Rhodamine-123: A p-glycoprotein marker complex with sodium lauryl sulfate

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Abstract: Aim of this study was to investigate the role of sodium lauryl sulfate (SLS) as P- glycoprotein inhibitor. The everted rat gut sac model was used to study in-vitro mucosal to serosal transport of Rhodamine-123 (Rho-123). Surprisingly, SLS decreases the serosal absorption of Rho-123 at all investigated concentrations. Investigation reveals complex formation between Rhodamine-123 and sodium lauryl sulfate. Interaction profile of SLS & Rho-123 was studied at variable SLS concentrations. The SLS concentration higher than critical micelle concentration (CMC) increases the solubility of Rho-123 but could not help in serosal absorption, on the contrary the absorption of Rho-123 decreased. Rho-123 and SLS form pink color complex at sub-CMC. The SLS concentrations below CMC decrease the solubility of Rho-123. For further studies, Rho-123 & SLS complex was prepared by using solvent evaporation technique and characterized by using differential scanning calorimeter (DSC). Thermal analysis also proved the formation of complex between SLS & Rho-123. The P values were found to be significant (<0.05) except group comprising 0.0001% SLS, and that is because 0.0001% SLS is seems to be very low to affect the solubility or complexation of Rho-123.

Keywords: P-glycoprotein, surfactant, rhodamine-123, efflux transport, everted gut sac.

INTRODUCTION

Transporters play important role in drug absorption. The influx/ efflux, or both transporters plays an important role in intestinal absorption of therapeutic drugs. Influx transporters facilitate drug absorption while efflux transporters hamper the absorption (Vasiliou *et al*., 2009; Varma *et al*., 2010). The drug substrates of these transporters are prone to clinically relevant drug interaction with transport modulators. P-glycoprotein, a well-known efflux transporter present at apical membranes of absorptive cells, influences the oral bioavailability of many orally administered drugs. P-gp drives drug molecules towards lumen side of intestine wall and interferes in drug absorption. To curb this P-gp mediated bioavailability issue, many P-gp inhibitors have been identified (Bansal *et al*., 2009).

Pharmaceutical excipients such as diluents, surfactants, binders etc., were known as pharmacologically inert; but recent investigations have shown that some of these excipients may interfere with pharmacokinetics of narrow therapeutic indexed drugs, viz. absorption or metabolism or both. Excipients, especially surfactants have been reported to interfere with the intestinal P-gp and modulate the intestinal absorption of drug substrates (Cornaire *et al*., 2004). For example, at low concentration Labrasol® might inhibit the P-gp in the intestine, and thereby increases the intestinal absorption and bioavailability of P-gp substrate rhodamine123 (Lin *et al.*, 2007). D-alpha-tocopheryl polyethylene glycol 1000 succinate (0.04%), inhibited P-glycoprotein mediated transport of talinolol in Caco-2 cells (Bogman *et al*., 2005). The influx transport of Rho-123 was significantly increased in presence of Tween-80, while its secretory transport decreased depending on the concentration of Tween-80 (Li *et al*., 2008). Pluronic-PAA copolymer suppresses the P-glycoprotein (Bromberg 2008). Polymeric P-gp inhibitor (thiolated chitosan) remarkably increases the oral bioavailability of Rho-123 (Föger *et al*., 2006). PEGs (400, 2000, 20000) and their derivatives (monolaurate, monooleate, and monostearate) inhibit intestinal P-gp and thereby improve the absorption of Rho-123 (Shen *et al*., 2006). The cremophor-EL and polysorbate-80 modulate P-glycoprotein and improve the oral bioavailability of poorly absorbed drugs (Cornaire *et al*., 2000). Pluronic F68 enhances intestinal transport of celiprolol in in-vitro and also inhibits CYP3A4 catalyzed formation of 1'-hydroxymidazolam (Huang *et al*., 2008).

Aim of this work was to investigate the interaction of the P- glycoprotein substrate (Rho-123) and surfactant, where the surfactant is an anionic surfactant, sodium lauryl sulfate (SLS). The role of intestinal transporters in drug absorption has been investigated through various in-vitro as well as in-vivo models (Acra *et al*., 1991). Everted gut sac technique was used as in-vitro model to investigate interaction between surfactant and P-gp substrate (Cornaire *et al*., 2004). The in vitro everted intestinal sac experiment was introduced by Wilson *et al*. in 1954, after that it has been extensively explored in pharmacokinetic investigations (Wilson *et al*., 1954).

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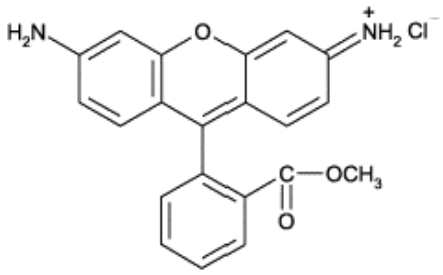
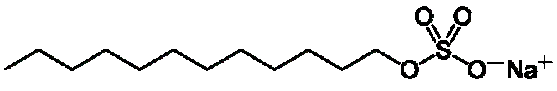
Rhodamine-123 (6-amino-9-(2-methoxycarbonylphenyl) xanthen-3-ylidene]azanium chloride) is a lipophilic, cationic, fluorescent dye. It is a model substrate of P-glycoprotein and has been used as a selective marker for studying the functional activity of P-gp and to asses P-gp related drug interactions (Nare *et al*., 1994; Hong *et al*., 2006; Ando *et al*., 2001; Pavek *et al*., 2003; Yumoto *et al*., 1999). Since Rho-123 is not a substrate of CYP3A (metabolizing enzyme) (Yumoto *et al*., 2001), this is one of the attribute which is important for the investigation of P-gp mediated interactions. The molecular weight of Rho-123 is 380.82 and the melting point is 235°C. Chemical structure of Rho-123 is given below.



Sodium lauryl sulfate (SLS) is an anionic surfactant. The molecular weight and melting point of SLS are 288.38 & 204°C, respectively. SLS has a negatively charged sulfonate group as its "hydrophilic" end (polar group) and a saturated 12-carbon chain forms "lipophilic" end (non-polar group). SLS has been used as dissolution aid, to investigate the release of poorly water-soluble drugs (Rawat *et al*. 2010; Bajerski *et al*. 2010). Further, SLS has been used in many pharmaceutical formulations for different purposes (Ndesendo *et al*. 2008; Turpin 2002; Shidhaye *et al*. 2010). In an investigation conducted by Shidhaye SS, bilayered mucoadhesive buccal tablets of pravastatin sodium comprising 1% SLS showed improved permeation of drug through mucosa (Shidhaye *et al*., 2010). Shidhaye, 2010 and Shah, 2007 reported that SLS alone and in combination with other absorption enhancers has improved transepithelial permeation and bioavailability of ganciclovir (Shidhaye *et al*., 2010; Shah *et al*., 2007).



MATERIALS AND METHODS

Rhodamine-123 was purchased from Sigma Aldrich Inc., U.S.A.; Sodium lauryl sulphate GRG was purchased from Winlab, U.K.; NaCl (BDH Chemicals Ltd., England); KCl (Fluka Chemika, Switzerland); $MgCl_2$ (BDH Laboratory supplies, England); Glucose (Fluka Biochemika, Buchs); Na_2HPO_4 (BDH VWR International Ltd., England); NaH_2PO_4 (BDH Laboratory supplies, England); DSC-60 (Shimadzu, Japan); HPLC (Shimadzu, Japan); Column (Teknokroma, C18, 5µm, 15 × 0.46); Detector (RF-10AXL-Fluorescence detector).

Determination of Rho-123 transport across rat intestine

Gut sac preparation

The everted gut sac experiment was performed according to a technique described by Wilson TH, 1954 and Barthe L, 1998 (Wilson *et al*., 1954; Barthe *et al*., 1998). Male Sprague-Dawley rats weighing 200 to 250g were obtained from Animal Care Centre at King Saud University, and fasted overnight with free access to drinking water. Under ether anesthesia, the ileum part of intestine was rapidly removed and divided into four segments (5-6 cm. each). Each segment was washed with cold oxygenated Kreb's solution of pH 6.5. The composition of Kreb's solution includes 0.34g/l potassium chloride, 7g/l sodium chloride, 0.207g/l sodium dihydrogen phosphate, 0.251g/l disodium hydrogen phosphate, 46.8 mg/l magnesium chloride and 1.8g/l glucose (Al-Mohizea 2010). The washed and cleaned intestine segments were immediately placed in warm (37°C) oxygenated (O_2/CO_2, 95%: 5%) Kreb's solution (media) and then gently everted with the help of glass rod. One end of segment was tied with a silk braided suture and then filled with 600µl of Kreb's solution (37 °C) by using a micropipette. The filled segment was sealed with a second knot at other end. The filled segments were kept in individual incubation tubes having 15ml of oxygenated Kreb's media at 37°C.

Incubation Assays

To study the effect of SLS on the serosal transfer of Rho-123, the series of Kreb's solution containing 1.0, 0.1, 0.05, 0.01 and 0.0001% w/v SLS was prepared, and transferred to the incubation tubes. Rho-123 was added to the incubation tubes to make final concentration 5µg /ml. Everted sacs filled with Krebs solution were placed in incubation tubes comprising 15ml oxygenated media comprising 5µg/ml Rho-123 (with or without SLS). Incubation tubes without SLS named as control and the tubes having SLS were coded as test. The Kreb's media was maintained at 37°C and aerated with O_2/CO_2 (95%:5%). At the 90th minute of sampling time intestinal sacs were removed from incubation tubes, washed and blotted dry. The dried sacs were cut to open and the serosal fluid was drained into Eppendorf Snap-Cap Microcentrifuge Safe-Lock Tubes (Capacity: 1.5ml). Eppendorf tubes were centrifuged to precipitate suspended matter. The supernatant was diluted and analyzed by using HPLC.

SAMPLE ANALYSIS

Rhodamine-123 has been analyzed by using high performance liquid chromatography. The mobile phase consisted of methanol and aqueous solution of 15 mM dibasic sodium phosphate in the ratio of 80:20, v/v (pH

6.0). The flow rate was adjusted at 1.0ml/min. The 50µl of diluted samples were injected into C18 column and eluted at an excitation wavelength of 505 nm (Jiang *et al*., 2008). The retention time of the peak was 4.0 min.

Interaction study of Rho-123 in SLS solutions

Series of aqueous solutions of sodium lauryl sulphate (0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001% w/v) were prepared by serial dilution. Ten ml of each solution was transferred to glass tubes and 50 µg of Rho-123 was transferred to each glass tube to make final concentration 5µg/ml. The tubes were held over vortex until Rho-123 was uniformly distributed through the solution and physically observed for color change by naked eye. The 5ml sample from each tube was filtered and analyzed, and remaining 5ml was kept overnight at room temperature.

Preparation of complex

The molar ratio of Rho-123 and SLS (1:26) was calculated on the basis of interaction study. Rho-123 was transferred into the aqueous solution of SLS and the solution was diluted slowly with distilled water until it turned brown/ pinkish from fluorescent green. The solvent was evaporated in an oven at 40°C. The dried mass was scrapped, powdered and again dried for over 48 hr.

Differential Scanning calorimetry (DSC)

DSC analysis was performed for sodium lauryl sulfate, Rho-123 and their complex by using a Shimadzu DSC-60 instrument. The aluminum pans were filled with 2-6mg of sample in each. The filled aluminum pans were crimped and hermetically sealed. Nitrogen gas was supplied at 40ml/min flow rate. Samples were heated at the rate of 10°C/min, in the range of 25 to 400°C.

RESULTS

The Rho-123 transport through everted intestine sac was measured on serosal side. Fig. 1 shows the effect of different concentrations of surfactant (SLS) on the serosal transfer of Rho-123 through everted intestinal sac. In fig. 1, first column represent the control, i.e. absorption of Rho-123 in absence of SLS, while the next column represent test, where the absorption of Rho-123 is in presence of SLS. The data labelled at the columns represent the quantity of Rho-123 absorbed in nano-gram/600µl. Outcomes of study were against general trend. The *in vitro* absorption of Rho-123 was found to be decreased in the presence of sodium lauryl sulfate. The decreased in absorption was noted at above as well as below the critical micelle concentration of SLS.

Solubility of Rho-123 was increased at above critical micelle concentrations, but a solubility decreasing trend was observed at sub-critical micelle concentrations. The interaction study of Rho-123 with SLS reveals the formation of less soluble complex. The maximum interaction was observed at 0.01 and 0.005% w/v SLS. Fig. 2 represents the interaction profile of Rho-123 in presence of different concentrations of SLS. The label put on the columns represents the quantity of Rho-123 (µg/ml) in solution in presence of SLS. Solubility of Rho-123 at SLS 0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001% w/v concentration was 3.28, 4.59, 3.73, 3.34, 3.15, 2.19, 0.85, 1.39, 2.53, 2.60, 2.94 µg/ml respectively.

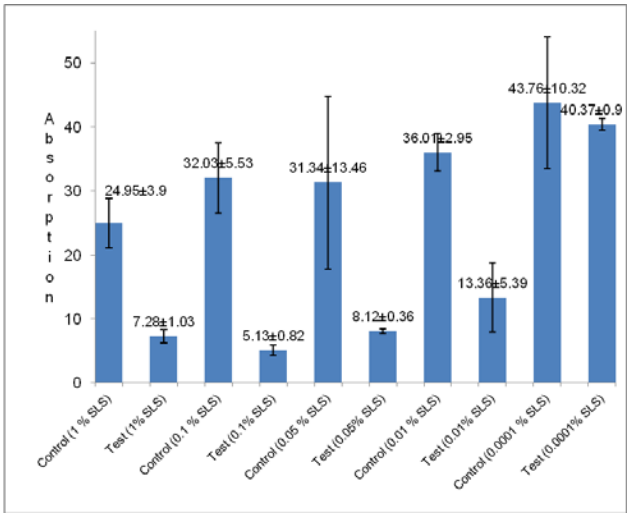


Fig. 1: Rho-123 absorption through everted sac in presence of different concentrations of SLS.

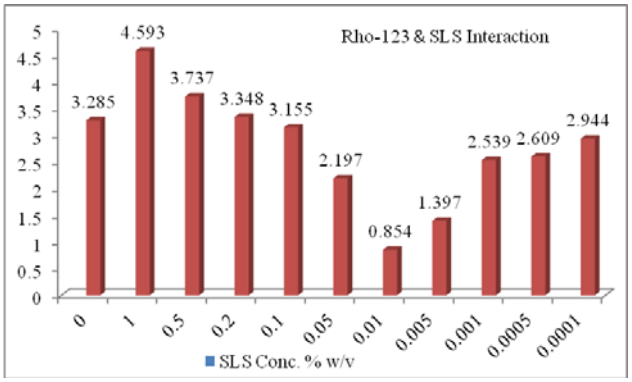


Fig. 2: Solubility profile of Rho-123 in presence of different concentrations of SLS.

The results of thermal analysis also support the findings of interaction study. DSC thermo grams of SLS, Rho-123 and their complex are shown in the fig. 3. The SLS thermograme shows its melting peak at 184.31°C. The endothermic peak of Rho-123 is at 212.20°C. No endothermic peak of SLS or Rho-123 was reported in the endotherm of complex (Rho-123 & SLS).

DISCUSSION

Surfactants are reported to be one of the non-medicinal P-gp inhibitors, and expected to enhance bioavailability of

P-gp substrates (Cornaire *et al*., 2004; Lin *et al*. 2007). The utmost objective of P-gp substrate and modulator interaction studies is to investigate the pharmacokinetic changes and to predict clinical outcome. In the present study we tried to investigate the impact of different concentrations of sodium lauryl sulphate on in-vitro absorption of P-gp substrate (Rho-123). Results of everted gut sac investigations are given in fig. 1. The concentration of Rho-123 was measured on serosal side, inside everted sac. Presence of SLS showed great influence on in-vitro absorption of Rho-123. Different concentrations of SLS have different impact on the absorption of Rho-123, through different mechanism. In general, presence of SLS hampers the absorption of Rho-123, 30% drug was absorbed in the presence of 1% SLS, as compared to control (fig. 1). Approximately 16% Rho-123 was absorbed in presence of 0. 1% SLS (fig. 1), 25% Rho-123 was absorbed in presence of 0.05% SLS (fig. 1), 37% Rho-123 was absorbed in presence of 0.01% SLS (fig. 1), and 92% Rho-123 was absorbed in presence of 0. 0001% SLS (fig. 1), in comparison to control. It was surprising that drug absorption was decreased in the presence of SLS, even at higher concentration (1% w/v SLS), which is against normal trend (Shidhaye *et al*., 2010; Shah *et al*., 2007). The P values for groups comprising 1%, 0.1%, 0.05%, 0.01% and 0.0001% SLS were found to be 0.01, 0.01, 0.06, 0.01 and 0.34; respectively. The P values were found to be significant except for group comprising 0.0001% SLS, and that is because 0.0001% SLS is seems to be very low to affect the solubility or complexation of Rho-123.

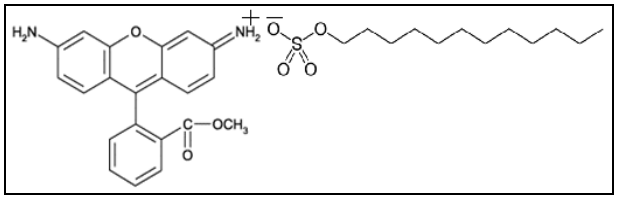


Fig. 3: The proposed structural diagram of Rho-123 & SLS complex.

During investigation we observed that there was quick color change in the solutions of 0.1%, 0.01% and 0.05% SLS upon mixing with Rho-123. The color of control medium (Kreb's solution) comprising Rho-123 was fluorescent green, and the color of test mediums (Kreb's solution & SLS) comprising 1% & 0.0001% SLS was also fluorescent green in presence of Rho-123. Color of test mediums (Kreb's solution & SLS) comprising 0.1%, .01% and 0.05% w/v SLS in the presence of Rho-123 was brown/ pink. The solutions were kept overnight at 30°C, pink precipitate as well as pink suspended particles were observed in test mediums comprising 0.1%, 0.01% and 0.05%w/v SLS. Hereafter a physical interaction/ complexation was expected and to prove this a series of solutions comprising SLS (0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001% w/v) was prepared, and to them Rho-123 (5µg/ml) was added. The color of Rho-123 solutions comprising 0, 1.0, 0.5, 0.2, 0.0005 and 0.0001% w/v SLS was fluorescent green, but the color of solutions comprising 0.1, 0.05, 0.01, 0.005 & 0.001% w/v SLS was brown/ pink. The presence of precipitate in the samples indicates the formation of insoluble or less soluble compound. The solutions were filtered through Watman filter-1, and were analyzed for their solubility. The solubility/ interaction profile of Rho-123 with SLS concentration series is "V- shape" and is shown in fig. 2. Compared to the control, the solubility of Rho-123 was increased in presence of SLS concentration higher than critical micelle concentration, which is quite obvious. But the solubility of Rho-123 in presence of SLS concentration below CMC (0.2% w/v) was decreased to a much low level, compared to control (0% SLS), and which was surprising. Solubility of Rho-123 at 0.01 % w/v SLS concentration was least (0.85 µg/ml). So SLS concentration 0.01% w/v was selected for further investigations.

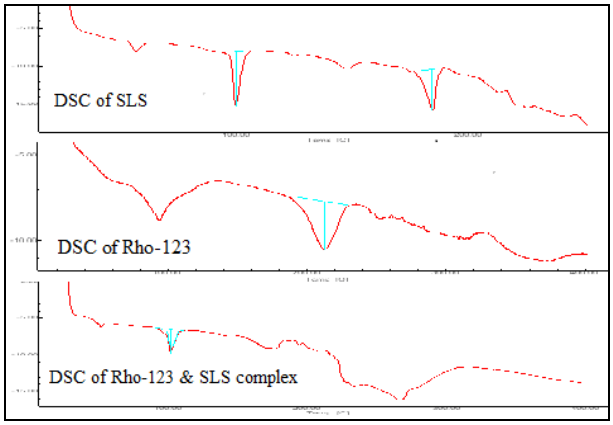


Fig. 4: DSC thermo grams of SLS, Rho-123 and their complex.

Interestingly, the results obtained through in vitro investigations were against general rule; where surfactants are supposed to increase the absorption of drugs, either through P-gp inhibition or through increased permeation or via solubilization mechanism (Lin *et al*. 2007; Shah *et al*. 2007; Torchilin 2001). The solubility of Rho-123 was increased up to 1.398 fold at 1% w/v SLS, despite this, it did not increase in-vitro absorption, and on the contrary it decreases the absorption of Rho-123. The absorption profile of Rho-123 was SLS concentration dependent, the maximum absorption takes place at 0.0001% w/v SLS concentration, and even this was less than control. The probable reasons for poor absorption in the presence of SLS (1.0 % w/v) may be the formation of micelle (Shaik *et al*. 2009), where Rho-123 is under the influence of micelle structure and is not available in free molecular form for better absorption. Further, it is hypothesized that SLS molecules at concentrations lower than CMC (sub-CMC concentrations 0.1 & 0.05% w/v),

are in free form as well as in aggregates. The free surfactant molecules are available in the medium to react with Rho-123 and form insoluble/ less soluble complex, and some Rho-123 is aligned with available aggregates of SLS molecules and then less drug is available for absorption. The solutions of SLS concentration 0.01% w/v shows maximum decrease in solubility and expected to form higher level of complex in amongst the concentrations tested, but still there was some drug available for the absorption. Least or no aggregate of micelle are expected at SLS concentrations 0.001, 0.0005, 0.0001% w/v; and SLS is available in free molecular form which reacts with Rho-123 and forms insoluble complex; but the quantity of SLS was not sufficient to complex with all Rho-123 molecules, so free Rho-123 was available in the medium for absorption (Zhao *et al*., 2004; Huang *et al*., 1998; Serbanescu *et al*., 2005).

Complex of Rho-123 & SLS was prepared at a molar ratio of 1: 26. The proposed structural diagram of Rho-123 & SLS complex is represented in fig. 3. The thermal analysis is used as a common approach for characterization of physical complexes and raw materials in solid state. Complex was characterized by using differential scanning calorimeter. In DSC thermograme, characteristic of peak (sharp, broad, flat) and its melting point/ heat of transition have been used as fingerprint for a particular solid compound or composition. DSC thermo grams of individual component (SLS & Rho-123) and complex of SLS & Rho-123 are shown in the fig. 4. The thermograme of SLS was a typical of crystalline substance showing a sharp endothermic peak at 184.31 °C, which corresponds to the melting point of SLS and here considered as fingerprint of SLS (fig. 4). The thermograme of Rho-123 showed as sharp endothermic peak at 212.20°C, which also corresponds to its melting point and considered as fingerprint for Rho-123 (fig. 4). The representing thermo grams of SLS & Rho-123 complex showed interaction between Rh-123 & SLS. Fig. 4 correspond to the DSC thermograme of complex prepared between SLS and Rho-123, no sharp endothermic peak was identified in the thermograme of complex, which clearly show absence of free Rho-123 as well as SLS. The endotherm of complex was flat & wavy which is the characteristic of amorphous/non-crystalline compounds. On the basis of DSC analysis we can conclude there is interaction between SLS and Rho-123 at molecular level and a less soluble complex is formed. The endothermic peak at 90-100°C in all samples indicates dehydration or loss of water associated with the solid material.

CONCLUSION

Present investigations open a new discussion for the role of surfactants as absorption or penetration enhancer. Further investigations can be done at molecular and cellular level to find out the role of SLS as transport modulator. As the SLS complexes with Rho-123 and form pink colored less soluble complex, some other P-gp substrate which does not complexes with SLS can be selected for further studies to establish the role of SLS as P-gp modulator.

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