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Absorption of progabide from aqueous solutions in a modified recirculating rat intestinal perfusion system

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Summary

The absorption rate of progabide from aqueous solutions was determined in a modified in situ recirculating rat intestinal loop. The absorption rate was found to be very rapid from the upper small intestine with a half-life of 8.1 min. The addition of Tween 80 drastically reduced the absorption rate of progabide. In comparison, the addition of β-cyclodextrin had only a slight negative effect.

Introduction

Previously, the oral bioavailability of progabide was investigated in the rabbit as an in vivo animal model (Farraj et al., 1987a). The plasma concentration–time profiles obtained have limited application in elucidating the absorption kinetics from the gastrointestinal tract. This is because the data obtained by such an "appearance" procedure can be affected by physiological processes such as gastric emptying, enterohepatic circulation or liver metabolism. The use of a "disappearance" method in which the concentration in the lumen of the gastrointestinal tract is measured against time avoids a number of the above complications although with this procedure, gastrointestinal adsorption or metabolism will be misinterpreted as part of the absorption process. Thus, perhaps the ideal model is one that concomitantly combines both of the above methods of analysis. Although this can be done, for example, in the Thiry and Vella loop procedure (Markowitz et al., 1964), the use of the two methods separately is still more common. Of the "disappearance" methods available, the in situ rat ligated intestinal loop was chosen to assess the absorption kinetics of progabide. Further, Tween 80 and β-cyclodextrin were found previously (Farraj et al., 1987b) to improve the aqueous solubility and stability of progabide. However, their presence may decrease the absorption rate of progabide by reducing its thermodynamic activity and hence limit their in vivo use. On that basis, it was decided to investigate the effect of these complexing agents on the intestinal absorption rate of progabide.

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Materials and Methods

Choice of the technique

Several versions (Schanker et al., 1958; Doluisio et al., 1969a; Levine et al., 1955; Levine and Pelikan, 1961) of the in situ rat intestinal loop have been used with varied conditions that resulted, in some cases, in unrealistic absorption rates. There are a number of aspects that warrant consideration if the integrity and physiological viability of the tissue is to be maintained and if reliable absorption data are to be obtained. These aspects are: (1) Choice between a static, single pass, or recirculating perfusion system. (2) Choice of a suitable perfusion rate. (3) Choice of a suitable water flux marker. A static intraluminal solution presents the drug with an exceptionally thick boundary layer to traverse before reaching the absorptive membrane. For drugs with high partition coefficient, such as progabide (Farraj et al., 1987c), the aqueous boundary layer has been shown (Winne, 1975, 1978; Desai, 1976; Park, 1976) to play a critical role in determining the absorption rate. On that basis, the static perfusion systems were ruled out. The recirculating perfusion system was chosen in preference to the single-pass system because it offered certain advantages. With the latter, each animal yields only one datum point and the assumption is made that the absorption follows first-order kinetics, which may not always be the case. In comparison, the former system yields a number of data points which can be used to ascertain both the rate and order of the absorption process (Tsuji et al., 1977). Many workers in this field have adopted the Doluisio method (Doluisio et al., 1969a), which is a static perfusion system but with the intraluminal perfusion fluid reciprocated between two syringes connected to either end of the gut for sampling purposes. Although this technique offered clear advantages over the totally static perfusion systems, it was not chosen for use here because it involved abrupt changes in the hydrodynamics used and also because the perfusion solution was pumped, for part of the experiment, in a direction opposite to the normal flow of the gastrointestinal contents. As an alternative, a recirculating perfusion system was developed which was based on the method of Tsuji et al. (1977) but which incorporated several added modifications to overcome the limitations and shortcomings of the previously used systems. One of the important improvements introduced was that the majority of the perfusion fluid was within the gut at any moment in time, i.e., a large fraction of the drug in the system was available for absorption, a feature not found in many present-day systems.

Savina et al. (1981) investigated the effect of the flow rate on the variability in the mean absorption clearance of a model compound in an attempt to define an optimal perfusion rate for the in situ rat intestinal loop. A parabolic relationship was established between the coefficient of variance and the flow rate with the minima occuring at an optimal perfusion rate of 0.594 ml/min. An increase in the clearance was observed (Savina et al., 1981; Winne, 1979) when the flow rate was increased. This was attributed to the flattening of the concentration gradient down the segment and a reduction of the effective unstirred layer thickness, since the surface area was shown to be little affected (Winne, 1979) until flow rates above 10 ml/min were used (Lewis and Fordtran, 1975). This optimal flow rate of about 0.6 ml/min was adopted in our system.

Because of the possibility of water movement across the intestinal mucosa, a flux marker is incorporated into the drug solutions, which can be recovered and assayed at suitable intervals. The two most widely used markers are polyethylene glycol 4000 and Phenol red. An ideal water flux marker must be inert, non-absorbable and non-adsorbable and non-degradable by metabolic or chemical processes within the lumen of the gastrointestinal tract. In a review of the application of the above two compounds as liquid phase markers in nutrition research, Clemens (1982) demonstrated that, although neither marker totally fulfilled the above criteria, a greater erratic recovery was observed with Phenol red; PEG 4000 was 93 to 100% recoverable whereas Phenol red recovery was consistently about 3–8% below that of PEG. The difference was attributed to the greater adsorption and absorption losses of phenol red compared with PEG 4000. On that merit, the latter marker was chosen for use in our studies.

Many of the technical difficulties associated with the analysis of PEG 4000 by turbidometric methods (Clemens, 1982) were avoided by using ^{14}C-labelled PEG 4000, in association with a radioisotopic method of analysis.

Overall, the validity of the developed in situ recirculating perfusion system was ensured by using it to evaluate the intestinal absorption rate of a well-documented reference compound, namely, salicyclic acid.

Materials and animals

Progabide (lot 11129; L.E.R.S.), β-cyclodextrin (Sigma) and Tween 80 (Honeywill-Atlas) were used as received. Salicyclic acid, sodium chloride, citric acid, sodium citrate and toluene were all of AnalaR grade. Scintran (Butyl-PBD) was obtained from BDH and 2-ethoxyethanol of scintillation grade was purchased from Fisons. [^{14}C]PEG 4000 (Amersham International) was provided as a 7.4 MBq/ml aqueous solution containing 3% ethanol.

Pentobarbitone sodium, supplied as a 60 mg/ml injectable solution (Sagatal; May and Baker), was used to induce animal anaesthesia. Polycarbonate membranes (Nuclepore) of 0.4 μm were used for filtration purposes.

Male albino Wistar rats, of weight range 200–240 g, were maintained on a solid diet (Thomson rat cubes; L.A. Pilsbury) and water, both ad libitum.

Instrumentation

The perfusion system consisted of a Grant SE 50 water bath, an LKB 2115 Multiperpex peristaltic pump, a submersible stirrer (Rank Brothers) with a PTFE-coated micro spinbar (Technilab Instruments), a 5-ml glass vial as a drug reservoir, a stainless-steel tray (20 cm × 25 cm), and a heat lamp to maintain the temperature of the anaesthesized animal. PTFE tubing of 0.74 mm i.d. and 2.16 mm i.d. (Jencons) was used as the circuit tubing except inside the pump where silicone tubing of 1.3 mm i.d. (LKB) was used. The proximal and distal cannulae (Fig. 1) were constructed of the above two types of tubing together with a commercially available polyester-covered cotton thread for ligation.

The osmolality of the prepared drug solution was measured on an advanced 3D II digital osmometer (Advanced Instruments), previously calibrated with standard sodium chloride solutions of 100 mOsm/kg and 900 mOsm/kg. A Dawe 7532B soniprobe was used in preparing the saturated solutions of progabide, prior to filtration.

The reversed-phase high-performance liquid chromatography (RP-HPLC) system used in the analysis of the collected samples of progabide and salicylic acid was described previously (Farraj et al., 1987d). [^{14}C]PEG 4000 counting was performed in disposable scintillation vials, of 20 ml nominal capacity (Packard Instruments), on a Kontron SL4000 liquid scintillation counter.

Methods

Animal preparation. Prior to surgery, the animal was fasted for 12–16 h but drinking water was readily accessible. Longer fasting periods were not used to avoid possible deviations in absorption (Doluisio et al, 1969b). Anaesthesia was induced by an i.p. injection of pentobarbitone sodium at a dose of 60 mg/kg, about 10 min before surgery.

Preparation of the drug solutions. A 0.4 mg/ml solution of salicylic acid in citrate buffer (Dawson et al., 1986) of pH 6.2 was prepared and adjusted to iso-osmolality (291 mOsm/kg) by the addition of sodium chloride.

For progabide, all the solutions were prepared immediately before use. Aqueous buffered solutions of about 15 μg/ml were prepared by first sonicating excess powdered progabide with the citrate buffer of pH 6.2 for about 1 min followed by filtration, at room temperature, on the 0.4 μm polycarbonate membrane filter. The aqueous solutions were adjusted to iso-osmolality by the addition of sodium chloride. Equimolar solutions of progabide were also prepared in 0.5% w/v Tween 80 in pH 6.2 buffer and in 1.0% w/v β-cyclodextrin in the same buffer. The osmolality of these solutions did not require any adjustment.

The ^{14}C-PEG 4000 was supplied as a 7.4 MBq/ml solution containing 3% ethanol. A stock solution of the marker, of 74000 Bq/ml, was prepared in distilled water. The prepared drug solutions were spiked immediately before use with

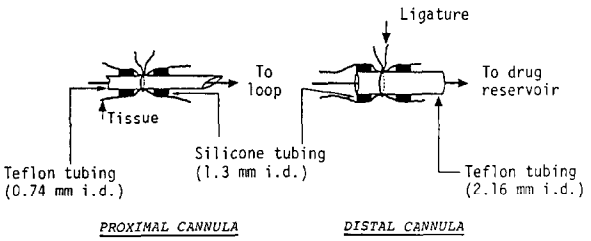

Fig. 1. Exploded views of the proximal and distal cannulae.

the above solution to produce a final isotope concentration of 740 Bq/ml. The concentration of ethanol remaining in the final solution was insignificant after the above dilution was performed.

In situ recirculating perfusion system. Fig. 1 shows exploded views of the proximal and distal cannulae used. These were designed to form a water-tight seal at either end of the intestinal loop without obstructing the perfusate flow. The concentric rings of silicone tubing, fitted onto the PTFE tubing, formed a 5 mm groove between them into which the tissue was secured, by ligation, using a polyester-covered cotton thread. The stainless-steel tray was placed on the surface of the water bath, at 37 °C, to provide a heated operating platform. Two holes were drilled into one side of the tray via which the circuit tubing was threaded to and from the drug reservoir at 37 °C. In this manner, the length of the circuit was minimised and no hydrostatic pressure head was applied to the loop. The overall resultant volume of the circuit was a mere 0.45 ml.

Intestinal absorption procedure. Three rats were used for each experiment. The small intestine was exposed by a midline abdominal incision through the linea alba. Two small slits were made in the intestinal wall, 2 cm and 25 cm below the pylorus, and the proximal cannula was secured in position by ligation. The small intestine was also ligated at a point immediately below the distal slit. The formed in situ loop was gently flushed with 30 ml of the iso-osmotic buffer solution (pH 6.2), warmed to 37 °C, until the effluent was clear and the segment was free of waste and debris. The flushing process, performed manually by a syringe attached to the proximal cannula, was implemented slowly so as not to expand the intestine overly and cause damage due to hydrostatic pressure. The remainder of the buffer solution in the loop was then carefully expelled by air from the attached syringe. This was followed by securing the distal cannula in position and the loop was returned to the abdominal cavity making sure that no kinks were formed in the segment. Care was taken in handling the small intestine and in reducing surgery to a minimum in order to maintain an intact blood supply. To that end, the thread used in the ligatures was passed underneath the anastomosis of an individual mesenteric vessel before the ligature was fastened in position. 3.5 ml of the appropriate drug solution was transferred into the 5 ml reservoir and the solution was continually stirred with the magnetic spinbar. The free ends of the proximal and distal cannulae were connected to the perfusion circuit and the drug solution was circulated through the lumen of the loop in the normal direction of digesta flow at a rate of 0.6 ml/min. The abdominal incision, as well as any exposed intestine, was covered with a gauze pad moistened with warmed normal saline to help maintain the integrity of the tissue. After 2 min of perfusing the drug solution, and at subsequent suitable intervals, 100 μl samples were removed from the reservoir for analysis. For the progabide experiment, the samples were collected onto crushed ice and immediately analysed following the experiment. The pH of the solution in the reservoir was checked at the end of each experiment. The animal was then sacrificed and the intestinal segment was removed and its length confirmed. As an added precautionary measure, the segment was blotted dry and its weight checked.

The cannulated upper small intestine loop ranged from 22 to 24 cm in length and at any time only about 1.5 ml of the drug solution was outside the loop.

Analysis of salicylic acid samples. A modified RP-HPLC assay procedure from that of Williams et al. (1980) was used. The column was eluted with the mobile phase at 1.7 ml/min with a pressure of 150 bar. The mobile phase was composed of acetonitrile/0.032 M hydrochloric acid (40 : 60, v/v). The detector was fixed at 302 nm with an AUFS of 0.2.

30 μl of the collected sample (100 μl) was

mixed with 30 μl of 0.1M hydrochloric acid and 20 μl of the resultant were injected onto the column by filling the fixed-volume loop in the injection port assembly.

The first analysed sample at 2 min was taken to represent zero time and all the other sample times were similarly corrected. The area under the peak of each subsequent sample, AUC_t, was expressed as a fraction of the peak area at "zero" time, AUC_0. The values were then corrected for water flux, as given below, and the data were plotted in accordance with first-order kinetics.

Analysis of progabide samples. 20 μl aliquots of the collected samples were analysed by RP-HPLC (Farraj et al., 1987d). As with salicylic acid, the areas under the peak were corrected for water flux and plotted in accordance with first-order kinetics.

Correction for water flux across the intestinal wall. 50 μl aliquots of the samples collected were transferred into scintillation vials, each containing 10 ml of a scintillation cocktail consisting of toluene/2-ethoxyethanol/Butyl-PBD (67 : 33 : 0.6, v/v/w). The ^{14}C count per minute (cpm) of each vial was determined on the scintillation counter set to the automatic external standardization mode. The cpm of a blank sample, carried through the same procedure, was subtracted from the cpm of the samples. For each experiment, the corrected cpm of the samples at time t, cpm_t, was expressed as a fraction of the corrected count of the initial sample at "zero" time, cpm_o.

The percentage of drug remaining in the loop was corrected for water flux by using:

$$\% \text{ remaining} = [(AUC_t/AUC_o) \cdot (CPM_o/CPM_t)] \cdot 100$$

The linearity of the scintillation counter was confirmed by constructing a calibration curve of corrected counts vs quantity of ^{14}C isotope, in the range 8–55 Bq.

Results and Discussion

The modified RP-HPLC assay of salicyclic acid produced peaks of excellent symmetry and of high efficiency, as illustrated by the example of Fig. 2A. For progabide, the analysis (Fig. 2B) indicated the presence of its degradation product, SL79.182, which appeared in small quantities initially and then rapidly decreased with time as a result of possible intestinal absorption. Further, it became clear from this study that progabide was not metabolised, within the lumen of the upper small intestine, to yield its acid metabolite, PGA. In fact, the metabolite was only present initially as an impurity of the progabide powder (Fig. 2B).

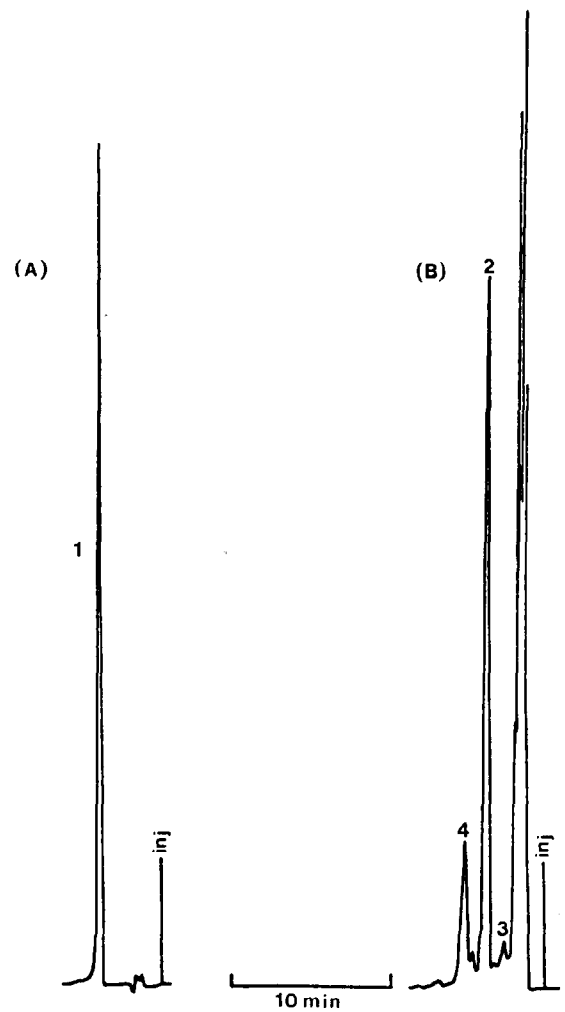

Fig. 2. Typical examples of the RP-HPLC chromatograms obtained upon analysis of the salicyclic acid perfusion solution (A) and of the progabide perfusion solution (B) for the rat intestinal loops; 1, salicyclic acid; 2, progabide; 3, PGA, 4, SL79.182

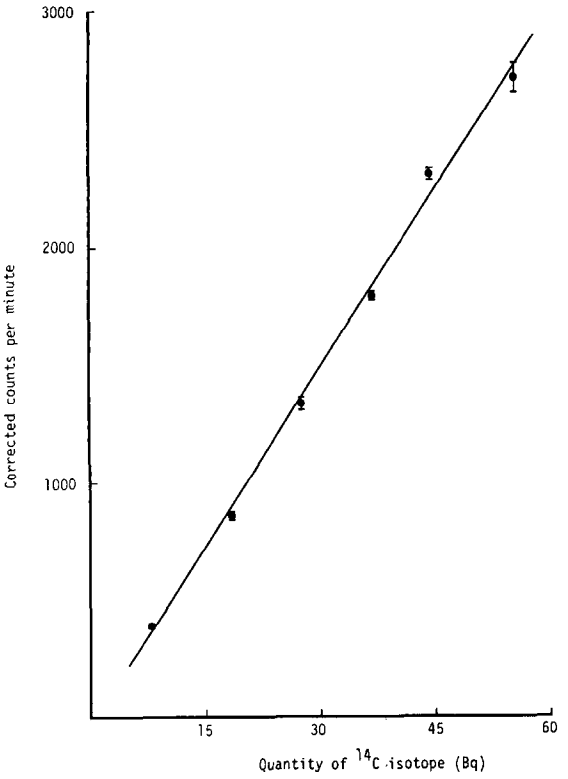

Fig. 3. Calibration curve of ^{14}C-PEG 4000.

The calibration curve for the ^{14}C counts was linear with a correlation coefficient of 0.998 for the best-fit line (Fig. 3).

The validity of the developed perfusion system was confirmed by examining the absorption kinetics of salicyclic acid. Doluisio et al. (1969a) reported a first-order absorption rate constant of 0.085 min^{-1}, with a corresponding half-life of 8 min. Similar results were obtained in this study; salicylic acid was found to be absorbed by first-order kinetics as shown in Fig. 4. By linear regression analysis, the rate constant of absorption was found to be 0.101 min^{-1} with a corresponding half-life of 6.9 min. During the experiment, the pH showed a slow upward drift but this was less than 0.1 unit and so its effect on the absorption of salicylic acid ($pK_a = 2.98$) can be ignored.

For progabide, Fig. 5 shows the disappearance kinetics of the drug from 3 different solutions. As with salicyclic acid, the upward pH shift of about 0.1 unit was ignored. The disappearance curves of the drug for the 3 solutions tested could be described by first-order kinetics. The disappearance rate constants were 0.0908, 0.0758, and 0.0180 min^{-1} for the buffer solution, the 1% β-cyclodextrin solution, and the 0.5% Tween 80 solution, respectively. Since these rate constants are composed of a first-order degradation component and a first-order absorption component, the first-order absorption rate constants can be evaluated by subtracting the appropriate value of the degradation rate constant from the composite values given above. From previous results (Farraj et al., 1987b and e), the degradation rate constants can be estimated at 5.432×10^{-3}, 2.282×10^{-3}, and 4.692×10^{-3} min^{-1} for the above solutions in that order. It therefore follows that the first-order absorption rate constants of progabide for the above solutions are 85.37×10^{-3}, 73.52×10^{-3}, and

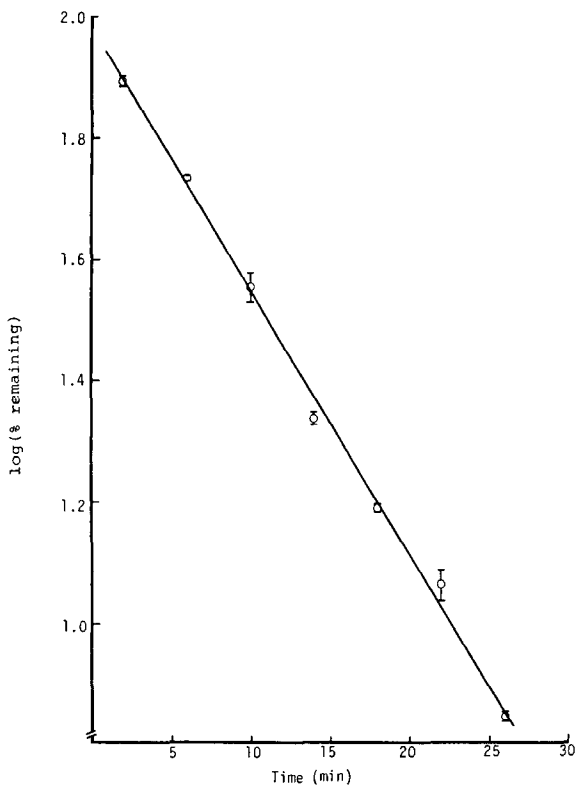

Fig. 4. Semilogarithmic plot of the percentage of salicylic acid remaining in the intestinal loop with time. Each point represents the mean ± S.E.M.

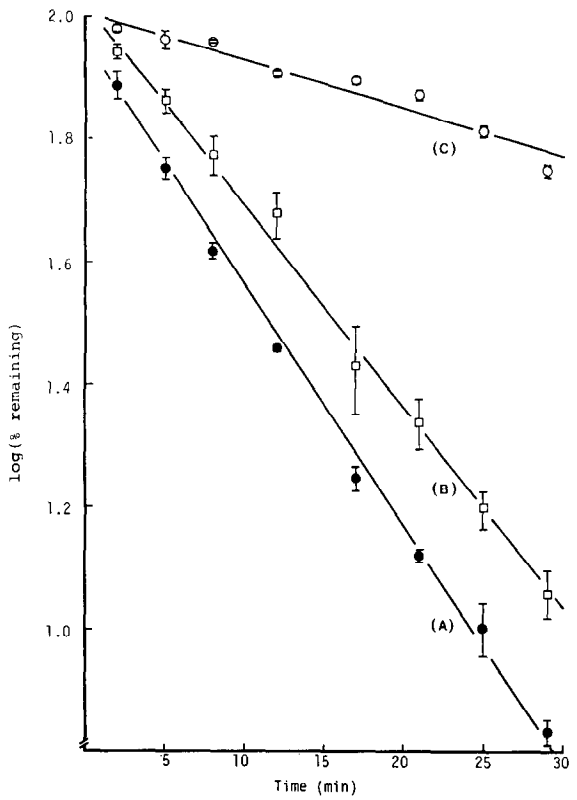

Fig. 5. Semilogarithmic plots of the percentage of progabide remaining in the intestinal loop with time, in the absence of any additive (A), and in the presence of 1% w/v β-cyclodextrin (B) or 0.5% w/v Tween 80 (C). Each point represents the mean ±S.E.M.

13.31×10^{-3} min^{-1} with corresponding half-lives of 8.1, 9.4 and 52.1 min.

Clearly, the absorption of progabide from aqueous solution in the upper small intestine of the rat is extremely rapid. The concomitant presence of complexing species reduced the absorption rate of the drug to varying degrees; Tween 80 resulted in an extreme reduction in the absorption of progabide whereas β-cyclodextrin had only a slight effect. Expressed as percentages, Tween 80 produced an 84% reduction in the absorption rate constant compared with only 14% produced by β-cyclodextrin.

The higher degree of reduction in progabide absorption produced by Tween 80 is probably caused by the strong association between the drug and the micelles in solution, with an association constant of 2122 M^{-1}. In contrast, the stability constant of the progabide/β-cyclodextrin inclusion complex is only 353 M^{-1} (Farraj et al., 1987b).

The possibility therefore exists for exploiting the favourable increase in the solubility and stability of progabide produced by β-cyclodextrin without seriously diminishing its intestinal absorption rate. An additional merit of β-cyclodextrin complexes is that they can be isolated in the solid phase by solvent removal. This offers the possibility of formulating the drug as the β-cyclodextrin complex of improved solubility and dissolution characteristics and avoids the need to form the required complex on site within the gastrointestinal tract. Undoubtedly though, the formation of soluble complexes with endogenous bile salts may result in, and perhaps in part account for, the rapid intestinal absorption of progabide (Farraj et al., 1987a). This is in contrast with the effect of exogenous surfactants, and is related to the different mechanisms by which endogenous surfactants take part in the intestinal absorption process (Westergaard and Dietschy, 1976; Thomson, 1980; Muranushi et al., 1980; Di Colo et al., 1981).

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