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New absorption promoter for the buccal delivery: Preparation and characterization of lysalbinic acid

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Abstract

Drug delivery across the buccal mucosa is convenient and safe transport method. The efficiency of the buccal system of peptide delivery is, however, not yet satisfactory. To improve the buccal transport new absorption promoters should be developed to be sufficiently active and at the same time causing no side effects like irritation or unpleasant taste. We have found that lysalbinic acid, a product of the alkaline hydrolysis of egg albumin and a mild detergent, meets those requirements. The preparation and some physicochemical properties of lysalbinic acid are described. Hamster cheek pouch was used as a model for the penetration process studies lysalbinic acid was shown to increase significantly an oral mucosa permeability for α-interferon and insulin. So this substance of the natural origin can be applied as an absorption enhancer for the buccal delivery of peptide drugs.

Keywords: Lysalbinic acid; Absorption promoters; Buccal delivery; Buccal mucosa; Detergent

1. Introduction

There are various methods for the medicines administration. The commonly used injection method leads to the rapid increase of drug concentration in blood that causes toxic or autoimmune responses. Another way, oral delivery, has been used only for non-protein drugs. The main problem of oral route is that only compounds stable in the gastrointestinal tract can be used. Over the time, numerous attempts have been made to explore alternative routes for systemic drug administration. Development of non-injection way for proteins introduction is currently the most attractive approach. Rectal, nasal, transdermal, pulmonary and other non-injection ways for medicines introduction are popular methods now. However, all these approaches have some essential limitation, such as low bioavailability, immunoreactivity or fluctuation of drug concentration in the blood.

The buccal method is one of the most attractive ways to deliver drugs into the organism (Kamimori et al., 2002; Bousquet et al., 1992; Rygnestad, 2002; Moller et al., 2000; Sagar and Smyth, 1999; Nielsen and Rassing, 2002). It can be employed for protein drugs introduction as well (Meritet et al., 1999; Hubbard et al., 1989; Sakane et al., 1995; Squier et al., 1978; Venugopalan et al., 2001). The delivery of peptide drugs across the buccal mucosa is more convenient and safe approach than most other delivery methods. It was shown that the buccal administration of proteins like insulin, interferons, interleukins has some advantages and reduces many related side effects. For example, buccal way provides constant, predictable level of drug concentration in blood. Venugopalan et al. have shown that buccally administered insulin provided a significant hypoglycemic response without any detectable fluctuation in blood glucose profile and risk of hypoglycemia (Venugopalan et al., 2001). However, the efficiency of the buccal delivery is not currently able to compete with injection methods.

From this point of view, the role of absorption promoter for protein buccal transport is crucial. Many substances can function as absorption promoter, the most popular being detergents such as bile acids salts, sodium lauryl sulfate, etc. But many detergents have some side effects. Often they cause irritation of buccal mucosa. An additional problem is a taste of buccal composition. The most efficient absorption promoters—bile acids salts—have a strong bitter taste, so regular use of compositions containing bile acids is hardly acceptable. That is why new absorption

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promoters should be found. A good candidate for the effective absorption enhancer seems to be lysalbinic acid.

Lysalbinic acid was first described by German chemist Paal in 1902 (Paal, 1902a). It is a product of alkaline hydrolysis of albumin (egg albumin (Inoue, 1937), serum albumin (Schulz, 1941) or casein (Tyalbji, 1949)). As a protein substance, which does not contain strong cationic and anionic functional groups, lysalbinic acid can be considered a non-ionic detergent. Originally lysalbinic acid was applied as the stabilizer of metal sols for gold, silver and mercury (Tyalbji, 1949; Paal, 1902b,c,d,e; Wolvekamp, 1921, 1922; Makino, 1989). Later it was used as soft detergent in some washing liquids. Some elite cosmetic compositions contain analogues of lysalbinic acid (Bennert, 1921; Jabalee, 1976; Rousso and Wallace, 2000; End et al., 1997; Varco, 1991; Marsh et al., 1980).

Surfactant properties have caused our interest to lysalbinic acid as possible absorption enhancer for the buccal transfer of proteins. The protein nature of a preparation allowed to assume that its irritating action would be minimal, and the composition taste to be neutral. The aim of the present study was to evaluate chemical and transport enhancer properties of the lysalbinic acid.

2. Materials and methods

2.1. Preparation of lysalbinic acid

Lysalbinic acid was prepared by modification of the original method (Paal, 1902a). One hundred grams of egg albumin powder (Biolar, Latvia) was added slowly with stirring and water bath heating to the solution of 15 g NaOH in 500 ml of water. The resulting viscous solution was heated at boiling water bath for 1 h, cooled to the ambient temperature and filtered through paper filter. Diluted sulfuric acid (40–50% aqueous solution) was added slowly to the filtrate until pH reached 5. The precipitate was filtered off through paper filter and washed by water (2×20 ml). The filtrate was dialysed (Spectra/Por 6 membrane, Cole-Parmer) against water for 3 days. To the dialysate containing lysalbinic acid sulfate, aqueous solution of barium hydroxide was added carefully by small portions until the filtered probe contained neither SO_4^{2-} nor Ba^{2+} ions. $BaSO_4$ was filtered off, and the foamy solution of free lysalbinic acid was lyophilised at $-20\,^{\circ}C$ at Jouan Heto DW8-85 freeze drier. The obtained powder was washed with ethanol (2×10 ml) and dried in vacuum. Typical yield of lysalbinic acid is 20–25 g per 100 g of albumin.

2.2. Determination of molecular weight of lysalbinic acid

It was carried out by gel filtration on Sephadex G-75 (Reanal, Hungary) (2.5 cm × 53 cm, $V_o = 70$ ml). Column was calibrated using following molecular weight markers: glucose (180 Da), actinomycin (1200 Da), myoglobin (17.8 kDa), bovine serum albumin (68 kDa). Electrophoresis in 16% polyacrylamide gel in the presence of molecular markers (Fermentas, Lithuania) was, also, used for molecular weight determination.

2.3. Surfactant characteristics of lysalbinic acid

They were obtained by standard physicochemical methods (Dominguez et al., 1997). Concentration dependence of the surface tension (σ) and electric conductivity (x) of lysalbinic acid solutions were determined at ambient temperature. Surface tension and conductivity data were plotted versus the concentration of the surfactant, and critical micelle concentration (CMC) of the substance was found from the plots (Fig. 2). Reported data represent the results of three measurements. The standard deviation in the experiments was below 5%.

2.4. The effect of lysalbinic acid on protein buccal penetration

It was investigated on hamster cheek pouch model. Cheek pouches were removed from ether–anaesthetised hamsters and washed with a Ringer solution (0.154 M NaCl, 0.154 M KCl, 0.11 M $CaCl_2$, 0.154 M KH_2PO_4, 0.154 M $MgSO_4$, 0.154 M $NaHCO_3$) (Dawson et al., 1986). The cheek pouch was turned inside out, filled with 1 ml of Ringer solution and dipped into 10 ml of Ringer solution containing a substance to be studied (peroxidase, α-interferon or insulin). Incubation was carried out at 37 °C with constant slow stirring. The amount of protein permeating from the mucosal side of the cheek pouch to serosal side was measured over time. Probes were taken from a cheek pouch in certain time intervals (1–15 min) and analysed by colorimetric, electrophoretic or fluorescence methods.

2.5. Probe analysis by colorimetric method

The concentration of peroxidase (Sigma) was determined using the reaction with 3,3′,5,5′-tetramethylbenzidine (TMB, Amersham) following the manufacturer's protocol. Starting peroxidase concentration in the Ringer solution was 4.5×10^{-5} mM, that of lysalbinic acid was in the range 1–10%. Peroxidase solution without lysalbinic acid was used as a control. A 50 μl probe was mixed with an equal volume of TMB solution and in 1 min reaction was quenched by 50 μl of 0.05 M sulfuric acid. Concentration was determined at Multiscan MCC/340P instrument at 450 nm.

2.6. Probe analysis by gel electrophoresis

Quantitative analysis of α-interferon was performed by disc electrophoresis in 16% polyacrylamide gel according to the published protocol (Westermeier, 1997). Initial interferon concentration in Ringer solution was 3.1×10^{-4} mM, lysalbinic acid content was 0.1–10%. Interferon solution without lysalbinic acid was used as a control. A 50 μl probes were taken for the analysis. Gels were stained with Coomassie R250 and scanned with HP Deskjet Professional scanner. Protein amounts were determined using TotalLab 2.01 software.

2.7. Probe analysis using fluorescent labeling

Insulin (Indar, Ukraine) was labeled with fluorescein isothiocyanate (FITC, isomer I, Serva) according to the FluoReporter FITC Protein Labeling Kit Protocol (F-6434). Excess FITC reagent was separated by gel filtration on Sephadex G-25 (Pharmacia) equilibrated with phosphate buffer (PBS tablet, ICN, pH 8.0).

FITC–insulin concentration in the Ringer solution was 3.44×10^{-4} mM, lysalbinic acid content was 0.3%. FITC–insulin solution without lysalbinic acid was used as a control. A 200 μl probes were taken from the pouch for analysis. Insulin concentration was determined with Cary Eclipse spectrofluorimeter (Varian, Australia, exciting at 496 nm, emission detection at 517 nm).

Luminescent microscopy was used for the visualisation of FITC-labeled insulin permeation pathways in the buccal epithelium (Lomo MP-49 microscope, objective 90×, ocular 7×, excitation at 380 nm).

3. Results and discussion

3.1. Physicochemical characteristics of lysalbinic acid

Lysalbinic acid can be easily prepared by alkaline hydrolysis of egg albumin followed by dialysis. The obtained material was an amorphous white or yellowish powder insoluble in alcohol and other organic solvent and well soluble in water forming foamy solutions. The pH values were measured at a wide range of lysalbinic acid concentration (0–5%) and were in the range 4.8–5.3. Above 0.05% pH becomes almost constant (about 4.8).

The chemical structure of lysalbinic acid is not completely clear, however, presumably it is a mixture of peptides formed during the process of albumin degradation. We have found by gel filtration experiments that the material consists of three peptide fractions with molecular weight about 600, 6000 and 11 000 Da. Results obtained by gel electrophoresis confirm these data. As can be seen from Fig. 1, the substance consists of two heterogeneous peptide fractions with molecular weight around 6000 and 11 000 Da. The fraction with MW ca. 600 Da is not observed in the gel. However, this is not surprising since its molecular weight is low and short peptides diffuse easily out of the gel during the gel staining.

The fact that lysalbinic acid is not a homogeneous compound with exact molecular weight is in accordance with early data. Cryoscopic method provided the average molecular weight of lysalbinic acid to be 800–1200 Da (Paal, 1902a). The difference between MW values published in Paal (1902a) and in our experiments can be explained by the difference in the preparation method, first of all the depth of albumin hydrolysis and dialysis conditions.

3.2. Surfactant properties of lysalbinic acid

There are no specific parameters in the literature allowing adequate characterization of the surface properties of proteins. So we used here standard methods applicable to low-molecular

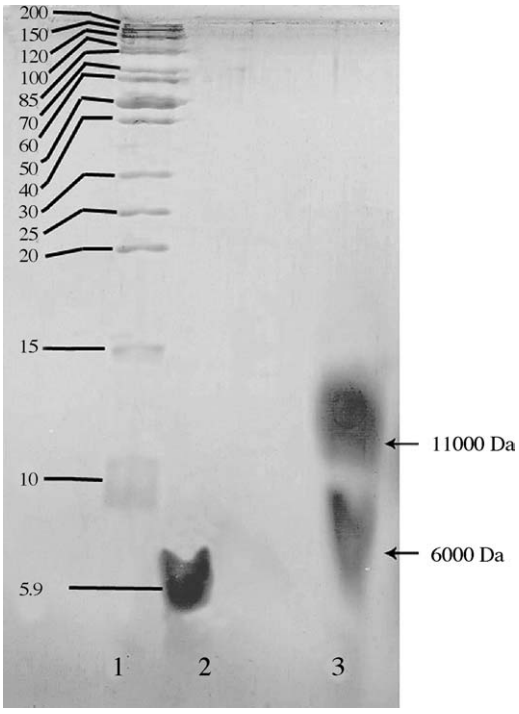

Fig. 1. Gel electrophoresis of lysalbinic acid. (1 and 2) Molecular weight markers; (3) lysalbinic acid.

weight surfactants. The experimental data showed that lysalbinic acid has surfactant activity. Many properties of surfactant solutions, if plotted against the concentration, appear to change at a different rate above and below the point known as the critical micelle concentration (CMC), a key parameter of surface-active compounds. For the evaluation of surface properties of lysalbinic acid, we used two basic characteristics—surface activity and electroconductivity. CMC of lysalbinic acid was determined by measuring a change in the surface tension and electric conductivity of its solutions, as the concentration of the surfactant increased. Fig. 2 shows plots of two physicochemical parameters against lysalbinic acid concentration, which are characteristic for surfactants. Both plots show breaks with clear inflection points. Two almost straight lines observed on these plots were extrapolated to find the point of intersection. The CMC values obtained by two methods were identical, with break points observed at concentration 0.19% (m/v). Due to the heterogeneous character of the lysalbinic acid sample, it is impossible to get the corresponding molar concentration. CMC for individual fractions was not determined, and the above value is the average for the mixture. It was interesting to observe the broad diffuse character of the bands of lysalbinic acid on electrophoregrams. This effect is, also, typical for the surface-active materials (Fig. 1).

3.3. In vitro buccal transport studies

The studies of buccal transport are important to found new efficient ways for drug delivery. One of the main targets of investigation is the development of the suitable model simulating the penetration process where the activity of various absorption

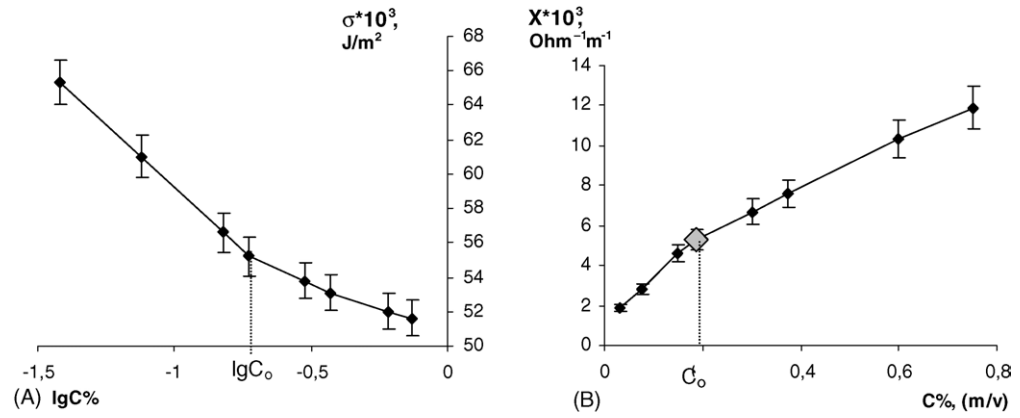

Fig. 2. Concentration dependence of surface tension (σ, A) and electric conductivity (x, B) of lysalbinic acid solutions. C_0, critical micelle concentration.

stimulators can be estimated. Currently the absorption levels are typically determined by measurement of drug concentration in blood, urine, etc. (Sakane et al., 1995; Venugopalan et al., 2001). Other authors evaluated absorption rates of labeled proteins by luminescent microscopy studies (Xu et al., 2002). Difficulties and high cost of such approaches are obvious. Development of simple adequate experimental model for investigation of proteins buccal permeability is needed.

We used hamster cheek pouch as a simple and convenient model for the transport studies. The use of keratinized mucosa of rodents may provide data that cannot be directly extrapolated to human buccal mucosa due to significant difference in the nature and organization of intercellular lipids between these species. However, we supposed that freshly isolated hamster cheek pouch could be employed at initial step of investigation for the fast screening of new compounds that could act as absorption enhancers. Of course, the future research related to the protein transport in humans should employ more adequate models (e.g. porcine, dog or rabbit mucosa).

This animal model allows an investigation of permeability of high-molecular substances and, also, can evaluate an efficiency of various absorption enhancers. We studied the permeability of buccal mucosa for several proteins of various molecular weight: peroxidase (45 kDa), α-interferon (16 kDa) and insulin (5.8 kDa). The effect of lysalbinic acid on buccal absorption level of these proteins was determined.

It was established that the buccal mucosa effectively retained proteins with molecular weight over 30–40 kDa. Peroxidase was not found in detectable amounts in hamster cheek pouch after 30 min incubation. The addition of lysalbinic acid to the studied sample in concentration from 0.2 to 2% did not result in the change of protein permeability. Peroxidase enzymatic activity in probes did not differ noticeably from the control background. The obtained results are in full agreement with literature data where it was shown that peroxidase could not transfer through the buccal external barrier (Squier et al., 1978). Peroxidase is perhaps too large protein that requires physical damage of the mouth mucous for buccal penetration.

The decrease of protein size led to the increase of buccal permeability. The experiments clearly demonstrated the efficiency of lysalbinic acid as absorption enhancer for α-interferon and FITC–insulin. In the absence of absorption enhancer, α-interferon was detected in hamster cheek pouches after 15–30 min of incubation only in trace amounts. This result is in a good agreement with literature data where it was shown that after buccal administration without absorption enhancer α-interferon was found in blood in the concentration below 1% of the applied dose (Bayley et al., 1995). Co-administration of lysalbinic acid in concentration 1% resulted in five-fold increase of the permeability of α-interferon through the mucous in 10 min. Addition of 5% of the same surfactant increased protein transport for 6 and 9 times in 2 and 10 min, respectively (Fig. 3).

Fluorescein-modified insulin was used for the study of peptide transport via fluorescence measurement technique. In this case, it was shown that buccal mucosa is able to transfer insulin in the presence of absorption enhancer. Without enhancer FITC–insulin was found inside hamster's bag in trace concentration. Lysalbinic acid in the concentration 0.3% increased buccal permeability of FITC–insulin for above five times just in 10 min.

The important issue is the understanding of the mechanism of action of lysalbinic acid in protein permeation enhancement. There are two main permeation pathways for the passive drug transport across the oral mucosa: paracellular and transcellular routes (Shojaei et al., 2001; Shojaei, 1998; Senel et al., 2001). Since intercellular space is less lipophilic than the cell

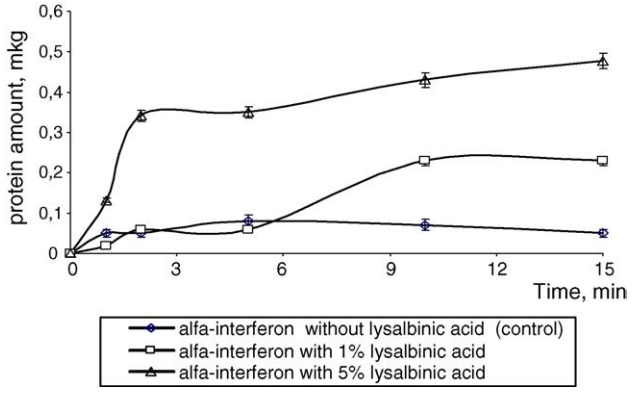

Fig. 3. Permeability of α-interferon (16 kDa) through the mouth cavity mucous at various concentrations of absorption promoter.

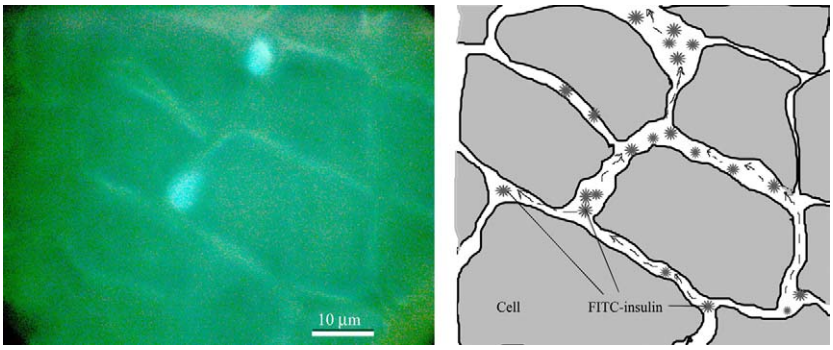

Fig. 4. (A) Fluorescence photography of buccal epithelium after incubation with FITC–insulin in the presence of lysalbinic acid. Lysalbinic acid concentration, 0.3%; incubation time, 15 min. (B) Schematic explanation for (A).

membranes, hydrophilic compounds are better soluble in this environment. The cell membrane, on the other hand, is highly lipophilic, and hydrophilic substances have great difficulties permeating the membranes. As a result, lipophilic molecules predominantly use the transcellular route whereas their passive transport via intercellular space is limited. At the same time, cell membranes are the major transport barrier for hydrophilic compounds. Since the oral epithelium is stratified, drug permeation may involve a combination of these two routes. The route that predominates is generally the one that provides the least amount of hindrance to the passage. Using luminescent microscopy, we have found that paracellular route for the FITC-labeled insulin is the major pathway through the buccal epithelium (Fig. 4). The obtained data are in good accordance with literature (Shojaei, 1998; Senel et al., 2001; Junginger et al., 1999).

4. Conclusion

We have studied lysalbinic acid as a new absorbance promoter for the buccal delivery of protein drugs. Hamster cheek mucosa was used as a simple animal model for the initial evaluation of absorption promoters. Although the obtained results not necessarily can be directly extrapolated to human, the experimental data suggest that using our method it is possible to determine quickly and qualitatively composition of the formulation to be used for the buccal delivery of large medicinal molecules. It was shown that co-administration of lysalbinic acid with relatively small proteins (6–16 kDa), such as α-interferon and insulin, can significantly increase their absorption via the buccal epithelium. We demonstrated that paracellular routes for the studied proteins are the major pathways through buccal epithelium. Thus, lysalbinic acid has been shown to increase significantly a permeability of the hamster oral mucosa for peptide compounds of low- to middle-molecular weight. The transport properties of the individual fractions of lysalbinic acid (MW 600, 6000 and 11 000) are the subject of further studies. Our preliminary experiments suggest that all three fractions possess surfactant activity. The molecular mechanism by which lysalbinic acid increases transport is not completely clear yet, but it may be similar to that of other detergent enhancers (SDS, bile salts, etc.) based on intercellular lipid solubilization, the lipids being the major hindrance to buccal transport. Before we have, also, demonstrated by histological investigation of rat mucosa that lysalbinic acid has no irritating or sensibilizing effects upon buccal use (Starokadomskyy, 2003). So this easily available mild detergent of the natural origin can be recommended as an absorption enhancer for the development of novel buccal peptide drugs.

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