



Research article

Functional characterization of L-tryptophan transport across mammalian cornea

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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 present research work is to investigate the transport characteristics of L-tryptophan (L-try) across goat cornea. Transport of L-try was investigated using a glass diffusion cell for effect of concentration, pH, presence of other amino acids or metabolic inhibitor or dipeptide and tripeptide. The amount of L-try transported increased as the pH of L-try aqueous solution increased from 5 to 9. Inhibition was observed in L-try transport in absence of sodium ions where L-try solution was made isotonic with dextrose. Amino acids like L-histidine, L-arginine, L-lysine (cationic), L-glutamic acid, L-aspartic acid (anionic), glycine and L-proline (neutral) inhibited the L-try transport as compared to control (L-try alone). In presence of sodium azide and Ouabain the inhibition in L-try transport across goat cornea was observed while no marked inhibition was observed on L-try transport across goat cornea in presence of aspartame and glutathione. The L-try transport was favored up to concentration 1% w/v and at higher pH in presence of sodium ions through excised goat cornea. Functional presence of a sodium dependent L-try transport system as inhibited by ouabain having affinity to cationic and neutral amino acid is evident on goat cornea.

Keywords: Cornea; Amino acid; Tryptophan; 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]. In mammalian cells multiple systems exist for transport of amino acids, and these transport systems differ markedly in their substrate specificity, substrate affinity, sodium dependence, energy and pH dependence. Numerous amino acid transport systems have been characterized at the molecular level including L, γ^+ L, A, ASC, asc, $b^{0,+}$, $B^{0,+}$ and x^- , Gly, N, and T [2-8]. In last few years importance of these

transporters have 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 [11-13]. 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, cell lines often are not considered to be epithelial, but it rather shows fibroblast like properties [14]. 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.

Tryptophan is one of the eight essential amino acids in the human diet. Diabetic ketoacidosis has been found to deplete plasma tryptophan level [15]. Tryptophan deficiency is known for long time to cause cataract in rats. Deficiency of the amino acid arrests chromatin breakdown in secondary lens fibers of rats [16]. Characterization of tryptophan transport through corneal membrane may open new sights for better ocular drug delivery through cornea through complex formation of amino acids with the drugs.

In view of the above information the objective of the studies is to characterize the corneal transport of L-tryptophan, with respect to concentration dependence, sodium ion dependence, pH dependence, effect of metabolic inhibitors and competitive inhibition by selected amino acids. Corneal transport of DL-tryptophan and D-tryptophan was also investigated to know the stereospecificity of the amino acid transporter.

Materials and methods

L-try, DL-try and D-try were procured from Lancaster, Chennai. Ouabain was obtained from Sigma Aldrich, Bangalore. 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 from 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 [17]. 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 cm². 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°C 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°C). 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 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 tryptophan content in UV-Visible spectrophotometer at absorption maxima of 280 nm. At the end of the experiment each cornea was weighed, soaked in methanol for 3-4 minutes, dried at 80°C 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 test solutions

Concentration dependence studies

L-Tryptophan aqueous solutions of increasing concentrations of pH 7.2 made isotonic with sodium chloride

Required quantity of L-tryptophan was added in 50 ml volumetric flask and calculated amount (sodium chloride equivalent method) of sodium chloride was added. After adding desired amount of distilled water the pH of the solution was adjusted to 7.2 using 0.1 N hydrochloric acid (HCl) or 0.1 N sodium hydroxide (NaOH) and final volume was made up to 50 ml with distilled water to have solutions of 0.1, 0.25, 0.5, 0.75, 1.0, 1.5% (w/v). Similarly aqueous solution of D-tryptophan 1% w/v was also prepared.

Sodium Ion Dependence Studies

L-Tryptophan aqueous solution 1.0 % (w/v), pH 7.2 made isotonic with dextrose or/and sodium chloride (NaCl).

L-tryptophan (0.5 g) dissolved in water, pH adjusted to 7.2 (with 0.1 N HCl or 0.1 N KOH), made isotonic with dextrose (2.05 g) or decreasing amount of dextrose (1.91g or 1.77g or 1.49g or 0.932g) and increasing amount of NaCl (50 mg or 100 mg or 200 mg or 400 mg) and volume made up to 50 ml with distilled water.

L-tryptophan (0.5 g) dissolved in water, pH adjusted to 7.2, made isotonic with NaCl and volume made up to 50 ml with distilled water (Control).

Effect of pH

L-Tryptophan 1.0 % (w/v) aqueous solution of different pH

Required amount of L-tryptophan (0.5 g) dissolved in water made isotonic with sodium chloride and pH adjusted with 0.1 N HCl or 0.1 N NaOH to pH 5 or 6 or 7.2 or 9 and volume made up to 50 ml with distilled water in a 50 ml volumetric flask.

Competitive Inhibition studies

L-Tryptophan 1.0 % (w/v) aqueous solution with equimolar amount of different amino acids

L-Tryptophan (0.5 g) dissolved in water along with glycine (182.8 mg) or L-glutamic acid (360.5 mg) or L-aspartic acid (326.1 mg) or L-proline (282.0 mg) or L-hydroxyproline (322.7 mg) or L-lysine (358.0 mg) or L-histidine (380.1) or L-arginine (426.8 mg) made isotonic with sodium chloride and pH was adjusted at 7.2 with 0.1 N HCl or 0.1 N NaOH. Then volume was made up to 50 ml with distilled water in a 50 ml volumetric flask.

Effect of Dipeptide on corneal transport of L-Tryptophan

L-Tryptophan (0.5 g) dissolved in water along with aspartame (a dipeptide) in equimolar amount and made isotonic with sodium chloride then pH was adjusted to 7.2 with 0.1 N HCl or 0.1 N NaOH. Then volume was made up to 50 ml with distilled water in a 50 ml volumetric flask.

Effect of Tripeptide on corneal transport of L-Tryptophan

L-Tryptophan (0.5 g) dissolved in water along with glutathione (a tripeptide) and made isotonic with sodium chloride then pH was adjusted to 7.2 with 0.1 N HCl or 0.1 N NaOH. Then volume was made up to 50 ml with distilled water in a 50 ml volumetric flask.

Effect of Metabolic Inhibitors

L-Tryptophan 1.0 % (w/v) aqueous solution with 25 or 50 micromole (µM) sodium azide

L-Tryptophan (0.5 g) dissolved in water in a 50 ml volumetric flask and required volume (1 ml) from prepared stock solution of sodium azide (2500µM or 5000µM) was added to flask, made isotonic with sodium chloride and pH adjusted to 7.2 with 0.1 N HCl or 0.1 N NaOH and volume made up to 50 ml with distilled water.

L- Tryptophan Solution (1 % w/v) with 50 micromole (μ M) Ouabain (octahydrate)

L-Tryptophan (0.5 g) dissolved in water in a 50 ml volumetric flask and required volume (10 ml) from prepared stock solution of ouabain (500 μ M) was added to flask, made isotonic with sodium chloride and pH adjusted to 7.2 with 0.1 N HCl or 0.1 N NaOH and volume made up to 50 ml with distilled water.

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. 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. The present study aimed to study the transport behavior of L-tryptophan that would help in understanding the complex behavior of amino acid transport across mammalian cornea.

Transport of L-tryptophan (L-try) was studied through excised goat cornea for concentration dependence, pH dependence, sodium ion dependence. Effect of metabolic inhibitors and competitive inhibition by acidic, basic and neutral amino acids was studied. Effect of a dipeptide (aspartame) and tripeptide (glutathione) on corneal transport of L-try was also investigated.

Permeation of L-try through goat cornea was studied from 0.1, 0.25, 0.5, 0.75, 1, 1.5 and 2 % (w/v) aqueous solution (pH 7.2) made isotonic with sodium chloride (NaCl). The permeation data (Table 1) reveals that there was an increase in amount of L-try permeated when concentration increased up to 1 % (w/v); after that no increase was observed (Figure 1).

Table 1. Corneal transport characteristics of L-tryptophan from 0.1 %, 0.25%, 0.5 %, 0.75 %, 1%, 1.5 % and 2 % (w/v) aqueous solution through excised goat cornea.

Concentration (% w/v)	Amount permeated (mg)	% Permeation	Corneal hydration %
0.1	0.008067 \pm 0.000367	0.806667 \pm 0.036667	74.27233 \pm 1.095207
0.25	0.0132 \pm 0.000635	0.528 \pm 0.025403	78.56667 \pm 0.731057
0.5	0.0199 \pm 0.000551	0.398 \pm 0.011015	79.63333 \pm 0.554777
0.75	0.037767 \pm 0.001322	0.503333 \pm 0.017704	79.56333 \pm 0.097011
1.0	0.0781 \pm 0.00254	0.781 \pm 0.023567	78.53 \pm 0.621217
1.5	0.0594 \pm 0.00254	0.395 \pm 0.016743	79.05667 \pm 0.581502
2.0	0.05885 \pm 0.00275	0.294 \pm 0.011431	79.635 \pm 0.085732

The amount of L-try permeated from 1 % (w/v) was 0.0781 mg and further investigations on transport of L-try through cornea were performed at this concentration.

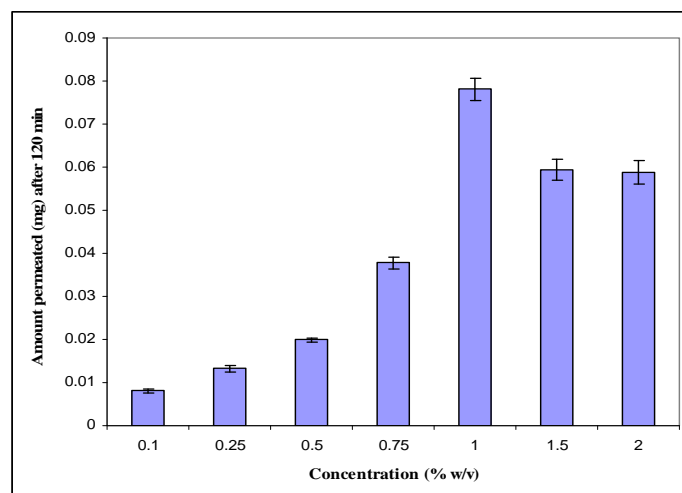


Figure 1: Corneal transport characteristics of Tryptophan from 0.1 %, 0.25%, 0.5 %, 0.75 %, 1%, 1.5 % and 2 % (w/v) aqueous solution through excised goat cornea.

Permeation of L-try was investigated from 1% L-try aqueous solution having pH 5 or 6 or 7.2 or 9 that was made isotonic with sodium chloride. The data (Table

2) revealed that transport of L-try increased gradually from 0.0561 to 0.0781 mg as pH of the donor solution increased from 5 to 7.2 respectively. However the increase was not much higher above pH 7.2. Hence further investigations were carried out at pH 7.2.

Table 2. Effect of pH on Corneal Transport of L-Tryptophan (1% w/v) through Excised Goat Cornea.

pH	Amount permeated (mg) After 120 min	% Permeation	% Corneal hydration
5	0.0561± 0.00191	0.561± 0.01905	75.0467± 2.97761
6	0.0671± 0.00191	0.671± 0.01905	78.58± 1.06096
7.2	0.0781± 0.00254	0.5467± 0.23567	78.53± 0.621
9	0.07847± 0.00194	0.78467± 0.0194	81.9533± 1.03747

The transport of solutes through cornea is the result of sum of passive and active transport. At pH above isoelectric point (P_i 5.9) tryptophan remains in deprotonated form and increased permeation seems to be due to combined active and passive transport. At pH 9.2 corneal damage was observed revealed by hydration level (81.95%) which was higher than the normal value. Membrane Transporters are some times distinguished on the basis of their sodium ion dependence. Transport of L-try through paired corneas of same animal was studied for sodium ion dependence. L-try (1 %) solution was made isotonic with either NaCl or dextrose. One cornea was treated with donor solution made isotonic with NaCl while the other of the same animal was treated with solution made isotonic with dextrose. The amount of L-try permeated from solution made isotonic with sodium chloride was 0.0715 mg that decreased to 0.04217 mg in presence of dextrose as tonicity modifier (Figure 2). Further, when solution of tryptophan made isotonic with dextrose and increasing quantity of NaCl, amount of permeated L-try through cornea increased gradually up to 0.066 mg (Table 3). The amount of L-try permeated reduced remarkably in absence of sodium ions. The results of sodium ion

dependence studies suggested involvement of a transporter that is sodium ion dependent.

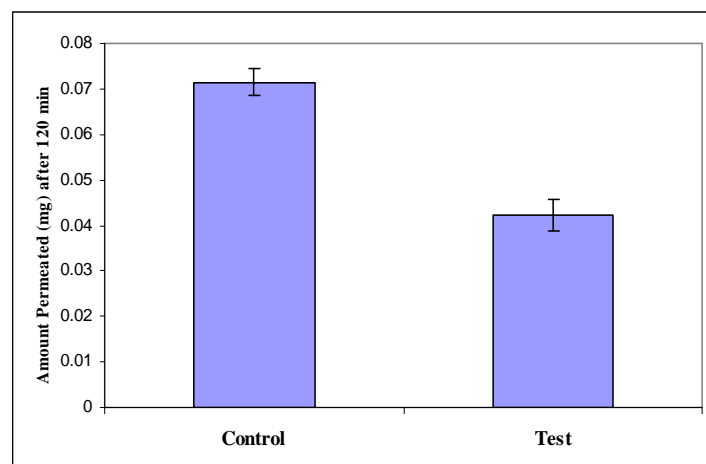


Figure 2. Effect of Sodium Ions on Corneal Transport of L-Tryptophan (1.0 % w/v) at physiological pH of tears (7.4) through excised goat cornea.

Effect of competitive inhibition on transport of L-tryptophan was investigated in the presence of equimolar acidic amino acids (L-glutamic acid,GA; L-aspartic acid, AA), basic amino acids (L-lysine,lys; L-histidine,his; L-arginine,arg) and neutral amino acids (glycine, L-proline, L-hydroxy proline).

Table 3. Corneal Transport of L-Tryptophan (1.0 % w/v) solution made isotonic with dextrose and/or increasing amount of NaCl at physiological pH of tears (7.2) through excised goat cornea.

Aqueous solution of L-tryptophan	Amount permeated (mg) After 120 min	% Permeation	Corneal hydration %
Control ((isotonic with sodium chloride))	0.0781± 0.00254	0.5467± 0.23567	78.53± 0.621
50 mg NaCl (isotonic with dextrose)	0.0528± 0.0011	0.5283± 0.011	81.92 ± 1.105
100 mg NaCl (isotonic with dextrose)	0.0603± 0.0007	0.6036± 0.007	78.4166± 0.51988
200 mg NaCl(isotonic with dextrose)	0.066± 0.0006	0.66± 0.006	81.04± 1.007

Amount of L-try permeated in the presence of GA, AA, Lys, His, Arg, Gly, Pro and Hydpro was 0.0627, 0.057567, 0.0462, 0.0421, 0.0407, 0.040333, 0.0451 and 0.044 mg respectively. All amino acids showed inhibition of L-try transport through goat cornea, however the magnitude of inhibition may be ranked as Gly>Arg> His>Hydpro>Proline>Lys>AA>GA (Figure 3).

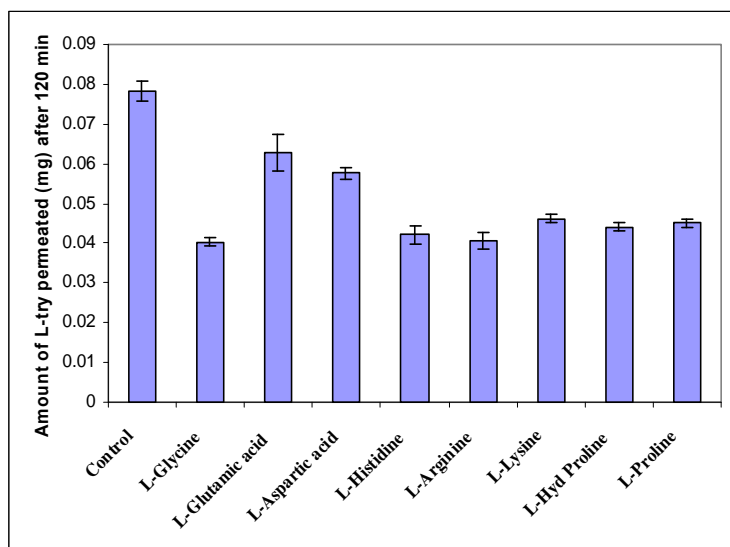


Figure 3. Corneal transport of L-tryptophan 1 % w/v solution in the presence of equimolar amount of various amino acids through excised goat cornea.

Neutral amino acids (Gly, Proline and Hydpro) and basic (cationic) amino acids showed inhibition to greater extent as compare to anionic amino acids like AA and GA. These results indicate possible sharing of transporters by these substrates. The transporter system responsible for transport of L-Try is also having affinity to neutral, cationic and anionic amino acids.

Effect of metabolic inhibitors (sodium azide) and a sodium potassium ATPase pump inhibitor (ouabain) was studied next. Sodium azide in concentration of 25 or 50 μ M and ouabain (50 μ M) inhibited the L-try transport and decreased the amount of permeated L-try from 0.0781 mg to 0.0429, 0.04766 and 0.03556 mg respectively (Figure 4). L-try transport was significantly inhibited in the presence of sodium azide and ouabain. The results suggest energy dependent

transport of L-try which is favoured in the presence of sodium ion.

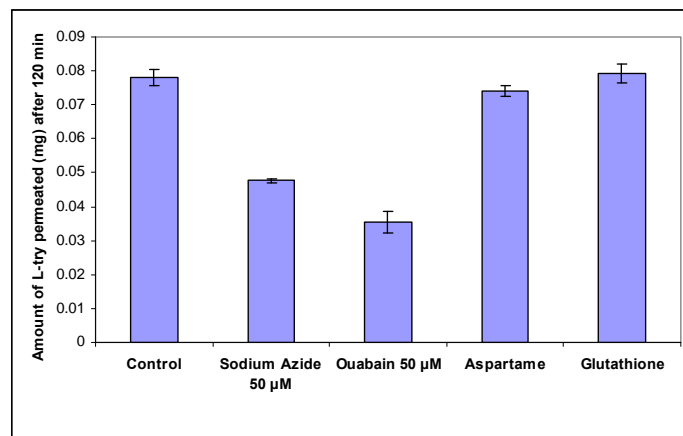


Figure 4: Corneal transport of L-tryptophan from aqueous solution (1% w/v) in presence of metabolic inhibitors (sodium azide and ouabain), aspartame (a dipeptide) and glutathione (a tripeptide).

Transport of L-try was also studied in presence of dipeptide (aspartame) and tripeptide (glutathione reduced) in equimolar concentration of L-try. The amount of L-try permeated in presence of aspartame and glutathione was 0.07393 and 0.0792 mg respectively as compared to control which was 0.0781 mg. There was no markable difference in transport of L-try in the presence of dipeptide and tripeptide. The results suggested that the transporter involved is specific for transport of amino acids only and not for dipeptide or tripeptide.

To know the stereo specificity of the involved transporter the permeation of D isomer of tryptophan at 1 % concentration was studied that was found to be 0.0194 mg as compared to L isomer 0.0781 mg (Figure 5). The result suggests more affinity of transporter to L-isomer as compared to D isomer of tryptophan. The study also suggested more involvement of active transporter system than passive transport.

Conclusion

The results of above studies indicates the functional presence of a sodium dependent stereospecific transporter system on goat corneal epithelium that

having more affinity to L-tryptophan and mediate transport of neutral, cationic and anionic amino acids in competitive manner.

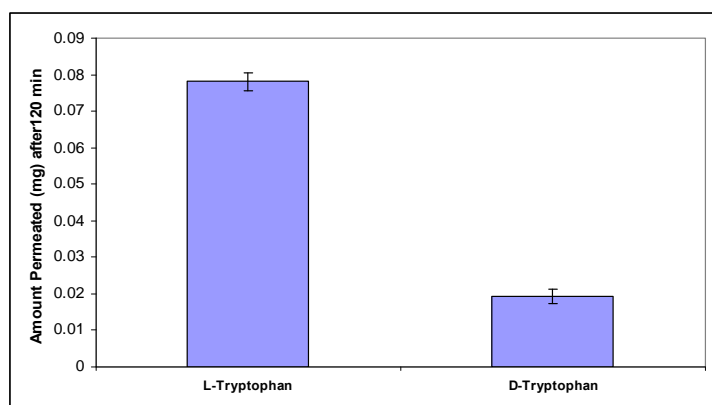


Figure 5. Effect of stereospecificity and transport of L-try and D-try from aqueous solution 1% w/v through excised goat cornea.

The research work reveals for the first time the functional characterization of tryptophan transport across goat cornea. The studies would be certainly helpful in identifying the amino acids with which the amino acid compounds may be synthesized for amino acid transporter targeted drug delivery through cornea for better availability.

References

- Christensen H.N. Role of amino acid transport and counter transport in nutrition and metabolism. *Physiol Rev.* 1990;70:43-77.
- Christensen H.N., Albritton L.M., Kakuda D.K. Gene-product designations for amino acid transporters. *J Exp Biol.* 1994;196:51-57.
- Broer S., Wagner C.A., Lang F. Function and structure of heterodimeric amino acid transporters. *Am J Physiol.* 2001;281:C1077-C109.
- Palacin M., Estevez R., Bertran J. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev.* 1998;78:969-1054.
- Deves R., Boyd C.A. Transporters for cationic amino acids in animal cells: discovery, structure, and function. *Physiol Rev.* 1998;78:487-545.
- Kekuda R., Torres Z.V., Fei Y.J. Molecular and functional characterization of intestinal Na⁺-dependent neutral amino acid transporter B⁰. *Am J Physiol.* 1997;272:G1463-G1472.
- Torrents D., Estevez R., Pineda M. Identification and characterization of a membrane protein (γ^+L

- amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity γ^+L : a candidate gene for lysinuric protein intolerance. *J Biol Chem.* 1998;273:32437-32445.
- Pfeiffer R., Rossier G., Spindler B. Amino acid transport of γ^+L -type by heterodimers of 4F2hc/CD98 and members of the glycoprotein-associated amino acid transporter family. *EMBO J.* 1999;18:49-57.
- Anand B.S., Mitra A.K. Mechanism of corneal permeation of L-valyl ester of acyclovir: targeting the oligopeptide transporter on the rabbit cornea. *Pharm Res.* 2002;19:1194-1202.
- Jain V.B., Pal D., Gunda S., Nashed Y., Ganapathy V., Mitra A.K. Identification of a Na⁺-dependent cationic and neutral amino acid transporter, B^{0,+} in human and rabbit cornea. *Mol Pharm.* 2004;1:338-346.
- Anand B.S., Katragadda S., Nashed Y.E., Mitra A.K. Amino acid prodrugs of acyclovir as possible antiviral agents against ocular HSV-1 infections: interactions with the neutral and cationic amino acid transporter on the corneal epithelium. *Curr Eye Res.* 2004;29:153-66.;
- Majumdar S., Nashed Y.E., Patel K., Jain R., Itahashi M., Neumann D.M., Hill J.M., Mitra A.K. Dipeptide monoester ganciclovir prodrugs for treating HSV-1-induced corneal epithelial and stromal keratitis: in vitro and in vivo evaluations. *J Ocul Pharmacol Ther.* 2005;21:463-74.
- Nielsen C.U., Vabeno J., Andersen R., Brodin B., Steffansen B. Recent advances in therapeutic applications of human peptide transporters. *Expert Opin Ther Patents.* 2005;15:153-166.
- Niederhorn J.Y., Meyer D.R., Ubelaker J.E., Martin J.H. Ultrastructural and immunohistological characterization of the SIRC corneal cell line. *In Vitro Cell Dev Biol.* 1990;26:923-930.
- Carl G.F., Hoffman W.H., Blankenship P.R., Litaker M.S., Hoffman M.G., Mabe P.A. Diabetic Ketoacidosis depletes plasma tryptophan. *Endocr Res.* 2002;28:91-102.
- Vrensen G.F., Van M.J., Jonges R., Voorhout W., Breipohl W., Wegener A.R. Tryptophan deficiency arrests chromatin breakdown in secondary lens fibers of rats. *Exp Eye Res.* 2004;78:661-672.
- Rathore M.S., Majumdar D.K. Effect of Formulation Factors on In Vitro Transcorneal Permeation of Gatifloxacin From Aqueous Drops. *AAPS PharmSciTech.* 2006; 7: E1-E6.