In Vivo Immunomodulatory, Cumulative Skin Irritation, Sensitization and Effect of d-Limonene on Permeation of 6-Mercaptopurine through Transdermal Drug Delivery

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Using skin as a port for systemic drug administration, transdermal drug delivery has expanded greatly over the last two decades. Our aim was to formulate the single layer drug-in-adhesive transdermal patch for 6-mercaptopurine (6-MP). In vitro permeation study was carried out using modified Franz diffusion cell with and without of different concentration of d-limonene in human cadaver skin. In vivo immunomodulatory was carried out in mice, cumulative skin irritation, sensitization and patch adherence study was done in both mice and human subjects. 6-MP flux increased from 43±12.2 μg/cm²h (control) to 162.8±32.2 μg/cm²h (6% w/v d-limonene) data was significant (p<0.05), with decrease in the lag time to 35±9.3 min compared to control of 90±15.3 min. In vivo immunomodulatory effect was shown in the Balb/c mice with 100 μmol/kg/body wt of animal for 5d (one dose/d) of d-limonene. WBC count of 13469 cells/mm peak was observed on 12th day, bone marrow cells of 26.3×10⁶ cells/femur and α-esterase positive cells of 1259±328.4 cells/4000 bone marrow cells. Cumulative skin irritation, sensitisation and patch adherence in animals and human subjects showed no skin irritation and sensitization. Patch adhesion was greater than 90.0% respectively in both human subjects and mice. The percentage of human subjects with adhesive residue was significantly less with scores of zero. d-Limonene proved as good chemical enhancer by increasing in the skin permeability with shortened the lag time. It proved that therapeutic amount of 6-MP can be delivered through transdermal drug delivery.

Key words transdermal; 6-mercaptopurine; d-limonene; immunomodulatory

The rewards for effective transdermal drug delivery (TDD) is large. Drug delivery using skin patches has grown into a multimillion dollar industry, with multiple commercial and clinical successes for a variety of small drugs.1) Patients like patches because of their convenience there is no need to remember to take frequent pills and no pain from hypodermic injections.2) Doctors like patches because of their efficacy. Transdermal delivery avoids the complications of poor absorption, enzymatic degradation, hepatic first pass metabolism associated with oral delivery and eliminates the peaks and valleys of drug concentration in the blood associated with bolus injections.3) Advantages of TDD include non-invasiveness, prolonged drug levels in the blood stream, reduced side effects, improved bioavailability, better patient compliance and easy termination of drug administration.4) Pharmaceutical companies are interested in patches because of their profitability, patches are not only preferred by their customers but can often be used, in effect, to extend the patent life of a drug through new and improved delivery.5) These advantages have motivated the research community to overcome the primary challenge of transdermal delivery. The skin’s outer layer of stratum corneum is an extremely tough barrier that generally only permits entry of small, lipophilic drugs and uniformly excludes large, hydrophilic proteins. Incorporation of chemical penetration enhancers into transdermal applications has been used since the sixties.6,7) Various chemical enhancers, such as ethanol and surfactants, have been used to increase skin permeability to small molecules.8) Single-layer drug-in-adhesive (DIA) method was employed for the formulations, medical-grade pressure-sensitive adhesive (PSA), was used in the development of transdermal patches,9) which is widely used for different drugs such as, physostigmine10) and trimеgestone.11) The PSA fulfills the adhesion-to-skin function and serves as formulation foundation, medical grade PSA adheres strongly to the skin but can be easily removed with little or no trauma and without adhesive residues on skin.12)

6-Mercaptopurine (Fig. 1a, 6-MP) is an antiproliferative drug widely used in the treatment and prophylaxis of acute lymphoblastic leukemia and acute myelogenous leukemia,13) ulcerative colitis,14) polycythemia vera,15) crohn’s disease and in inflammatory bowel disease.16) It has a short elimination half-life of 60±90 min, exhibits high first-pass metabolism after oral administration and variation in the oral bioavailability from 5—80%,17) with side effects such GI irritation and hepatotoxicity, the most important being the hepatic toxicity associated with myelosupression, anorexia, diarrhea and jaundice.18) The delivery of a 6-MP via the transdermal route offers the advantage of prolonged and consistent blood concentration of drug with, reduced adverse effects, the physico-chemical property of 6-MP i.e. low molecular weight, (170.19), pK_a (7.77), non-irritant to skin and lipophilic properties motivates 6-MP as ideal candidate for TDD. In the present study, we aimed at the in vitro permeation of 6-MP through human cadaver skin, primary skin irritation studies in animals, human subjects and study the permeation enhanc-

Fig. 1. (a) 6-Mercaptopurine and (b) d-Limonene

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Materials and Methods

Materials 6-Mercaptopurine and d-limonene was purchased from Sigma-Aldrich, Polyethylene glycol, Sodium hydroxide was purchased from Rankem chemicals (Mumbai, India), DuroTak® 387—2082 (Poly vinyl acrylate) was received as gift sample from National starch and chemical company (Netherlands), Backing membrane CoTran (9726-Ethylene Vinyl acetate (EVA) Film), Release liner 3M Scotch (9742 Release Liner- Fluoropolymer Coated Polyester films) were received as gift sample from (3M St. Paul, U.S.A.) and all the remaining chemicals were of laboratory grade with high quality and used with out further purification. All the human experiments were conducted according to the rules and conditions of the declarations of Helsinki for humans. The Institutional human Ethical Committee (IHEC) approved the study, informed consent was obtained from each volunteer. All the animal studies were conducted according to the guidelines of Institutional animal ethical committee (IAEC) and Govt. of India.

Drug Assay A primary stock solution was prepared with 50 mg of 6-mercaptopurine in 100 ml volumetric flask, 10 ml of above solution was pipetted and made up to 100 ml in volumetric flask (working standard). From the above standard 0.4 to 4 ml was pipetted into a series of 10 ml of volumetric flasks. Finally the volume was made up to 10 ml with 0.1N NaOH to get a concentration range 2 to 20 μg/ml. The absorbance was noted at 321 nm using a UV–visible spectrophotometer (UV1700 Shimadzu, Japan) 0.1N NaOH as blank. The experiment was repeated three times and the average values were taken and standard deviation was calculated. The absorbance at 321 nm showed no interference from water-soluble extracts of the skin.

Preparation of Transdermal Patches in Drug in Adhesive Approach and Drug Loading

The drug-in-adhesive patches were made of flexible backing, the accurate amount of the drug per 10 cm² was loaded based on the pharmaco kinetic parameters, such as volume of distribution (Vd), total body clearance (Cl), and therapeutic plasma concentration (or) minimum effective concentration. Desired flux = clearance (11±4 ml/min/kg) (for 60 kg = 660 ml/min) (minimum effective concentration (MEC) (0.04 μg/ml)/ surface area (10 cm²) = 158.4 μg/cm²·h. The approximate dose per day = Flux (158.4 μg/cm²·h×24 h×surface area (10 cm²) = 38.016—40 mg. The medical grade adhesive i.e. poly vinyl acrylate was mixed in n-hexane after the complete solubility, d-limonene which was dissolved in PEG-400 was slowly added, 6-MP which was dissolved in NaOH was then added and followed by glycerin, the complete preparation was carried with stirring speed of 100 rpm for 30 min. The compositions of the polymeric mixtures used in the preparation of drug-in-adhesive patches is shown (Table 1). The release liner (3M Scotch 9742 Release Liner- Fluoropolymer Coated Polyester film) was held in place on a flat surface, a sample of each polymeric adhesive mixture was placed across the top edge of the release liner, the mixture was casted onto the release liner by drawing a multiple clearance film applicator AR 5315 (Pacific Scientific, Sliver Spring, MD, U.S.A.). The accurate amount of drug per 10 cm² was determined by varying matrix thickness. The wet adhesive film was dried at 75 °C for 30 min. The backing membrane (CoTran 9726-Ethylene Vinyl acetate (EVA) Film) was placed on the top of the coatings. The transdermal films were cut into circular pieces having 3.6 cm diameter corresponding to a 10 cm² surface area, stored in air tight container prior to the day of use. The release liner was removed before in vitro permeation studies and animals experiments.

Preparation of Human Epidermal Membrane

Full thickness abdominal skin samples were obtained from cadaver, department of forensic medicine (Victoria Hospital, Bangalore). Skin samples were sealed in evacuated resealable plastic bags and stored frozen at −20 °C until used (≤24 h). The donor was an Asian with natural death, male with an average age of 45±9 (mean±S.D.) years. The thickness of these specimens was approximately 1.5 cm, which was based on average skin layer thickness including the stratum corneum, the epidermis, dermis and some subcutaneous fat. The subcutaneous fat was trimmed off with a scalpel and sheets of stratum corneum plus attached epidermis were prepared from the whole skin by a heat separation technique. Skin samples trimmed of fatty tissues were immersed in water at 600°C for 2 min, after which the epidermal membranes were teased off the underlying dermis. The membranes were floated on water, cut into square pieces, and then placed dermal side down on filter papers. Then epidermal membranes were washed with distilled water, dried at room temperature (RT) and stored at −20°C in a sealed petridish. The epidermis was allowed to thaw at RT and rehydrated by immersing in water for 1 h before being used. Prior to use, each specimen was checked for its leakage.

Apparatus for Permeation Studies

In vitro passive per-
mation studies were carried out using modified Franz diffusion cells, made up of borosilicate glass, with 2.01 cm² diffusion surface area. The diffusion cells were designed in our laboratory (slight modifications in the Franz diffusion cell). The height was 50±5 mm and internal diameter of 20±1 mm and maximum capacity of 20 ml for receptor compartment, respectively. Square pieces of isolated epidermis were mounted securely between the two halves of the diffusion cell with the dermal side exposed to the receptor compartment. A spring-loaded clamp was used to hold the donor and receptor compartments together. The assembled cells were connected of circulating water bath (Equitron, Mumbai, India) maintained at 37±0.5°C. All the permeation studies were done at physiological pH 7.4. The solutions in receptor compartments were constantly stirred by a star head-shaped magnetic bead (Serwell instruments, Bangalore, India) rotating at 100 rpm.

Evaluation of the Physicochemical Parameters of Transdermal Patches Thickness: The thickness of the patch was measured by vernier caliper (Mitotoyo, Japan) at three different points on the film.

Drug Content: Drug loaded Polymeric films of 1 cm² was taken from three different locations of the 10 cm² film, dissolved in the 2 ml of methanol and sonicated for 10 min, volume was made up to 10 ml using 0.1N NaOH absorbance was measured at 321 nm using UV–visible spectrophotometer (UV 1700 Shimadzu, Japan).

Folding Endurance: The folding endurance value can be defined as ‘the number of times a film can be folded at the same place without breaking’. This test is an index of the brittleness of the film, the lower the folding endurance value, the more brittle the film. It is important test to assess the integrity of the film. The folding endurance was determined according to method reported by Ali and co-worker.21) Film of (2×4 cm²) was folded in center between finger and thumb and then opened. This is called as ‘one folding’. The procedure was repeated until the film showed breakage or cracks in the center. The total number of folding operations was termed as the ‘folding endurance value’.

Flatness: An ideal transdermal patch should possess a smooth surface and should not constrict over time after application on skin. For this a flatness study was done for the patches, using (2×4 cm²) square glass molds, and strips were cut out and measured. Percent flatness is determined as follows. % Flatness=L₁−L₂/L₁×100 which, L₁ and L₂ are the initial length and final lengths of each strip, respectively.

Design of Velcro Protection Jackets The major problem encountered during experiments was how to protect the applied transdermal patch from being licked off, scratched off and/or rubbed off once applied on to the shaved dorsal surface of mice skin. The Velcro jacket was designed with small modifications as reported earlier.22) Velcro jacket was made to cover the entire trunk of the mice and opening at the top, which was designated for application of transdermal patch. A Velcro jacket protected the transdermal patch and allowed for good ventilation, details of the Velcro protection jackets are illustrated in Fig. 3. It serves its purpose quite well and the mice were able to lead a normal life pattern with wearing this jacket.

Cumulative Skin Irritation Study and Sensitisation in Animals and Human Subjects Sample size: 30 subjects, exclusion criteria: dermatologic disease that might interfere with the evaluation of test site reaction, duration of study: 22 d. Study design: a randomized, controlled, repeat patch test study that compares the test patch to the innovator patch. Placebo (transdermal patch without 6-MP) transdermal patches of DIA technique were applied on each subject. Patches were applied for 23 h (plus or minus 1 h) daily for 21 d to the same skin site. At each patch removal, the site was evaluated for reaction and the patch reapplied. Application of a test patch was discontinued at site if predefined serious reactions occur at the site of repeated applications. Application at a different site was subsequently initiated. All applications were initiated on the right side of forearm for all the subjects, the patches were applied on a clean, dry area of the forearm. All subjects were examined for signs and symptoms of skin irritation, and patch adherence was evaluated immediately prior to the removal of the patches. Evaluations: Scoring of skin reactions and patch adherence were performed by a trained and blinded observer at each patch removal, using an appropriate scale as described below. Skin irritation was planned and done according to United States Food and Drug Administration (USFDA) shown in Table 2.

Determination of the Effect of L-Limonene on the Hematological Parameters Two groups of Balb/c mice (6 nos/group) were used in this study. Group 1 animals were treated with five doses for 5 d (one dose per day) of 100 μmol/kg body wt/dose/animal through the intraperitoneal injection, group II was kept as untreated control. Blood was collected from tail vein and parameters such as total WBC count (Haemocytometer) differential count (Leishman’s stain) and body weight was recorded prior to the drug treatment and every 3rd day for one month.

Determination of the Effect of L-Limonene on the Bone Marrow Cellularity and Ω-Esterase Positive Cells Balb/c mice (6 nos/group) were treated with five doses for 5 d (one dose per day) of l-limonene of 100 μmol/kg body wt/dose/animal, the animals were sacrificed 24 h after the last dose of drug treatment. Bone marrow cells from femur were collected and made into a single cell suspension and the number of the cells were determined using a haemocytometer. The Ω-esterase positive cells were determined by azo dye coupling method. A smear of bone marrow cells from the above the preparation was made on the clean glass slides, air dried, stained with Ω-naphthyl acetate and pararosaniline hydrochloride and counter stained with heamatoxilene. The number of Ω-esterase positive cells were expressed out of 4000 cells.

Statistical Analysis The results are expressed as mean ± S.D. statistical evaluation of the data was done using Student’s t-test using Sigma Stat 3.0®.

RESULTS AND DISCUSSION

Effect of L-Limonene on in Vitro Skin Permeation of 6-Mercaptopurine The flux data is shown in (Table 2) with and with out l-limonene. l-Limonene concentration was varied from (2 to 10%) with fixed dose of 6-MP of 40 mg in the patch. In order to elucidate the action of l-limonene on drug permeation, abdominal skin from a single donor (45 years old) was used so as to eliminate inter- and intra-individual differences. l-Limonene is a hydrocarbon terpene
(lipophilicity indicated by a log $P$ is 4.53 ± 0.23), most of the studies suggest that hydrophilic terpenes (alcohol, ketone and oxide terpenes) are more effective in enhancing the permeation of hydrophilic drugs, whereas hydrocarbon terpenes ($d$-limonene and cymene) are more effective in promoting percutaneous permeation of lipophilic drugs.23) In the present study the flux was achieved with $d$-limonene concentration of 6% w/v in the formulation D (desired flux 158.4 mg/cm²·h and obtained flux 162.8 ± 24.36 mg/cm²·h), with decrease in the lag time to 35 ± 9.3 min and control (without $d$-limonene) of 90 ± 15.3 min to achieve the steady state concentration (data was statistically significant $p<0.05$). It has also been reported that terpenes increase the drug percutaneous permeation mainly by disrupting the intercellular packing of stratum corneum lipids.24,25) Here, the results have proved hydrocarbon $d$-limonene is a potential chemical enhancer and more effective for 6-MP TDD. The effectiveness of hydrocarbon $d$-limonene has also been demonstrated for the other lipophilic drugs such as ketoprofen, indomethacin and estradiol.26) The present study shows that $d$-limonene has low skin irritancy and sensitization, it has also proved that when administered with a pretreatment method, it allows a reversible change in the skin structure.27) The enhancement of 6-MP permeation through human cadaver skin by $d$-limonene suggests that there are possible multiple mechanisms that could have resulted in a more permeable intercellular pathway for 6-MP. They include an increased 6-MP solubility in skin, partial extraction of SC lipids,28) phase separation within the SC lipid lamellae23) and $d$-limonene-PG synergy.29) Ohara et al.30) have reported that $d$-limonene exhibits a convex curvature, barrier function of the non-polar pathway in the skin with $d$-limonene pretreatment. According to lipid partitioning theory the terpenes did not exert their action by increasing partitioning of the drug into the membranes as illustrated by stratum corneum: water partitioning studies. The penetration enhancers increased drug diffusivity through the membranes, an effect which correlated empirically with the enhancer activities. The principal mode of action of these accelerants may be described by the lipid-protein-partitioning theory: the terpenes interact with intercellular stratum corneum lipids to increase diffusivity, and the accelerant effects were not due to partitioning phenomena. Keratin interaction was assumed negligible.

**Physicochemical Parameter Evaluation of Transdermal Patch** Patches were made with a drug loading of 3.9 ± 0.12 mg/cm². More over knowing the influence of the amount of penetration enhancer in determining the flux, the drug was loaded in adhesive matrix (40 mg/patch) from which the MEC and flux of 6-MP can be achieved with $d$-limonene 6% w/v (acts as penetration enhancer) through human skin. The drug content were found to be 3.8 ± 0.32 mg ($n=3$). The flatness study indicated a 100% flatness of patch, and it showed that it will remain as such without constriction for a long period of time, 100% flatness means that there is no constriction. The thickness of the patches was in the average of the three values and it was 200 ± 0.32 mm ($n=3$). The folding endurance test, an index of the brittleness of the film, showed more than 500 foldings ($n=3$), the films were found to be more elegant, smooth, transparent and flexible which can be attributed to the plasticizer. At the time of skin scoring for cumulative skin irritation and sensitization, velcro protection jacket was temporarily detached, for the treatment, with finite dose formulations were applied onto skin of the dorsal surface of animal (i.e., the treatment would start
on day after the tumor inoculation and continue for ten consecutive days).

**Effect of d-Limonene on the Hematological Parameters**

Immunomodulator are materials, which can modify the body’s defense mechanism either by enhancing or by controlling immune response. Total WBC count in Balb/c mice was increased with $13469 \text{cells/mm}^3$ and the peak was observed on 12th day (Fig. 2). There was no appreciable change in the differential count, and body weight. *d*-Limonene is a naturally occurring monoterpane has chemopreventive and chemotherapeutic activity against many rodent solid tumor types.\(^{31}\) The chemopreventive activity of *d*-limonene during initiation can be attributed to the induction of phase I and phase II enzymes with resulting carcinogen detoxification.\(^{32}\)

**Effect of d-Limonene on Bone Marrow Cellularity and α-Esterase Positive Cells**

The effect of *d*-limonene on the bone marrow cellularity and α-esterase positive cells, showed significant changes, the *d*-limonene treated groups showed increase in the bone marrow cells compared to control animals ($15.4 \times 10^6 \text{cells/femur}$), in *d*-limonene treated it was $26.3 \times 10^6 \text{cells/femur}$ respectively. The number of α-esterase positive cells was found to be increased significantly in the animals with $1520 \pm 328.4 \text{cells/4000 bone marrow cells}$, with control animals $1259 \pm 136.5 \text{cells/4000 bone marrow cells}$. Bone marrow serves as the major source of all blood cells including lymphocytes. Bone marrow is not only the source of all blood cell classes but also provide micro-environment for the antigen independent differentiation of B cells. Administration of *d*-limonene increased the bone marrow cellularity and α-esterase positive cell indicating its effect on the stem cell proliferation. Both the innate and adoptive immunity depends upon the activity of WBCs. Innate immunity largely involves granulocytes and macrophages. Adoptive immunity depends upon the lymphocytes, which provide life long immunity that can follow exposure to diverse antigens.

**Cumulative Skin Irritation Study, Sensitisation and Adherence in Animals and Human Subjects**

At each evaluation during the study, the sites treated with patches exhibited less irritation. Scores of 1—2 was observed among the 2 subjects. Subjects showed the score=0, confirming that there was no skin irritation and sensitization. Patch adhesion was greater than 90.0% with scores of scores 0 and 1 in both human subjects and animals. The score of one was observed in 2 mice of which designated as patch adhered of 75—90% in mice. There was no sensitization or local skin reactions such as pruritus, scaling and oedema. Patient compliance and effective patch adhesion confirmed the efficacy to the transdermal patch adherence. In conclusion *d*-limonene proved as a good chemical enhancer and by increase the skin permeability shortened the lag time and proved to deliver the therapeutic amount of 6-MP delivery by *in vitro*. Additionally *d*-limonene demonstrated immunomodulatory activity; further studies need to be conducted in order to elucidate the exact mechanism of action of this naturally occurring monoterpane, *d*-limonene in stimulation of the immune system. Velcro protection jacket served our purpose quite well and the mice were able to lead a normal life pattern wearing this jacket.

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