HINNAO™
TECHNOLOGY

HINNAO™ High Stability Liposomal Vitamin D3 with HPLC Analysis

Analysis Conducted By CC Research (P) Ltd. for HINNAO™

20 White Church Lane, London, E1 7QR

HIGH STABILITY LIPOSOMAL VITAMIN D3 DIFFUSION STUDIES

Transdermal drug delivery:

An Introduction

The idea of delivering drugs through skin is old, as the use is reported back in16th century B.C.Today the transdermal drug delivery is well accepted for delivering drug to systemic circulation. Until recently, the use of transdermal patches for pharmaceuticals has been limited because only a few drugs have proven effective delivered through the skin typically cardiac drugs such as nitroglycerin and hormones such as estrogen.

Definition: Transdermal therapeutic systems are defined as self-contained discrete dosage forms which, when applied to the intact skin, deliver the drug(s), through the skin, at controlled rate to the systemic circulation.

The first Transdermal drug delivery (TDD) system, Transderm-Scop developed in 1980, contained the drug Scopolamine for treatment of motion sickness. The Transdermal device is a membrane-moderated system. The membrane in this system is a microporous polypropylene film. The drug reservoir is a solution of the drug in a mixture of mineral oil and polyisobutylene.

This study release is maintained over a one-day period.

Non-medicated patch markets include thermal and cold patches, nutrient patches, skin care patches (a category that consists of two major sub-categories therapeutic and cosmetic), aroma patches, weight loss patches, and patches that measure sunlight exposure.

Transdermal drug delivery has many advantages over conventional drug delivery and can be discussed as follows.

Advantages

1. They can avoid gastrointestinal drug absorption difficulties caused by gastrointestinal pH, enzymatic activity, and drug interactions with food, drink, and other orally administered drugs.
2. They can substitute for oral administration of medication when that route is unsuitable, as with vomiting and diarrhea.

3. They avoid the first-pass effect , that is, the initial pass of drug substance through the systemic and portal circulation following gastrointestinal absorption, possibly avoiding the deactivation by digestive and liver enzymes.
4. They are noninvasive, avoiding the inconvenience of parenteral therapy.
5. They provide extended therapy with a single application, improving compliance over other dosage forms requiring more frequent dose administration.
6. The activity of a drugs having s short half-life is extended through the reservoir of drug in the therapeutic delivery system and its controlled release.
7. Drug therapy may be terminated rapidly by removal of the application from the surface of the skin.
8. They are easily and rapidly identified in emergencies (e.g., unresponsive, unconscious, or comatose patient) because of their physical presence, features, and identifying markings.
9. They are used for drugs with narrow therapeutic window.

At the same time transdermal drug delivery has few disadvantages that are limiting the use of transdermal delivery.

Disadvantages

1. Only relatively potent drugs are suitable candidates for transdermal delivery because of the natural limits of drug entry imposed by the skin's impermeability.
2. Some patients develop contact dermatitis at the site of application from one or more of the system components, necessitating discontinuation.
3. The delivery system cannot be used for drugs requiring high blood levels.
4. The use of transdermal delivery may be uneconomic.

For better understanding of transdermal drug delivery, the structure of skin should be briefly discussed along with penetration through skin and permeation pathways.

Advances in Transdermal Permeation Enhancement

To achieve and to maintain a plasma drug concentration above the minimum therapeutic drug level, the barrier properties of the skin must be overcome before the effective transdermal controlled delivery of drugs can be successfully accomplished, the following

approaches have been shown to be potentially promising for accomplishing the goals of reducing skin barrier properties and enhancing the transdermal permeation of drugs.

Generally, methods to enhance transdermal drug permeation can be grouped into two categories: Chemical methods and Physical methods.

Diffusion: Movement of a fluid from an area of higher concentration to an area of lower concentration. Diffusion is a result of the kinetic properties of particles of matter. The particles will mix until they are evenly distributed.

Diffusion is, by definition, the random movement of molecules through a domain driven by a concentration gradient, from high concentration to low concentration. In vitro diffusion is generally passive diffusion of a permanent from a vehicle in the donor chamber, through an artificial or biological membrane into a receptor fluid in a receptor chamber, disregarding delivery systems such as iontophoresis and microneedles. The permeant is the molecular species moving through or into the tissue/membrane. Permeation is the movement of the permeant through the membrane that encompasses first partitioning the membrane and then diffusion through the membrane. Penetration can occur into the membrane without necessarily diffusing, or passing through, the membrane. Flux is the amount of permeant crossing a membrane per unit area into the circulating system per unit time, and for in vitro permeation this “system” is the receptor chamber, expressed in units of mass/area/time. Similarly, accumulation is the amount of permeant crossing a membrane within a certain time, expressed in units of mass/area. Diffusivity is a property of the permeant and is a measure of how easily it penetrates a specific membrane expressed in units of area/time. The permeability coefficient (Kp) describes the rate of permeant penetration per unit concentration expressed in distance/time.

Franz’s diffusion cell:

Franz showed an excellent correlation between in vitro and in vivo studies. Majority of In-vitro experiments are conducted in animal skin i.e. hairless mouse, guinea pig, rabbit etc. Although these exist a number of similarities there is as yet no animal skin that completely mimics the penetration characterization of human skin.

In 1975 Franz developed a static diffusion cell which is now one of the most commonly used in vitro systems in the research of skin penetration. The system has a simple design and is inexpensive to use. Human as well as animal skin can be mounted on the metal grid which divides the donor chamber and the receptor chamber. The skin is set placing the dermis in contact with the receptor fluid below. The skin can be either full-thickness or split-thickness skin. The skin thickness will affect the experimental results as elaborated under Flow-through system.

The receptor chamber of the cell is placed in circulation water in a water bath with a temperature of 37 ºC keeping the temperature at the skin surface at 32° to imitate a real life skin condition as much as possible. The receptor fluid is kept homogenous in concentration and in temperature by a magnetic stirring bar. The fluid in the receptor chamber is manually sampled at predefined time intervals. Any type and any amount of vehicle (that will fit into the donor chamber) may be applied to the skin.

The components of the diffusion cell are shown below

Donor Compartment:

- Easy access to deliver the penetrant to the skin.
- Stirred where possible.
- Temperature controlled (32o ¬ C ± 1o C) Control of evaporation for vehicles and penetrants.

Membrane:

- For the study of penetration kinetics, only human skin should be used.
- For vehicle/device release studies other barrier may be used.
- The skin sample should contain both stratum corneum and viable epidermis.
- A molecule of known penetration kinetics should used prior to the test molecule, to assess barrier function. Where applicable metabolic viability of epidermis may be assessed

Receptor Compartment:

- Either, flow – through or static
- Temperature controller (32o ¬ C ± 1o C) Sufficient volume to maintain infinite sink conditions.
- Stirred without obvious formations of boundary layers.

Receptor Fluid:

- Should not compromise barrier function.
- Should be of favorable partitioning.
- Capable of maintaining epidermal viability wherever necessary.
- Must be contained once collected.

When testing different substances it is important to be aware of the solubility of the substance. The solubility of a substance influences the sink capacity and is therefore of great importance when it comes to choosing the right sampling frequency and receptor chamber dimension. The size of the receptor chamber determines when the receptor fluid achieves a certain degree of saturation.

The barrier integrity of the skin can be evaluated by capacitance measurement. This value indicates the ability of the skin to separate electrical charge. Skin samples with a high capacitance are unable to act as capacitors, which mean that the skin is damaged. The measurements are carried out at the beginning and at the end of the study to give an accurate evaluation of the skin barrier.

Preparation of pbs buffer solution:

Measure a volume of 800 ml of ddH 2 O with a graduated cylinder and transfer to an Erlenmeyer flask. Add a magnetic stir bar to the Erlenmeyer flask and place the flask on a magnetic stir plate

Adjust the speed of the magnetic stir bar so that oxygen is not introduced into the solution while it is rapidly mixed.

Transfer to the flask:

8g of NaCl, 0.2 g KCl | 1.44 g of Na 2 HPO 4 | 0.25 g of KH 2 PO 4

Allow the solutes to dissolve for 3 to 5 min. Ensure that there are no remaining particles of undissolved salts in the solution before adjusting the pH. If particles are present, continue stirring vigorously. Reduce the speed of the magnetic stir bar so that the solution is gently mixing.

Ensure that the pH meter has been properly calibrated and rinse the pH probe with double distilled H 2 O. Remove the excess water from the probe tip (without touching the probe tip) with a clean paper towel. Place the pH probe into the solution. Slowly add 1 M HCl dropwise with a transfer pipette and allow the HCl to fully dissolve into the solution. Stop stirring the solution.

Measure the pH with the pH meter. Repeat until the pH of the solution is 7.4. Pour the solution into a fresh graduated cylinder and adjust the final volume to 1 liter with double distilled H 2 O. Store the PBS solution at room temperature. The PBS Solution is sterile; when using the PBS Solution, ensure that sterile techniques are employed.

Drug Diffusion Study:

The drug diffusion studies through membrane experiments were conducted by using vertical type diffusion cell (Franz type) having receptor compartment 60ml volume with 10.18cm 2 area. The receptor compartment was filled 60 ml of phosphate buffer pH 7.4; the activated dialysis membrane was mounted on the flange of the diffusion cell receptor compartment. The prepared Transdermal patch with surface area 10.18cm 2 placed on center of membrane, the donor compartment was then placed in position and the two valves of the cell clamped together. The whole assembly was kept on a magnetic stirrer and solution in the receptor compartment was constantly and continuously stirred using a magnetic bead and at 37 ± 1 o C maintained.

Permeation study of gel through the sheep mucosal membrane was performed using Franz diffusion cell and membrane assembly , at 37°C ± 1°C and 50 rpm. This temperature and rpm was maintained by magnetic stirrer. The tissue was stored in Krebs buffer at 4°C upon collection.

After the buccal membrane was equilibrated for 30 min with the buffer solution between both the chambers, the receiver chamber was filled with fresh buffer solution (pH 7.4), and the donor chamber was charged with 5mL (1mg/mL) of drug solution. Aliquots (5mL) were collected at predetermined time inter wells up to 45 min and the amount of drug permeated through the mucosa was then determined by measuring the values at 215 nm using HPLC method. The medium of the same volume (5 mL), which was pre-warmed at 37°C, was then replaced into the receiver chamber .

The experiments were performed and values were used to calculate flux (J) and permeability coefficient (P).

$$J = \frac{dQ/dt}{A\ t}$$

$$P = \frac{dQ/dt}{\Delta CA}$$

Where, **J** is Flux (mg.hrs-1cm-2)
P is permeability coefficient (cm/h)
dQ/dt is the slope obtained from the steady state portion of the curve
ΔC is the concentration difference across the mucosa and
A the area of diffusion (cm2)

CALIBRATION CURVE FOR VITAMIN D3 USING HPLC:

CHROMATOGRAPHIC CONDITIONS:

Column	:	INERTSIL ODS C 18 150*4.6mm,5 µ particle Size.
Mobile Phase	:	Acetonitrile: water (70:30 v/v).
Flow rate	:	0.6 ml/min
Injection Volume	:	10 µl
Wavelength	:	251 nm
Temperature	:	5 o C ± 2
Runtime	:	6 mins
Rt	:	2.3 mins.

Preparation of Linearity Solutions:

Weigh Accurately 10 mg of Vitamin D3 and take it into a 10 ml of volumetric flask to this add 3 ml of diluent and make up the solution upto the mark with same solution.(1000 µg/ml) From the above stock solution take 0.1 ml into a 10 ml of volumetric flask make up the solution with diluent upto the mark. (10 µg/ml).

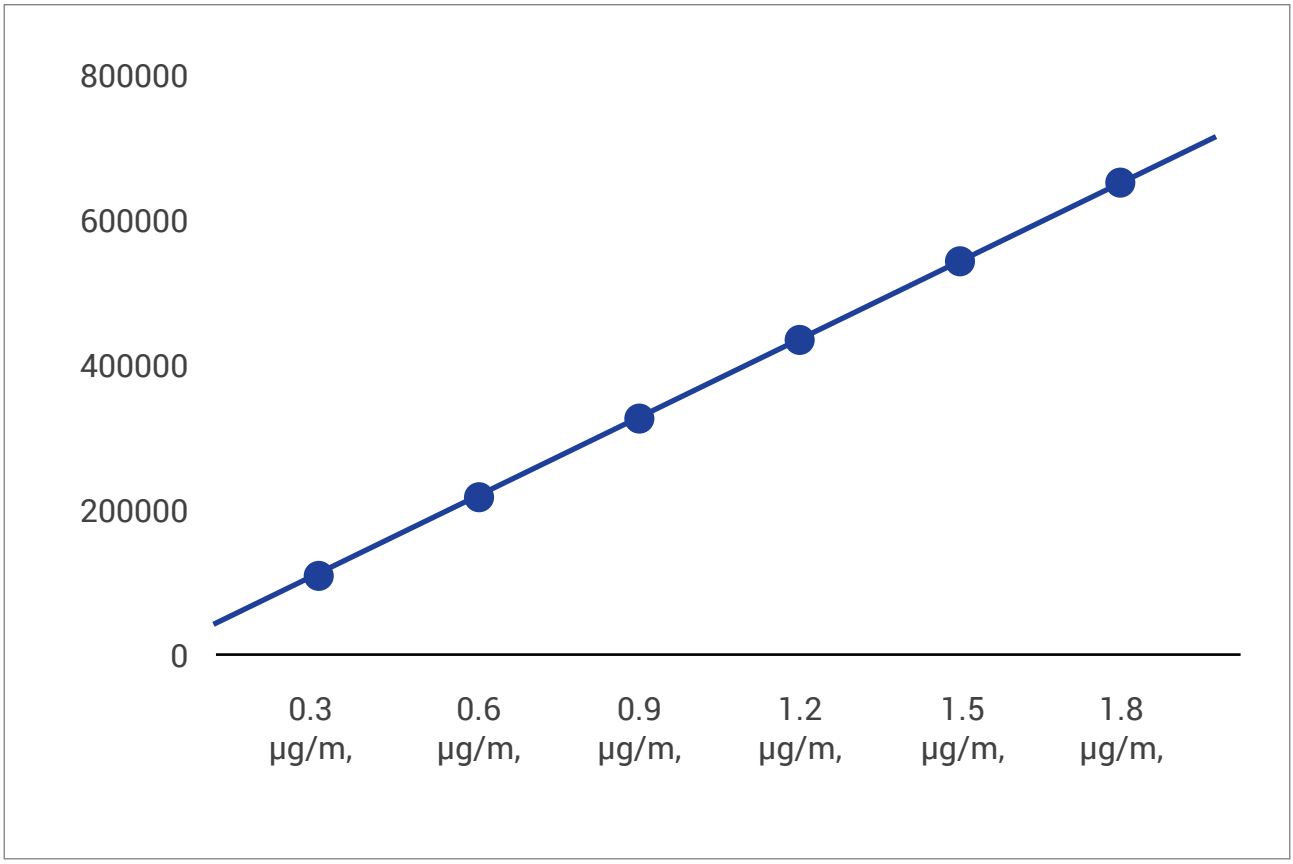
From the Above solution take a series of solutions 0.3ml,0.6ml,0.9ml,1.2ml,1.5ml,1.8 ml into different 10 ml volumetric flasks and make up the solution with diluent to get a concentration range of 0.3 µg/ml to 1.8 µg/ml.

(*mobile phase is used as a Diluent)

Results :

S.no	Concentration	Area
1	0.3 µg/ml	101234
2	0.6µg/ml	202468
3	0.9µg/ml	303699
4	1.2 µg/ml	404489
5	1.5 µg/ml	505366
6	1.8 µg/ml	606159

Calibration curve for HINNAO™ Vitamin D3:



NOTE: The diffused samples are injected into HPLC by maintaining above chromatographic conditions, from the data obtained area of the peak were calculated and the % drug release.

Time(sec)	% Drug diffused F1	% Drug diffused of F2	% Drug diffused F3
0	0.000	0.000	0.000
10	10.64865561	10.59874226	10.69856895
20	21.00210943	21.13758851	21.75080961
30	23.4906462	23.60473385	24.31065115
40	31.99017579	33.05974746	33.82983907
50	36.98151027	37.80151523	37.93699431
60	40.23300817	41.3168408	41.61632087
70	56.78997772	57.72407031	58.08059421
80	62.57279525	61.97383511	62.78670958
90	64.07732607	64.71906908	65.11124536
120	65.44637782	65.88846744	66.00968557
150	66.47316663	67.40725922	67.6069126
180	66.52307997	67.69960881	67.67821738
210	68.65509284	69.82449121	70.38066848
600	69.46796732	70.25231988	70.14536271
900	69.70327309	70.54466947	70.51614756
1500	76.49148799	77.98175786	78.03167121
2700	77.70366923	78.43097796	78.52367418

Permeability coefficient calculation (cm/h):

Time (secs)	Time (hrs)	F1	F2	F3
10	0.002	0.658769562	0.655269542	0.662273988
20	0.005	1.494239235	1.506836891	1.564483384
30	0.008	1.733825028	1.74523946	1.816747899
40	0.011	2.707123751	2.850185819	2.956564077
50	0.013	3.424130617	3.555038792	3.577064032
60	0.016	3.968570931	4.166212194	4.222384962
70	0.019	8.394514109	8.779529015	8.932423078
80	0.022	11.19945888	10.85519755	11.32608195
90	0.025	12.13438068	12.56701785	12.84239039
120	0.033	13.08467917	13.41459677	13.50718213
150	0.041	13.86998283	14.64639633	14.82074928
180	0.05	13.90990808	14.90276009	14.88377406
210	0.05	15.78961428	16.98933251	17.61011588
600	0.16	16.60908381	17.46375454	17.34323224
900	0.25	16.85854912	17.79994341	17.76670037
1500	0.41	27.79704488	31.85261579	32.00592836
2700	0.75	31.02123058	33.28005338	33.58851278

Conclusion: Percentage drug diffusion results appear to be increased over time tested (10 Sec- 2700 Sec). These results suggest increased % drug diffusion using VitD3 nanoparticles in this batch. However, these results of VitD3 require to be continued beyond time points above 2700 Sec. to establish peak and stationary phase.

QC Reviewed and Approved By: FINAL CALCULATED VIT D3 CALCULATION CROSS CHECKED BY DR.K.SOMESHWAR