Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 9, pp. 737–745, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00

Determination of physicochemical properties, stability in aqueous solutions and serum hydrolysis of pilocarpic acid diesters

TOMI JÄRVINEN,*† PEKKA SUHONEN,‡ HELI NAUMANEN,† ARTO URTTI‡ and PEKKA PEURA†

†*Department of Pharmaceutical Chemistry and* ‡*Department of Pharmaceutical Technology, University of Kuopio, P.O. Box 1627, SF-70211 Kuopio, Finland*

Abstract: New alkyl and aralkyl pilocarpic acid diesters, prodrugs of pilocarpine, were synthesized with the aim of improving the bioavailability of pilocarpine by increasing its corneal permeability. These esters were several orders of magnitude more lipophilic than pilocarpine as determined by their apparent partition coefficients between 1-octanol and phosphate buffer (pH 7.40) (log *P*). Good correlation between log *P* and HPLC capacity factors of the compounds was observed. All the compounds are stable in acidic aqueous solution; in serum, however, pilocarpic acid diesters are hydrolysed enzymatically to pilocarpic acid monoester, which undergoes spontaneous cyclization to active pilocarpine and inactive isopilocarpine. The half-lives of the diesters in serum varied from 6–232 min. In addition to the direct effects of the R_2, R_1 moiety had a remarkable effect on the rate of enzyme-catalysed hydrolysis taking place in moiety R_2. The formed pilocarpine was analysed with a new HPLC method which allowed good resolution of pilocarpine, isopilocarpine, pilocarpic acid and isopilocarpic acid. Rates for pilocarpine formation were both determined by experiment and calculated using the STELLA™ simulation programme with known degradation rate constants of pilocarpic acid diesters and monoesters. Since the simulations were in good agreement with the experimental results, it is concluded that STELLA™ simulation programme is useful in predicting pilocarpine formation.

Keywords: *Prodrug; pilocarpic acid diester; LC; partition coefficient; stability; enzyme hydrolysis; STELLA™ simulation.*

Introduction

Pilocarpine, a direct acting cholinergic agent, is used topically in the eye to control the elevated intraocular pressure associated with glaucoma. However, the drug has significant delivery problems; owing to rapid precorneal removal the ocular bioavailability of pilocarpine is low [1], which necessitates administration several times daily. This results in a pulsatile concentration profile in the eye, side-effects related to peak concentrations [2] and poor patient compliance [3]. Ocular bioavailability can be improved by increasing the contact time of the drug on the cornea and by increasing drug permeability in the cornea [4].

The residence time of pilocarpine in the conjunctival sac can be extended using either water-soluble polymers in eye-drop formulations [5] or polymeric inserts [6, 7]. However, several problems are associated with these methods for improving ocular bioavailability. Only modest improvements in ocular bioavailability are achieved with viscous solutions and patient acceptance is often poor with polymeric inserts [8].

Transcorneal drug penetration has been reported to be at maximum when the octanol–water partition coefficient (P) of the drug is 100–1000 (log P = 2–3) [9]. Pilocarpine is a hydrophilic compound (log P = 0.01) and hence its ocular bioavailability may be improved by preparing more lipophilic derivatives. Accordingly, quaternary ammonium salt prodrugs [10] and pilocarpic acid diesters [11–14] have been shown to improve ocular absorption of pilocarpine. Pilocarpic acid diesters are very stable in aqueous solution but undergo enzymatic hydrolysis to pilocarpic acid monoester in the presence of esterases in the cornea. Thereafter pilocarpic acid monoester hydrolyses by spontaneous lactonization to active pilocarpine or inactive isopilocarpine [11, 13].

Although there are several HPLC methods with an ODS column for the separation of pilocarpine and its degradation products (isopilocarpine, pilocarpic acid, isopilocarpic acid)

*Author to whom correspondence should be addressed.

[15–18], these methods all show incomplete resolution between pilocarpine and isopilocarpine or a long analysis time. Consequently, these methods have been modified by changing the column to obtain a new efficient method for the separation and analysis of pilocarpine and its degradation products.

In the first part of this study a series of new alkyl and aralkyl diesters of pilocarpic acid were prepared and characterized [19]. In the present study physicochemical properties and hydrolytic and enzymatic degradation kinetics of the compounds have been investigated. General structure–degradation relationships and especially the importance of R_1-substituent have been studied. A novel simulation software STELLA™ has been used for the prediction of rates of pilocarpine formation from the prodrugs and the predicted rates have been compared with experimentally determined rates.

Materials and Methods

Materials

All the diesters of pilocarpic acid **1–11** (Fig. 1) were synthesized and identified as previously described [19]. Pilocarpine hydrochloride was kindly supplied by Huhtamäki Oy Leiras (Tampere, Finland). Isopilocarpine nitrate was obtained from Aldrich (Steinheim, Germany). Pilocarpic acid and isopilocarpic acid were prepared by the method of Bundgaard *et al.* [11]. Monobasic potassium phosphate, disodium phosphate dihydrate, sodium bicarbonate, sodium nitroprusside and 1-octanol were purchased from Merck (Darmstadt, Germany). Sodium chloride and methanol (HPLC-grade) were from J.T. Baker (Deventer, The Netherlands).

Equipment

HPLC was performed with a system consisting of a Beckman programmable solvent module 116, a Beckman programmable UV-detector 166 (set at 215 nm), a System Gold data module (Beckman Instruments Inc., San Ramon, USA), a Marathon autosampler (Spark Holland, AJ Emmen, The Netherlands) equipped with a column thermostat and a Rheodyne 7080-080 loop (20 μl) injector. Deactivated Supelcosil LC8-DB (150 × 4.6 mm i.d., 5 μm) and LC18-DB (250 × 4.6 mm i.d., 5 μm) reversed-phase columns (Supelco, Bellefonte, USA) were used.

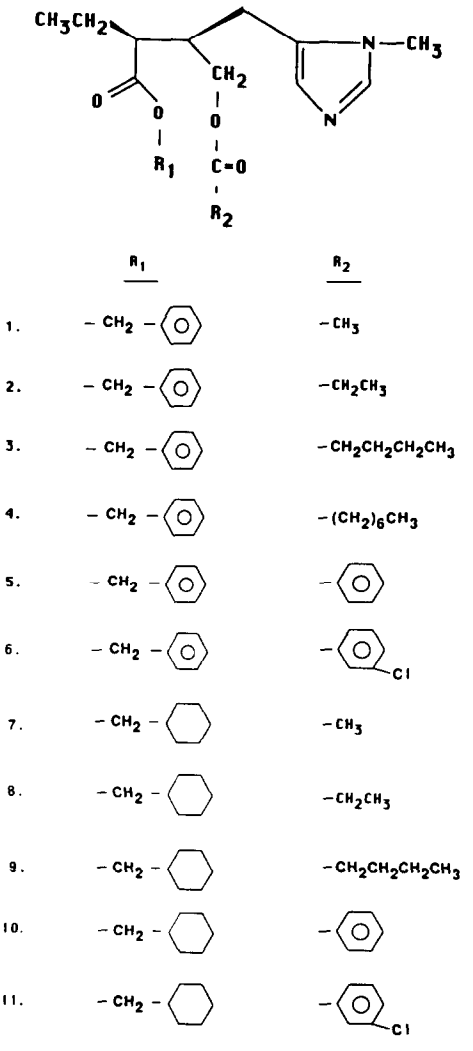

Figure 1
Structures of pilocarpic acid diesters studied.

An Orion SA 520 pH meter (Boston, USA) equipped with a combination pH electrode was used for pH determinations.

A STELLA™ computer programme was purchased from High Performance Systems Inc. (Hanover, USA).

Determination of dissociation constants

The dissociation constants (pK_a values) of pilocarpine and pilocarpic acid diesters **1–11** (as free base) were determined by titration of 2 mM solutions of the compounds in water–ethanol (50:50, v/v) with 0.5 M hydrochloric acid at room temperature.

Measurement of partition coefficients

The apparent partition coefficients (P) for pilocarpine and pilocarpic acid diesters **1–11**

were measured using a 1-octanol–buffer system. Phosphate buffers (0.16 M) of pH 2.50, 4.20 or 7.40 and ionic strength (μ) of 0.5 M (adjusted with potassium chloride) were used. Buffer and 1-octanol phases were saturated with each other by stirring vigorously for 24 h at room temperature; the phases were separated and the compounds were dissolved in the aqueous phase at suitable concentration. Phase volumes and the pH of aqueous phase were selected in each case to ensure reliable determination of the diester concentration changes in the aqueous phase. The buffer–1-octanol mixtures were shaken for 60 min to achieve equilibrium. After separation of the aqueous and 1-octanol phases, the aqueous phase were centrifuged at 4000 rpm for 5 min in order to achieve complete separation.

The concentration of the pilocarpic acid diesters in aqueous phase before and after distribution were measured by reversed-phase HPLC. The quantitation of the compounds was achieved by measuring the peak area in relation to standard solutions subjected to chromatography under the same conditions. The column was LC8-DB Supelcosil thermostated at 40°C; the isocratic solvent system was 0.02 M KH_2PO_4 (pH 4.5)–methanol (29:71, v/v), and the flow rate was 1.0 ml min^{-1}. The proportion of methanol can be increased or the pH of the aqueous phase decreased for very lipophilic compounds to shorten the retention time without losing separation. The column effluent was monitored at 215 nm. The apparent partition coefficients (P) were calculated from equation (1):

$$P = \left(\frac{C_i - C_a}{C_a}\right)\left(\frac{V_a}{V_o}\right), \qquad (1)$$

where C_i and C_a represent the initial (i) and equilibrium (a) solute concentrations of the aqueous buffer phase; V_a represents the volume of the aqueous phases and V_o the volume of the 1-octanol phase. Triplicate apparent partition coefficient determinations were made for each compound.

The lipophilicity of the compound was also estimated by reversed-phase HPLC using the method described for the determination of partition coefficients. The capacity factor (k') of each compound was calculated from equation (2):

$$k' = (t_r - t_o)/t_o, \qquad (2)$$

where t_r is the retention time of the compound; and t_o is the elution time of sodium nitroprusside, which was used as the t_o marker (a compound which is not retained in the column).

Hydrolysis in aqueous solutions

The hydrolysis of the pilocarpic acid diesters **1–11** was studied in aqueous buffer solutions (pH 4.20, 6.00, 7.40, 9.00) at 37°C. The hydrolysis of compound **2** was also studied at temperatures of 50, 60 and 70°C. Phosphate buffers (0.16 M) at an ionic strength (μ) of 0.5 M were used.

Solutions of pilocarpic acid diesters were prepared by dissolving the appropriate amount of the compound in 2.0–5.0 ml of ethanol and diluting to 50.0 ml with the pre-heated buffer. Ethanol was used for solubility reasons. The solutions were placed in a thermostated water-bath and at suitable intervals 1.0-ml samples were withdrawn and mixed with 2.0 ml of 0.1 M phosphate buffer (pH 6.0). The mixture was analysed by HPLC using the method described for the measurement of partition coefficients.

The pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$) for the degradation of the pilocarpic acid diesters were determined from the slopes of the linear semilogarithmic plots of remaining diester against time.

Enzyme hydrolysis in human serum

Pilocarpic acid diesters (2–10 μmol) were first dissolved in 4.0 ml of phosphate buffer (0.16 M, pH 7.40) at 37°C. Then 16.0 ml of pre-heated human serum was added and the solutions were kept in a water-bath at 37°C. At suitable intervals 1.0-ml samples of the serum–buffer mixture were withdrawn and added to 3.0 ml of ethanol in order to deproteinize the serum. The clear supernatant was analysed for remaining diester by the HPLC method described above. The pseudo-first-order rate constants and half-lives for degradation were calculated from the slopes of linear plots of the logarithm of remaining pilocarpic acid diesters versus time. The rate data for pilocarpic acid monoesters were determined by the same procedure.

The formed pilocarpine and its degradation products in serum solutions were determined by a different method. A 1.0-ml sample of the clear supernatant was evaporated to dryness under a stream of air. The residue was dis-

solved in 500–750 μl of mobile phase (see later), mixed and analysed by HPLC. The column was LC18-DB Supelcosil thermostated at 40°C; the isocratic solvent system was 5% KH_2PO_4 (pH 2.50, adjusted with phosphoric acid)–methanol (97:3, v/v) and the flow-rate was 1.5 ml min^{-1}. The column effluent was monitored at 215 nm. This procedure also permits the measurement of isopilocarpine, pilocarpic acid and isopilocarpic acid with complete separation. The pseudo-first-order rate constants (k_f) for the formation of pilocarpine and the times when 50% of total pilocarpine was formed ($f_{50\%}$) were determined from the slopes of linear plots of the logarithm of pilocarpine yet to be formed ($pilocarpine_{max}$–$pilocarpine_t$) against time.

STELLA™ simulation

A STELLA™ computer program with a fourth-order Runge–Kutta algorithm was used to calculate the formation of pilocarpine according to the scheme shown in Fig. 2. The model was based on the experimentally determined rates of degradation of pilocarpic acid diester and pilocarpic acid monoester in 80% (v/v) human serum.

Results and Discussion

Dissociation constants of pilocarpic acid diesters

The dissociation constants (pK_a values) for pilocarpine and pilocarpic acid diesters (free base) are given in Table 1. Owing to the inadequate water-solubility of many compounds the samples were titrated in a water–ethanol mixture.

Table 1
pK_a values, apparent partition coefficients (P) and reversed-phase LC capacity factors (k') of pilocarpine and pilocarpic acid diesters

Compound	pK_a*	log P†	k'
Pilocarpine	6.30	0.01	0.41
1	6.18	2.87	1.09
2	6.20	3.30	1.42
3	6.12	4.43	2.59
4	6.10	7.70	7.34
5	6.10	4.57	2.29
6	6.08	5.64	3.55
7	6.25	3.86	2.36
8	6.12	4.40	3.15
9	6.12	5.56	5.96
10	6.20	5.47	5.01
11	6.20	7.32	7.78

*The pK_a of compounds were determined in ethanol–water (50:50, v/v).

†The apparent partition coefficient between 1-octanol and phosphate buffer (pH 7.40).

pK_a values of pilocarpic acid diesters were in the range 6.08–6.25. Parke and Davis [20] reported that many organic bases show decreased pK_a values with increasing concentrations of ethanol in the titration solution. In the present experiments the pK_a of pilocarpine decreased from 7.00 to 6.30 with an increase in the ethanol concentration from 0 to 50%. The pK_a values of pilocarpic acid diesters were slightly lower than the pK_a of pilocarpine in 50% (v/v) ethanol (Table 1). Hence it was estimated that the pK_a values of the pilocarpic acid diesters in water are about 6.9. The R_1- and R_2-groups do not seem to influence the pK_a values (Table 1, Fig. 1). The small differences in the pK_a values are probably caused by experimental variation.

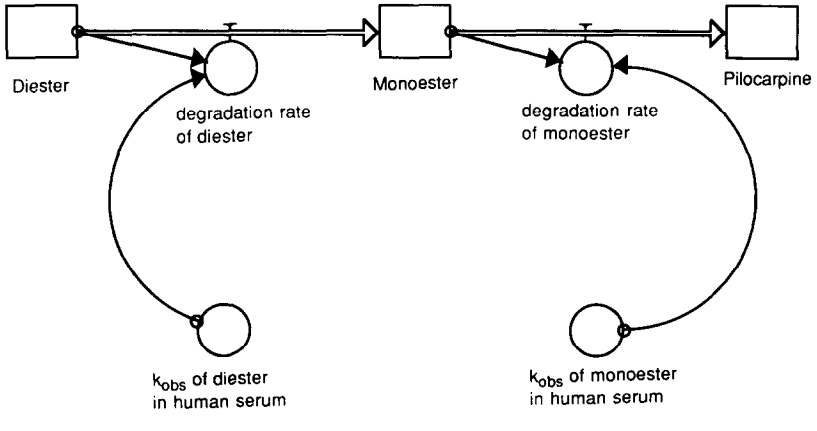

Figure 2
The STELLA™ model in the prediction of the formation of pilocarpine.

The lipophilicity of pilocarpine and pilocarpic acid diesters

The apparent 1-octanol–buffer partition coefficients of pilocarpic acid diesters and pilocarpine (Table 1) show that pilocarpic acid diesters are more lipophilic than pilocarpine and that their lipophilicity can be varied by changing R_1 and R_2. Owing to the poor aqueous solubility of the diesters at pH 7.40 it was not possible to determine the apparent partition coefficients at pH 7.40. Partitioning was measured using phosphate buffers of pH 4.20 (diesters **1–3** and **5–11**) or pH 2.5 (diester **4**). As described earlier [13] the apparent partition coefficient at pH 7.40 was estimated using equation (3):

$$P_{pH7.40} = P_{pH} \frac{1 + 10^{(pK_a - pH)}}{1 + 10^{(pK_a - 7.4)}}, \quad (3)$$

where $P_{pH7.40}$ is the apparent partition coefficient at pH 7.40; P_{pH} is the apparent partition coefficient at the other pH; and pK_a is 6.9 for all the pilocarpic acid diesters. The validity of this procedure was tested by measuring the apparent partition coefficient at pH 7.40 and by calculating the apparent partition coefficient from the results in acidic pH using equation (3). For compound **1** the apparent partition coefficient (log P) obtained at pH 7.40 was 2.75 and the corresponding value obtained by equation (3) was 2.87. For compound **2** these values were 3.24 and 3.30 and for compound **3** 4.23 and 4.43, respectively.

The lipophilicity of the pilocarpic acid diesters was also estimated as capacity factors (k') of reversed-phase HPLC (Table 1). It can be seen easily from these data that all the derivatives are more lipophilic in comparison with pilocarpine. The linear relationship between log P and log k' is shown in Fig. 3 and the best fit was obtained by the equation: log P = 5.34 log k' + 2.27 (r = 0.97). Thus, reversed-phase liquid chromatography can be used as an alternative method in measuring the lipophilicity of pilocarpic acid diesters.

Stability in aqueous solution

All pilocarpic acid diesters exhibited pseudo-first-order degradation kinetics in the aqueous solutions.

The influence of pH and temperature on the pseudo-first-order rate constant (k_{obs}) of *o*-propionyl pilocarpic acid benzyl diester is shown in Fig. 4. The lowest pH used was 4.20

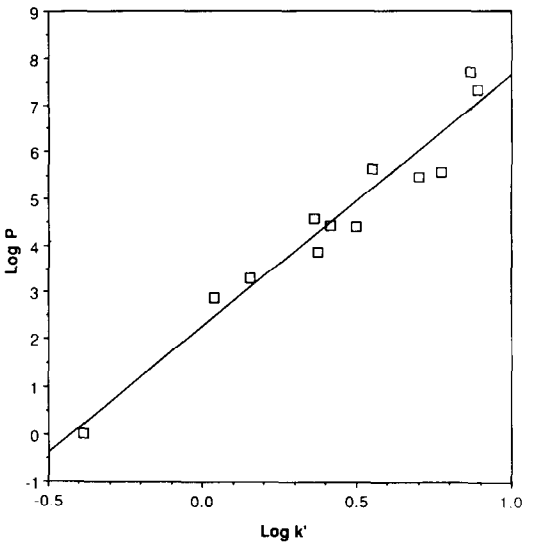

Figure 3
Relationship between log P values and log k' values of pilocarpic acid diesters.

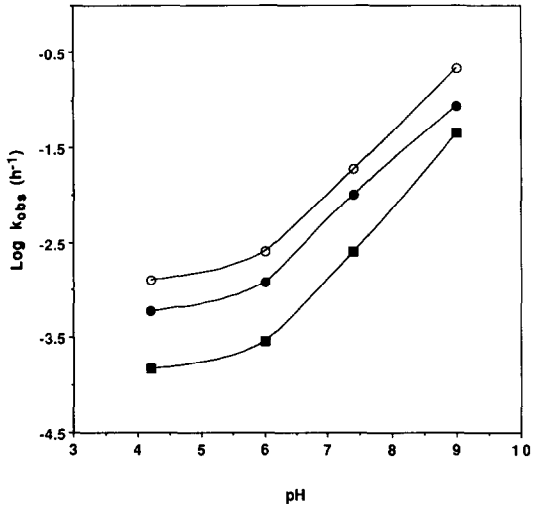

Figure 4
Log rate constant–pH profiles for degradation at 50°C (■), 60°C (●) and 70°C (○) of pilocarpic acid diester **2**.

because the pH of an eye-drop formulation should be preferably more than 4.0. In the pH range investigated, the degradation rates of all pilocarpic acid diesters increased with increasing pH and temperature.

The stability of *o*-propionyl pilocarpic acid diester was determined under different storage conditions. The rates of hydrolysis were determined at different values of pH and at three or four different temperatures. The rate constants obtained for pilocarpic acid diester **2** at different temperatures were plotted according to the Arrhenius equation (4):

$$\log k = \log A - \frac{E_a}{2.303R}\frac{1}{T}, \qquad (4)$$

where A is the frequency factor, E_a is the apparent energy of activation, R is the gas constant, and T is the absolute temperature in K. Shelf-lives ($t_{10\%}$, the time required to degrade 10% of the compounds) of 42 years, 5 years, 48 days and 31 days at 4°C were predicted from Arrhenius plots at pH 4.20, 6.00, 7.40 and 9.00, respectively. The corresponding values at 25°C were 674, 142, 7 and 2 days. Thus, *o*-propionyl pilocarpic acid diester cannot be formulated as a stable, ready-to-use solution at neutral pH but an acceptable shelf-life can be achieved in the pH range 4–6. However, the ocular bioavailability of weak bases at this pH is less than at neutral pH [21].

Hydrolysis in human serum

The rates of hydrolysis of the pilocarpic acid diesters **1**–**11** were determined in 80% (v/v) human serum. All derivatives exhibited pseudo-first-order degradation kinetics for several half-lives in serum.

The pseudo-first-order plots for compounds **2**, **7** and **11** are shown in Fig. 5. The half-lives ($t_{1/2}$) and the pseudo-first-order rate constants (k_{obs}) for degradation of the diesters in 80% human serum at 37°C are listed in Table 2 with

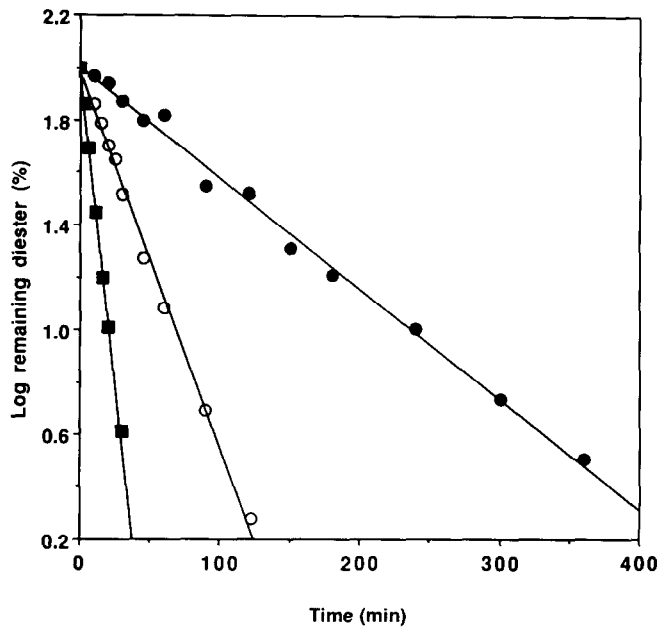

Figure 5
Pseudo-first-order plots for hydrolysis of pilocarpic acid diesters **2** (■), **6** (○) and **7** (●) in 80% human serum at 37°C.

Table 2
Rate data for hydrolysis of various pilocarpic acid diesters in aqueous solution and in human serum at pH 7.40 and 37°C

Compound	$t_{1/2}$ (h) phosphate buffer	$t_{1/2}$ (min) 80% human serum	k_{obs} (h^{-1}) 80% human serum
1	547	16	2.612
2	784	6	6.481
3	1169	9	4.726
4	*	38	1.534
5	2043	18	2.280
6	3052	23	1.879
7	503	72	0.580
8	829	33	1.257
9	274	28	1.492
10	1646	116	0.359
11	851	232	0.180

*Not determined owing to the poor water-solubility of the compound.

the half-lives for degradation of the diesters in phosphate buffer (pH 7.40) at 37°C for the purpose of comparison. In contrast to earlier assumptions both R_1 and R_2 substituents affected the enzyme-catalysed hydrolysis (Table 2). When the aromatic ring (benzyl, compounds **1–6**) was changed to a non-aromatic ring (cyclohexylmethyl, compounds **7–11**) the half-lives of enzymatic hydrolysis became considerably longer although the enzymatic hydrolysis takes place only in the position of R_2. The half-lives ($t_{1/2}$) for pilocarpic acid benzyl monoester and pilocarpic acid cyclohexylmethyl monoester in 80% human serum at 37°C were 19 and 546 min, respectively.

Pilocarpic acid diesters **1**, **2**, **3**, **5** and **6** undergo almost complete conversion to pilocarpine involving enzymatic hydrolysis of the R_2-moiety followed by spontaneous lactonization of the pilocarpic acid monoester. In most cases only minor formation of isopilocarpine was found. In some experiments formation of isopilocarpine was exceptionally high but in these cases the pure pilocarpine standard also undergoes epimerization to isopilocarpine in the same proportion. Thus the epimerization was due to the serum matrix but no explanation was found.

Since double prodrugs are converted to the parent drug via a two-step reaction, neither of the degradation rate constants adequately describes the rate of parent drug formation. The actual rate of parent drug formation was determined in this study and expressed as the time when 50% of total pilocarpine was formed ($f_{50\%}$). These times ($f_{50\%}$) are listed for compounds **1**, **2**, **3**, **5** and **6** in Table 3. The results show that these compounds may be promising pilocarpine prodrugs because of the suitable rate of conversion to pilocarpine. Formation of pilocarpine from pilocarpic acid diester **4** was not determined owing to the poor aqueous solubility of the diester at pH 7.40. The enzymatic hydrolysis of diesters **7–11** and the spontaneous lactonization of pilocarpic acid cyclohexylmethyl monoester was very slow. Consequently, pilocarpine formation was difficult to monitor because during long experiments the formed pilocarpine may epimerize to isopilocarpine or hydrolyse to pilocarpic acid.

HPLC determination of pilocarpine

The formation of pilocarpine was studied by a reversed-phase HPLC method which allows also determination of isopilocarpine, pilocarpic acid and isopilocarpic acid. The method was modified from previous reports [15–17]. However, better resolution and peak shape was achieved by using a Supelcosil LC-18-DB column which has been designed especially for the separation of basic compounds. Complete chromatographic separation was achieved in 22 min (Fig. 6). The selectivity factor (a) was 1.16 for pilocarpine–isopilocarpine and 1.22 for pilocarpic acid–isopilocarpic acid. The resolution (R) was 2.9 for pilocarpine–isopilo-

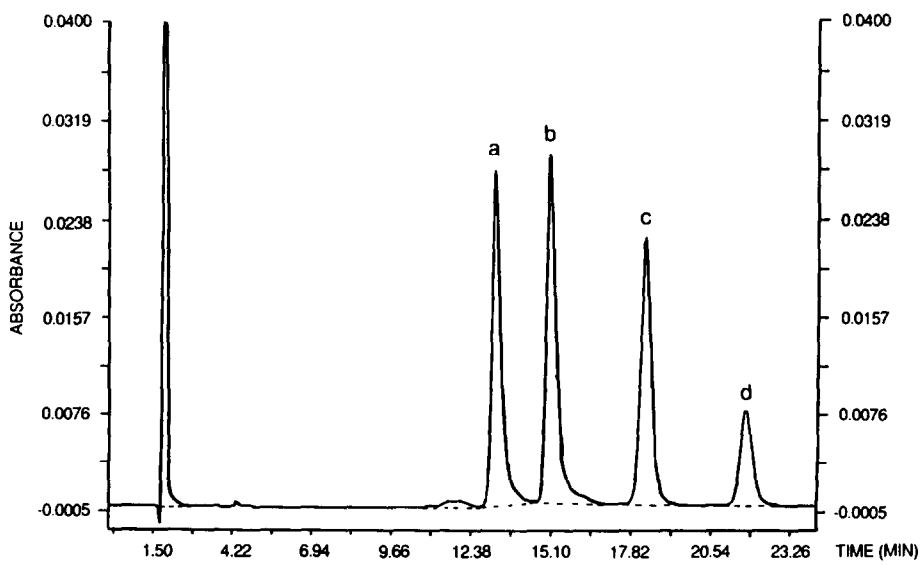

Figure 6
HPLC separation of isopilocarpine (a), pilocarpine (b), pilocarpic acid (c) and isopilocarpic acid (d).

carpine and 4.1 for pilocarpic acid–isopilocarpic acid. The tailing factor ($T_{0.05}$) for isopilocarpine, pilocarpine, isopilocarpic acid and pilocarpic acid was 1.29, 1.25, 1.00 and 1.00, respectively. The achieved selectivity is perceptibly better than the minimum claims in the USP XXI [22]. By using a special cyano column Gomez-Gomar *et al.* [23] were able to determine pilocarpine and its degradation products with a resolution of 1.8 between pilocarpine and isopilocarpine [23].

STELLA™ simulation

Separate analytical methods have to be used for the prodrug and for pilocarpine and its degradation products. The analysis of pilocarpine and its degradation products by HPLC is a time-consuming procedure. Also, in some cases the hydrolysis test is very difficult owing to the poor aqueous solubility of prodrugs at pH 7.40 and/or slow hydrolysis of the compound. Therefore the suitability of STELLA™, a graphical interphase simulation program, was tested to predict the formation of pilocarpine.

The simulated and observed $f_{50\%}$ values for different pilocarpic acid diesters in 80% human serum are listed in Table 3. The determined k_{obs} of diesters in human serum, used in the STELLA™ simulation, are listed in Table 2; the k_{obs} for pilocarpic acid benzyl monoester and pilocarpic acid cyclohexylmethyl monoester in 80% human serum at 37°C were 2.183 and 0.076 h^{-1}, respectively. The simulated values are very close to the experimental values for diester **1**, **2**, **3**, **5** and **6**. The results show that the STELLA™ simulation program can be used to predict the formation of pilocarpine from pilocarpic acid diesters. Due to the reasons explained earlier the values of $f_{50\%}$ for diester **4** and **7–11** were determined only by the STELLA™ programme and the results are shown in Table 3. Pilocarpic acid diesters **7–11** are not suitable pilocarpine prodrugs because of the slow formation of pilocarpine. The determination of the $f_{50\%}$ of pilocarpine is time-consuming for pilocarpic acid diesters; in addition to saving time, the STELLA™ programme may give in some cases (e.g. compounds **7–11**) a more reliable estimate of pilocarpine formation than that given by practical laboratory tests because analytical inaccuracies can be avoided.

Table 3
Rate data for formation ($f_{50\%}$) of pilocarpine from various pilocarpic acid diesters in human serum at pH 7.40 and 37°C

Compound	Experimental data (min)	STELLA™ simulation (min)
1	44	42
2	26	29
3	31	33
4	—*	55
5	46	45
6	47	50
7	—*	658
8	—*	596
9	—*	589
10	—*	730
11	—*	908

* Not determined owing to the poor aqueous solubility of pilocarpic acid diester or to the slow formation of pilocarpine.

Conclusions

This paper describes methods to determine the physicochemical behaviour of pilocarpic acid diesters. The described HPLC method allows the determination of pilocarpine in the presence of isopilocarpine, pilocarpic acid and isopilocarpic acid. The STELLA™ simulation program was shown to be a useful method for the prediction of pilocarpine formation and it can decrease the need for laboratory tests. As double prodrugs, pilocarpic acid diesters possess sufficient aqueous stability for an eyedrop formulation and controlled and optimum formation of pilocarpine can be achieved. In contrast to an earlier report it was found that the R_1 moiety has a remarkable effect on the rate of enzyme-catalysed hydrolysis in moiety R_2. The results obtained indicate that for an eyedrop formulation it may be difficult to dissolve these compounds at an acceptable pH and at sufficient concentration.

Acknowledgements — The authors would like to thank Huhtamäki Oy Leiras (Finland), the Technology Development Centre (Finland), Jenny and Antti Wihuri Foundation and the Finnish Cultural Foundation for financial support. We also thank Leiras for research cooperation and Ms Päivi Varis for her technical assistance.

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[Received for review 4 April 1991; revised manuscript received 22 July 1991]