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Research Paper

Hemolytic activity and solubilizing capacity of raffinose and melezitose fatty acid monoesters prepared by enzymatic synthesis

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ABSTRACT

The hemolytic activity and solubilizing capacity of two families of non-reducing trisaccharide fatty acid monoesters have been studied to assess their usefulness as surfactants for pharmaceutical applications. The carbohydrate-based surfactants investigated included homologous series of raffinose and melezitose monoesters bearing C_{10} to C_{18} acyl chains prepared by lipase-catalyzed synthesis in organic media. The hemolytic activity was determined *in vitro* using a static method based on the addition of the surfactants to an erythrocyte suspension and subsequent spectrophotometric determination of the released hemoglobin. The effect of the carbohydrate head group, the acyl chain length and the regioisomeric purity was investigated. In all cases, the carbohydrate monoester surfactants decreased their hemolytic activity (with respect to their critical micelle concentration) when increasing the length of the acyl chain. A very similar behaviour was observed either the carbohydrate head-group (raffinose and melezitose) or regardless of the regioisomeric purity. Interestingly, decanoyl (C_{10}) and lauroyl (C_{12}) monoesters were just marginally hemolytic at their critical micelle concentrations while the longer palmitoyl (C_{16}) and (C_{18}) stearoyl monoesters become hemolytic at concentrations much higher than their respective *cmc*. The palmitoyl and stearoyl monoesters also displayed higher solubilization capacity than the shorter acyl chain monoesters in a solubilization assay of a hydrophobic dye as a model drug mimic. These results suggest that raffinose and melezitose monoesters with long-chain fatty acids (C_{16} to C_{18}) are promising surfactants for pharmaceutical applications and could be an alternative to the use of current commercial nonionic polyoxyethylene-based surfactants in parenteral formulations.

1. Introduction

Surfactants have several applications in pharmaceutical products and systems [1,2]. Their main use is as solubilizing agents of poorly water-soluble drugs in aqueous formulations [3] but they are also employed in solid dosage forms, topical formulations and also in biopharmaceutical preparations (both liquid or lyophilized). The most extensively used surfactants in pharmaceutical formulations are polyoxyethylene-based products. Nevertheless there are some drawbacks associated with them. The commercial technical products typically employed are comprised of complex polydisperse mixtures with varying degree of ethoxylation, which complicates chemical analysis and product specification handling. They are not considered environmentally friendly due to the lack of biodegradability and their petrochemical origin. Additionally they may cause some adverse responses such as release of histamine [4]. Sugar-based surfactants are promising alternatives to traditional polyoxyethylene surfactants [5,6]. This important type of non-ionic amphiphiles is constituted of carbohydrates as polar head groups conjugated with long-chain alcohols, fatty acids or other hydrophobic molecules. They are environmentally friendly due to their biodegradability and their manufacture from renewable sources [7].

Carbohydrate fatty acid esters are one of the most important classes of sugar-based surfactants [8,9]. Carbohydrate esters are produced industrially by acid-catalyzed esterifications or transesterifications [10] rendering mixtures with variable degree of substitution. Monoesters, which have better solubility in water than higher substituted derivatives, are comprised of a mixture of regioisomers due to the nature of the chemical process

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[11,12]. Nevertheless, enzymatic catalysis allows the regioselective esterification of carbohydrates [13]. The effect of the sugar, the fatty acid chain length and even the acylation position on the surfactant and self-organizing properties of carbohydrate fatty acid monoesters has also been studied [14–17]. Sucrose esters are the main class in this surfactant family [18] and have been used in a variety of pharmaceutical applications such as the solubilization of poorly water soluble drugs [19,20], drug delivery in transdermal therapeutic systems [21–23] or protein microencapsulation [24] among others. The closely related trehalose fatty acid esters have been proposed as a promising alternative for polysorbates in protein formulations [25] and maltose fatty acid monoesters having comparable properties to sucrose esters are claimed to be applicable in similar uses [26]. Recently, we have expanded the repertoire of sugar esters by developing a regioselective enzymatic oligosaccharide acylation methodology [27,28] which have allowed us to prepare new homologous series of non-reducing trisaccharide fatty acid monoesters [29]. The use of the trisaccharides raffinose and melezitose as polar head groups allows access to long-chain derivatives which display much higher solubility in water at room temperature than for example monopalmitoyl and monostearoyl esters of sucrose [30]. The new families have been proved to be promising detergents for membrane protein studies [29].

In the current work we have studied the potential pharmaceutical application of these new surfactants. We have investigated their usefulness as solubilizing agents in parenteral formulations by assessing both their hemolytic activity and solubilizing capacity. Since strongly hemolytic surfactants are not biocompatible, the measurement of this parameter is essential. To get a critical assessment of performance, the results obtained have been compared with those reported for other sugar surfactants derived from mono- and disaccharides including alkyl gluco- and maltopyranosides and lauroyl monoesters of both sucrose and lactose [31,32].

2. Materials and methods

2.1. Materials

The hydrophobic dye 1-(2-Pyridylazo)-2-naphthol (PAN) was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). The homologous series of non-reducing trisaccharide fatty acid monoesters included the following surfactants: 6-*O*-decanoylraffinose (R6D), 6-*O*-lauroylraffinose (R6L), 6-*O*-myristoylraffinose (R6M), 6-*O*-palmitoylraffinose (R6P), 6-*O*-stearoylraffinose (R6S), melezitose monodecanoate (MD), melezitose monolaurate (ML), melezitose monomyristate (MM), melezitose monopalmitate (MP) and melezitose monostearate (MS). All surfactants were prepared by lipase-catalyzed synthesis as previously described [29]. Raffinose monoesters were regioisomerically pure (6-*O*-acylraffinose > 99%) while each melezitose monoester was a mixture of regioisomers (61% of 6-*O*-acylmelezitose, 33% of 6″-*O*-acylmelezitose and 6% of other regioisomers) [29]. The structures of the surfactants are sketched in Fig. 1.

2.2. Solubilization capacity

Solubilization of hydrophobic dyes has been employed for the colorimetric determination of the *cmc* of surfactants [33]. In this work, the dye PAN was employed as model drug mimic since its aqueous solubility is negligible [34]. For the determination of the solubilization capacity, each surfactant was dissolved in normal saline solution (0.9% NaCl in water) at different concentrations above the corresponding *cmc*. A saturated solution of PAN was prepared in ethanol. For each point of the solubilization curves, 300 μL of the dye solution in ethanol was dried down in 1.5 mL Eppendorf tubes by evaporation to form a film of dye. 300 μL of the corresponding surfactant solution was added and after initial vortexing the tubes were incubated in a shaker at 25 °C for 16 h (there was a high excess of dye in all tubes). After incubation, the tubes were centrifuged to pellet any non-solubilized dye dispersed in the solution, and 250 μL of each supernatant was transferred to 96 microtiter well plates for measuring the absorbance at 462 nm in a microplate reader. The corresponding calibration curve of absorbance vs. dye concentration in solution was prepared with serial dilutions of PAN dissolved in water:ethanol (1:1) prepared from an stock of known concentration.

The solubilization capacity (κ) was estimated as the slope of the solubilization curve in the linear region: $\kappa = \Delta[\text{PAN}]/\Delta[\text{surfactant}]$, where [PAN] and [surfactant] are the concentrations of PAN and surfactant respectively.

2.3. Hemolytic activity

The technique employed for the hemolysis assays was based on the one described by Kondo and Tomizawa with erythrocytes [35]. The protocol was adapted from those reported for other non-ionic surfactants [36], including polyoxyethylene [32] and maltopyranoside [31] surfactants.

Fresh group 0 positive human blood was obtained from the Centro Regional de Transfusión Sanguínea-SAS (Granada, Spain) and kindly provided by Dr. Luis Miguel Ruiz Pérez (IPBLN-CSIC, Granada). The erythrocytes were separated by centrifugation at 2000*g* for 10 min at 20 °C. The plasma was decanted and replaced by an equal volume of normal saline solution (0.9% NaCl in water) in which the erythrocytes were re-suspended. The washing procedure was repeated three times. The resulting erythrocytes suspension was employed for evaluating the hemolytic activity of

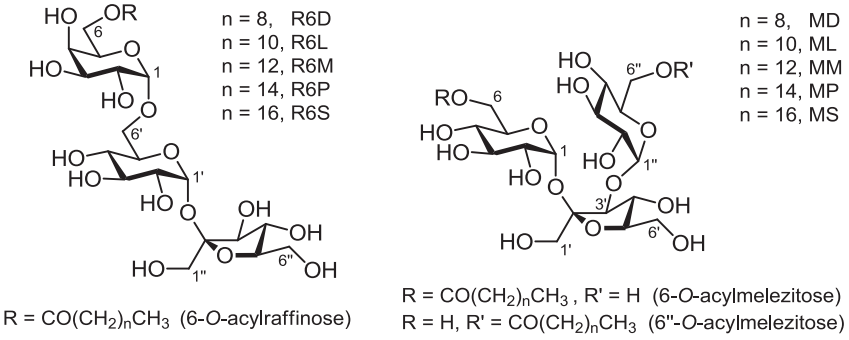

Fig. 1. Structure of the non-reducing trisaccharide fatty acid monoesters with different acyl chain lengths. Raffinose monoesters are regioisomerically pure (6-*O*-acylraffinose) while each melezitose monoester is comprised of a mixture ca. 2:1 of the 6-*O*-acyl/6″-*O*-acyl derivatives.