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Physicochemical and biopharmaceutical characterization of BTA-243, a diacidic drug with low oral bioavailability

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Abstract

This investigation has examined possible causes of the poor bioavailability of the beta$_3$-adrenoceptor agonist BTA-243. The aqueous solubility of BTA-243 is pH dependent with a solubility minimum at pH 1.5. However, the dissolution rate of the disodium salt of BTA-243 is similar at both pH 2.0 and 7.4 indicating that dissolution rate is unlikely to be the controlling factor in the absorption of BTA-243. The apparent permeability coefficient of BTA-243 across Caco-2 monolayers at pH 6 was lower than that of mannitol and therefore the epithelial permeability of the molecule in vivo is predicted to be very low and potentially bioavailability limiting. Apparent permeability coefficients were not dependent on BTA-243 concentration over the concentration range 0.5 to 12 mM, indicating that epithelial transport is unlikely to occur via a saturable mechanism. They were of similar magnitude in both directions across the monolayers, indicative of no significant effluxing of BTA-243 by components of the cell membrane. Apparent octanol/water distribution coefficients increased with decrease of pH between 2 and 6; the relatively low values at pH 4 and 6 suggest that the limited intestinal absorption predicted in vivo will occur predominantly via paracellular passive diffusion. Everted gut sac experiments performed at pH 2.0 and 6.8 suggest that at pH 2.0 a significant proportion of the BTA-243 transport occurs via the transcellular route confirming that the ionization state of the BTA-243 molecule influences the route and rate of epithelial permeability.

Keywords: BTA-243; Solubility; Dissolution rate; Caco-2 cells; Permeability coefficients; Distribution coefficients; Everted gut sac

1. Introduction

New chemical entities often fail in early clinical studies because of low bioavailability resulting from poor absorption. The absorption characteristics of new chemical entities delivered via the oral route are influenced by the physicochemical and biological properties of the molecule. Unfavourable physicochemical properties include inadequate aqueous solubility, slow dissolution and poor membrane permeability. Biological properties influencing the overall epithelial permeability

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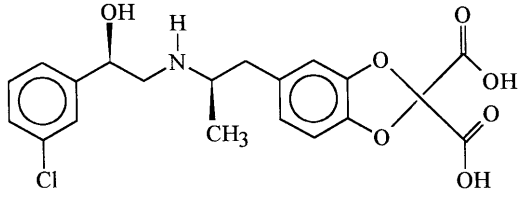
of a drug molecule include the ability of the drug molecule to act as a substrate for nutrient transporters and efflux pumps.

BTA-243 is a pharmacologically active beta$_3$-adrenoceptor agonist and is of potential use in the treatment of diabetes. The drug is associated with a low oral bioavailability following administration in a solid dosage form. The BTA-243 molecule contains three ionizable groups, one amine group and two acid groups .



BTA-243

In a previous study (Brown et al., 1998) we determined the ionisation and aggregation characteristics of this surface active molecule. Potentiometric titration gave a pK_a value of 8.29 ± 0.06 for the amine group. Observation of the chemical shift changes of protons close to the ionizable groups of BTA-243 with change of apparent pH in D_2O at 25°C, using NMR techniques, showed no change in the ionization of the two acidic groups above pH 4. Hence the ionization of the two acid groups occurs at pH values of less than 4 and it may be inferred that between pH 4 and 6.3 the BTA-243 molecule exists in a triple charged ionization state. In 0.2 M sodium acetate at pH ≈ 5 micelles are formed at a critical micelle concentration of approximately 0.01 M with an aggregation number of between 80 and 105 (Anacker et al., 2000).

The aim of the present work is to investigate solubility, dissolution and epithelial permeability of BTA-243 as parameters which may be responsible for the observed poor bioavailability in vivo.

2. Materials and methods

2.1. Materials

BTA-243 disodium salt was donated by Wyeth-Ayerst Research. All other chemicals were purchased from Sigma–Aldrich Ltd. Acetonitrile was HPLC grade and octanol was spectrophotometric grade. Water used to prepare all solutions was doubly distilled.

pH stability studies have demonstrated that the BTA-243 molecule is chemically stable in acidic solutions of pH 1 for up to 96 h at 25°C and is therefore unlikely to undergo any significant degradation in the reported experiments.

2.2. Assay of BTA-243

The concentration of BTA-243 in aqueous solution was measured using a Cecil CE 292 Series II UV spectrophotometer at a wavelength of 287 nm.

HPLC analysis was performed using a Hewlett Packard 1090 Series II HPLC system and a C8 analytical column with a particle size of 5 μm (Phenomonex, prodigy column). The mobile phase consisted of 80% aqueous phase and 20% organic phase (acetonitrile). The aqueous phase was prepared by adding 20 ml triethylamine to 1400 ml water and adjusting to pH 2.5 with concentrated phosphoric acid. BTA-243 was detected at a wavelength of 287 nm, its retention time at a flow rate of 1 ml min^{-1} was 6–8 min.

2.3. Solubility studies

An excess of BTA-243 disodium salt was added to 10 ml volumes of distilled water in 20 ml glass vials and the pH adjusted to a specified value by the addition of 1 M or 0.1 M hydrochloric acid. The solutions were placed in a shaking water bath maintained at 25 ± 0.5°C and shaking at 120 strokes per minute. The pH was readjusted after the initial dissolution of drug and also, if necessary, after 8 h. Aqueous phase was removed at specified time intervals, filtered through Millipore filters (0.45 μm), and assayed spectrophotometrically after dilution in 0.1 M NaOH. Equilibrium solubility was considered to be achieved when the concentration of dissolved drug in solution after 24 and 48 h was found to be constant to within ± 5%. Solubility determinations were performed in triplicate.

2.4. Dissolution studies

Non-disintegrating discs (diameter 19.05 mm, thickness 2.14–2.33 mm) were prepared by compressing 0.56 g BTA-243 disodium salt at 350 kg cm^{-2} for 30 s using a Brookman hydraulic press. Discs were positioned inside a precision-engineered titanium disc holder and inserted into the base of a Perspex dissolution cell (80 mm deep with a flat base of radius 47.5 mm) such that only one face of the disc was uncovered and this face lay flush with the dissolution cell base. The cell was immersed in a water bath at a temperature sufficient to maintain the contents of the cell at 25 ± 0.1°C. Two hundred millilitres of Clark and Lubs KCl–HCl buffer (pH 2) or Sørensen's phosphate buffer (pH 7.4) at 25°C were added to the cell and stirred by a paddle attached to a centrally-positioned, 60 rpm asynchronous electric motor (Crouzet Ltd.). Two millilitre samples of dissolution medium were removed at regular time intervals and replaced by an equal volume of buffer to maintain the dissolution volume and sink conditions. The samples were filtered through Millipore filters (0.45 μm), diluted in 0.1 M NaOH, and assayed spectrophotometrically. The dissolution experiments were performed in triplicate.

2.5. Partitioning studies

Forty millilitres of octanol saturated with water were added to 4 ml aliquots of a 2 mM solution of BTA-243 disodium salt at a specified pH (adjusted with 0.1 M NaOH or 0.1 M HCl) prepared in water saturated with octanol. The flasks were gently inverted ten times before being placed on an orbital shaker inside an incubator maintained at 25°C.

At regular time intervals after the start of the equilibration, the aqueous phase of each flask was separated from the organic phase and its pH was adjusted, if necessary, by the addition of 0.01 M HCl or 0.01 M NaOH before returning it to the flask. The volumes of acid or base added over the duration of the partitioning experiment never exceeded 100 μl and, therefore, did not significantly alter the aqueous phase volume or phase ratio.

Aqueous and organic phases were assayed spectrophotometrically at the end of the equilibration period, after centrifugation at 3500 rpm for 20 min and dilution in 0.1 M NaOH (aqueous phase only). The difference in the BTA-243 content of the aqueous phase before and after partitioning was used to determine the BTA-243 transfer into the organic phase and hence the apparent distribution coefficient. Mass balance calculations were performed to confirm that drug had not been lost from the two-phase system during the partitioning process. In all experiments at least 97% of the drug initially added to the aqueous phase was detected in the aqueous and organic phases at the end of the experiment.

Partitioning experiments were performed in triplicate with equilibration times of 48, 72 and 96 h. Partition coefficients, D, attained an equilibrium value after 48 h; $\log D$ remained constant to within 0.3 units over the period of measurements at each pH.

2.6. Caco-2 cell culture

Caco-2 cells (American Tissue Culture Collection Rockville, MD) were maintained at 37°C in 95% relative humidity with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 1% nonessential amino acids and 2 mM glutamine.

For transport experiments, the cells were seeded onto the upper surface of Millicell-PCF culture plate inserts (diameter 30 mm, pore diameter 0.4 μm) at a target density of 1.2×10^5 viable cells cm^{-2}. Cells of passage 30–40 were used throughout. The cells were allowed to grow and differentiate for 22–25 days before use. The cells were provided with 2.5 ml of supplemented DMEM on both the apical and basolateral sides of the filter. Transport experiments were also performed with mannitol as a passive permeability marker. Mannitol is assumed to be transported by the paracellular route (Artursson et al., 1996).

2.7. Drug transport in Caco-2 cell monolayers

The transport experiments were performed in Hanks' Balanced Salt Solution (HBSS, Sigma). Prior to assay, differentiated, confluent Caco-2 monolayers on Millicell-PCF inserts were equilibrated in HBSS (pH 7.3) for 15 min. The transport experiment was started by positioning the inserts into clean wells containing 2.5 ml of fresh HBSS (pH 7.3) and adding a solution of 2.5 ml HBSS containing drug or ^{14}C-mannitol to the donor side of the cell monolayer. The pH of the dosing solution was preadjusted to pH 6.0 with 1 M HCl or 1 M NaOH. Transport rates of BTA-243 were determined in both directions across the cell monolayer.

Two hundred microlitre samples were withdrawn from the receiving chamber at regular time intervals and immediately replaced by 200 μl of fresh HBSS. Samples were also taken from the donor chamber at the start and end of the experiment. The monolayers were incubated at 37°C on a plate shaker operated at 20 rpm to avoid aqueous boundary layer contributions to drug transport.

The samples taken from the BTA-243 transport experiments were analysed using HPLC. Samples containing ^{14}C-mannitol were mixed with 5 ml Ecolite (ECN) scintillation fluid and the radioactivity measured in a liquid scintillation counter (Beckman LS 6000).

2.8. Everted gut sac model

2.8.1. Preparation of everted gut sacs

Male Sprague–Dawley rats weighing between 300 and 400 g were starved overnight and then killed by cervical dislocation. A 25-cm portion of the jejunum was removed from a position 10 cm distal to the pyloric sphincter, flushed through several times with normal saline at room temperature and placed into a bath of aerated TC199 medium maintained at 37°C. The intestine was carefully everted over a steel rod (2.5 mm diameter) and one end of the tissue was closed using a steel clip to create a sac. The sac was filled with freshly oxygenated TC199 medium (pH 7.4) to give a physiological distension and then sealed using a second clip. The resulting large gut sac was divided into smaller gut sacs of 2.5 cm length using braided silk.

2.8.2. Drug transfer in everted gut sacs

Each everted gut sac was placed in a 50 ml plastic beaker containing 10 ml of pregassed TC199 solution containing 2.0 mM BTA-243 at pH 6.8 or 2.0. The sacs were maintained at 37°C in a shaking water bath operating at 60 strokes per minute. At selected time intervals, a gut sac was removed from its incubation medium, washed four times in saline, dried on a paper towel and weighed. After weighing, the sacs were cut open and the contents of the serosal cavity were drained into a vial for subsequent HPLC analysis. Each sac was then reweighed to permit accurate calculation of the volume of fluid in the sac, and the surface area of the gut sac was measured using a grid system.

2.8.3. Extraction of BTA-243 from intestinal tissue

Intestinal tissue was washed three times in 0.9% saline, dried on a paper towel and weighed. The tissue was homogenised with an equal mass of 0.1 M NaOH, and 1 ml methanol was added to 0.5 g samples to precipitate the protein component. The tissue samples were mixed, cooled for 5 min and then centrifuged for 10 min. One millilitre of the supernatant was removed, dried under nitrogen and reconstituted in 400 μl of mobile phase prior to analysis by HPLC.

3. Results and discussion

3.1. Solubility

The solubility of BTA-243 in water exceeded 20 mg ml^{-1} in the pH range 3–12 and is therefore unlikely to represent an absorption-limiting factor over this pH range. Fig. 1 shows the variation of solubility between pH 1.0 and 2.8. The solubility minimum close to pH 1.5 corresponds to the isoelectric point at which the amine group is completely protonated and exactly 50% of the carboxylic acid groups are ionized. The solubility

of the BTA-243 molecule at physiologically relevant pHs is therefore closely related to the ionization state of the acid groups.

3.2. Dissolution

The possibility that the low solubility of the BTA-243 species at low pH could cause slow dissolution rates in acidic media such as the stomach contents was examined by comparison of the intrinsic dissolution rate under conditions of high and low drug solubility. The intrinsic dissolution rates for the disodium salt of BTA-243 at pH 2.0 and 7.4 calculated from the gradients of linear plots of amount of drug dissolved as a function of time are 9.26 ± 1.20 and 10.85 ± 1.64 mg min^{-1} cm^{-2}, respectively. The similarity of the dissolution rates at these two pHs shows that the low solubility of BTA-243 at pH 2 does not significantly retard the dissolution rate of the disodium salt at this pH, suggesting the creation of a basic microenvironment favourable for dissolution as the disodium salt dissolves. The absorption of BTA-243 disodium salt is therefore unlikely to be dissolution rate limited.

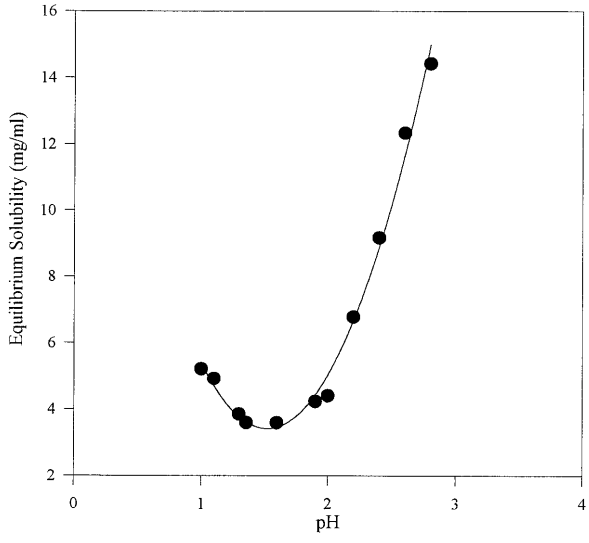


Fig. 1. Equilibrium solubility of BTA-243 as a function of pH in aqueous solutions at 25°C.

Table 1
Octanol/water distribution coefficients, D, for BTA-243 as a function of pH

pH	Log D	Standard deviation
2	0.24	0.005
3	−0.74	0.009
4	−1.8	0.11
6	−3.3	1.2

3.3. pH-Partition studies

Table 1 shows the logarithm of apparent distribution coefficient D, defined as the ratio of all related species, ionized and neutral, between nonpolar and aqueous phases (Stewart et al., 1997), in the pH range 2–6. Log D values increase as the pH of the BTA-243 solution is reduced from pH 4 to 2 reflecting increased lipophilicity of the BTA-243 molecule brought about by protonation of the diacid group and a reduction in the negative charge in this region of the molecule. At pH 4 and 6, the apparent distribution coefficient is relatively low indicating that BTA-243 in solution at these pHs will penetrate cell membranes to only a limited extent.

3.4. Caco-2 permeability

Caco-2 transport experiments were performed to assess the permeability of BTA-243 across cultured epithelial cells at pH 6 and to determine if the BTA-243 molecule is a substrate for an active carrier mediated transport mechanism in either the serosal-to-mucosal or mucosal-to-serosal directions.

Drug transfer rates are conventionally reported and compared by the calculation of an apparent permeability coefficient (P_{app}) according to Eq. (1):

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A\ C_0} \quad (1)$$

where dQ/dt is the permeability rate, C_0 the initial concentration in the donor chamber and A the surface area of the monolayer. Fig. 2 shows the influence of drug concentration and direction of

transfer on the apparent permeability coefficients calculated for BTA-243. The apparent permeability coefficient of mannitol in the apical-to-basolateral direction is included as a reference.

Concentration of drug in the donor compartment was observed to have no significant effect on the mean BTA-243 apparent permeability calculated at the three concentrations considered. The active membrane transport proteins such as the dipeptide transporter are known to transport several hydrophilic drugs, including cephalexin and enalapril, across the apical membrane of epithelial cells in the small intestine (Walter et al., 1996). Active transporter mechanisms are saturable and therefore the contribution of a passive route to the overall transport is expected to increase with increasing substrate concentration above the saturation level. For drugs such as BTA-243 with low passive permeabilities, saturation of the carrier would be expected to result in a lowering of apparent permeability coefficient with increasing concentration. Consequently, the absence of any significant effect of drug concentration on apparent permeability is consistent with BTA-243 being transported across the monolayer predominantly by a passive diffusion mechanism.

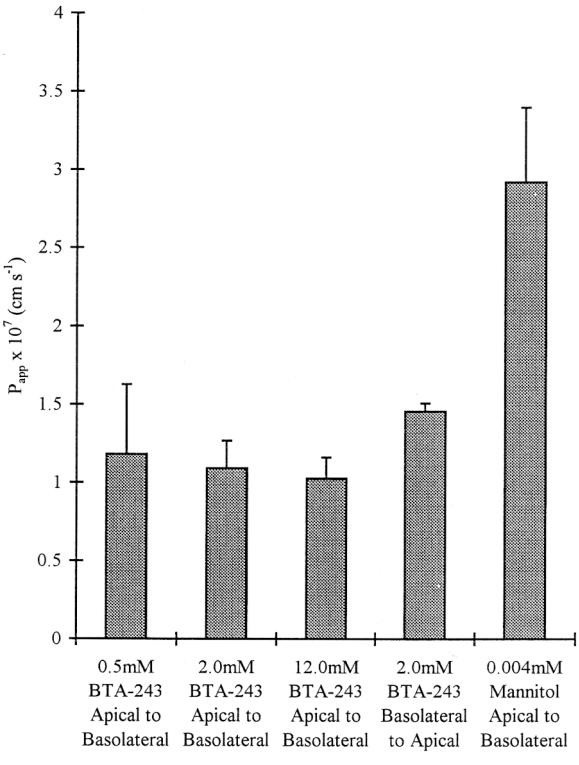


Fig. 2. Apparent permeability coefficients P_{app} of BTA-243 and mannitol across Caco-2 monolayers in the direction specified.

The mean apparent permeability coefficient in the basolateral-to-apical direction was 34% greater than that in the opposite direction. The difference in mean P_{app} recorded in either direction was not found to be significant at the $p = 0.05$ level and therefore it is concluded that at the concentrations studied, BTA-243 was not significantly effluxed into the apical medium.

The mean apparent permeability coefficient calculated for BTA-243 in the apical-to-basolateral direction from solutions with concentrations of 0.5, 2 and 12 mM is 0.135×10^{-6} cm s^{-1}; $\sim 50\%$ of that observed for the paracellular marker mannitol (0.29×10^{-6} cm s^{-1}). In vivo studies have indicated that 16% of a mannitol dose is absorbed (Artursson, 1991) and hence the low permeability coefficient of BTA-243 suggests its poor in vivo absorption occurs as a consequence of a poor epithelial permeability at intestinal pH.

3.5. Transfer of BTA-243 across everted gut sacs from solutions of pH 2.0 and 6.8

The pH-partitioning study showed that BTA-243 distributed into the octanol phase to a greater extent at pH 2 than at pH 6. In vivo, the more lipophilic species present at pH 2 may penetrate epithelial membranes to a greater extent making greater use of the transcellular route and increasing the mucosal-to-serosal transfer rates across gastrointestinal epithelia. The everted gut sac experiment was employed as an ex vivo model to investigate this hypothesis. Fig. 3 shows a smaller amount of BTA-243 transferred across everted gut sacs from bathing solutions adjusted to pH 2.0 than from solutions at pH 6.8. Calculation of the rates of transfer from the gradients of the linear best fit lines for each set of data showed a lower transport rate to the serosal cavity at pH 2.0 (0.31 nmol cm^{-2} min^{-1}) compared with that at pH 6.8 (0.86 nmol cm^{-2} min^{-1}). However, the amount of BTA-243 accumulated over 60 min in

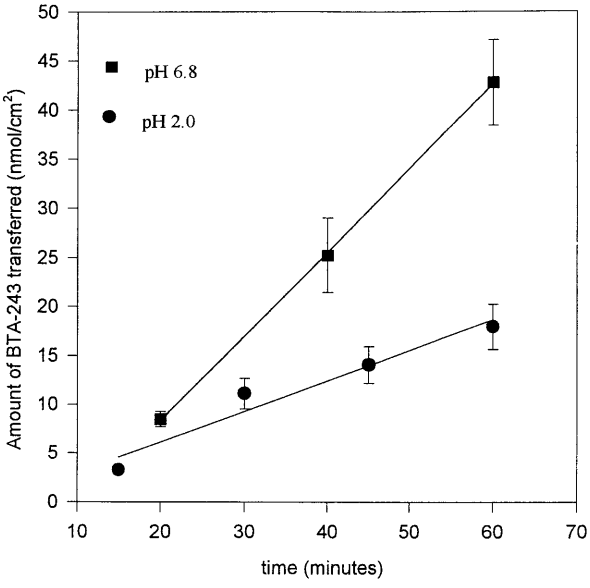


Fig. 3. Amount of BTA-243 transported across everted gut sacs from aqueous solutions at ● pH 2.0 and ■ pH 6.8. Vertical bars represent standard error ($n = 3$ at pH 2.0 and $n = 8$ at pH 6.8).

the gut tissue from solutions at pH 2.0 (1.92 ± 0.48 μmol g^{-1} wet tissue, $n = 3$) was approximately ten times that from a solution at pH 6.8 (0.20 ± 0.08 μmol g^{-1} wet tissue, $n = 3$).

The combination of a low overall transfer rate and a high level of tissue accumulation at pH 2.0 may indicate an interaction of the drug molecule with some mucosal components leading to retardation of transfer and accumulation in the tissue. A similar accumulation has been reported previously with propranolol in the Ussing chamber experiment (Yamashita et al., 1997).

4. Conclusions

This investigation has examined possible causes of the poor bioavailability of the beta$_3$-adrenoceptor agonist BTA-243.

The apparent permeability coefficient of BTA-243 across Caco-2 monolayers at pH 6 was lower than that of mannitol and therefore the epithelial permeability of the molecule in vivo is predicted to be very low and potentially bioavailability limiting. The lack of dependence of the apparent permeability coefficient on BTA-243 donor concentration over the concentration range 0.5–12 mM suggests that epithelial transport is unlikely to occur via a saturable mechanism such as a nutrient transporter. The absence of polarised transport at a concentration of 2 mM indicates that BTA-243 is not significantly effluxed when presented to a cell membrane at this concentration. The partition coefficient measured for BTA-243 at pH 6 suggests that at this pH membrane penetration will be low and it is concluded that intestinal absorption of BTA-243 is likely to occur to a limited extent via a predominantly paracellular passive diffusion mechanism. Everted gut sac experiments performed at pH 2.0 and 6.8 suggest that at pH 2.0 a significant proportion of the BTA-243 transport occurs via the transcellular route confirming that the ionization state of the BTA-243 molecule influences the route and rate of epithelial permeability.

Although the equilibrium solubility of BTA-243 is pH dependent with a solubility minimum at pH 1.5, the dissolution rates of the drug in its disodium salt form are similar at both pH 2.0 and 7.4 indicating that the absorption of BTA-243 disodium salt is unlikely to be dissolution rate limited. It may, therefore, be concluded that the low epithelial permeability of the molecule at pH 6 is chiefly responsible for the low oral bioavailability of this drug.

Acknowledgements

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