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The effect of mucin on the bioavailability of tetracycline from the gastrointestinal tract; *in vivo, in vitro* correlations

M. P. BRAYBROOKS, B. W. BARRY AND E. T. ABBS

School of Pharmacy, Portsmouth Polytechnic, King Henry I Street, Portsmouth, PO1 2DZ U.K.

The bioavailability of tetracycline in the presence and absence of a porcine gastric mucin dispersion (1% w/v) has been studied by *in vivo* perfusion of the rat small intestine, the rat everted gut, a diffusion cell technique and the Sartorius Absorption Simulator Apparatus. In the presence of the mucin preparation an approximate 50% reduction in the numerical values of each of the parameters used to measure the tetracycline movement across the membrane was found. The physiological significance of each technique and the methods themselves are discussed. Interaction of tetracycline with the porcine gastric mucin preparation is considered in relation to the molecular structure of mucus. The results suggest the drug is bound in some way, as yet undefined, to the mucin macromolecule.

Although knowledge of the components of the mucus film which lines the gastrointestinal epithelium is limited, it is generally accepted that the film functions as a physical barrier against mechanical and chemical injury (Zaus & Fosdick, 1934; Florey, 1955; Houck, Bhayana & Lee, 1960). The mucus film, synthesized and secreted by the mucosal epithelial cells of the gut, must however, permit the secretion and absorption of many molecules through the gastrointestinal mucosa. Macromolecules, including gastric mucin, were reported to depress drug transfer rates in *in vitro* models (Block & Lamy, 1969). Tetracyclines bind to porcine gastric mucin (Saggers & Lawson, 1966) and increase the viscosity of bronchial mucus (Kellaway & Marriott, 1973). The viscosity increase caused by the addition of gastric mucin could not account for the extent of the depression of drug transfer rates (Block & Lamy, 1969). We have examined the effect of a mucin preparation upon the absorption of tetracycline in an attempt to elucidate the possible role of the mucus lining. Four experimental procedures were used and the results of the *in vivo* and *in vitro* methods were compared. This paper augments a communication presented at the British Pharmaceutical Conference (Barry & Braybrooks, 1974).

MATERIALS AND METHODS

Materials. Tetracycline hydrochloride B.P. (supplied by Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex and Cyanamid, Gosport, Hants) was converted to the phosphate by reacting with excess sodium phosphate; it was filtered, dried and redissolved in phosphate buffer pH 6·3. The sodium dihydrogen phosphate and disodium hydrogen phosphate (buffer ingredients), the ethyl carbamate (anaesthetic) and hydrochloric acid were laboratory grade. Crude mucin from porcine stomach (Sigma Chemical Co., St. Louis, Missouri, Lot No. 51C–2290), a *readily available 'model'*

mucus material, was suspended in the phosphate buffer pH 6·3. Dialysis bags and diffusion cell membranes were Visking Tubing (The Scientific Instruments Centre Ltd., London) and were soaked in hot water and then in distilled water for three days at 4°.

The Sartorius Absorption Simulator (loaned by V. A. Howe, London) was used with the unspecified membrane-forming chemicals and materials as supplied by the manufacturer (Sartorius-Membronfilter, GmbH, Gottingen, W. Germany). Initial scanning for the transmittance peak was with a Pye Unicam SP800 Spectrophotometer and samples were assayed at the fixed wavelength on a SP600.

Assay of tetracycline. An acid colorimetric method (modified from Grove & Randal, 1955) used 1·0 part sample with 2·5 parts of 2N HCl in 10 ml volumetric flasks, heated in a boiling water bath for 5 min cooled and made up to volume with distilled water. Transmittance was read at 440 nm and a standard graph was prepared using solutions of known concentrations of tetracycline. Known tetracycline concentrations were also assayed in the presence of buffer, the mucin preparation and other additives used to ascertain their effects at 440 nm.

Preparation of mucin dispersions. A 2% suspension of mucin in phosphate buffer pH 6·3 precipitated. Microscopy revealed large undispersed masses in this primary suspension. The dry powdered mucin was hydrophobic and even stirring for several hours did not thoroughly disperse it. As agglomerates could provide sites for surface adsorption of charged molecules, such as tetracycline, they were removed by spinning in a M.S.E. 25 Centrifuge at the radial centrifugal force (RCF) of 23 600g and the supernatant fluid was passed through a sintered glass filter No. 3 with a Metasil A filter bed, but this procedure inevitably also removed some of the insoluble fraction, but gave a suitably stable dispersion. Aliquots were dried to constant weight in an oven at 100° and the bulk dispersion was adjusted to 1% w/v by adding extra buffer as necessary. All mucin dispersions were prepared from the same stock batch and were aged at 2° for at least five days to allow hydration to occur.

Methods

Intestinal perfusion technique—in vivo. The method was a modification of that described by Schanker, Tocco & others (1958). Male Wistar rats, 200–300 g, were anaesthetized with ethyl carbamate (1·5 g kg^{-1}, ip) and maintained at 36–38°. A midline abdominal incision was made and a section of small intestine was exposed below the duodenal loop. The upper and lower ends of a 15 cm length were tied and cut, maintaining the blood supply. The segment was washed with normal saline at 35° and drained. A polyethylene cannula, (0·5 mm i.d.) was secured into the duodenal end of the segment and connected to a pH monitor (Beckman Microblood Assembly with E. I. L. Meter). Another cannula (1·0 mm i.d.) was similarly tied into the distal end of the segment and connected to a reservoir. A peristaltic pump, delivering 0·15 ml min^{-1}, circulated the solution around the system (Fig. 1). The abdominal incision was closed and kept moist. 2·0 ml test solution, in buffer pH 6·3, was pipetted into the reservoir and 0·2 ml samples of its contents were taken at 20, 40 and 60 min. An estimate of the fluid absorption from the buffer perfusing the segment was obtained by adding phenol red to the buffer in the presence and absence of the mucin dispersion and measuring the dye concentration after 20, 40 and 60 min of perfusion (Schedl, 1966; Schanker & others, 1958). The apparent percentage absorption of tetracycline phosphate in phosphate buffer pH 6·3 was found by assaying for changes in the con-

centration of the drug in the reservoir contents. The apparent percentage absorption was also determined in the presence of 1·0% w/v mucin dispersions.

Everted gut technique—in vitro. The apparatus was based on that reported by Crane & Wilson (1958). It consisted of a Pyrex tube with a bung through which was inserted a blunted No. O Luer hypodermic needle with a polyethylene tubing extension over which the everted gut was positioned. The gut was suspended in the tube in a solution of 0·9% sodium chloride and 0·3% glucose plus tetracycline phosphate 0·6 mg ml^{-1} and was continuously aerated by 5% carbon dioxide in oxygen via further openings in the bung. Male Wistar rats, 200–300 g, were killed and a 35 cm length of

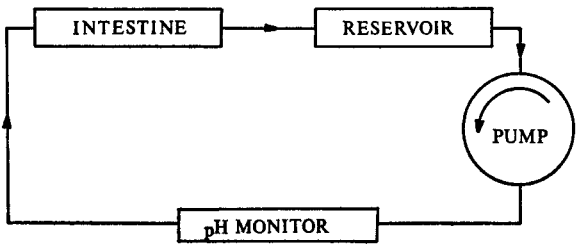

FIG. 1. Flow diagram for *in vivo* absorption apparatus.

gut was removed from below the duodenal loop. The segment was rinsed with warmed glucose-saline solution (37°) and everted (Wilson & Wiseman, 1954). Three 10 cm lengths were cut, each was ligated at one end and attached at the other end to the tubing extension so that the sac had a used length of approximately 9 cm. The three lengths of gut randomly arranged were set up on each occasion and the entire apparatus was suspended in a waterbath at 37° ± 0·5°. 2 ml of phosphate buffer pH 6·3 (artificial serosal fluid) was introduced into the sac via the cannula and after 20 min the entire contents of one sac were removed and a 0·5 ml sample analysed for its tetracycline content. The process was repeated on the other sacs at 40 and 60 min respectively. The passage of tetracycline phosphate through the everted gut was also investigated in the presence of the 1% w/v mucin dispersion.

Diffusion cell technique. The cells were a modified design of those of Patel & Foss (1964). The diffusion cell block was constructed from two sheets of 1·9 cm Perspex measuring 20 by 8 cm, suitably milled to provide three cells, each of 44 cm^3 total capacity. The cellulose acetate membrane (Scientific Instrument Centre, London) was held securely between the two halves of the unit with eight brass bolts with wing nuts, and the sampling ducts were closed with rubber bungs. The cells were maintained at 37° ± 0·5° in a shaking water bath. The donor solution was tetracycline phosphate dissolved in phosphate buffer pH 6·3 with the buffer alone as the receptor solution. Five ml samples of receptor solution were taken 6, 8, 10, 15, 20, 25, 30, 35, 40, 50 and 60 min, in the steady state region of solute flux. Once a sample was taken the cell was abandoned so as to overcome diminishing volume or dilution errors. The investigation was repeated in the presence of 1% w/v mucin dispersion added to the donor solution.

Sartorius Absorption Simulator. (Stricker, 1971, 1973). The apparatus consisted of a diffusion chamber with artificial lipid barrier of area 40 cm^2 representing an artificial intestinal wall. A peristaltic pump circulated the donor and receptor solutions

from containers held at 39° ± 1° (to allow for a drop to 37° at the artificial membrane) through the corresponding compartment of the chamber (Fig. 2). The donor solution consisted of artificial intestinal juice (phosphate buffer pH 6·3) and the receptor solution was artificial plasma (phosphate buffer pH 7·5). For one series of experiments tetracycline phosphate was dissolved in the artificial intestinal juice and in the second series the process was repeated in the presence of the 1% w/v mucin dispersion which was prepared in the same buffer solution. 3 ml samples from both donor and receptor solutions were taken at 0, 1, 2, 3, 4, 5 and 6h. The artificial lipid barrier caused turbidity when acid was added for the tetracycline assay. The turbidity was removed by warming the test solution to 45° and spectrophotometrically assaying the solution at that temperature. Another tetracycline concentration graph was drawn to allow for the increase in assay temperature.

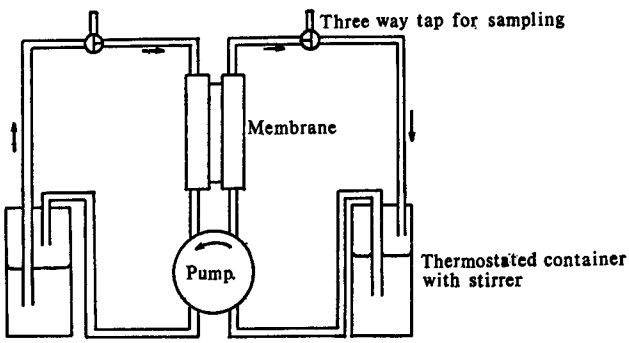

FIG. 2. Flow diagram of Sartorius Absorption Simulator.

THEORY AND RESULTS

The apparent percentage absorption (% Aapp) quoted in the *in vivo* absorption method was calculated using the method of Schanker & others (1958)

$$\% \text{ Aapp} = 100 - \left(100 \frac{C_{\text{DRUG t}}}{C_{\text{DRUG to}}} \times \frac{C_{\text{IND to}}}{C_{\text{IND t}}}\right) \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where $C_{\text{DRUG t}}$ = concentration of drug at sampling time t min, $C_{\text{DRUG to}}$ = concentration of drug at zero time, $C_{\text{IND to}}$ = concentration of dye indicator at zero time, $C_{\text{IND t}}$ = concentration of dye indicator at sampling time, t min, and the results were quoted with standard error of the mean.

The average concentration of tetracycline on the serosal side of the everted gut was also quoted with standard error of the mean.

Apparent permeability coefficients (Pa) for the diffusion cell technique were calculated after Lovering & Black (1973); during steady state

$$Q = \frac{Dc}{L}\left(t - \frac{L^2}{6D}\right) \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where D is the diffusion coefficient, L the thickness of the membrane, c the concentration of the drug in the membrane at the absorbing side and Q is the quantity of drug transferred across the membrane of unit area, at time t.

c is proportional to the concentration of the drug in solution available to pass into the membrane i.e. c′

$$c = kc' \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

Thus substituting in equation 2

$$Q = \left(\frac{Dkc'}{L}\, t\right) - \frac{kc'L}{6} \quad .. \quad .. \quad .. \quad (4)$$

When q (total quantity passed) is plotted against t, upon reaching steady state the slope (dq/dt) is given by (Dkc′/L)

$$\text{Thus } Pa = \frac{(dq/dt)}{c'A} L \quad .. \quad .. \quad .. \quad (5)$$

since Pa = kD where k is a constant for a given set of experimental conditions. A is the area of the membrane in contact with the drug solution.

Using the Sartorius Absorption Simulator the Sartorius diffusion rate constant (kd) was calculated from the initial diffusion rates according to the method of Stricker (1973) as given in the apparatus handbook.

$$kd = \frac{q}{t} \frac{1}{co} \frac{Vo}{A} \quad .. \quad .. \quad .. \quad .. \quad (6)$$

where q/t was the slope of the graph of concentration of tetracycline in the receptor solution (corrected for diminishing volume errors due to sampling) against time, co was the analysed initial concentration of tetracycline in the donor solution, Vo was the initial volume of receptor solution and A the effective barrier area.

As each technique provided a different experimental parameter the percentage changes in the results were quoted to show the effect of repeating the experiments in the presence of mucin dispersion.

Table 1. *The effect of mucin dispersion upon the bioavailability of tetracycline, examined by four techniques.*

Method	*In vivo* perfusion			Everted gut			Diffusion cell	Sartorius Absorption Simulator
Parameter	% App. Absorption			average serosal concn (μg)			App. perm. coef. $cm^2\ s^{-1}$	Diff. rate constant $cm\ min^{-1}$
No. of replicates	20			12			6	5
Time (min)	20	40	60	20	40	60		
Tetracycline	17·2*	30·3	43·3	18·1	77·2	202	$1{\cdot}6 \times 10^{-7}$	$4{\cdot}6 \times 10^{-4}$
	±1·2	±1·3	±1·4	±1·7	±7·0	±10·0		
Tetracycline + 1% mucin dispersion	8·20	16·1	24·1	20·2	45·1	88·8	$9{\cdot}7 \times 10^{-8}$	$2{\cdot}4 \times 10^{-4}$
	±0·70	±0·90	±1·2	±1·6	±2·1	±7·2		
% Reduction in numerical value of parameter	52	47	45	−10**	42	56	41	49

* Value quoted with error of mean.
** % Reduction non-significant.

DISCUSSION

The *in vivo* recirculatory perfusion technique provides a means of studying the intestinal absorption of slowly absorbed drugs. With the perfusion technique, keeping the tubing bore and the total volume as small as practicable with slow circulatory speeds, each portion of the perfusate had a possibility of passing along the intestine at least four times within the hour. Most of the perfusate at any given time was either in the reservoir or in the intestine, so the sampling method gave a reasonable indication of the absorption occurring. The apparent percentage absorption of tetracycline was calculated as a direct comparison of drug concentration at zero time with that at the sampling time and allowing for the fluid absorption from the buffer. The figures thus obtained were directly comparable, even when different drug concentrations were used, as long as the length of the intestinal segment was constant.

The everted gut technique suffers from the lack of a blood supply. The drug therefore has to diffuse through all the structures which constitute the entire intestinal wall. As a result, absorption rates are unrealistically slow. However, the method requires no unusual apparatus and gives reproducible results. The same concentration of tetracycline was used throughout and the gut length was kept constant. Thus the results were calculated as the average concentration of drug on the serosal side at the various sampling times. A 1·0% w/v mucin dispersion hindered the transport of tetracycline from the mucosal to the serosal side of the everted gut (Table 1).

The animals we used were not fasted, because this has been shown to cause unexpected physiological and biochemical changes; fasting times exceeding 20h generally hinder the absorption process (Doluisio, Tan & others, 1969).

With the diffusion cells the mucin dispersion reduced the apparent permeability constant (Pa) of tetracycline by 41%, a reduction similar to that found by the other methods. The Pa figure gave an indication of the rate of movement of the drug from the donor to the receptor solutions with time. But in methods employing artificial membranes the rate of transfer across the barrier depends upon the membrane-aqueous phase drug partition coefficient. Thus, this method is best used to compare permeation rates of a series of similar drugs, giving results which should correlate in rank order with *in vivo* results.

The Sartorius Absorption Simulator was developed for primary absorption studies of drugs and new pharmaceutical formulations. The manufacturers claim that it reduces the number of animal experiments required since results obtained by analogous *in vivo* experiments agreed with Sartorius results "to a reasonable extent". We therefore accepted the offer of the loan of the apparatus to check its results against our other methods. The Sartorius rate constant of tetracycline diffusion (kd) from artificial intestinal juice through an artificial lipid barrier into artificial plasma was therefore measured. The barrier consisted of a membrane filled with a lipid phase. The *in vivo* significance of any results so obtained is always questionable although with passively transferred drugs, reasonable correlation between the *in vitro* constants and corresponding *in vivo* absorption rate constants is claimed (Stricker, 1971).

The time scale of diffusion was greatly extended compared with the *in vivo* experiment; equivalent amounts of drug left the intestinal lumen approximately fifty times as fast *in vivo* as they appeared in the receptor solution of the Simulator. Measurements were taken in the first period of diffusion when the drug molecule passage was largely in the donor to receptor direction. Equations for a simultaneous back

diffusion were not required in this instance because of the relatively short experimental time involved. Using the kd figure, a rate of absorption (ki) can be calculated. The claim is made that this correlates with either rat or human absorption rates for the drug in question from either the stomach or the small intestine. To date no proportionality factor has been published for the rat small intestine, so no ki figure can be quoted in this case. A complete range of these proportionality factors would certainly make the results from the apparatus more meaningful. A 1% w/v dispersion of mucin was used and found to reduce the rate constant of tetracycline diffusion through the artificial lipid membrane (Table 1).

The results of Perrin & Vallner (1970) indicate that tetracycline is absorbed by a passive diffusion process. Pindel, Cull & others (1959) found that it was absorbed to varying degrees along the entire length of the intestine with extremely rapid, peak serum concentrations being obtained when it was passing through the small intestine, even though it must generally pass through the mucus film.

Schrager & Oates (1968) suggested that gastric, bronchial and salivary mucus all have the same basic protein-carbohydrate structure and that differences may be due to other structures present. These workers proposed that the principal protein carbohydrate complex contains a single protein linked to carbohydrate side chains which apparently consist of alternating molecules of glucosamine and galactose and vary in length from two to about twelve units. The molecular weight was shown to be $2{\cdot}04 \times 10^6$ and it was deduced that there are between 1000 and 1500 side chains with branching of the side chains. Using light scattering techniques, Schrager (1969) showed that the macromolecules exist as rigid rods of up to 10000 Å in length and suggested that the gel structure of the bulk material arises from the overlapping of these long rods. The large number of hydroxyl groups on the carbohydrate chains provides the possibility of hydrogen bonding to suitably orientated molecules.

Although endogenous mucus is highly viscous the use of mucin dispersion of similar viscosity proved impractical for the present work. Preliminary experiments demonstrated that a 2% w/v dispersion was the practical upper limit for perfusion. Above this level the viscosity of the dispersion rapidly increased with concentration. High viscosity may slow the passage of drug through the denser environment. If the macromolecular structure of mucin is as Schrager suggests, then at low concentrations little interlocking and overlapping of the rods occurs so there is minimal resistance to the passage of small drug molecules. Lower absorption rates are explicable by possible binding of the drug to the mucin molecule.

In our work the mucin dispersion we prepared reduced by approximately 50% the figures of the parameters used to measure the tetracycline movement across the various barriers. As the permeation of the drug through cellulose acetate membrane was affected by the presence of the mucin dispersion this suggests an attachment between drug and mucin dispersion. If the presence of mucin altered only the passage of tetracycline through the intestinal wall, but not through synthetic membrane, the effect might be due to an interaction between the mucin and the cellular surface rather than binding. Such a weak attachment may be due to for example hydrogen bonding, or ionic interactions, since both mucin and tetracycline have suitable sites, or it may be connected with the solubilization of the drug in a portion of the mucin molecule as found with serum lipoproteins (Powis, 1974).

As in all the methods used a mucin dispersion hindered the passage of tetracycline,

the endogenous highly viscous mucus film can be assumed to play a part in the passive absorption of this and other drugs. It may simply slow the rate of drug passage or it may loosely bind and concentrate the drug at the membrane surface which may increase the passive diffusional rate. Since mucin dispersion was present throughout the donor phase in these techniques only the hindering of drug passage was shown.

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