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Inhibition of skin sphingosine synthesis: enhanced percutaneous permeation of 5-fluorouracil

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The present study was designed to investigate the role of skin sphingosine inhibition in enhancing the transcutaneous permeation of 5-fluorouracil (5-FU), a hydrophilic drug, across rat skin. Ethanol-perturbation significantly reduced the sphingosine content as compared to that in normal skin until 24 h of perturbation ($p < 0.05$). To maintain the low content of sphingosine for a longer time, β-chloroalanine (β-CA), a selective inhibitor of serine palmitoyl transferase, was used for inhibiting the sphingosine synthesis in viable skin. Application of β-CA (600 μg or 1200 μg/7cm^2) to viable skin perturbed with ethanol significantly reduced the sphingosine content untill 48 h as compared to that in perturbed viable skin ($p < 0.05$). However, the sphingosine content in viable skin perturbed with ethanol and treated with lower doses (200 or 400 μg/7cm^2) of β-CA, returned significantly close to that in ethanol-perturbed viable skin at 36 h ($p < 0.05$). Skin sphingosine synthesis inhibition efficacy of 1200 μg β-CA was insignificantly different to that of 600 μg dose of β-CA at 36 h or 48 h ($p < 0.05$). The systemic delivery of percutaneously applied 5-FU across ethanol-perturbed rat skin treated with either 600 μg or 1200 μg β-CA was significantly greater as compared to that obtained after oral administration or after application of lower percutaneous doses of β-CA ($p < 0.05$). Higher C_{max}, MRT, AUC and maintenance of effective plasma concentration of 5-FU for 46 h was achieved by a single topical application of a formulation containing 5-FU and 600 μg β-CA to ethanol-perturbed skin.

1. Introduction

5-Fluorouracil (5-FU) is an antineoplastic agent extensively used in chemotherapy of solid tumors including advanced gastrointestinal and colorectal tumors. 5-FU has a short biological half-life of 15–20 min (Barberi-Heyob et al. 1992). In addition, its oral availability is only 28% due to high presystemic and systemic first-pass metabolism. Due to these reasons, 5-FU is preferably administered parenterally (Blanco et al. 2000). The transdermal route seems to offer an alternative means for systemic delivery of 5-FU. However, 5-FU is a hydrophilic drug, exhibiting a log $K_{o/w}$ of −0.86 (Yamaguchi et al. 1997). Since the intact skin acts as an excellent barrier for percutaneous permeation of polar drug molecules, there is a need to increase the percutaneous permeation of 5-FU.

Ceramides are the main polar lipids and account for up to 50% of the intercellular lipid milieu in the stratum corneum (Elias and Menon 1991). They play a distinct role in maintaining permeability and barrier properties of stratum corneum (Holleran et al. 1991a). Ceramides are synthesized by acetylation of sphingosine with fatty acid in the lowest layers of the epidermis like stratum granulosum (Hedberg et al. 1988). The synthesis of sphingosine involves condensation of palmitoyl CoA with L-serine. This rate-limiting step is catalyzed by the enzyme serine palmitoyl transferase. β-chloroalanine (β-CA), a selective inhibitor of serine palmitoyl transferase enzyme, has been reported to block this rate-limiting step (condensation of palmitoyl CoA with L-serine) in the synthesis of sphingosine, thereby resulting in a decreased ceramide content in the skin (Medlock and Merrill 1988). As a consequence, abnormal lamellar bodies and abnormal extracellular membrane structures appear in the ultrastructure of skin that lead to an abnormal permeability barrier homeostasis (Behne et al. 2000). Solvents that remove sphingolipids (and other neutral lipids) from the stratum corneum are also capable of abrogating the permeability barrier (Grubauer et al. 1989b). This is accompanied by an increased sphingolipid synthesis that starts after 5 h of solvent treatment leading to normalization of the barrier function within 24 h (Holleran et al. 1991a). Specific lipid synthesis inhibitors that delay the recovery of these lipids after solvent perturbation of the skin have been reported to enhance transcutaneous permeation of drugs (Tsai et al. 1996; Babita et al. 2002).

The present study investigates the role of sphingosine (a precursor of ceramide) synthesis inhibition by topical application of β-CA (a selective inhibitor of serine palmitoyl transferase) on the *in vitro* and *in vivo* percutaneous permeation of 5-FU, a polar drug.

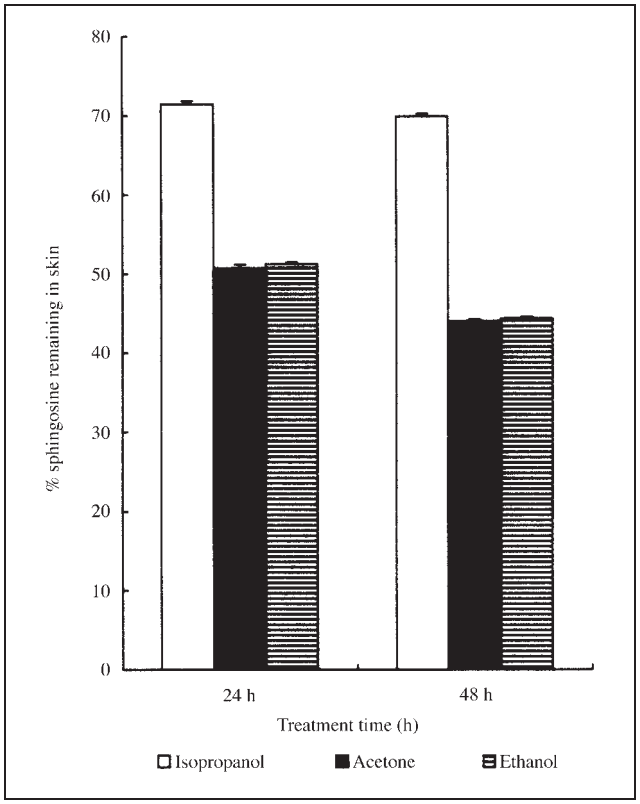

Fig. 1: Sphingosine extraction ability of various solvents in excised rat skin (n = 5).

2. Investigations and results

The intact stratum corneum is impervious to most of the drug molecules. Treatment with solvents perturbs the skin due to extraction of skin lipids. Such solvent perturbation is reported to impair the barrier homeostasis of skin that may be hypothesized to enhance transcutaneous permeation of drug molecules. Three solvents with different dielectric constants were screened for their ability to extract sphingosine from rat skin.

2.1. Screening of solvents for sphingosine extraction ability from skin

It is evident from Fig. 1 that both acetone and ethanol are more effective than isopropanol in extracting sphingosine from excised rat skin. Both acetone and ethanol extracted a significantly greater quantity of sphingosine in 48 h than in 24 h ($p < 0.05$). However, there was no significant difference in % sphingosine remaining in the skin after perturbation with either acetone or ethanol at both 24 h and 48 h ($p < 0.05$).

A significant difference in sphingosine content of normal and ethanol-perturbed viable skin ($p < 0.05$) was found until 24 h of skin treatment. But, the sphingosine content in ethanol-perturbed skin returned to a level insignificantly different from that of normal skin ($p < 0.05$) after 36 h.

2.2. Influence of β-chloroalanine (β-CA) on skin sphingosine content

Application of either dose of β-CA (600 or 1200 μg/7cm^2) resulted in a significant inhibition of sphingosine synthesis up to 48 h ($p < 0.05$) as compared to ethanol-perturbed skin (Fig. 2).

Fig. 3 shows that sphingosine synthesis could be maintained at a significantly low level ($p < 0.05$) up to 48 h by

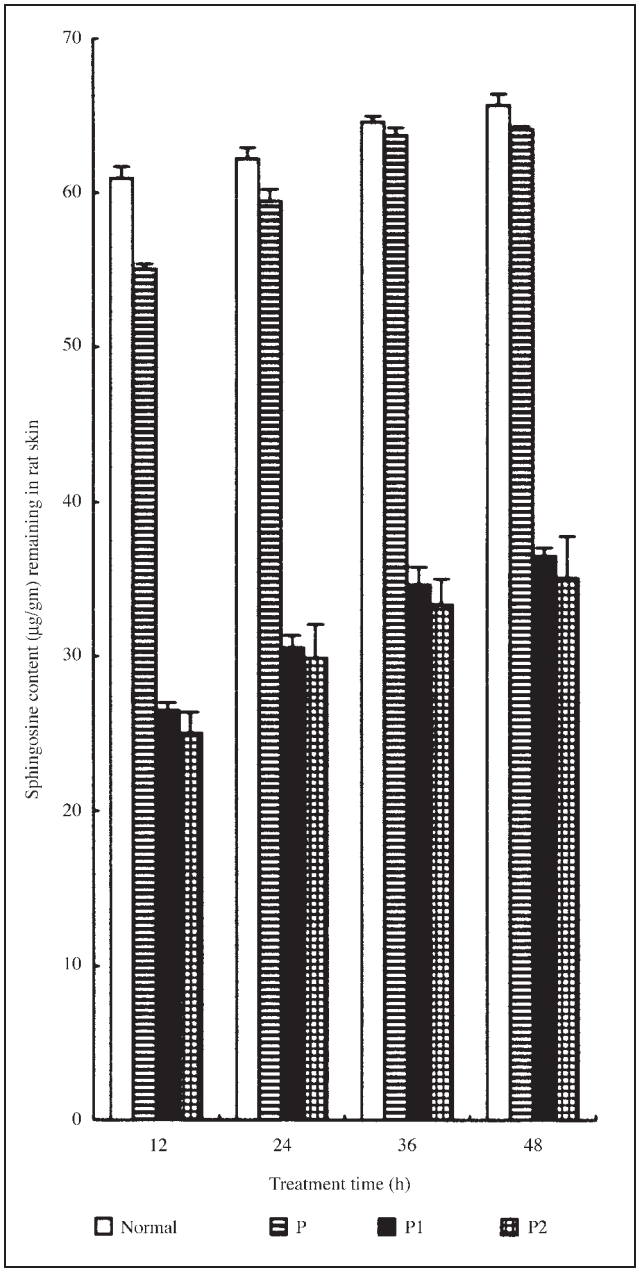

Fig. 2: Sphingosine content (μg/gm) remaining in viable rat skin excised after different treatments (P: skin treated with ethanol for 10 min; P1: skin treated with ethanol + β-CA (600 μg); P2: skin treated with ethanol + β-CA (1200 μg).

application of either dose of β-CA (600 μg or 1200 μg) to ethanol-perturbed viable skin but not by lower doses of β-CA (200 μg or 400 μg). Furthermore, sphingosine synthesis inhibition efficacy of both, 600 and 1200 μg/7cm^2 dose of β-CA at 36 h is not significantly different to that at 48 h ($p < 0.05$). In addition, sphingosine synthesis inhibition efficacy of 1200 μg dose of β-CA was insignificantly different than that of 600 μg dose of β-CA at both 36 h and 48 h ($p < 0.05$).

2.3. Influence of skin treatment on in vitro permeation of 5-FU

The *in vitro* flux of 5-FU across ethanol-perturbed skin treated with β-CA (600 μg/7cm^2) and excised after 48 h was found to be significantly higher than that across ethanol-perturbed skin ($p < 0.05$). The enhancement ratio

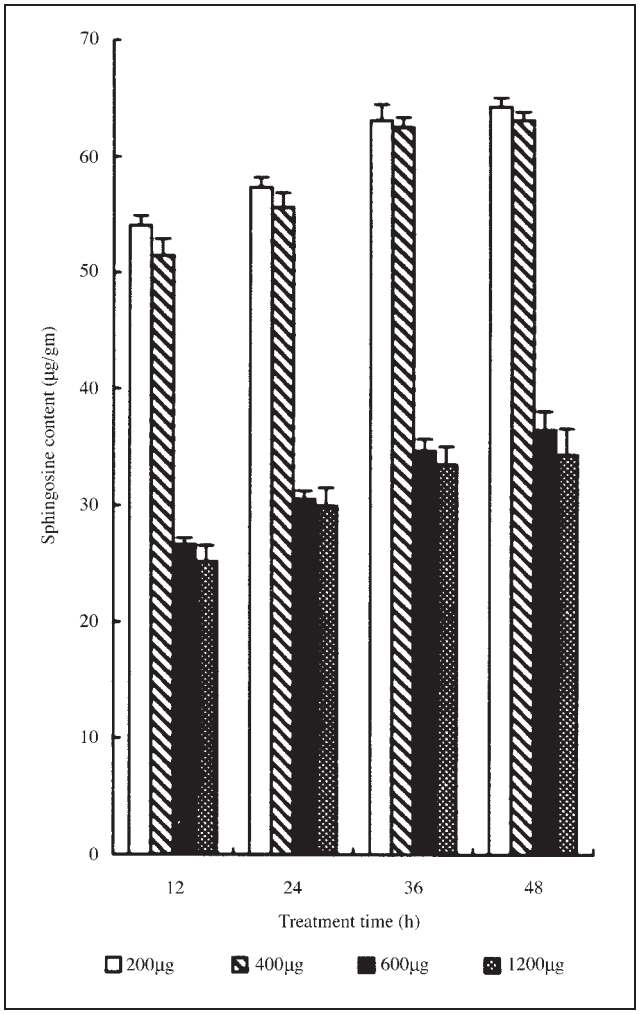

Fig. 3: Sphingosine content (μg/gm) remaining in viable rat skin excised after treatment with different doses of β-CA for various time periods.

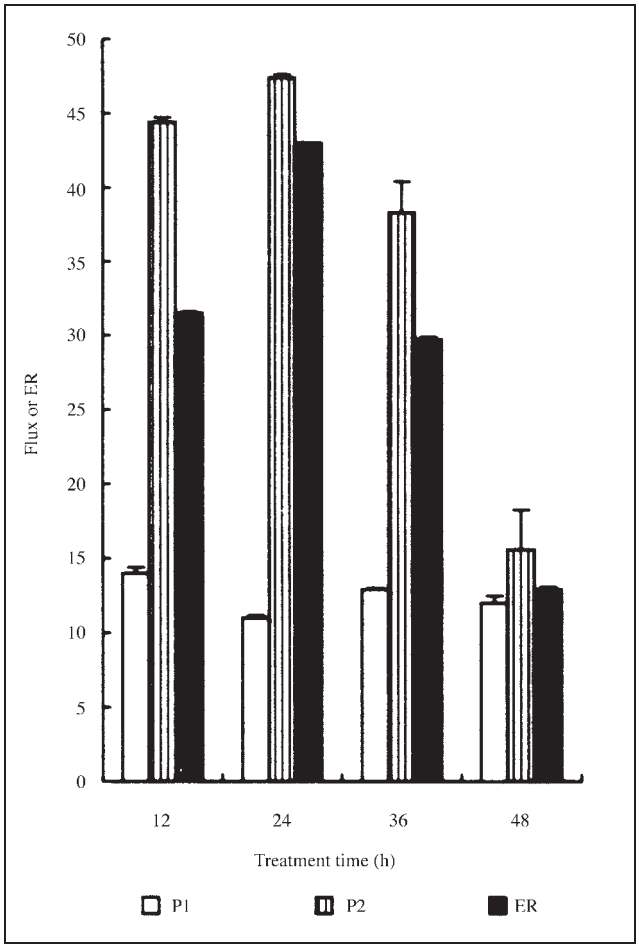

Fig. 4: *In vitro* flux and enhancement ratio (ER) of 5-FU across rat skin excised after ethanol perturbation and treatment with β-CA for different time periods (P1: ethanol-perturbed skin; P2: ethanol-perturbed + β-CA treated skin; actual ER = ER × 0.1).

(ER) of 5-FU across skin excised after ethanol-perturbation and treatment with β-CA for different time periods followed the order: 24 h > 12 h > 36 h > 48 h ($p < 0.05$), indicating an inverse relationship of permeation with sphingosine content of skin (Fig. 4).

2.4. Systemic delivery of percutaneously applied 5-FU

A pharmacokinetic study in rats (Fig. 5) revealed significantly higher systemic delivery of 5-FU ($p < 0.05$) from transdermal formulations containing any dose of β-CA (Gr. II–Gr. V) than that after oral administration (Gr. I).

3. Discussion

Skin treatment with solvents is known to perturb the skin due to their ability to extract skin lipids (Yang *et al.* 1995). The highest quantity of sphingosine remained unextracted in rat stratum corneum (Fig. 1) after treatment with isopropanol (lowest dielectric constant). Ethanol was the most effective in extracting sphingosine from excised rat stratum corneum and hence was used as perturbation solvent in further experiments.

The sphingosine content in ethanol-perturbed skin returned back to a level insignificantly different from that of normal skin ($p < 0.05$) after 36 h of perturbation (Fig. 2). This seems to be due to increased synthesis of sphingosine that is reported to start after 5 h of solvent perturbation in a bid to restore the barrier status of skin (Holleran et al. 1991a). Hence, there is a need to inhibit sphingosine

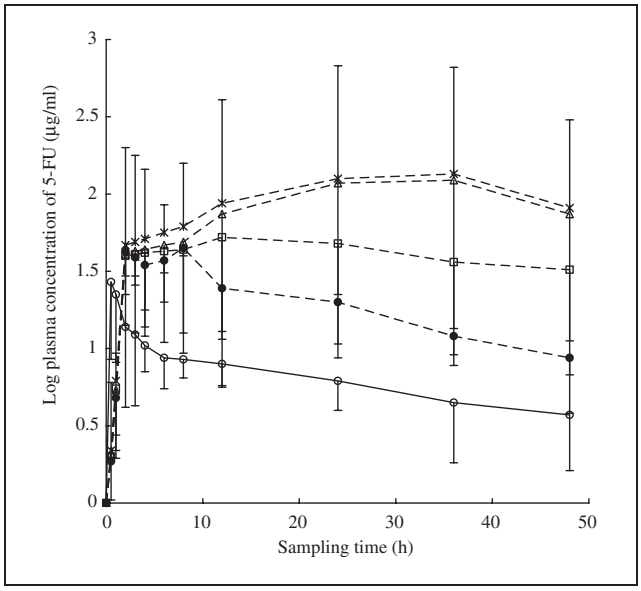

Fig. 5: Systemic delivery of 5-FU in rats after oral administration and percutaneous application of formulations containing different doses of β-CA (for details see Experimental). —○— Gr. I, –●– Gr. II, –□– Gr. III, –△– Gr. IV, –×– Gr. V

Table 1: Pharmacokinetic parameters of 5-FU after oral administration and transdermal application

Group	T_{lag} (h)	$T_{duration}$ (h)	AUC(μg/ml · h)	MRT (h)	C_{max} (μg/ml)	T_{max} (h)	K_a(h)	K_e(h)	$t_{1/2}$ (h)
I	0.5	0.75	338.56 ± 30.07	17.86 ± 0.493	27.06 ± 3.19	0.5	42.48 ± 12.30	6.35 ± 1.51	0.113 ± 0.031
II	2.0	22.0	1009.08 ± 93.83	18.00 ± 0.360	44.83 ± 3.62	8.0	20.85 ± 10.08	1.31 ± 0.19	0.535 ± 0.074
III	2.0	46.0	2017.06 ± 141.51	22.93 ± 0.242	53.20 ± 4.61	12.0	18.27 ± 1.94	1.39 ± 0.31	0.516 ± 0.132
IV	2.0	46.0	4400.00 ± 284.53	27.10 ± 0.064	124.38 ± 9.31	36.0	49.00 ± 11.76	9.55 ± 1.00	0.072 ± 0.007
V	2.0	46.0	4813.87 ± 204.28	26.90 ± 0.069	135.23 ± 4.95	36.0	52.67 ± 4.00	10.17 ± 0.30	0.068 ± 0.002

synthesis in order to maintain its low level for a longer duration. Application of β-CA, a selective inhibitor of serine palmitoyl transferase, to intact skin (unperturbed) is reported not to inhibit the enzyme due to its limited permeability across intact skin (Holleran et al. 1991b). Thus, β-CA was applied to the skin after perturbation with ethanol for inhibiting *de novo* sphingosine biosysnthesis. No significant difference was found at either 36 h or 48 h ($p < 0.05$) in sphingosine synthesis inhibition efficacy (Fig. 3) between the higher doses of β-CA (600 μg or 1200 μg). This indicates that application of a dose of 600 μg/7cm^2 β-CA after ethanol-perturbation is capable of inhibiting the increase in sphingosine synthesis for 36 h.

The *in vitro* flux and enhancement ratio (ER) of 5-FU across skin excised after perturbation with ethanol and treatment with β-CA (600 μg/7cm^2) for various time periods followed the order ($p < 0.05$): 24 h > 12 h > 36 h > 48 h (Fig. 4). It is important to note that although the sphingosine content remaining in skin 24 h after β-CA treatment is greater than that after 12 h, the permeation ER of 5-FU is also greater across skin treated for 24 h as compared to that treated for 12 h. This is due to the fact that the sphingosine content in ethanol-perturbed skin (control) recovers close to the normal level within 24 h of perturbation. This leads to a reduction in the flux of 5-FU across the control skin thus, increasing the enhancement ratio (ratio of flux of 5-FU across perturbed-β-CA treated skin to that across ethanol-perturbed skin) of the skin treated for 24 h. The pharmacokinetic investigation in rats showed an initial slow increase until 8 h followed by a sudden increase in plasma 5-FU concentration after application of transdermal formulations containing 400, 600 or 1200 μg β-CA (Gr. III, Gr. IV and Gr. V, respectively). This seems to be due to the reported time lag in onset of inihibition of sphingosine synthesis by β-CA (Holleran et al. 1991a). However, the plasma 5-FU concentration was observed to continuously decline after 8 h following the application of the formulation containing 200 μg dose of β-CA (Fig. 5). Inhibition of sphingosine synthesis does not alone seem to influence the systemic delivery of 5-FU in the initial phase because the synthesis of both cholesterol and fatty acids is also reported to accelerate after perturbation (Grubauer et al. 1989a; Holleran et al. 1991a). In the later phase, when the sphingosine content rose significantly higher at 36 h as compared to 24 h, the 5-FU plasma concentration did not increase significantly ($p < 0.05$).

An analysis of various pharmacokinetic parameters revealed that 5-FU remained above the effective concentration ($T_{duration}$) only for 0.75 h after oral administration. However, after transdermal application, effective concentrations remained in plasma for 46 h (Table). Accordingly, AUC and MRT increased manyfold after application of transdermal formulations containing β-CA. It is important to note that despite a very high K_a, oral administration showed the lowest AUC, MRT and $T_{duration}$. This can be ascribed to the very high presystemic and systemic first-pass metabolism of 5-FU. The literature does not reveal any specific C_{eff} concentration for 5-FU. A peak concentration of 11.2 μM has been reported after continuous i.v. infusion to cancer patients (Chabner et al. 2001) which is equivalent to 2 μg/ml of 5-FU. It is important to note that C_{max} of 5-FU after application of 1200 μg dose of β-CA was found to be ∼68 times higher than the reported C_{ss} (Chabner et al. 2001).

The results of this investigation indicate that application of β-CA to ethanol-perturbed skin is an effective approach for enhancing percutaneous delivery of 5-FU. However, keeping in view the observed high C_{max}, it seems imperative to study approaches for controlling the rate of percutaneous absorption of 5-FU while using a β-CA-ethanol enhancer system.

4. Experimental

4.1. Materials

D-Sphingosine and β-chloro-L-alanine were purchased from Sigma Chemicals, USA. 5-Fluorouracil and sulphanilamide were generous gift samples from Dabur Research Foundation, India and Panacea Biotech. Ltd., India, respectively. Water, methanol and ethyl acetate were of HPLC grade and were purchased from S.D. Fine Chemicals, India.

Purity of 5-FU was calculated from the ε value of 7070 at 266 nm in 0.1 N hydrochloric acid (Budavari 1996). The purity of gift sample of 5-FU was found to be 100% which is within I.P. limit of 98.5–101.0% (Pharmacopoeia of India 1996).

Albino wistar rats of either sex, weighing between 175–225 g, maintained on a standard laboratory diet and tap water *ad libitum* were used in the present study.

4.2. Optimization of solvent and treatment time for sphingosine extraction ability (in vitro)

Freshly obtained epidermal skin was used for the study (Williams and Barry 1991). The epidermal skin was mounted on a vertical Keshary-Chien diffusion cells with stratum corneum facing the donor compartment and treated with 0.5 ml of solvent (ethanol, 2-propanol or acetone). Receptor fluid (phosphate buffer, pH 7.4 maintained at 37 ± 2 °C) was stirred at 300 rpm and the treated skin was removed from diffusion cell at the end of experiment. Five rats were used for each experiment.

Sphingosine extraction from the treated epidermal skin was done according to the procedure used by Babita et al. (Babita et al. 2002). Spingosine content was dertermined at an excitation wavelength of 340 nm and an emission wavelength of 455 nm, as reported by Sabbadini et al. (Sabbadini et al. 1993).

4.3. Dose dependent influence of β-CA on sphingosine content in viable rat skin

Three patches (7 cm^2) were prepared on dorsal skin surface of rats by shaving with an electric razor. One patch was left unperturbed (control) and the second patch was perturbed by ethanol treatment (0.5 ml for 10 min). The third patch was perturbed by ethanol followed by immediate application of β-CA (200, 400, 600 or 1200 μg) solution prepared in 1.0 ml of a propylene glycol: ethanol mixture (1 : 1). The animals were sacrificed after 12, 24, 36 or 48 h and treated skin patches were excised. The patches were dried to constant weight and subjected to sphingosine content determination. Skin obtained from five rats was used for each treatment period.

4.4. Permeation studies (in vitro)

4.4.1. Dose designing of 5-FU

To achieve an effective plasma concentration (C_p) of 2 μg/ml (Chabner et al. 2001), the amount of 5-FU to be loaded in the donor compartment was calculated by using the formula $C_p \times V_d \times K_e$ (2 μg/ml × 0.25 L/kg × 3.84 h^{-1}). Hence, ∼93 mg of drug was used in the donor compartment to obtain a release rate of 1.920 mg/hr for 48 h.

4.4.2. Permeation of 5-FU across ethanol perturbed β-CA treated excised skin

Two patches were prepared, one on either side of the spinal cord, by shaving with an electric razor. One patch was perturbed by ethanol treatment (0.5 ml, 10 min) and served as control. The other patch received ethanol treatment followed by immediate application of β-CA (600 μg). The animals were sacrificed 12, 24, 36 or 48 h after application of β-CA and epidermal skins obtained from these excised patches were used for studying the *in vitro* permeation of 5-FU. Four groups, one for each time period, were used in the study. Each group consisted of five rats.

4.4.3. In vitro permeation studies of 5-FU

Permeation of 5-FU across excised skin was investigated by employing the Keshary-Chien diffusion cell. The receptor compartment fluid contained phosphate buffer saline I.P. (pH 7.4) and sodium azide (0.05% w/v) as preservative and was stirred at 300 rpm. 5-FU (93 mg) dispersed in propylene glycol (2 ml) was loaded in the donor compartment and sealed with aluminium foil. The amount of 5-FU permeated into the receptor compartment was analyzed spectrophotometerically at 266 nm.

4.5. Pharmacokinetic studies

5-FU (93 mg) dissolved in propylene glycol:ethanol (1 : 1) mixture (1.0 ml) was applied to shaved dorsal skin (7 cm^2) of rats. Treatment given to various groups, each comprising five animals, can be summarized as: Group I, oral administration (15 mg/kg) as aqueous dispersion; Group II, ethanol perturbation (0.5 ml, 10 min.) + β-CA application (200 μg); Group III, ethanol perturbation + β-CA application (400 μg); Group IV, ethanol perturbation + β-CA application (600 μg); Group V, ethanol perturbation + β-CA application (1200 μg). Blood samples (0.5 ml) were withdrawn from the tail vein in heparinized syringes at various intervals. Only one blood sample was withdrawn from each rat and enough satellite groups of rats were kept ready for sampling. Blood samples were centrifuged at 2500 rpm for 10 min at 4 °C. An equal volume of 4% w/v trichloroacetic acid was added to the separated plasma and centrifuged at 2500 rpm for 10 min. The deproteinized plasma was collected and stored at −20 °C until analyzed by HPLC. This deproteinized plasma was mixed with 0.25 M sodium dihydrogen phosphate buffer (0.1 ml) and extracted with ethyl acetate (6.0 ml). After centrifugation at 5000 rpm for 5 min, the supernatant ethyl acetate layer was aspirated and evaporated to dryness. The residue was reconstituted in water and analyzed by HPLC at 270 nm employing 0.1 M sodium acetate buffer (pH 4.0, adjusted with glacial acetic acid) as mobile phase with a flow rate of 1.0 ml/min. The retention times for 5-FU and sulphanilamide (internal standard) were found to be 2.4 and 3.6 min, respectively. The extraction efficiency of the extraction process was 75% and this was taken into account while calculating the actual plasma concentrations of 5-FU.

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