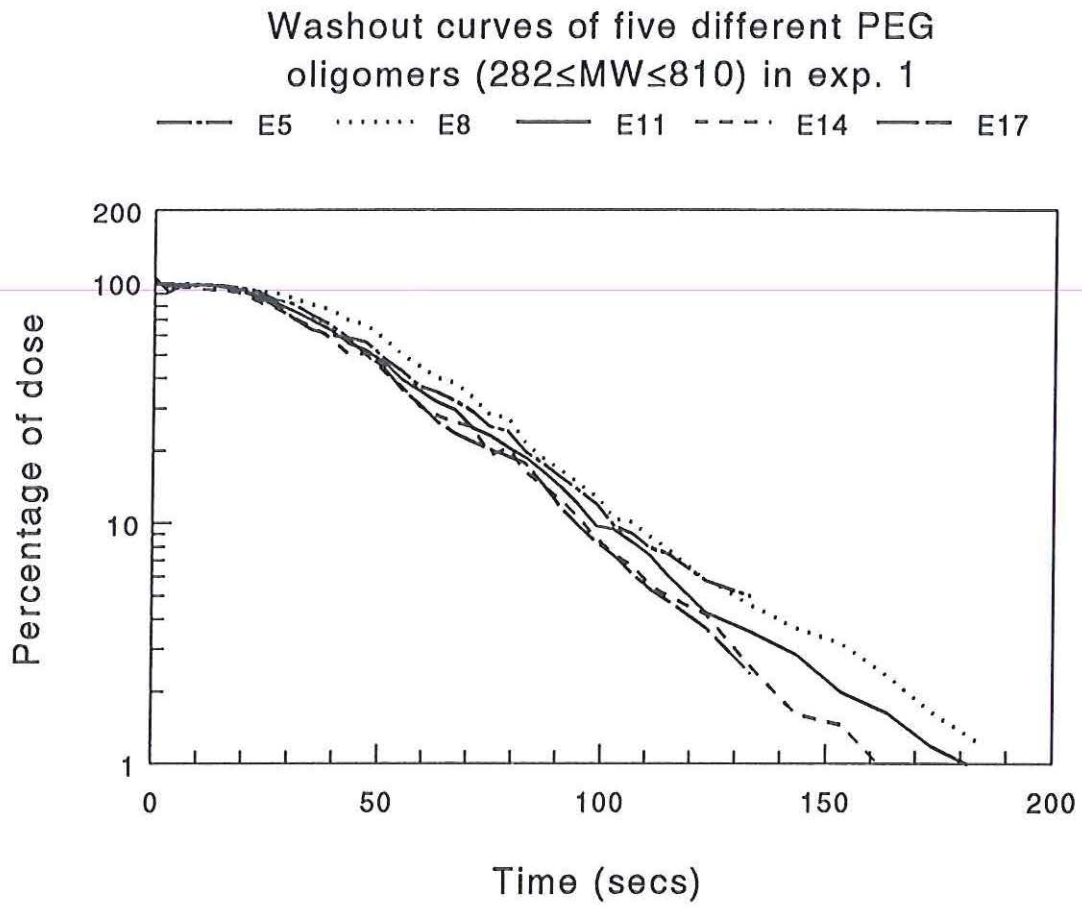
ANNEX 16. Elution profile of four different oligomers after the first bolus of ^{14}C-labelled PEG400 in the first rat liver perfusion.



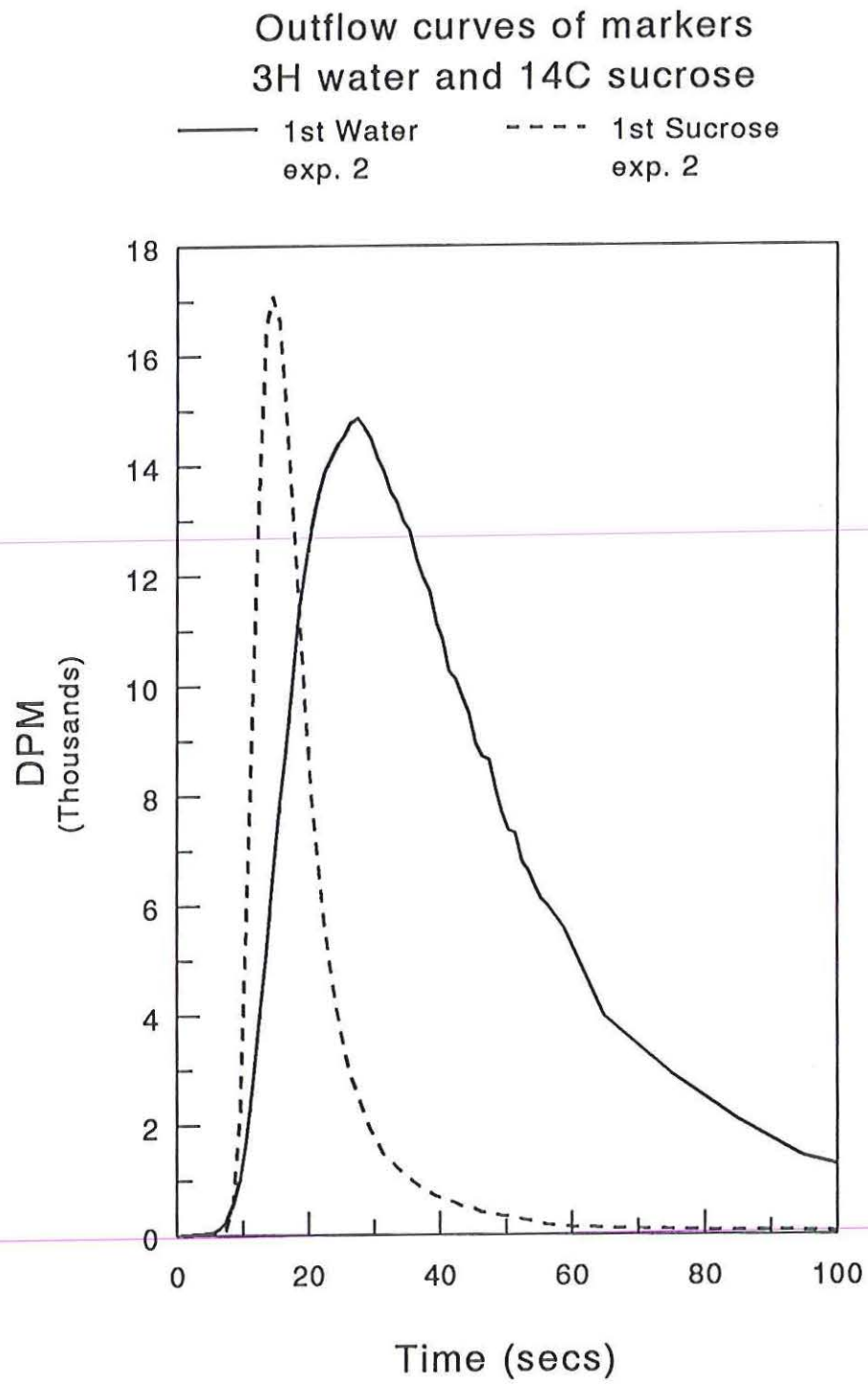
ANNEX 17. Elution profile of four different oligomers after the second bolus of ^{14}C-labelled PEG400 in the first rat liver perfusion.



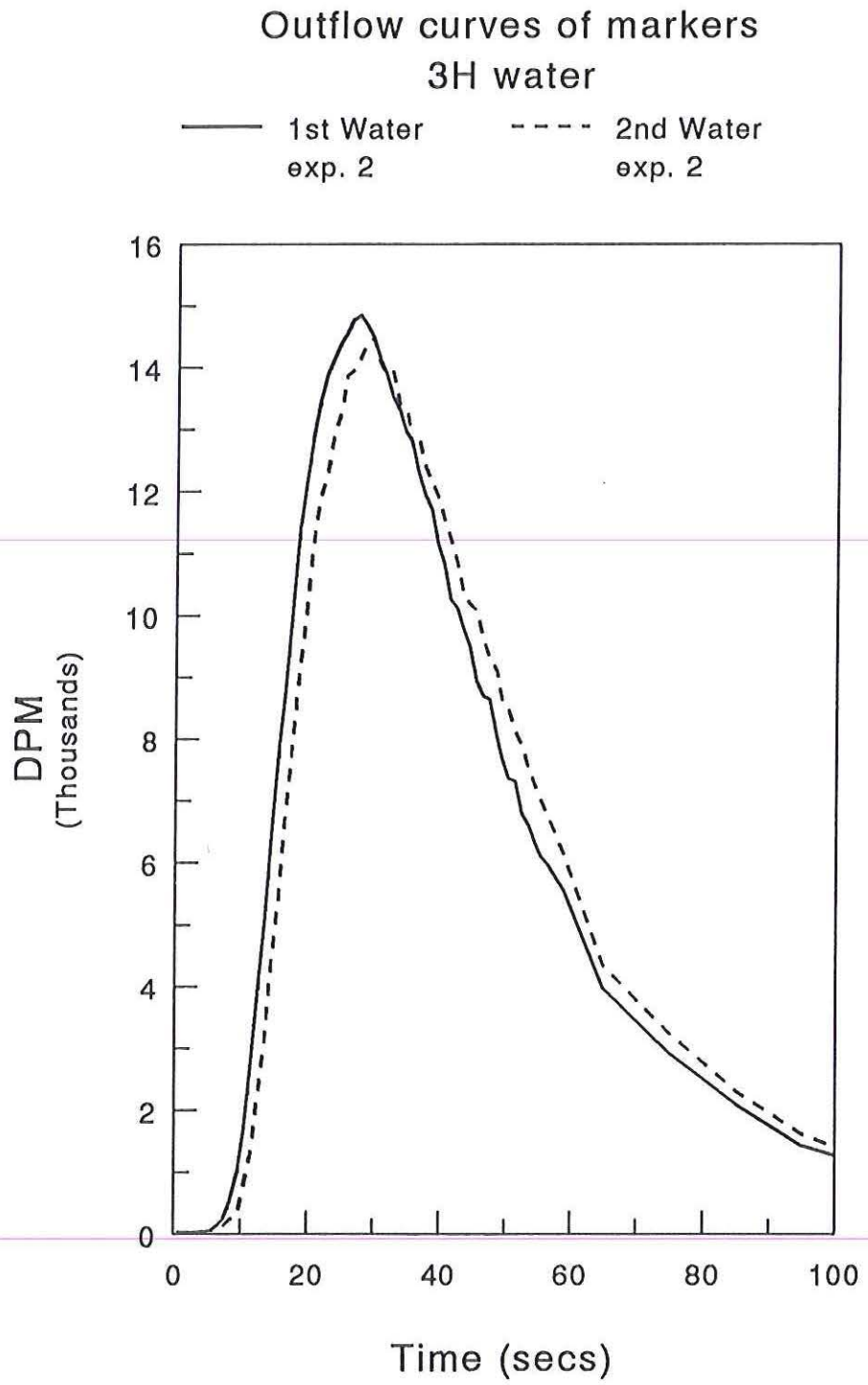
ANNEX 18. Washout curves of five different oligomers in the first rat liver perfusion.



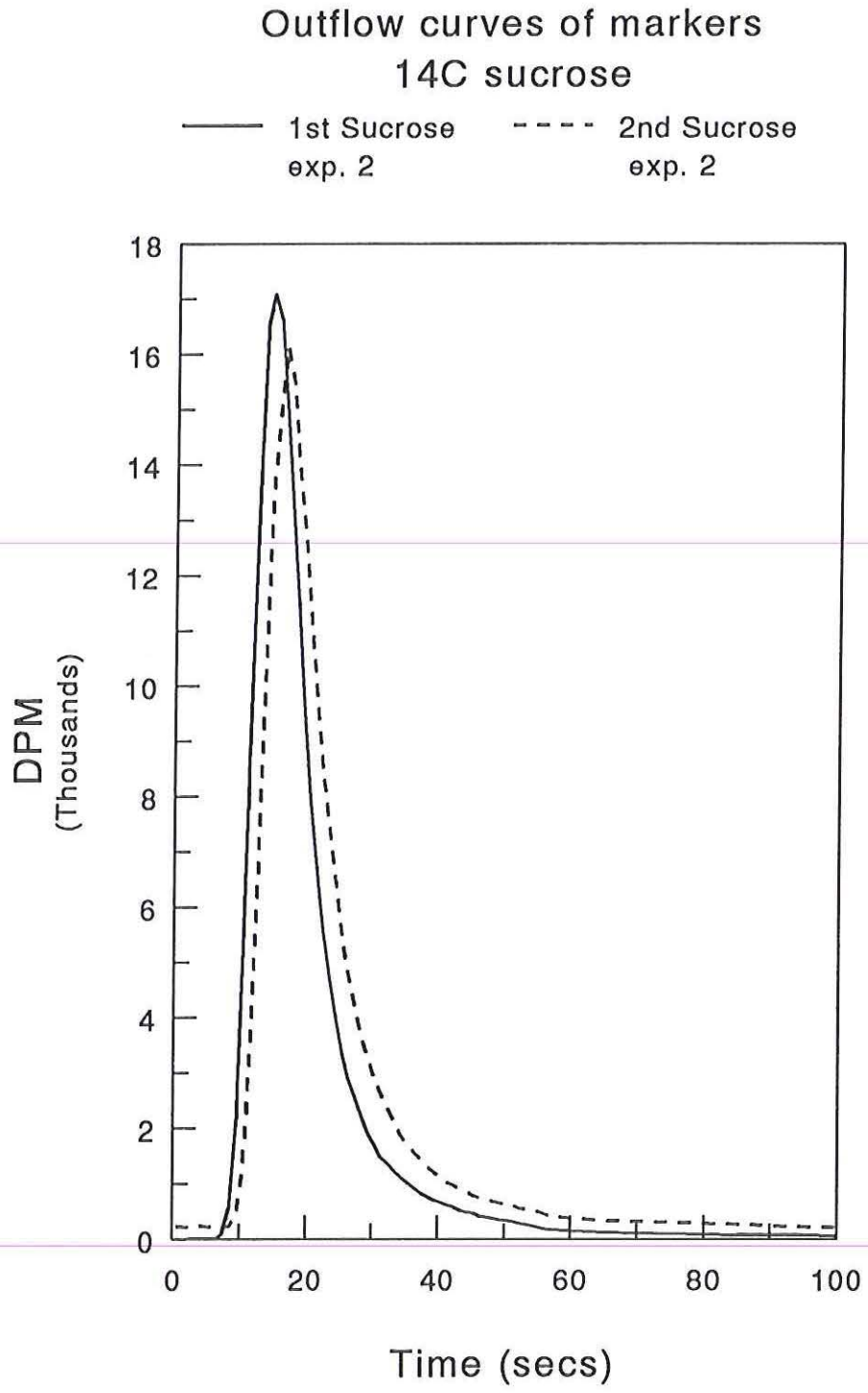
ANNEX 19. Outflow curves of the first bolus of tritiated water and ^{14}C-labelled sucrose in the second rat liver perfusion.



ANNEX 20. Outflow curves of the first and second bolus of tritiated water in the second rat liver perfusion.



ANNEX 21. Outflow curves of the first and second bolus of ^{14}C-labelled sucrose in the second rat liver perfusion.



ANNEX 22. Washout curves of five different oligomers in the second rat liver perfusion.