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Functional Characterization of Amino Acid Transport System for Transport of Phenylalanine on Mammalian Cornea for Better Ocular Drug Delivery

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Abstract:
In last few years transporter targeted drug delivery has drawn attention of research to identify and explore various nutrient transport systems including amino acid transporters for better drug delivery. The aim of the present study was to investigate and characterize the transport of L-phenylalanine across goat cornea. Transport of L-phenylalanine (0.5 % w/v aqueous solution) through excised goat cornea using modified Franz diffusion cell was investigated to characterize the effect of concentration, pH, presence of sodium ions, presence of metabolic inhibitors like sodium azide, presence of sodium potassium ATPase pump inhibitor like ouabain and presence of other amino acids. Effect of presence of aspartame (a dipeptide) and tripeptide (glutathione) was also investigated. To characterize the effect of stereo specificity, corneal transport of DL-phenylalanine and D-phenylalanine was studied. The results of studies revealed that transport of L-phenylalanine is concentration dependent, pH independent, sodium independent and inhibited by cationic and neutral amino acids along with the dipeptide aspartame. Cationic and neutral amino acids seem to share the same transporter for corneal transport. The transporter identifies and prefers the transport of L form of amino acid as compared to DL and D form. All the result are consistent with the previous reports on transport of L-phenylalanine through mammalian epithelial membrane and suggests functional presence of L-type of amino acid transport system on goat cornea.
Keywords: amino acid, cornea, phenylalanine, transport

Introduction:

Amino acids are basic constituents of a cell structure. Their importance in anatomy and physiology of the cell is thoroughly studied. Amino acids require specialized transport systems to cross the plasma membrane [1]. Special transport systems exist on mammalian cell for transport of amino acids. These transport systems are classified on the basis of their characteristics like substrate affinity, dependence on sodium ions, energy, pH. Several amino acid transport systems have been identified and characterized on molecular level. These includes L, y^+L, A, ASC, asc, $b^{0,+}$, $B^{0,+}$ and x^-, Gly, N, and T type of systems[2-8]. In last few years importance of these transporters in pharmacokinetics has been recognized. Drug permeation through various biological barriers has been studied and the role of active transport is established. In some studies improved bioavailability of amino acid linked compound is reported. Various ocular tissues like conjunctiva, retinal-pigmented epithelium, are known to have presence of some of these transporters. Limited information is available on the presence of amino acid transporters on corneal epithelium. Existence of oligopeptide transporter has been reported in rabbit corneal epithelium [9]. A Na^+ dependent cationic and neutral amino acid transporter $B^{0,+}$ has been identified recently in human and rabbit corneas [10]. Few efforts have been made successfully to improve topical ocular drug delivery through cornea by targeting these transporters by means of amino acid linked drug derivative approach [11-14]. Majority of studies have been conducted on rabbit cornea and rabbit corneal cell line (SIRC, Statens Serum institute rabbit corneal cells). Human cornea and cell lines have also been employed in investigations. However, procuring human cornea is difficult while the cell lines often are not considered to be epithelial, but it rather shows fibroblast like properties [15]. Data on mammalian corneas with respect to transport of amino acids is still sparse. In ocular therapeutics, so far not many people have utilized the corneal transporter targeted drug delivery approach to improve topical drug delivery. More studies on mammalian amino acid transporters will help in understanding the complex process of amino

acid transport and their role in better drug delivery.

Phenylalanine is one of the eight essential amino acids in the human diet. The amino acid plays important role in various cellular metabolic activities.

In view of the above information the objective of the studies is to characterize the corneal transport L-phenylalanine (L-phe), DL-phenylalanine (DL-phe) and D-phenylalanine (D-phe) with respect to concentration dependence, sodium ion dependence, pH dependence and effect of metabolic inhibitors.

Materials and Methods:

L-phe, DL-phe and D-phe, choline chloride were procured from Lancaster, Chennai. Ouabain was obtained from Sigma Aldrich, Banglore. Sodium azide amino acid kit was supplied by SD Fine Chem Ltd, Mumbai. Other chemicals used were of either LR or AR grade from SD-Fine Chem-Ltd, Mumbai. Fresh whole eye balls of goat were collected form local butcher shop (Sanjeet Naka, Mandsaur) immediately after slaughtering and preserved in cold Dulbecco's phosphate buffer saline (DPBS, pH 7.4) in order to prevent any sort of deterioration.

Corneal transport studies of Amino acids:

Diffusion apparatus

In vitro permeation studies were carried out in an all glass modified Franz diffusion cell containing a donor and receptor cells as described by Rathore and Majumdar [16]. The donor cell was clamped over the receptor, which was provided with a side arm for sampling and had an internal capacity of 11ml. The area available for the corneal transport was 0.785 cm^2. The receptor contained Dulbecco's phosphate buffer saline (DPBS). Donor solution (1ml) was placed on the epithelial surface of the cornea and placing a glass cover slip over the opening of the donor cell retarded evaporation of donor solution. In experiments to study sodium ion dependence the sodium chloride in DPBS composition was replaced with choline chloride [10]. Water at 32^0C was circulated through the water jacket surrounding the receptor cell. A teflon coated magnetic bead was placed at the bottom of the receptor cell to ensure homogeneity of the receptor cell solution. The whole assembly was placed on a magnetic stirrer. The donor compartment represented the conjunctival sac of the eye where as the receptor compartment represented the anterior segment of the eye.

Preparation of Cornea

Fresh whole eyeball of goat, collected immediately after slaughtering of the animal, was brought to the laboratory in cold DPBS (4^0C). The cornea was carefully removed along with 2-4 mm of surrounding scleral tissue and washed with cold normal saline, till the UV absorbance of the washing became zero. Care was taken not to traumatize the tissue while handling it.

Experimental Procedure:

Fresh cornea was mounted by sandwiching the surrounding scleral tissue between clamped donor and receptor compartment. The receptor compartment was filled with freshly prepared normal saline and all air bubbles were expelled from the receptor by inverting the diffusion cell then allowing the bubbles to travel out of the sampling port. An aliquot (1ml) of donor solution was placed on the cornea, while the receptor fluid was kept under stirring and permeation was continued for 120min. Sample was withdrawn from the receptor and analyzed for phenylalanine content in UV-Visible spectrophotometer (Shimadzu,Japan) at absorption maxima of 213 nm. At the end of the experiment each cornea was weighed, soaked in methanol for 3-4 minutes, dried at 80^0C for overnight. From the differences of weights corneal hydration (%) was calculated by the following formula

$$\% \text{ Hydration} = (1- W_d/W_w) \times 100$$

Where W_d= Weight of dried cornea and
W_w= Weight of wet cornea

Preparation of standard curve of L-Phenylalanine in DPBS pH 7.4

Solutions ranging from 1 to 10 µg/ml concentration were prepared and absorbance was read in a UV- Visible spectrophotometer at absorption maxima of 213 nm. Standard curve was plotted between concentration and absorbance. The amount of phenylalanine transported through cornea was read from the standard curve.

Preparation of test solutions

1. L-Phenylalanine aqueous solutions of increasing concentrations of 0.1 or 0.2 or 0.3 or 0.4 or 0.5 or 0.6 or 0.8 or 1.0 % (w/v) at pH 7.2 made isotonic with sodium chloride were prepared.
2. L-phenylalanine aqueous solution 0.5 % (w/v), pH 7.2 made isotonic with dextrose or sodium chloride (NaCl) were prepared.
3. L-phenylalanine aqueous solution 0.5 % (w/v) was constituted at pH 5 or 6 or 7.2 or 9.
4. L-Phenylalanine 0.5 % (w/v) aqueous solution with equimolar amount of L-L-glutamic acid or L-aspartic acid or L-proline or L-lysine or L-histidine or L-arginine were constituted at pH 7.2 and made isotonic with sodium chloride.
5. L-Phenylalanine aqueous solution with 0.030 mM aspartame was prepared and made isotonic with sodium chloride and pH was adjusted at 7.2
6. L-Phenylalanine solution 0.5 % w/v with 0.030 mM glutathione was prepared and made isotonic with sodium chloride at pH 7.2
7. L-Phenylalanine 0.5 % (w/v) aqueous solution with 25 or 50 micromole (µM) sodium azide or 50 µM Ouabain (octahydrate) was also prepared at pH 7.2
8. Aqueous solutions of DL-phenylalanine (0.5 %) and D-phenylalanine (0.5 %) were prepared and made isotonic with sodium chloride at pH 7.2.

Results and Discussion:

Transport of amino acids through mammalian cells is governed by multiple transporters. These transport systems differ in their substrate specificity and affinity, pH and ion dependence and tissue expression pattern. A number of transporter systems have been identified in mammalian cells. Gene expression of several amino acid transporters (LAT1, ATB0,+, ASCT1) have been found in the cornea and the active transport of L-phenylalanine, L-arginine and L-alanine across isolated rabbit cornea was shown[17]. Amino acid transport consists of various carrier systems with different affinities towards anionic, cationic and neutral amino acids. However, the transporters may vary with the animal species and among mammals may express different type of transport systems. Also, Limited information is available on the expression of transporters on mammalian corneal epithelium. Transport of L-Phenylalanine (L-Phe) was studied through excised goat cornea for concentration dependence, pH dependence, sodium ion dependence. Effect of metabolic inhibitors like ouabain and sodium azide was investigated. Effect of a dipeptide (aspartame) and tripeptide (glutathione) on corneal transport of L-Phenylalanine were also studied.

Effect of concentration

The transport characteristics of L-phenylalanine were studied through isolated goat cornea. The amount of L-phenylalanine transported through cornea from isotonic aqueous solutions of different concentrations like 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1% (w/v). As the concentration of L-phenylalanine in aqueous solution made isotonic with sodium chloride increased from 0.1 to 1.0 %, the amount permeated increased up to 0.5 % beyond which no

increase was observed. The amount permeated was maximum at 0.5% concentration of L-phenylalanine i.e. 0.0451 mg. The transport of L-phenylalanine seems to be concentration dependent. The results are shown in figure 1.

Sodium ion dependence

To investigate the effect of dependency of sodium ions on transport process of L-phenylalanine paired goat corneas were used. L-phenylalanine solution of 0.5 % w/v were prepared and made isotonic with either sodium chloride or dextrose. The receptor compartment was filled DPBS that were devoid of sodium ions. Amount permeated of L-phenylalanine was 0.0451 mg when tonicity was adjusted with sodium chloride while that of with dextrose was 0.0405 mg. Study indicates that there was no significant difference in transport from both the solutions through goat cornea. Transport process seems unaffected by sodium ions. Results depicted in figure 2.

Effect of pH

Transport characteristics of L-phenylalanine (0.5 % w/v made isotonic with sodium chloride) was investigated on pH 5 or 6 or 7.2 or at pH 9. There was no significant difference in transport from pH 5 to 7.2. Slight decrease was observed at pH 9 as presented in figure 3. From the results it seems that L-tryptophan transport through goat cornea at 0.5 % w/v concentration is independent of pH of the corneal epithelium site.

Competitive inhibition studies in presence of other amino acids

Corneal transport of L-phenylalanine 0.5 % w/v made isotonic with sodium chloride was investigated in the presence of anionic (L-glutamic acid and L- aspartic acid), cationic (L-histidine, L-arginine and L-lysine) and neutral amino acids (glycine and L-proline) through freshly excised goat cornea. All amino acids inhibited the transported of L-phenylalanine to varied extent as compared to control 0.0451 mg. Data is presented in figure 4. In presence of L-lysine maximum inhibition was observed followed by L-arginine, L-histidine, L-proline L-aspartic acid, L-glutamic acid and least inhibition was observed with glycine. Study reveals that the transport system is also having affinity to the L-forms of the above amino acids. Among all the cationic amino acids showed maximum inhibition of L-phenylalanine hence may be considered as preferred substrates over others for the transport system responsible to transport of L-phenylalanine. Corneal hydration was in normal range except with glutamic acid, aspartic acid proline, and lysine that was beyond 80 %.

Competitive inhibition studies with di and tripeptide:

Corneal transport of L-phenylalanine form aqueous solution of 0.5 % w/v made isotonic with sodium chloride was studied through cornea in presence of aspartame and glutathione. The amount of L-phenylalanine transported through cornea was 0.0297 mg in presence of aspartame and that was 0.0451 with control (without aspartame) as shown in figure 5. Aspartame is L-aspartyl-L-phenylalanine methyl ester and synthesized by esterification of aspartic acid and phenylalanine. L-phenylalanine is a constituent of this dipeptide. The study indicates that aspartame is sharing the same transport system involved for the transport of L-phenylalanine. The reduced permeation of L-phenylalanine may be attributed to competitive sharing of the same transporter.

With tripeptide glutathione no significant difference was observed in transport process of L-phenylalanine through paired goat cornea (figure 6). The result indicates that L-phenylalanine transport system recognizes alike dipeptides but not tripeptides.

Effect of metabolic inhibitors

Corneal transport of L-phenylalanine form aqueous solution of 0.5 % w/v made isotonic

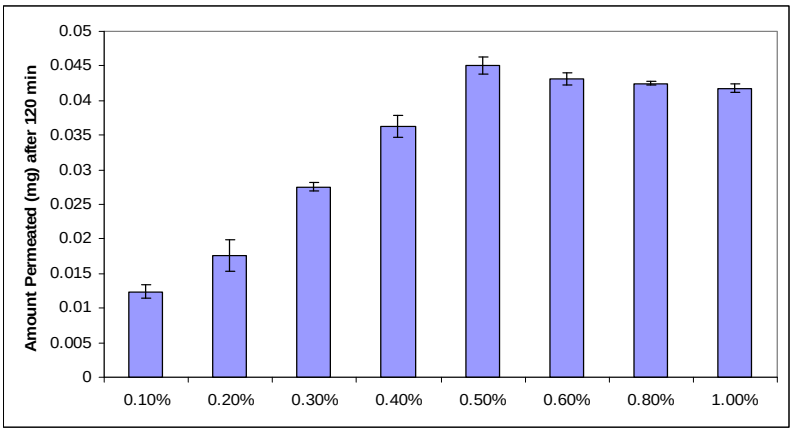

Figure 1: Corneal transport characteristics of L- Phenylalanine from 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0% w/v aqueous solution through excised goat cornea

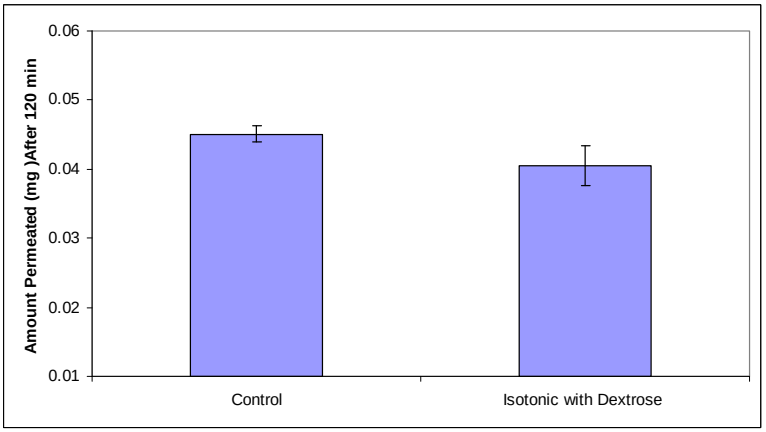

Figure 2: Effect of Sodium ions on transport characteristics of L-phenylalanine (0.5 % w/v) aqueous solution across goat cornea

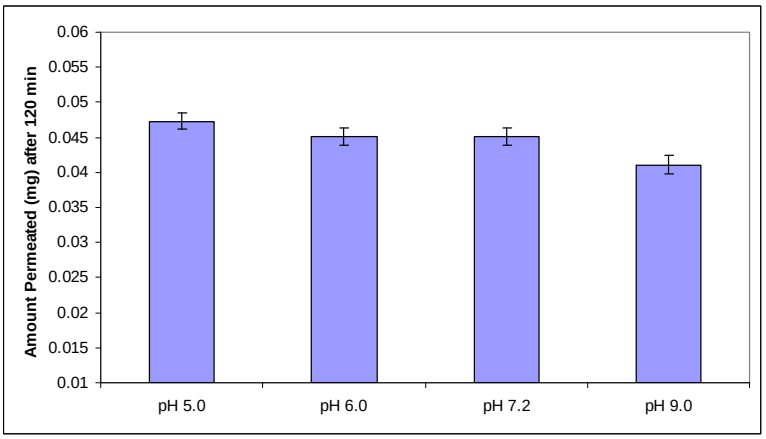

Figure 3: Effect of pH on corneal transport of L-phenylalanine through excised goat cornea

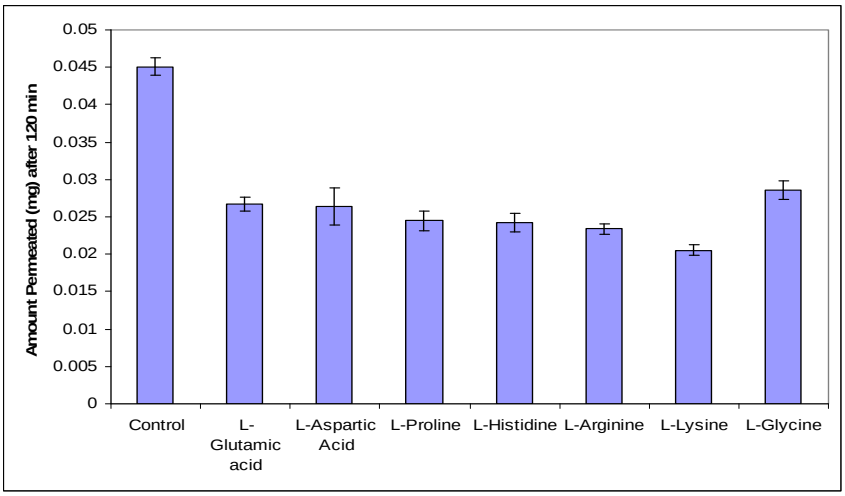

Figure 4: Effect of presence of different amino acids on corneal transport characteristics of L-phenylalanine aqueous solution (0.5 % w/v) through excised goat cornea

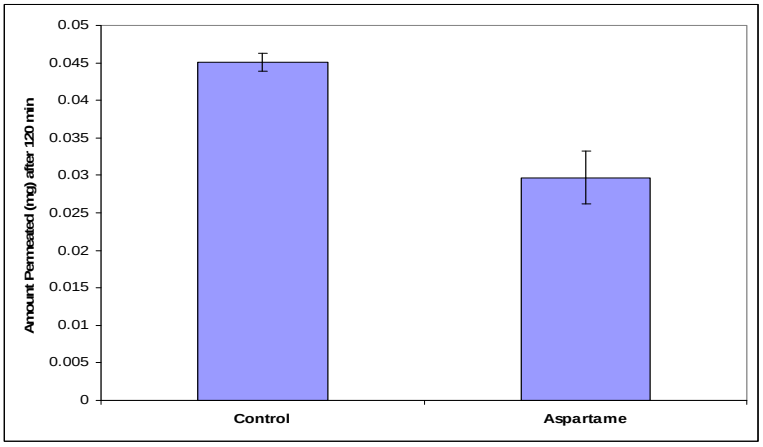

Figure 5: Transport characteristics of L-phenylalanine (0.5 % w/v) aqueous solution through excised goat cornea in presence of a dipeptide aspartame

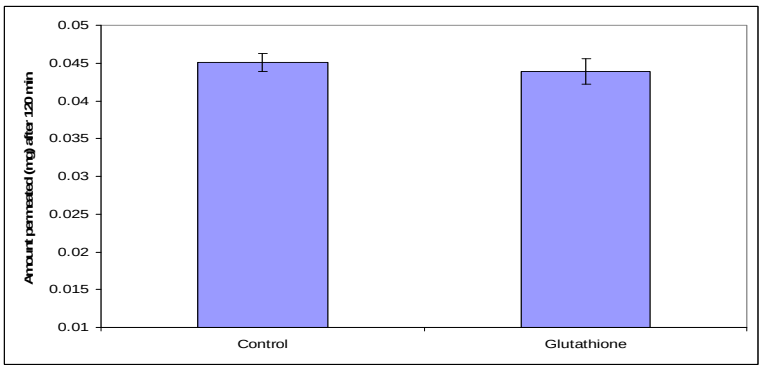

Figure 6: Transport characteristics of L-phenylalanine (0.5 % w/v) aqueous solution through excised goat cornea in presence of a tripeptide glutathione

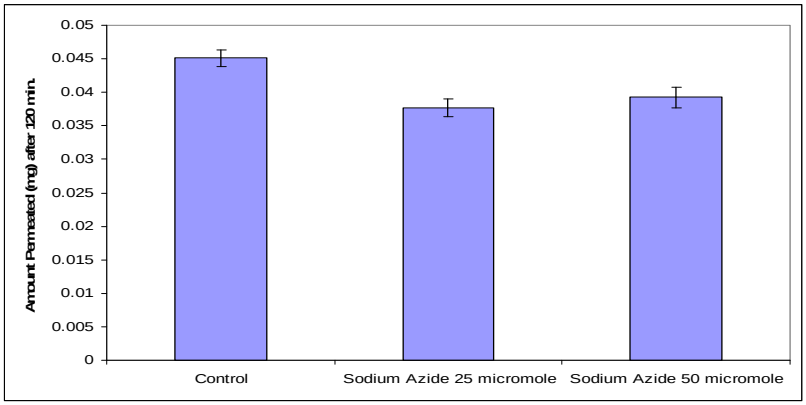

Figure 7: Effect of presence of sodium azide on corneal transport of L-phenylalanine through excised goat cornea

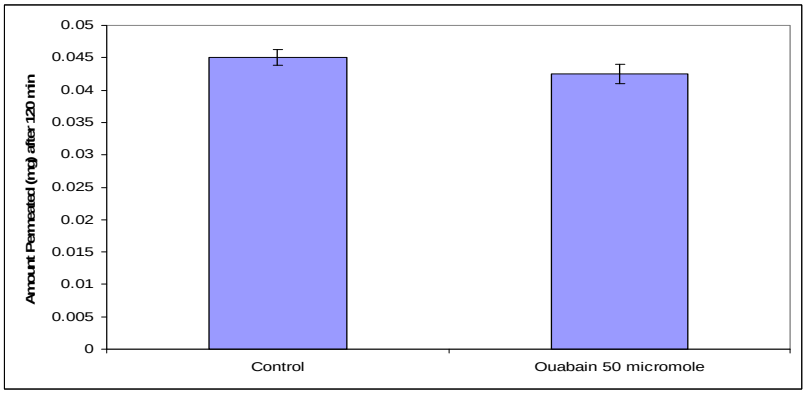

Figure 8: Effect of presence of ouabain on corneal transport characteristics of L-phenylalanine through excised goat cornea

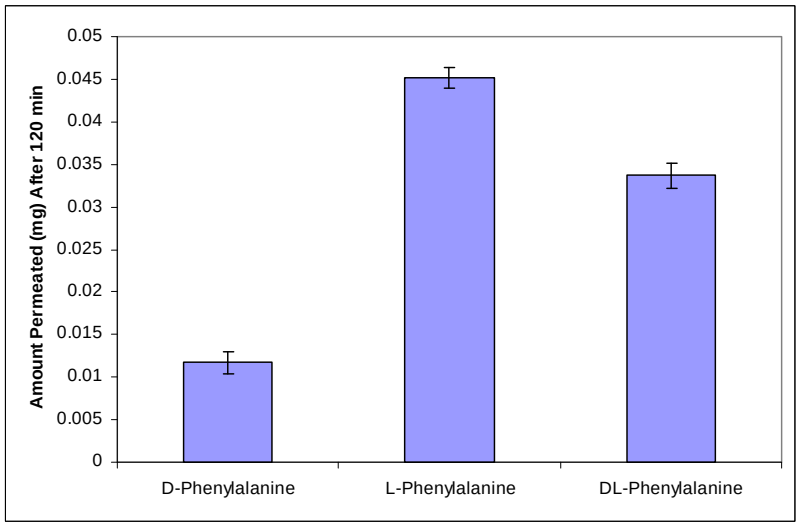

Figure 9: Relative transport characteristics of L-phenylalanine, DL-phenylalanine and D-Phenylalanine through excised goat cornea

with sodium chloride was investigated in the presence of metabolic inhibitor sodium azide (25 and 50 µM) and ouabain (50 µM). The permeation was slightly reduced in presence of the metabolic inhibitor while there was no significant difference observed in presence of ouabain. The result in figure 7 and 8 shows that the transport of L-phenylalanine across goat cornea is energy and sodium ion independent.

Corneal Transport of DL-phenylalanine

Cormeal transport of DL-phenylalanine at 0.5 % concentration was investigated. The amount of DL-phenylalanine permeated across cornea was 0.0337 mg after 2 hours of permeation (figure 9).

Corneal Transport of D-phenylalanine

To investigate the stereo specificity of the involved transporter the transport characteristics of D-phenylalanine was studied. The permeation of D-phenylalanine (0.011 mg) from 0.5 % w/v aqueous solution was significantly low as compared to L-phenylalanine (0.0451mg) or DL-phenylalanine (0.0337 mg) at same concentration figure . The results (figure 9) indicated that the transport system responsible for phenylalanine transport is stereo specifically selective and has more affinity to L-type as compared to D isomer.

On the basis of above studies on corneal transport of phenylalanine the following facts may be stated:

1. Transport of L-phenylalanine is concentration dependent. After 0.5% concentrations the transport does not increase with increase in concentration.
2. The transport is not dependent on pH.
3. The transport occurs in presence of sodium azide and ouabain that indicates the transport is not energy dependent and has not impact of sodium ions.
4. The transport system seems to have more preference to L-isomer compared to D-isomer of phenylalanine.
5. The transport is inhibited by L-forms of cationic (Arginine, histidine and Lysine) , anionic (glutamic acid and aspartic acid) and neutral (glycine and proline) amino acids
6. Transport system transports L-phenylalanine along with structurally similar dipeptide like aspartame across goat corneal epithelium.

SLC7 LAT. Nakauchi et al. [18] reported that LAT1 (SLC7A5) and LAT2 (SLC7A8) mRNAs are expressed in cultured human RPE cell line and mRNA expression of LAT2 was found also in ARPE-19 cells. Furthermore, Na+ independent transport system for L-phenylalanine, which shows characteristics similar to LAT2 was found in ARPE-19 cells [19].

The present study reports the functional presence of L type of transport system on goat cornea for the first time. In studies on rabbit corneal cell line L type of system is of two types i.e. LAT-1 and LAT-2 LAT1 recognizes and transports D-leucine, L- and D-phenylalanine, D-methionine, L-tyrosine, Lhistidine and L-tryptophan. LAT2 substrates include Lalanine, L-cysteine, L-glutamine, L-glycine, L-alanine L-serine. System L is also known to participate in drug transport. L-Dopa, methyldopa, gabapentin and melphalan are transported by this system [18]. There might be a possibility of presence of another type of L system since in the studies the transport system recognizes L-phenylalanine preferably along with and neutral amino acid glycine, proline; cationic amino acid arginine, lysine and histidine and anionic amino acids like aspartic and glutamic acid.

Conclusion:

With these studies functional presence of L-type of system is evident on goat corneal epithelium. For the first time ever the presence of a substrate specific (prefers L-

form of amino acid for transport) sodium and energy independent transport system for phenylalanine that is likely to cotransport the dipeptide with structural similarity like aspartame on goat corneal epithelium is reported. The identified transporter would certainly help in selecting the amino acid for preparation of amino acid compound of drugs that are likely to be transported through cornea for better ocular delivery and availability.

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