An In Vitro Method for Evaluation of the Irritancy of Anionic Surfactants

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Extraction of Coomassie Brilliant Blue R250 dye from gelatin by anionic surfactants was found to be proportional to the concentration, duration of extraction, and the nature of the surfactant. Under a given set of conditions, the decreasing order of dye extraction was SLS > LAS > sodium laurate > AOS ≅ SLES. This order agrees well with the decreasing order of irritancy of these surfactants. For a homologous series such as sodium salts of carboxylic acids, there was a sharp maximum in dye extraction for laurate. This coincides with the reported order of irritancy for soaps of fatty acids. Substitution of gelatin by agar or Coomassie Brilliant Blue by eosin abolished this specificity. Thus, the measurement of extraction of Coomassie Brilliant Blue R250 from gelatin is a reproducible and simple method for estimating the irritancy of anionic surfactants.

Anionic surfactants used in washing products are known to cause irritation when brought in contact with living tissue. The magnitude of the irritant response is dependent upon the structure of the surfactant, its concentration in use, the duration of the contact, and other environmental factors. Several test methods have been developed to assess the irritancy of surfactants (1).

The irritation caused by anionic surfactants is related to their ability to penetrate the living epidermis (2,3). Penetration of anionic surfactants into the epidermis is preceded by their adsorption to the protein in stratum corneum (4). A high correlation between the ability of anionic surfactants to cause protein denaturation and the compatibility of the surfactant with skin (5) has been found to exist. Several *in vitro* methods such as solubilization of Zein (6), adsorption on stratum corneum (7), and interaction of bovine albumin with surfactants (8) have been developed to quantitate the irritant action of anionic surfactants. These methods estimate the extent of the interaction between an anionic surfactant and a proteic substrate.

In this article we describe a simple and reproducible *in vitro* method for evaluating the potential irritancy of anionic surfactants. It has been one of our objectives to develop a method which can be adapted in most laboratories and does not involve the use of animals or tissues. The method involves measuring the amount of dye extracted by an anionic surfactant from a matrix of gelatin and Coomassie Brilliant Blue R 250 (CBB). Increased extraction of the dye is an indication of the increased irritancy of the surfactant. The order of irritancy of various surfactants estimated by this procedure coincides with those reported in literature.

EXPERIMENTAL PROCEDURE

Gelatin samples were procured from local suppliers (Food Grade, United Trade Corporation, Bombay; Pig Skin, 1st Extraction ex HLL Factory, Taloja; Photographic grade ex Indu Photofilms, Ootacamund, India). Eosin dye (ex SD's lab Chem. Industry), Coomassie Brilliant Blue R-250 (ex Fluka) and Agar (ex Difco) were used for this study.

Standardization of gelatin concentration to be used. Because commercial samples of gelatin are somewhat heterogeneous in terms of molecular weight, gel strength, etc., it was necessary to have a specification for the sample being used. By trial and error it was established that the concentration of gelatin that gives a viscosity of 10 cps (measured on a Brookfield Viscometer at 30°C, using spindle no. LV1, 60 rpm) gives optimum performance. Thus 6.66% of the Food Grade and 5.2% of the Pork Skin gelatins had viscosities of 10 cps.

Preparation of gelatin/agar solutions with CBB or eosin dye and setting of gel. An aqueous solution of gelatin (concentration determined as described above) or agar (2.5%, w/v) was prepared with eosin (0.5%, w/v) or CBB (0.1%, w/v) by heating and dissolving it at 60–65°C in a water bath. Ten ml of this solution was pipetted into 100 ml Erlenmeyer flasks which were kept in ice water and allowed to set for 30 min. It is essential that the flasks be kept stationary to ensure that a smooth surface to the gel is obtained.

Preparation of surfactant/sodium salts of fatty acid solutions. A 1% solution of surfactant or sodium soap of fatty acids was prepared by dissolving 1 g of sample of surfactant or soap in water (warming if necessary), and adjusting the pH to 9.0 before increasing the volume up to 100 ml.

Preparation of solutions of commercial toilet soaps. Under conditions of actual use, the concentration of soap has been estimated to be about 5%. Furthermore, the use of higher concentrations of soap would serve to magnify the response in the assay. Soap gratings (2.5 g) were extracted with 50 ml of water by shaking on a rotary shaker at 160–170 rpm for 15 minutes at room temperature. The supernatant was centrifuged at 10,000 × g for 20 min. The clear supernatant was used again after adjusting the pH to 9.0.

Measurement of dye extraction by surfactant solutions. The solution of soap/surfactant (10 ml) prepared as above was added to the gel of gelatin and CBB or agar and eosin and kept on a gyrotary shaker at 100–110 cycles/min at room temperature (23–25°C). The shaking was continued for 30 min, and then a 5 ml aliquot of the supernatant was transferred to a stoppered 50 ml test tube. A 5 ml aliquot of the original surfactant solution was also transferred to another 50 ml stoppered test tube and used as control. Both the solutions were extracted with ether:alcohol (3:1) (10 ml × 2) to remove fatty acids which would interfere with the color determination. If the emulsion was not separating, an additional 0.5 ml of alcohol was added. Then the ether layer was discarded and the aqueous layer was heated at 70°C to remove the last traces of solvent. The absorption of both blank and experimental tubes was read at 590 nm for CBB and at 515 nm for eosin.

RESULTS AND DISCUSSION

Several grades of gelatin are commercially available, and even in the same grade batch to batch variations were

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found to give inconsistent results. This could be normalized by taking that concentration of gelatin which had a viscosity of 10 cps. The results from Table 1 show that different samples of gelatin gave similar results. It must be added that if all the gelatins had been used at the same concentration, the values would have been radically different (data not shown).

The importance of the substrate and the dye used are demonstrated in the results shown in Tables 2 and 3. In Table 2, when agar was used as the substrate the extraction of dye was independent of the surfactant used since even water could extract the dye. Similarly, when eosin was used as the dye, even water could extract the dye from a gelatin substrate (Table 3). Only the gelatin and CBB combination was found to be selective. In this connection, it may be added that collagen as a substrate has been used for testing *in vitro* the skin compatibility of surfactants (5).

TABLE 1

Extraction of Coomassie Brilliant Blue from Different Samples of Gelatin by Three Anionic Surfactants

	A_{590}		
Gelatin Sample (Concn.)	AOS	LAS	SLS
Food Grade Gelatin Sample A (Experiment 1) (6.66%)	0.200	0.700	1.081
Food Grade Gelatin Sample A (Experiment 2) (6.66%)	0.192	0.597	0.960
Food Grade Gelatin Sample B (8.8%)	0.168	0.503	0.872
Pork Skin 1st Extrn. (5.2%)	0.177	0.513	0.854

TABLE 2

Extraction of Coomassie Brilliant Blue from Agar by Various Commercial Soaps

Soap	A_{590}
1	5.44
2	6.49
3	4.82
4	4.40
5	6.47
Water	6.08

TABLE 3

Extraction of Eosin from Gelatin by Various Surfactants

Surfactant	A_{515}
Sodium Laurate	25.15
Sodium Lauryl Sulfate (SLS)	20.05
Linear Alkyl Benzene Sulfonate (LAS)	22.15
Sodium Lauryl Ether Sulfate (SLES)	21.15
Alpha Olefin Sulphonate (AOS)	20.6
Water	25.10

Earlier *in vitro* methods involved either measurement of the solubilization of a protein (6) or changes in the physicochemical properties of the protein (8) following addition of surfactant. The magnitude of these changes are small, especially at low concentrations of surfactants. The present method serves to magnify these differences and is therefore easier to quantify.

Extraction of the dye from gelatin increased linearly up to 30 minutes and tapered off for two different surfactants (Fig. 1). Therefore, in all further studies, the optimum duration of extraction of 30 min was chosen.

Extraction of CBB from gelatin varied for different surfactants (Table 4). The decreasing order of dye extraction for typical anionic surfactants was Sodium Lauryl Sulfate (SLS) > Linear Alkyl Benzene Sulfonate (LAS) > Sodium Laurate > Alpha Olefin Sulfonate (AOS) ≅ Sodium Lauryl Ether Sulfate (SLES). This ordering also coincides with the decreasing order of irritancy of these surfactants established in animal studies (9). For a given surfactant, the extraction of dye was proportional to the concentration of the surfactant (Fig. 2).

The amount of dye extracted for different commercial soaps with their approximate coconut soap content is given in Table 5. It can be seen that the values were mostly

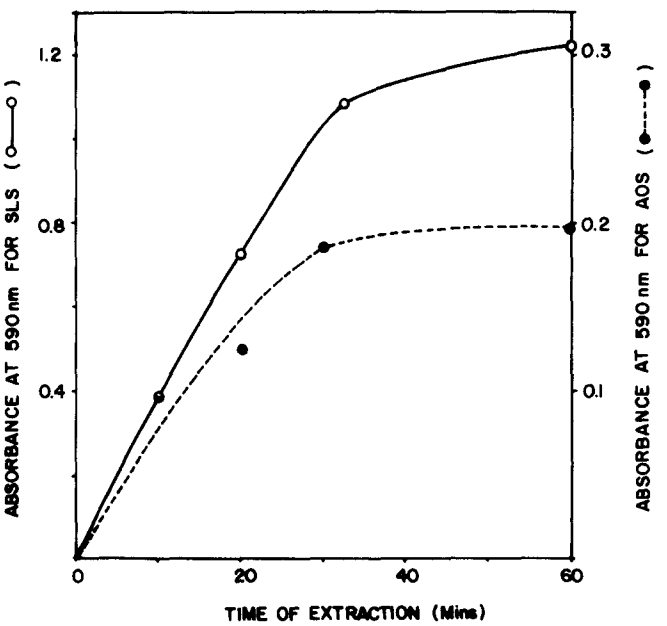

FIG. 1. Time dependence of dye extraction.

TABLE 4

Extraction of Coomassie Brilliant Blue from Gelatin by Various Anionic Surfactants

Surfactant	A_{590}
Sodium Laurate	0.289, 0.275, 0.278
Sodium Lauryl Sulfate (SLS)	1.016, 1.146
Linear Alkyl Benzene Sulphonate (LAS)	0.781, 0.615
Sodium Lauryl Ether Sulphate (SLES)	0.188, 0.142
Alpha Olefin Sulphonate (AOS)	0.233, 0.162
Water	0.007, 0.019

in the order of 0.2 to 0.4, with the highest value for a soap containing the highest amount of coconut soap. It is well established that the laurate and myristate in coconut are more of an irritant than other soaps.

This observation was further strengthened when the dye extraction by 1% solutions of the sodium salts of fatty acids from C4–C18 was determined (Fig. 3). A sharp maximum in terms of dye extraction was seen at C12—an observation that corresponds very well with the measured irritancy of the homologous series of soaps in rat studies (10). Thus, the gelatin-CBB method has distinguished between different anionic surfactants and this distinction closely parallels the observed irritancy of these surfactants in animal studies.

Soap containing 10 and 20% LAS was evaluated by the gelatin-CBB method. It was found that at 10% level, there was a substantial increase in dye extraction (Table 6), indicating that by this method, presence of irritant anionic surfactants in soaps can be detected.

In this study, gelatin has been chosen as a protein substrate because of some similarity to the principal protein in stratum corneum (i.e., keratin). Further, a solution of gelatin can be set into a gel and serves as a matrix which can be approximated to the proteinaceous matrix of stratum corneum. The other advantage in the use of gelatin is its ready availability.

The choice of the dye is equally important. This is because the interaction of the surfactant with the protein must displace a proportional quantity of the dye so that measurement of the absorbance in the supernatant is a measure of the adsorption of the surfactant to the protein. This would mean that the forces of interaction between the dye and the protein must be similar to those between surfactant and protein. In the case of gelatin and CBB the above criteria were presumably met, whereas in the case of gelatin-eosin or agar-CBB these criteria were not met.

Under conditions of actual use, anionic surfactants do not penetrate through the skin readily (11,12). Anionic surfactants have been shown to adsorb to the skin proteins and are only removed with difficulty by washing (1). Thus the action of surfactants on skin extends beyond mere contact time. Therefore, it follows that increased interactions between surfactants and skin proteins would prolong the contact and thus enhance penetration of the surfactant into the skin.

Several studies in the literature (9) have attempted to correlate the irritancy of surfactants to their ease of penetration of the stratum corneum. Using conductance measurement, Dugard and Scheuplein (11) found that the degree of penetration of stratum corneum was C12 > C14 > C16 > C10 > C8 for sodium salts of carboxylic acids. This order exactly parallels the order of irritancy found from our study (Table 4). In other words, displacement of CBB from gelatin by anionic surfactants is a measure of the degree of association between the surfactant and the protein. Furthermore, the greater this interaction the greater the permeability, and hence, more irritant the product.

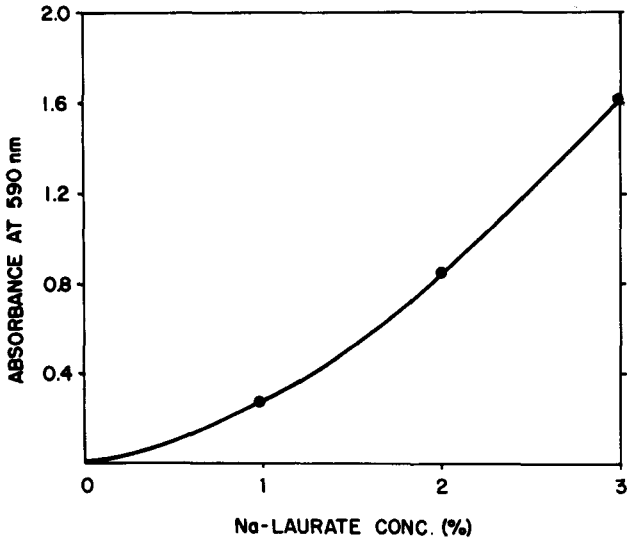

FIG. 2. Dose dependence of dye extraction on soap concentration.

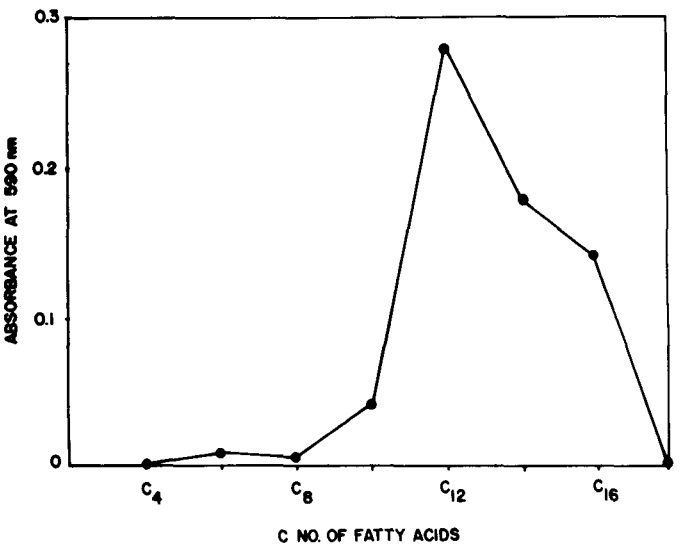

FIG. 3. Correlation between dye extraction and fatty acid chain length of soaps.

TABLE 5

Extraction of Coomassie Brilliant Blue from Gelatin by Different Commercial Soaps

Soap	Approx. coconut soap content	A_{590}
1	50%	0.470, 0.437
2	40%	0.28, 0.29
3	10%	0.24, 0.26
4	5-10%	0.22, 0.24

TABLE 6

Effect of Incorporating 10 and 20% LAS in Soap Base on Extraction of Coomassie Brilliant Blue from Gelatin

LAS content (%)	A_{590}
0	0.160, 0.170
10	0.735, 0.740
20	1.283, 1.266

The results obtained in this study demonstrate that extraction of CBB from gelatin by anionic surfactants is related to the extent of interaction between the surfactant and gelatin. Furthermore, this is related to the irritancy of the surfactants. Thus, the method described in this paper measures the potential irritancy of anionic surfactants.

REFERENCES

1. Frosch, P.J., in *Principles of Cosmetics for the Dermatologist*, Frost, P. and S.N. Horwitz, eds., The CV Mosby Co., St. Louis, MO (1982) pp. 5-12.
2. Howes, D.I., *J. Soc. Cosmet. Chem. 26*:47 (1975).
3. Prottey, C. and Ferguson, T.J., *Ibid. 26*:29 (1975).
4. Imokawa, G., Sumura, K. and Katsumi, M., *J. Am. Oil Chem. Soc. 52*:484 (1975).
5. Lindner, H. and Lindner, H., *Parfum Kosmet. 67*, 372 (1986).
6. Gotte, E., *Aesthet. Med. 15*:313 (1966).
7. Belaguer, F., Garcia Dominguez, J.J., Parvai, J.L. and Pelejerd, C.M., *Cosmet. Perfum. 90*:77 (1975).
8. Breuer, M. and Strauss, M.P., *J. Phys. Chem., 64*:252 (1960).
9. Kastner, W., in *Anionic Surfactants, Biochemistry, Toxicology, Dermatology*, C. Gloxhuber, ed., Marcel Dekker, Inc., New York (1980), pp. 139-307.
10. Prottey, C., Hartop, P. and Ferguson, T.F.M., *J. Soc. Cosmet. Chem., 24*:473 (1973).
11. Dugard, P.H. and Scheuplein, R.J., *J. Invest. Dermatol. 60*:263 (1973).
12. Scala, J., McOsker, D.E. and Reller, H.H., *Ibid. 50*:373 (1968).

[Received August 22, 1988; accepted March 12, 1989]
[JS-D5556]