Journal of Thermal Analysis and Calorimetry, Vol. 83 (2006) 1, 27–30

INTERACTIONS BETWEEN A SKIN PENETRATION ENHANCER AND THE MAIN COMPONENTS OF HUMAN STRATUM CORNEUM LIPIDS
Isothermal titration calorimetry study

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The study employed isothermal titration calorimetry (ITC) technique to investigate the interactions between a skin penetration enhancer, laurocapram, and three representatives of the human stratum corneum (SC) intercellular lipids, i.e., ceramide-3, cholesterol and behenic acid. Experiments were performed using a TAM2277 (Thermometric AB, Sweden) calorimeter. Results showed that, firstly, two laurocapram molecules were bound to one ceramide-3 molecule in propylene glycol solution. The binding was enthalpy-driven and system entropy decreased after binding. Secondly, the binding ratio between cholesterol and laurocapram molecule was one to one. It was mainly an entropy-driven process, suggesting the ordered cholesterol molecules in SC lamella were disturbed by the introduction of laurocapram. Hydrogen bonding might be the main force of the binding. The three different interaction modes showed that the chemical enhancers selectively interacted with the components of SC lipids so that the SC can still retain its barrier function.

Keywords: *ceramide, cholesterol, free fatty acid, ITC, laurocapram, stratum corneum*

Introduction

The stratum corneum (SC) is the outermost layer of the skin, which is the main rigorous layer maintaining human interior milieu. The SC consists of keratin-filled dead corneocytes, embedded in a crystalline lipid lamellar matrix. The lipids in the SC form the continuous domain. The envelope of the corneocyte is almost impermeable to diffusing substances so that the main penetration pathway of substances through the SC is located in the intercellular lipid lamella [1–5].

The lipid composition differs markedly from that of typical biological membranes. The predominant lipid classes are ceramides, cholesterol and free fatty acids, the percentage (mass/mass) of which are about 50, 25 and 10%, respectively [6–9]. Nine subclasses of ceramides have been identified in human SC. They are classified according to the different combination of sphingosine and fatty acid moieties joined by an amide bond, and numbered by ascending polarity. The fatty acid part of ceramide-1 and -4 are ω-OH fatty acid, esterified to linoleic acid, possess much longer chains than the other ceramides. Ceramide-1, found in both human and pig SC, is essential for the formation of the 13-nm lamellar pattern in the X-ray diffraction study of SC lipids. Ceramide-4, -6 and -8, having the 6-OH-spingosine moieties, present only in human SC lipids, may not be essential for barrier formation [2, 10, 11]. The ceramide composition (mass/mass) as determined by high performance thin-layer chromatography was about 10% (ceramide-1), 30% (ceramide-2), 20% (ceramide-3), 10% (ceramide-4), 15% (ceramide-5, -6 and -7), 15% (ceramide-8), according to the new numbering rules based on ascending polarity [9, 10]. Ceramide-3 (CAS entry [34354-88-6], *MW*=583.97, log*P*=13.65) was well characterized among all the SC ceramides [12, 13]. It is thus chosen for the study here. Free fatty acid constituents are ranging from C14:0 to C28:0, and the principle components are palmitic acid (C16:0), stearic acid (C18:0), behenic acid (C22:0), lignoceric acid (C24:0) and hexacosanoic acid (C26:0), which consist approximately of 10, 10, 15, 25 and 10% (mass/mass), respectively, out of all the free fatty acids [6, 7, 9].

Laurocapram (Azone) is an effective enhancer for many hydrophilic and lipophilic drugs [14]. It can solubilize the SC intercellular lipids and/or disturb the lipid lamella [15, 16]. The aim of this study is to investigate the molecular interactions between laurocapram and the three SC lipids, i.e., ceramide-3, cholesterol and behenic acid, respectively (Fig. 1). Propylene glycol is a suitable vehicle for transdermal drug delivery and used here as the medium to dissolve laurocapram or the lipids. Isothermal titration calorimetry (ITC) technique is used for the study. ITC can monitor the heat generated in any physical or chemical reactions initiated by the addition of titrant to titrand. When substances interact with each other, heat is either generated or absorbed. Measurement of this heat allows the determination of reaction parameters [17, 18].

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1388–6150/$20.00

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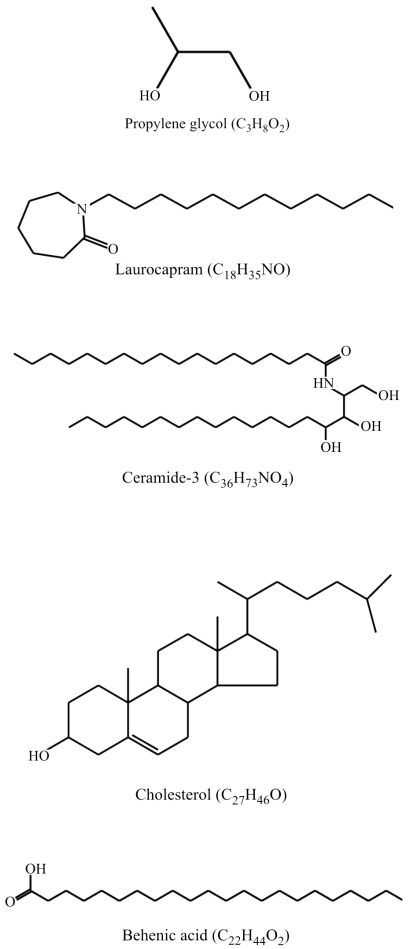

Fig. 1 The molecular structure of the solvent, propylene glycol; the skin penetration enhancer, laurocapram; and the three SC lipids, the ceramide-3, cholesterol and behenic acid

Experimental

The ITC experiments were performed using a TAM2277 (Thermometric AB, Sweden) calorimeter, with 4-mL stainless steel ampoules. Samples of laurocapram, cholesterol, behenic acid and ceramide-3 were prepared in propylene glycol, at concentrations of 71, 2, 0.667 and 0.667 mmol L^{-1}, respectively. Laurocapram solution of 0.12 mL was titrated consecutively into 2.7 mL of lipid solutions by 15 aliquots. The lipid solution was stirred with a turbine at 60 rpm. The system's temperature was maintained at 37°C. Control experiment was done by titrating pure propylene glycol into lipid solutions with the same experimental protocol as that used for the laurocapram solutions. ITC data were analyzed by Digitam® software (Scitech Software, Sweden) supplied with TAM2277 [19, 20].

The measured heat of laurocapram titrated into lipid solution by ITC technique includes the binding heat of laurocapram with the lipid, the dilution heat of laurocapram/the lipid, and other non-specific heat. Therefore, the binding heat was derived from the measured heat subtracting the heat of the control experiment in which laurocapram solution was replaced by pure propylene glycol for titration. The binding stoichiometry n, binding constant K, and enthalpy change ΔH were estimated from the non-linear regression analysis procedure of Digitam®. Free energy change ΔG and entropy change ΔS were calculated according to their relations with enthalpy change ΔH and binding constant K, i.e., $\Delta G = -RT\ln K = \Delta H - T\Delta S$, where R is the gas constant, 8.314 (J mol K^{-1}), and T is the temperature (K).

Results and discussion

Figure 2 shows the isothermal titration results of laurocapram solutions titrated into ceramide-3, cholesterol and behenic acid solution, respectively. Each of them is accompanied with the control titration result. The control study was conducted by titrating pure propylene glycol solution into the lipid solution. For non-linear regression analysis, the heat of the control experiment was deducted from the titration study. From Fig. 2a, it can be seen that heat was released during the titration process. This indicates the binding between laurocapram and ceramide-3 molecules is an exothermic process. It can be seen in Fig. 2b that the binding between laurocapram and cholesterol is also exothermic. Figure 2c shows the binding between behenic acid and laurocapram molecules is similar to that of laurocapram and cholesterol but without recognizable pattern.

Figure 3 summarizes the binding results fitted by non-linear regression analysis. The energy (integral) of each peak as in Fig. 2 was plotted as a function of the ratio of the moles of laurocapram added to the moles of the lipid in the ampoule. It can be seen in Fig. 3a that two laurocapram molecules bind one ceramide-3 molecule. The enthalpy change of the binding process was $-2.29 \cdot 10^5$ J mol^{-1}, with a decreased entropy change of –653 J mol^{-1} K^{-1}. These results show the binding between laurocapram and ceramide-3 is driven by enthalpy because the decreased enthalpy contributes largely to the decreased free energy. It can be seen in Fig. 3b that one laurocapram molecule binds one cholesterol molecule. The enthalpy change accounts for about only 1% of the free energy change, indicating that the process is driven mainly by entropy. The entropy increase suggests that after the binding, the system becomes less ordered than the initial state when they exist separately. This is different from the binding process between laurocapram and ceramid-3, where the entropy decreased after binding, indicating that the final state is more ordered than the initial state. Although in both

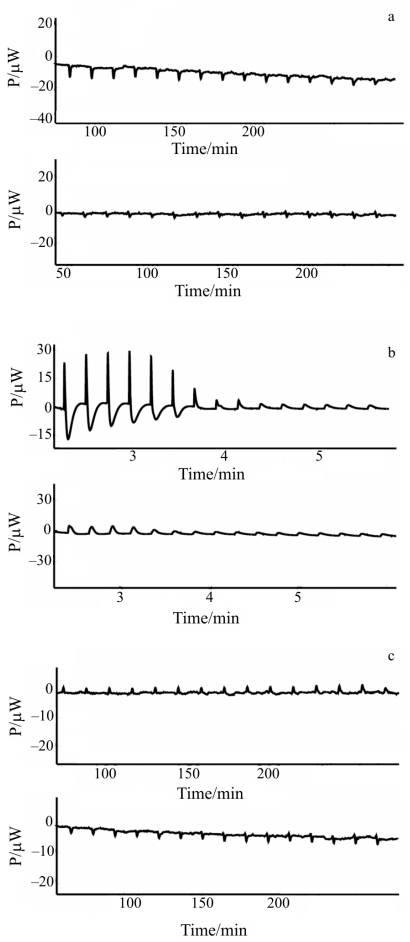

Fig. 2 Results obtained from ITC. The positive heat peak indicates an exothermic process; 0.12 mL of eihter laurocapram solution 71 mmol mL^{-1} (upper curve) or pure PG (lower curve) was titrated into a – ceramide-3, b – cholesterol, c – behenic acid solution by 15 aliquots. 2.7 mL of each lipid solution 0.667 mmol mL^{-1} was used

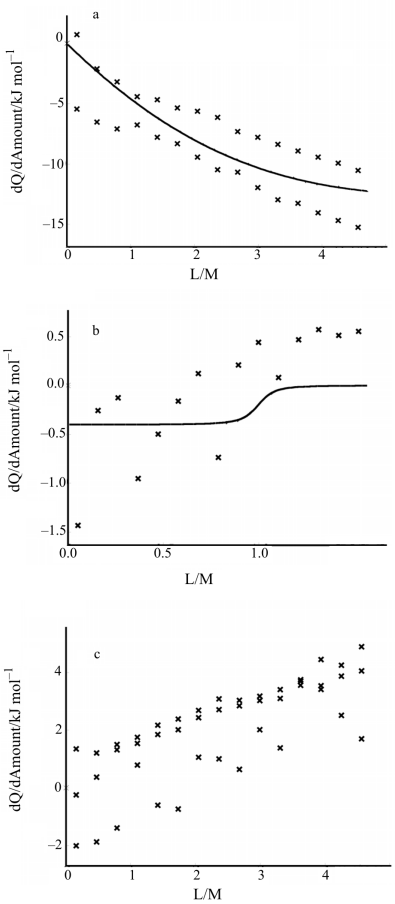

Fig. 3 Non-linear regression analyses to estimate the binding stoichiometry *n*, the binding constant *K*, and the enthalpy change ΔH with software Digitam®. Results of laurocapram titrated into a – ceramide-3 solution (2 replicates) with binding stoichiometry = 2, b – cholesterol solution (4 replicates) with binding stoichiometry = 1, c – behenic acid (3 replicates) with absence of or very weak interactions

cases the binding processes are product-favored, they are essentially different. The binding between laurocapram and ceramide-3 is enthalpy-driven while the entropy-driven binding between laurocapram and cholesterol made the system more chaotic in its final state than the initial state. The binding result between laurocapram and behenic acid is presented in Fig. 3c. The thermo profile showed no proof of binding between their molecules. This infers that the interaction/binding between the laurocapram molecules and the behenic acid molecules is non-specific, or, non-existent. Thus laurocapram does not exert its enhancing effect by interacting with behenic acid.

The binding between cholesterol and laurocapram makes the final state more disordered or random than the original state. This may suggest that cholesterol molecules were extracted from the SC lamella, where the lipid molecules are in crystalline or liquid crystalline phases [9]. The binding between laurocapram and ceramide-3 is an enthalpy-driven spontaneous process and the system entropy decreased upon binding. The decreased entropy may indicate laurocapram molecules became part of the ordered SC lipid phases. The divergence between cholesterol and ceramide-3 may be explained by their solubility in propylene glycol. The solubility of cholesterol in propylene glycol is about 4.62 mmol L^{-1} at 37°C [21]. The addition of laurocapram to propylene glycol can increase the solubility of cholesterol by more than 3 times but not applicable to ceramide-3. Therefore, the resultant complex of cholesterol and laurocapram can dissolve into propylene glycol more easily than the complex of ceramide-3 and laurocapram.

Inter- and intra-molecular hydrogen bonds may be the driving force for the binding. Both cholesterol and ceramide-3 are hydrogen bond donors while laurocapram molecules serve as acceptors (Fig. 1). Between one pair of laurocapram and cholesterol

molecules there is only one hydrogen bond. But one ceramide-3 molecule binds two laurocapram molecules, as shown by the results. It is plausible that an intra-molecular hydrogen bond was formed within the ceramide-3 molecule, between the hydroxyl and the neighboring carboxyl groups.

Conclusions

The chemical penetration enhancer, laurocapram, can exert its enhancing effect in propylene glycol by selectively extracting cholesterol, binding ceramides and leaving free fatty acids undisturbed. The reported SC lipid crystalline phase transition or disruption by spectrometry study is complementary to this ITC study [15]. Compared with the enhancing methods of circumventing the SC barrier by means of piercing multiple passages for transdermal drug delivery employed by many physical enhancing techniques, chemical enhancement may be safer. Chemical enhancers only disturb part of the SC lipids and leave the highly impermeable corneocytes unchanged, so the SC is still functional as an effective biological barrier [22–24].

Acknowledgements

The authors would like to thank Dr Peter Johansson from Thermometric AB (Sweden) for his technical assistance and valuable suggestions. The Cosmoferm BV (Delft, The Netherlands) is also highly appreciated for providing the ceramide-3 samples.

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DOI: 10.1007/s10973-005-7050-8