Effects of Breed, Season, Temperature, and Solvents on the Permeability of Frozen and Reconstituted Cattle Skin to Levamisole

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Abstract □ *In vitro* measurements have been made of the permeability of frozen and reconstituted cattle skins to levamisole. Breeds used were Red Poll cross, Hereford/Shorthorn cross, Hereford/Santa Gatrudis cross (or Brahman), Friesian (or Friesian/Jersey cross), and Hereford cattle killed in early fall, early summer, or winter. Inter- and intrabreed differences in skin permeability were small, but skin permeability in summer and fall was appreciably greater than in winter. Increases in skin temperature also increased skin permeability. The solvent properties of the skin toward neutral molecules appeared to be similar to those of water, suggesting that skin is a relatively polar barrier.

Keyphrases □ Levamisole—effects of breed, season, temperature, and solvents on permeability, frozen and reconstituted cattle skin □ Permeability—frozen and reconstituted cattle skin, effects of breed, season, temperature, and solvents, levamisole □ Skin, cattle—frozen and reconstituted, effects of breed, season, temperature, and solvents on permeability to levamisole

It has been shown recently (1) that the outer 1 mm of frozen and reconstituted skin from a Hereford calf (*i.e.*, stratum corneum, viable epidermis, papillary layer of the dermis, and associated appendages) acts as a homogenous barrier to the diffusion of molecules of levamisole (I). This observation highlights a significant difference between the barrier properties of cattle skin and those of similarly treated human skin in which the stratum corneum is the rate-determining barrier to diffusing molecules (2, 3). Consequently, it is probable that different mechanisms are involved in the transport of molecules across cattle and human skin, and different steps must be taken when formulating topical products for cattle than those taken when formulating topical products for humans.

Steps that can be taken to control topical absorption of drugs by humans have been reviewed previously (4, 5).

The effects of breed, season, skin temperature, and solvents on the *in vitro* permeability of frozen and reconstituted[1] cattle skin to I are reported here.

It is anticipated that the results of this study will be directly applicable to the development of liquid dosage forms that are intended to promote systemic drug delivery [*i.e.*, pour-ons (6)] to cattle.

EXPERIMENTAL

The methods used to harvest and store cattle skins were identical to those described earlier (1). Details of the cattle used and of the times when skins were harvested are in Table I.

Materials—Compound I (mp 61°), I hydrochloride (mp 228°), polyoxypropylene-15-stearyl ether, sorbitan sesquioleate, the nonaromatic hydrocarbon fraction, and 2-ethoxyethanol were used as received[2] as was formulation C (Table II). All other chemicals were analytical reagents unless otherwise stated. Formulations are listed in Table II.

Measurement of Skin Permeability—The procedures, including analytical methods, described previously (1, 7) were employed to determine the permeability of the skins to I. Skin penetration was followed for 300 min, and lag times varied from 4 min (for penetration from toluene) to 120 min (for penetration from aqueous and alcoholic solutions). In two studies, measurements were made of the penetration of tritiated water (^3H_2O) through cattle skin. Approximately 0.5 μCi of tritiated water was added to either the aqueous pH 8.9 solution or the pH 6.0 solution of I, and 1.0 ml of the mixture was applied to the epidermal surface of the skin. Samples (0.2 ml) of the donor solutions were taken at 0 and 300 min, and samples (1.0 ml) of the receptor solutions were taken at 300 min. The samples were adjusted to 1 ml with water and to each was added 10 ml of scintillation liquid (0.4% 2,5-diphenyloxazole in toluene–tyloxopol[3], 2:1 v/v). After thorough mixing, the samples were counted on a liquid scintillation spectrometer[4].

The temperature of the water bath in which all experiments, except those carried out to study variation of permeability with temperature, was 37.0 ± 0.1°. Thermistor measurements indicated that the temperature of the outer surface of the skin in these experiments was 30.6 ± 0.5°.

Statistical Analysis—Unless otherwise stated, a multiple comparison statistical technique previously described (8) and modified[5] was used to analyze the experimental results.

RESULTS AND DISCUSSION

As a consequence of the finding (1) that the outer 1 mm of cattle skin behaves as an homogenous barrier to the diffusion of molecules of I, it follows that:

1. The product of the permeability constant (k_p, cm min^{-1}) and the thickness of the skin specimen (r, cm, *i.e.*, k_pr cm^2 min^{-1}) is a constant for diffusion through skin specimens with thicknesses in the range of 0.3–1.0 mm;
2. The diffusion coefficient (D, cm^2 min^{-1}) of I in skin specimens with thicknesses in the range of 0.3–1.0 mm can be calculated from the lag time for diffusion (L, min) by:

$$D = r^2/6L \qquad \text{(Eq. 1)}$$

3. The partition coefficient (PC) for I from the formulation into skin specimens with thicknesses up to 1.0 mm can be calculated from:

$$PC = k_pr/D \qquad \text{(Eq. 2)}$$

The derivations of Eqs. 1 and 2 are discussed in a previous report (1).

Values of k_pr, D, and PC for penetration of I through the skins of a number of cattle from a single formulation and from several formulations are in Tables III and IV, respectively. The skin specimens used in all experiments had thicknesses in the range of 0.3–1.0 mm.

Intra- and Interbreed Variation in Skin Permeability—Table III contains mean values of k_pr, D, and PC for penetration of I through skins of four Hereford calves from formulation A. The calves were about the same age, and the skin was harvested in early fall.

Statistical analysis of the data indicates that there was no significant difference at the 1% level between the permeability of the four skins and suggests that intrabreed differences are likely to be small for animals with similar histories treated during the same season.

Two pieces of evidence suggest that interbreed differences (involving mainly European breeds) in skin permeability are also likely to be small for animals of similar ages that are treated during the same seasons.

Thus, the permeability of the skin of a Friesian calf to I from formu-

[1] Results to be published shortly from this laboratory establish that there are no significant differences at the 5% level in the permeability of fresh, frozen, and reconstituted cattle skin.
[2] ICI Australia Ltd.

[3] Triton X-100.
[4] Packard Tri-carb #544.
[5] Personal Communication, B. H. Kellett, Victorian College of Pharmacy.

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Table I—Details of Cattle and Time of Skin Harvesting

Calf Number	Approximate Age, months	Breed	Time of Harvesting
115	6	Red Poll Cross	Late August (winter)
110	9–11	Hereford/Shorthorn cross	Late August (winter)
109	6–8	Hereford/Santa cross Gatrudis or Brahman	
F1	4–6	Friesian (or Friesian/ Jersey cross)	Early December (early Summer)
16	10–12	Hereford	Mid March (early fall)
21	8	Hereford	Mid March (early fall)
29	8–10	Hereford	Mid March (early fall)
34	8–9	Hereford	Mid March (early fall)

lation A in early summer was similar (there was no significant difference at the 1% level) to that of skins of the four Hereford calves in early fall (Table III). Similarly, the results in Table IV show that interbreed differences for the permeability of the winter skins of a Red Poll (No. 115), a Hereford/Shorthorn cross (No. 110), and a Hereford/Santa Gatrudis cross or Brahman (No. 109) to I from formulation A and of those of calves Nos. 110 and 109 to formulations B and C, aqueous pH 8.9 and 6.0 solutions, were not significant at the 1% level.

Seasonal Variation—Comparison of the results in Table III for the permeability to I from formulation A of the skin of the four Hereford calves killed in early fall (Nos. 16, 21, 29, and 34) with similar data in Table IV for skins of calves killed in winter (Nos. 115, 110, and 109) indicate that the skin is significantly more permeable to I in the fall than in the winter (a two-tailed t test on the means for each group of results indicated that $p < 10^{-7}$).

This result is consistent with an observation that greater blood levels of I were achieved in cattle following application of a pour-on in summer than were achieved in winter[6].

Furthermore, while the values of both the diffusion coefficient and partition coefficient for I were greater in fall skin than in winter skin ($p < 10^{-2}$ and $p < 10^{-4}$, respectively), inspection of the data reveals that the increase in the value of the partition coefficient is much larger. The greater partition coefficient in fall indicates that for I fall skin is a better solvent than winter skin.

The solvent properties of the skin will be discussed further after considering the effect that different formulations and different temperatures have on skin permeability.

Formulation Variation—A noticeable feature of the data in Table IV is that I penetrated the skins of calves 110 and 109 ~30 times faster ($p < 10^{-2}$) from the aqueous pH 8.9 solution (where I existed to the extent of 90% as a neutral molecule[7]) than from the aqueous pH 6.0 solution (where I existed to the extent of 99% as a cation).

To test whether the pH value of aqueous buffers affected the intrinsic permeability of the skin, experiments were conducted using aqueous solutions of I buffered at pH 8.9 and 6.0 and containing tritiated water. Measurements were made of the rate at which both tritiated water and I penetrated the skin from each formulation. The results in Table V indicate that the percent of the applied amount of tritiated water penetrating the skin in 300 min was essentially the same at pH 8.9 and 6.0. This result is consistent with the conclusion that the variation of pH values from 8.9 to 6.0 does not significantly alter the intrinsic permeability of the cattle skin. In the same experiments, the percent of the applied dose of I that penetrated the skin in 300 min was ~ 10 times higher than the pH 8.9 buffer than from the pH 6.0 buffer.

The data in Table IV indicate that whereas the value of the diffusion coefficient for I in cattle skin is essentially the same from aqueous pH 8.9 and 6.0 solutions (differences are not significant at the 1% levels), the value of the partition coefficient of I from pH 8.9 solution into the skin is >10 times that from the pH 6.0 solution ($p < 10^{-2}$).

This result suggests that the skin has similar solvent properties to water for neutral molecules for I but is an inferior solvent for I cations.

Comparison of the data in Table IV for penetration of neutral molecules of I through the skins of calves Nos. 110 and 109 from formulations A (largely 2-ethoxyethanol), B (largely 2-ethoxyethanol), and C (largely 2-propanol) and water indicates that for I the skin permeability is greatest

[6] Personal Communication, D. Taylor, I.C.I. Limited, United Kingdom.
[7] The pK_a value of I is 7.94 at 37° (7).

Table II—Formulations

Designation	% w/v I	Formulation
Aqueous pH 8.9 solution	0.85	I HCl 1% Borate Buffer to 100% Final pH 8.9
Aqueous pH 6.0 solution	0.85	I HCl 1% Phosphate Buffer to 100% Final pH 6.0
80% Dimethyl sulfoxide	1	I 1% Water 20% Dimethyl Sulfoxide to 100%
Toluene	0.85	I to 0.85% Toluene to 100%
Formulation A[a]	10	I 10% Poloxypropylene-15-stearyl ether 12% Nonaromatic Hydrocarbons 15% 2-Ethoxyethanol to 100%
Formulation B	10	I 10% Sorbitan Sesquioleate 10% Nonaromatic Hydrocarbons 20% 2-Ethoxyethanol to 100%
Formulation C[a]	10	I 10% Spindle Oil 10% Isopropanol to 100%

[a] Bayer.

from water. Also, while the values of the diffusion coefficient for I from each of the above formulations are similar, the value of the partition coefficient for I into the skin is significantly greater from water than from the organic solvents ($p < 10^{-2}$).

Table IV also includes data for the penetration of neutral molecules of I through the skin of calf No. 109 from 80% dimethyl sulfoxide (in water) and from toluene. These results differ from those from the other formulations studied, in that the values of the diffusion coefficients of I in the skin were significantly larger ($p < 10^{-2}$) when 80% dimethyl sulfoxide and toluene were used as solvents. These results arose because the lag times for diffusion from these solvents were much less than those from aqueous pH 8.9 solution, aqueous pH 6.0 solution, or from formulations A, B, and C.

In the case of penetration from 80% dimethyl sulfoxide, the value of $k_p r$ was six times less than that from aqueous pH 8.9 solution, although the value of the diffusion coefficient in the former system was 8.6 times larger. The determining factor in this case was the fact that the value of the partition coefficient of I between the aqueous pH 8.9 solution and skin was ~60 times that between 80% dimethyl sulfoxide and skin.

The value of $k_p r$ for penetration from toluene was comparable to that from aqueous pH 8.9 solutions at the 1% level. In this case, although the partition coefficient of I from toluene into skin was 0.06 that from aqueous pH 8.9 solution, the value of the diffusion coefficient in the former case was 18.7 times that in the latter.

These results are difficult to explain at the present time, but it is probable that they arise because dimethyl sulfoxide and toluene dissolve away or displace certain components of the skin and thereby change its permeability characteristics. It is known (9) that the application of ether removes a significant proportion of the lipid and stratum corneum from cattle skin.

An important conclusion that can be drawn from these observations is that dimethyl sulfoxide may not be a universal promoter of penetration of neutral molecules through cattle skin to the same extent as it is for human skin penetration (10, 11). It has previously been shown (7) that dimethyl sulfoxide impedes rather than promotes the absorption of I through sheep skin.

Table III—Comparative Data[a] for Penetration of I from Formulation A through Hereford[b] and Friesian[c] Skins

Calf Number	Formulation	$10^6 k_p r$ ($\pm SD$) $cm^2\ min^{-1}$	$10^6 D$ ($\pm SD$) $cm^2\ min^{-1}$	Partition Coefficient ($\pm SD$)
16	A	17.8(4.5)	17(4)	1.13(0.42)
21	A	15.7(2.1)	28(3)	0.58(0.14)
29	A	20.8(7.2)	21(8)	1.19(0.79)
34	A	19.6(5.5)	19(5)	1.12(0.60)
F1	A	24.0(3.7)	23(6)	1.09(0.35)

[a] Mean values of $k_p r$, D, and PC from at least four experiments with skin sample thicknesses between 0.3 and 1 μm, together with standard deviations (SD). [b] Calves 16, 21, 29, and 34 harvested in early fall. [c] Calf F1 harvested in early summer.

Table IV—Comparative Data for Penetration of I from a Variety of Solvents through Winter Skin of Three Breeds of Cattle [a]

Calf Number	Formulation	$10^6 k_p r$ (±*SD*) cm² min⁻¹	$10^6 D$ (±*SD*) cm² min⁻¹	Partition Coefficient (± *SD*) cm² min⁻¹
115	A	1.8(0.2)	14(7)	0.15(0.05)
110	A	2.0(0.01)	10(4)	0.22(0.09)
109	A	1.8(0.5)	11(3)	0.18(0.10)
110	B	2.3(0.4)	10(3)	0.25(0.10)
109	B	1.2(0.3)	11(3)	0.11(0.02)
110	C	0.8(0.2)	14(5)	0.07(0.04)
109	C	0.6(0.1)	20(15)	0.05(0.03)
110	Aqueous pH 8.9 solution	11.3(3.6)	15(3)	0.79(0.24)
109	Aqueous pH 8.9 solution	10.2(3.1)	10(4)	1.10(0.47)
110	Aqueous pH 6.0 solution	0.4(0.1)	11(7)	0.04(0.05)
109	Aqueous pH 6.0 solution	0.3(0.1)	12(13)	0.06(0.06)
109	80% Dimethyl Sulfoxide [b]	1.6	86	0.02
109	Toluene	13.1(1.1)	188(14)	0.07

[a] As described in Table I. [b] Only one experiment was performed.

Table V—Relative Rates of Penetration of I and Tritiated Water through the Skin of Calf No. 115 at 37°

Formulation	Applied Dose of I Penetrating in 300 min, % (1)	Applied Dose of Tritiated Water Penetrating in 300 min, % (2)	(1)/(2)
Aqueous pH 8.9 solution + tritiated water	1.6	2.6	0.6
Aqueous pH 8.9 solution + tritiated water	1.7	1.3	1.3
Aqueous pH 6.0 solution + tritiated water	0.1	2.1	0.05
Aqueous pH 6.0 solution + tritiated water	0.2	2.0	0.08

Temperature Variation—Table VI contains values of $k_p r$, diffusion coefficient (D), and partition coefficient (PC) for penetration of I from formulation A through the skin of calf No. F1 at different temperatures.

As expected for a diffusional process, the rate of penetration, as reflected in the value of $k_p r$, increased with an increase in temperature. (Differences between values of $k_p r$ at the two lower temperatures and at the two higher temperatures were not significant at the 5% level, but $k_p r$ values at the higher temperatures were greater than those at lower temperatures.)

This result is important when taken in conjunction with the observation that the permeability of animal skin in early fall (and presumably summer) is greater than that in winter. Hence, the higher ambient temperatures that are likely to exist in fall as compared with winter are likely to contribute to very much greater permeability in fall (and summer) as compared with winter.

The complexity of the effect of temperature on skin permeability is highlighted by the fact that at both the 1 and 5% level there was no significant difference in values of D and PC as the skin temperature was raised from 24.1 to 32.7°.

Diffusion Model and Solvent Properties of Cattle Skin—It was postulated (1) that the major mechanism by which I, and presumably other neutral molecules, is transported across cattle skin involves diffusion into and down the hair follicles and into their associated sweat and sebaceous ducts to a capillary bed, at which point they enter the blood. It was also suggested (1) that the emulsified sebum [which is expelled from the common sweat and sebaceous ducts into the infundibulae of the hair follicles and which seeps up through the outer layers of the stratum corneum and down the hair follicle (12)] has a profound influence on skin permeability.

A potentially useful model for cattle skin penetration involves the following steps:

1. Drug molecules rapidly partition from applied topical formulations into the emulsified sebum which is associated with the skin. The partition coefficient for this step is the PC value calculated from *in vitro* diffusion experiments.
2. Drug molecules diffuse through the emulsified sebum and eventually pass down the hair follicles and reach the capillary beds where they pass into the blood. The parameter (D) that is calculated from *in vitro* experiments would be the diffusion coefficient for this process.

Results to date indicate that the value of D is not significantly affected by solvents such as water, 2-ethoxyethanol, and 2-propanol, but is affected by toluene and 80% dimethyl sulfoxide. It was suggested earlier that those latter solvents might modify the diffusion barrier (the emulsion) by dissolving or displacing a component.

A rationalization of the solvent properties of emulsified sebum, and consequently of values of PC, can be achieved by considering those features that determine what is a good or a poor solvent for I.

Table VII contains values of the partition coefficients for neutral molecules of I from aqueous pH 8.9 solution into liquid paraffin, toluene, and dimethyl sulfoxide. These results indicate that I is less soluble in water than in each of the other solvents, but that a complex relationship exists between the solubility of I and the polarity (dielectric constant ϵ) or solubility parameter (δ) of the solvent. For the purposes of these comparisons, liquid paraffin has been assigned the same values of ϵ and δ that are possessed by n-hexane (1.89 at 20° and 7.3 hr (13), respectively).

Hence, although the solubility of I in liquid paraffin, toluene, and dimethyl sulfoxide increases as both the dielectric constant increases [1.89 at 20° (13), 2.38 at 25° (13), and 45 (14)] and the solubility parameter increases [7.3 H, 8.9 H, 12.0 H (13)], it is least soluble in water, the solvent with the highest dielectric constant [78.54 at 25° (13)] and solubility parameter [23.4 H (13)]. It appears that specific dipole–dipole or other solvent–solute interactions are of at least equal importance to polarity in determining the solubility of I in a given solvent.

The PC values in Table IV that relate to partitioning of I between various solvents and the barrier (*i.e.*, the emulsion) suggest that the latter has similar solvent properties to water toward I, but that it is poorer than the alcohol-based solvents in formulations A, B, and C as well as toluene or 80% dimethyl sulfoxide. The results also show that while the barrier has similar solvent properties to water toward neutral molecules of I, it is a poorer solvent than water toward cations.

Table VI—Effect of Temperature on the Permeability of Cattle Skin [a] to I from Formulation A

Bath Temperature	Skin Temperature	No. Experiments	$10^6 k_p r$, cm² min⁻¹	$10^6 D$, cm² min⁻¹	Partition Coefficient
25.0°	24.1°(0.2)	6	8.2(1.7)	16(5)	0.59(0.29)
30.0°	27.3°(0.5)	5	13.2(3.1)	16(4)	0.90(0.39)
37.0°	30.6°(0.5)	11	24.0(3.7)	23(6)	1.09(0.35)
40.0°	32.7°(0.5)	2	22.6(1.1)	21(6)	1.19(0.40)

[a] The skin was from the dorsal region of calf F1. [b] The temperature of the skin was measured periodically using a thermistor.

Table VII—Partition Coefficients [a,b] for I at 37°

Solvent 2	Solvent 1	Partition Coefficient (*PC*), (±*SD*)
Liquid paraffin	aqueous pH 8.9 solution	2.3(0.6)
Toluene	aqueous pH 8.9 solution	5.2(2.1)
Dimethyl sulfoxide	aqueous pH 8.9 solution	14.4[c]

[a] *PC* = [I] solvent 2/[I] solvent 1. [b] Data are from Ref. 7. [c] Calculated by dividing *PC* from aqueous pH 8.9 solution into liquid paraffin (2.3) by *PC* from dimethyl sulfoxide into liquid paraffin (0.16).

The results in Tables III and IV reveal that the solvent properties of the skin toward I increase in the fall as compared with winter.

The complex nature of the solubility of I does not allow simplistic statements to be made. However, it is very likely that changes in the composition of the emulsified sebum, perhaps even phase changes, led to the observed changes in permeability.

There appears to be very little difference between the skin permeabilities of European breeds of cattle at any particular time, and intrabreed differences are small.

Because the permeability of cattle skin appears to be 10 times greater in early fall as compared with winter and to increase with increasing temperature, it can be predicted that the skin permeability of cattle in the field in mid-summer will be substantially higher than that in midwinter. Cattle skin is a relatively polar solvent with properties similar to water towards neutral organic molecules. Consequently, water or poorer solvents than water for particular drugs should be selected for topical formulations in preference to better solvents than water in order to maximize the rate of drug penetration.

Studies aimed at characterizing the solvent properties of domestic animal skins are currently underway.

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Determination of Antimicrobial Preservatives in Pharmaceutical Formulations Using Reverse-Phase Liquid Chromatography

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Received November 20, 1981, from *The Upjohn Company, Kalamazoo, MI 49001.* Accepted for publication April 15, 1982. *Present address: Westinghouse Electric Corporation, Bettis Atomic Power Laboratory, West Mifflin, PA 15122.

Abstract □ A specific stability-indicating reverse-phase high-performance liquid chromatographic analytical method has been developed to quantitate the antimicrobial preservatives methylparaben, propylparaben, butylparaben, sorbic acid, and benzoic acid in a series of typical pharmaceutical formulations. The mobile phase of this system is a water–acetonitrile mixture, modified by various acids and buffers. The proportions of water and acetonitrile as well as the type and amounts of modifiers are varied in order to achieve optimum chromatography. This method has been used successfully to quantitate preservatives in solutions, suspensions, creams, lotions, and ointments, and can be readily adapted to routine automated assays, either for routine product evaluation or stability programs.

Keyphrases □ Preservatives, antimicrobial—determination in pharmaceutical formulations using reverse-phase liquid chromatography □ Reverse-phase liquid chromatography—determination of antimicrobial preservatives in pharmaceutical formulations

Antimicrobial preservatives are materials added to formulations to protect the product from microbial contamination. A given preservative material can be used in a wide variety of products and also may be used in combinations with other preservatives. Separate testing methods for each product–preservative combination would not make efficient use of laboratory resources if the tests are to be performed frequently; thus a method which is generally applicable is desirable. However, the analytical methods should be specific to ensure that decomposition products and impurities are not inadvertently measured. Regulatory agencies have also shown interest in specific test methods for preservatives (1, 2). The challenge for the methods developer is to come up with a method that satisfies both criteria of assay efficiency and specificity.

It is currently of interest within the pharmaceutical industry to assure that specific, stability-indicating, and validated testing methods are available for antimicrobial preservatives. This study reports the development of a reverse-phase high-performance liquid chromatographic (HPLC) assay system that, with minor modifications in

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