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Drug delivery studies in Caco-2 monolayers. VI. Studies of enzyme substitution therapy for phenylketonuria—a new application of Caco-2 monolayers

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Abstract

Phenylalanine ammonia-lyase (PAL) which is an enzyme capable of converting L-phenylalanine to *trans*-cinnamic acid has been shown to be able to minimize the amount of L-phenylalanine transported across Caco-2 monolayers when administered apically. The impact of this is that a formulation of PAL, able to lower the transport of L-phenylalanine across epithelia, potentially could be applied in the oral treatment of phenylketonuria. Confluent monolayers of Caco-2 cells were shown to simplify the studying of the simultaneous events of conversion and transport across an epithelial barrier. © 1998 Elsevier Science B.V.

Keywords: Caco-2; Intestinal transport; Phenylalanine ammonia-lyase; Enzyme substitution therapy; Phenylketonuria

1. Introduction

Recently, the adenocarcinoma cell line Caco-2 has gained much attention for intestinal transport studies in pharmaceutical research (Hidalgo et al., 1989, 1990; Wilson et al., 1990; Hovgaard et al., 1994, 1995). The Caco-2 cells are well accepted as a transport model for studies relating physicochemical properties of compound series to passive diffusion and in some cases even to active transport. However, the number of reports in literature relating enzyme kinetic processes to epithelial transport in Caco-2 cells are very limited. The obvious advantage of expanding the application area of Caco-2 cells to include studies on pharmaceutical formulations is the simplicity of the system (Hidalgo et al., 1989, 1990; Wilson et al., 1990; Hovgaard et al., 1994, 1995).

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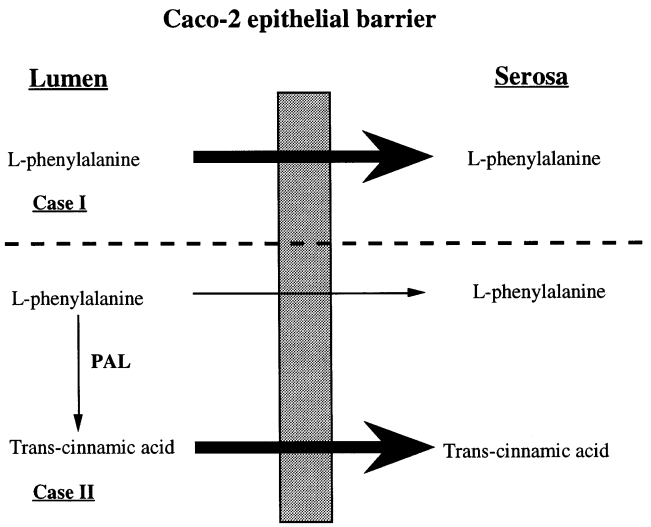

Fig. 1. Hypothetical cartoon illustrating transport of L-phenylalanine from the luminal to the serosal side of Caco-2 monolayers. Case I illustrates a situation where free L-phenylalanine is present alone. Case II illustrates the case where PAL is present in active form and can convert L-phenylalanine to *trans*-cinnamic acid which then is the dominant species being transported.

Phenylketonuria (PKU) is an inherited deficiency of the hepatic enzyme phenylalanine hydroxylase or various co-factors of the enzyme (Matalon and Michals, 1991). Phenylalanine hydroxylase is responsible for the elimination of L-phenylalanine from the blood via hepatic metabolism to L-tyrosine. The disease develops gradually from birth if not treated and causes severe mental retardation at early age. Today treatment of PKU-patients consists of a rigid and well monitored diet to maintain low plasma levels of L-phenylalanine and metabolites hereof. As this implies the replacement of most common foods for unsavory substitutes for proteins and amino acids, the dietary regime has serious social as well as psychological consequences for the patients. In order to ease the lives of PKU-patients, obviously, a better treatment strategy is needed. On a long term horizon PKU may be a candidate for gene-therapeutic treatment but at present enzyme substitution therapy appears the most promising option.

Phenylalanine ammonia-lyase (PAL), an enzyme derived from the red yeast *Rhodotorula glutinis*, has been shown to convert L-phenylalanine to cinnamic acid (Abell and Shen, 1987). The enzyme has been found in several organisms ranging from fungi and yeast to plants. It has been shown by Hoskins in 1980 that a simple oral formulation of PAL was able to produce a 25% lowering in the plasma levels of L-phenylalanine in an adult PKU-patient (Hoskins et al., 1980). The underlying mechanism for this was believed to be a conversion of free L-phenylalanine to *trans*-cinnamic acid prior to intestinal absorption. Moreover, the transport of L-phenylalanine was previously shown to be of an active nature in Caco-2 monolayers (Hidalgo et al., 1990).

The hypothesis for the effect of the enzyme phenylalanine ammonia-lyase on the transport of L-phenylalanine from the intestine is depicted in Fig. 1. In healthy humans as well as in PKU-patients L-phenylalanine is readily absorbed in large quantities from the small intestine. For the healthy human this is not a problem since phenylalanine hydroxylase in the liver metabolizes L-phenylalanine by converting it to L-tyrosine. However, in the PKU-patient an accumulation of L-phenylalanine will take place causing severe brain damage. An oral formulation of the enzyme PAL aims at the conversion of free L-phenylalanine to *trans*-cinnamic acid prior to the absorption. Thus, the harmless *trans*-cinnamic acid will be absorbed instead into the blood stream and

subsequently excreted through the kidneys (Hoskins et al., 1984).

This report shows that Caco-2 cells grown to fully differentiated monolayers on polycarbonate filters can be used as a valuable tool for studies on the above suggested mechanism for PAL. The enzymatic conversion of L-phenylalanine to *trans*-cinnamic acid was followed simultaneously with the transport of L-phenylalanine and *trans*-cinnamic acid.

2. Materials and methods

2.1. Materials

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), L-phenylalanine, *trans*-cinnamic acid and trizma base was obtained from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, L-glutamine, MEM non-essential amino acids, streptomycin, benzylpenicillin, trypsin and Hank's balanced salt solution (HBSS) were all obtained from Biological Industries (Israel). Cell culture flasks, 75 cm^2, were obtained from Greiner (Austria) and Transwell diffusion chambers from Costar (Cambridge, MA). Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Disposable Sephadex PD-10 size exclusion columns were obtained from Pharmacia (Sweden).

2.2. PAL purification

PAL was received in a glycerol solution from Sigma. Purified PAL enzyme was obtained by a chromatographic procedure. A PD-10 column was equilibrated according to manufactures recommendation. PAL dissolved in glycerol was then diluted to five times the volume in Tris-buffer pH 7.4. After fractionation the PAL containing fractions were identified by enzymatic assay (see Section 2.3). The activity of PAL was expressed in units and was measured by determining the conversion rate of L-phenylalanine to *trans*-cinnamic acid photometrically at 270 nm. Active fractions were pooled.

2.3. Enzyme conversion assay

For determination of enzyme activity, simple conversion studies of 1.75 mM L-phenylalanine in 0.15 M Tris-buffer (pH 8.5) were carried out in the presence of 0.3 U/ml of PAL at 37°C. The enzyme kinetics were followed by UV-spectroscopy at 270 nm continuously over 6 min.

2.4. In-vitro cell culture

Caco-2 cells were cultured as previously described (Hovgaard et al., 1994). In brief, the cells were maintained in DMEM containing 9% fetal calf serum, 1% L-glutamine, 1% nonessential amino acids and antibiotics (10 U/ml streptomycin and 100 U/ml benzylpenicillin) at 37°C and 5% CO_2. Cells were trypsinized every week split 1:7 in 75 cm^2 flasks and seeded onto microporous Transwell polycarbonate filters on which they were ready for transport experiments after 21–28 days. Caco-2 cells used in the present study were all between passage 25 and 35. The cells were at all times tested for possible mycoplasma infections and found not to be infected by Statens Veterinärmedicinska (Uppsala, Sweden). Moreover, the integrity of the monolayers was evaluated by measuring of the transepithelial electrical resistance (TEER) before and immediately after experiments. The TEER values were found to be from 267 to 336 Ω*cm^2 prior to experiment and from 306 to 499 Ω*cm^2 after. This indicates that the monolayers were not damaged during experiments.

2.5. Integrated enzyme conversion and transport assay

Detailed methods for transport experiments in Caco-2 cells are described elsewhere (Hovgaard et al., 1994). Solutions of L-phenylalanine and *trans*-cinnamic acid at concentrations of 3.5 mM in HBSS were investigated in transport experiments in the presence and absence of 0.3 U/ml of PAL. Volumes of 0.6 ml of the compound assessed for transport were applied on the luminal side of the cells and samples of 100 μl were removed from the serosal side for analysis by HPLC and re-

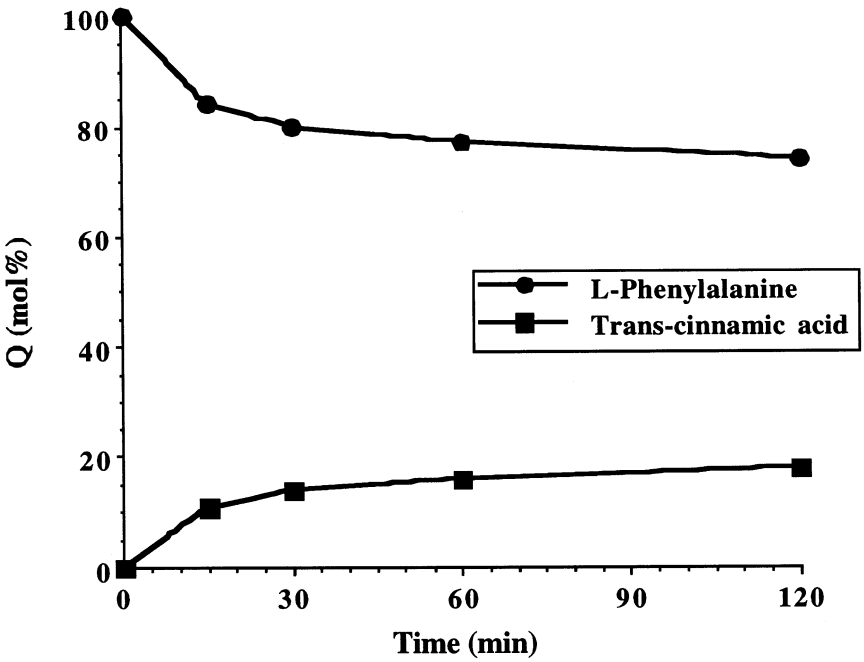

Fig. 2. Enzymatic conversion of L-phenylalanine to *trans*-cinnamic acid by PAL. Initial concentration of L-phenylalanine was 1.75 mM in 0.15 M Tris-buffer, pH 8.5. Amounts of L-phenylalanine is indicated by circles, *trans*-cinnamic acid by squares (average of $n = 2$).

placed with HBSS. Transport experiments were carried out for up to 180 min.

2.6. Analytical assessment of transport by HPLC

The analytical HPLC method was based on a gradient reversed-phase separation. The HPLC system consisted of a Hitachi-Merck gradient controller pump (Model L-6200), a Hitachi-Merck UV-detector (Model L-4000) and a Hitachi-Merck autosampler (Model 655A-40). Data acquisition and processing were carried out using the Hitachi-Merck HPLC-Manager (Model D-6000). The analytical column was a Knauer, Spherisorb ODS-1 (5 μm, 120 × 6.4 mm). The gradient used was composed of mobile phases A (equal parts of 0.02 M acetate pH 5 and acetonitrile) and B (0.01 M acetate pH 5). The gradient cycle was as follows: 0–10 min 5% A:95% B–60% A:40% B; 10–14 min 60% A:40% B; 14–16 min 60% A:40% B back to 5% A:95% B; 16–24 min 5% A:95% B. Retention times for L-phenylalanine and *trans*-cinnamic acid was found to be 4.2 and 11.1 min, respectively.

3. Results and discussion

When the enzymatic conversion of L-phenylalanine was studied at a concentration of 1.75 mM in the presence of PAL a rapid conversion to *trans*-cinnamic acid was observed initially. However, significant product inhibition was evident as the conversion rate declined dramatically early on, see Fig. 2. The experiment was carried out in a 0.15 M Tris-buffer at constant pH 8.5, so that pH change as an explanation for the enzymatic rate decline can be neglected. The amount of L-phenylalanine decreased rapidly from 100% initially to 85% after 15 min and at the same time the amount of *trans*-cinnamic acid increased from 0 to 15%. Hereafter, the profiles level off suggesting that the *trans*-cinnamic acid building up in the system inhibits PAL (Tipton et al., 1993). Obviously this potentially poses as a major problem for the application of PAL as an oral enzyme substitute since the conversion of L-phenylalanine in the gut may be shut of early on due to the inhibitory action of the formed product *trans*-cin-

Table 1
Apparent permeability coefficients of L-phenylalanine for transport across Caco-2 monolayers

Donor [3.5 mM]	P_{app} [cm/s] ($\times 10^{-5}$) ± S.D.
L-Phe	1.9 ± 0.32
L-Phe (+0.4 mM *trans*-cinnamic acid)	1.6 ± 0.07
L-Phe (+2.1 mM *trans*-cinnamic acid)	1.9 ± 0.14

Effect of the presence of *trans*-cinnamic acid ($n = 3$).

namic acid. However, using Caco-2 cell monolayers it was possible to evaluate the implications of the product inhibition kinetically and simultaneously to study the uptake of L-phenylalanine and *trans*-cinnamic acid.

In Table 1 the apparent permeability coefficients across Caco-2 monolayers of L-phenylalanine in presence of various amounts of *trans*-cinnamic acid is given. The apparent permeability coefficient of L-phenylalanine calculated as described elsewhere (Hovgaard et al., 1995) was found to be 1.9×10^{-5} cm/s. Unpublished data from our laboratories indicate that the transporter of L-phenylalanine is saturated at this concentraton. An increase in the donor concentration to 10 mM did not increase the transported amount of L-phenylalanine. Table 1 clearly depicts that the transport of L-phenylalanine is independent of and not stimulated nor inhibited by the presence of *trans*-cinnamic acid in relevant concentrations. Therefore, although it was shown that L-phenylalanine is transported actively in Caco-2 monolayers the enzyme inhibitory product *trans*-cinnamic acid does not interact with this carrier (Hidalgo et al., 1990).

Fig. 3. shows the transport profiles of L-phenylalanine across Caco-2 monolayers in the absence of PAL and the resulting L-phenylalanine and

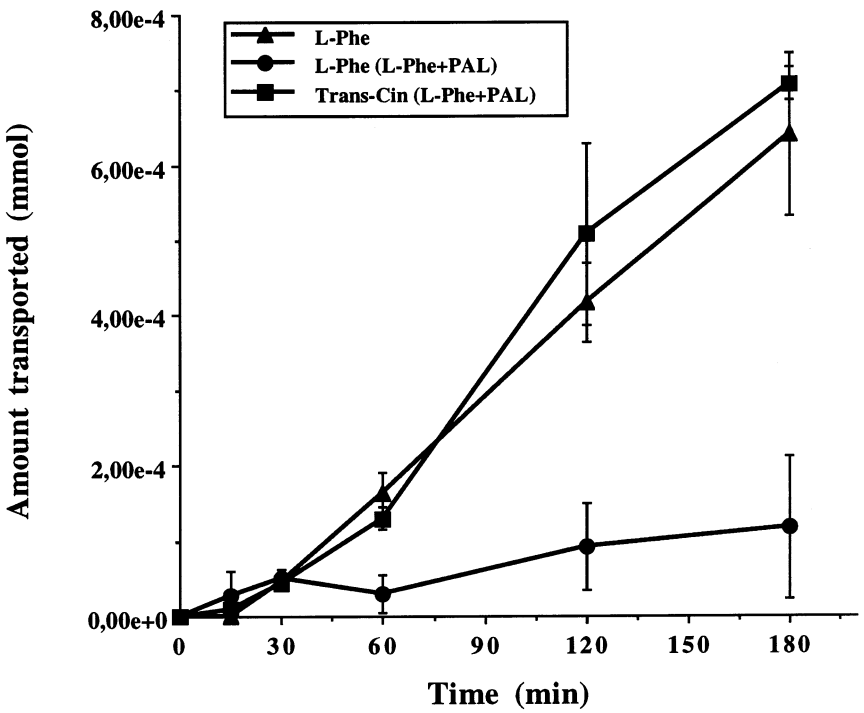

Fig. 3. Amount transported across Caco-2 monolayers of L-phenylalanine and *trans*-cinnamic acid after application of 3.5 mM L-phenylalanine alone or together with 0.3 U/ml PAL to the luminal side. Triangles represent plain transport of L-phenylalanine. Circles and squares represent L-phenylalanine and *trans*-cinnamic acid transported respectively when L-phenylalanine was applied together with PAL ($n = 3$, ± S.D.).

trans-cinnamic acid transport in the presence of PAL on the luminal side. Over a period of 180 min, ≈30% was transported across the Caco-2 monolayers in the absence of PAL. This net transport of L-phenylalanine was however lowered significantly when PAL was present. In that case less than 5% L-phenylalanine was transported. The transport of cinnamic acid therfore accounted for ≈35% of the applied L-phenylalanine. This can be explained by the rapid conversion of L-phenylalanine to *trans*-cinnamic acid. Of course this conversion reduces the concentration gradient for L-phenylalanine but at the same time builds a gradient for *trans*-cinnamic acid which is believed to be transported passively (own unpublished data). When PAL was absent a high transportability of the epithelial cells towards L-phenylalanine was observed. However, when PAL was present the picture changed to a high transport of *trans*-cinnamic acid. Therefore, it appears that the product inhibition by *trans*-cinnamic acid of PAL may not be a problem in-vivo since the product from the enzymatic conversion (*trans*-cinnamic acid) is effectively transported across the epithelium and thus removed from the luminal side.

In conclusion, this study has shown that Caco-2 cell monolayers are excellent for initial feasibility studies of pharmaceutical formulations. In this case a simple solution was studied but more advanced formulations could easily be applied. Moreover, the study is the first to discuss the complex enzyme kinetics, product inhibition and transport kinetics encountered in oral enzyme substitution therapy using PAL for PKU and at the same time giving a means to study this. Also we have shown that phenylalanine ammonia-lyase has a potential as an oral drug in the treatment of phenylketonuria. The conversion of L-phenylalanine to *trans*-cinnamic acid on the luminal side of an intestinal epithelium was found to be sufficiently rapid to significantly lower the transported amount of L-phenylalanine. At the same time the transport of *trans*-cinnamic acid was high and therefore, product inhibition appears not to be a problem. Although the studies presented here are promising still much needs to be investigated. At present, studies involving L-phenylalanine incorporated in various di-, tri- and higher oligopeptide forms are being carried out in our laboratories, since the activity of PAL is dependent on the presence of free L-phenylalanine.

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