Project Report

In vitro penetration studies of Fuse Science Formulations through human skin

Prepared for: Jeanne Hebert
Fuse Science Inc.
6135 NW 167th Street, Suite E-21
Miami Lakes, FL 33015

Prepared by: Genepharm, Inc.
1237 Midas Way
Sunnyvale, CA 94085

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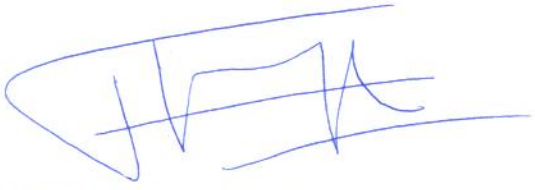
GENEPHARM
1237 Midas Way Sunnyvale, CA 94085 T 408.773.0106 F 408.773.1018 info@genepharminc.com www.genepharminc.com

Approval

Study Director



Hanafi Tanojo, Ph.D.
Vice President, Research and Development
Genepharm Inc.

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1. SUMMARY

Two Fuse Science formulations were tested in an *in vitro* penetration study through human epidermis. The first formulation tested was with “enhanced encapsulation”. It was tested with and without occlusion. When the enhanced encapsulation formula was tested with occlusion, parafilm was applied and it was kept under occlusion during the period of the study. The second formulation tested was with “reduced encapsulation”. Radiolabel [8-^{14}C]-caffeine was incorporated in each formulations prior to the application and used to analyze the samples. The penetration profile was assessed by collecting the receptor fluid samples at various time-points from the chamber below the epidermis. The selected time-points were 3 minutes, 10 minutes, 1 hour and 2 hours after the application of the formulations on the surface of the epidermal membrane. The results showed that Fuse Science Enhanced Encapsulation Formula delivered the most caffeine at all time-points. Occlusion of parafilm decreased the amount of caffeine penetrating across the epidermis. Fuse Science Reduced Encapsulation Formula yielded the least caffeine at the end of study period.

2. INTRODUCTION

Fuse Science formulations containing the same high dose of caffeine were to be tested in an *in vitro* penetration study through human epidermis. One formulation is with “Enhanced Encapsulation Formula”, whereas the other is with “Reduced Encapsulation Formula”.

The *in vitro* skin penetration assay has been utilized to effectively screen and investigate the formulations with a good correlation to the *in vivo* conditions. In particular, excised human skin or its portion, such as epidermis, is the most selected as the membrane to simulate topical drug delivery.

3. OBJECTIVE

The project aims to assess the penetration profiles of caffeine from Fuse Science formulations, with different encapsulation formulas; and in the case of the Enhanced Encapsulation Formula, with and without occlusion.

4. MATERIALS

4.1 Test Articles

The test articles were received from the Sponsor and identified as follows:

- Radioactive material:

- 2.5 mL of [8-^{14}C]-caffeine in ethanol solution under argon (Catalog# MC-499, Lot No. 195-132-0521-A-20100630-MW, Specific activity: 52.1 mCi/mmol; concentration 0.1 mCi/mL; 375.9 µg/ml)

was received on May 8, 2012 from Moravek Biochemicals Inc. 577 Mercury Lane, Brea, CA 92821; stored in a -20°C refrigerator upon arrival.

- Radiolabeled formulations:
Two formulations

- Fuse Science Enhanced Encapsulation Formula
- Fuse Science Reduced Encapsulation Formula

were delivered on May 9, 2012 from CURE Pharmaceutical, 1620 Beacon Place, Oxnard, CA 93033. Each formulation was mixed with radioactive caffeine which was dried overnight from 1 mL solution per 2 mL formulation. Therefore, each formulation contains radioactive caffeine of 50 µCi/mL. The mixture were stored at a 4°C refrigerator after preparation.

The Sponsor was responsible for any necessary evaluations related to identity, strength, purity, composition, stability and method of synthesis of the test material.

4.2 Excipients and Reagents

All excipients and reagents were obtained from vendors with noted quality and analytical reports.

4.3 Skin Donor

The skin used in the study was obtained from abdominal surgery on a single human donor, who gave a written consent.

5. EXPERIMENTAL PROCEDURES

5.1 Study Design

An open label, within donor, comparison study of topical formulation of the test article were performed. The formulations were tested on epidermal membrane using a finite dose application. The receptor solution was continuously stirred, bathing the lower surface of the membrane. At certain time-points, receptor fluid samples were collected by draining the receptor chamber. The chamber was fully replenished with fresh receptor fluid. The amount of radioactive caffeine was analyzed from the receptor fluid samples.

5.2 Topical Application

Each formulation was applied to the surface of the epidermal membranes at a target dose of 15.6 µL/cm^2 (= 10 µL/cell).

Test article/conditions:

- Fuse Science Enhanced Encapsulation Formula (no occlusion).
- Fuse Science Enhanced Encapsulation Formula; epidermis surface occlusion with parafilm.
- Fuse Science Reduced Encapsulation Formula (no occlusion).

Each test article/condition was tested on six replicates of epidermal membranes from one human donor.

5.3 Transmembrane Diffusion Study

The *in-vitro* delivery study was performed using Franz diffusion cells. The protocol for this experimental procedure is attached in **Appendix A**. Briefly, the diffusion cells were mounted on a holder with magnetic stirrer. The water jacket compartment of each cell was connected to a circulating water-bath. The temperature of the cells was maintained by flowing water from the water-bath controlled at 33°C. The human epidermal membrane was mounted between the donor chamber (where the formulation is applied) and the receptor chamber. The receptor chamber was filled with phosphate buffered saline (pH 7.4; 1 mM) as receptor liquid. The delivery through human epidermis was assessed for 2 hours after the application of the formulations. The receptor fluid was collected in a vial from the receptor chamber of the diffusion cell at 4 time-points: 3 minutes, 10 minutes, 1 hour, and 2 hours. The vial was weighed and saved for sample analysis. The skin membranes were stored. From the results of the analysis, the amount of caffeine penetrating through human epidermis was determined

5.4 Sample Analysis

All samples collected throughout the study period were analyzed for caffeine content by radioactive assay using Liquid Scintillation Counter for [C-14] isotope, after adding the appropriate liquid scintillation cocktails to them.

6. RESULTS

The data is presented in two parts: (i) per time-point and (ii) as cumulative amounts. Samples are named for the applied test articles/conditions and abbreviated as follows:

in:	Fuse Science Enhanced Encapsulation Formula (no occlusion)
in-o:	Fuse Science Enhanced Encapsulation Formula; epidermis surface occlusion with parafilm.
dc:	Fuse Science Reduced Encapsulation Formula (no occlusion)

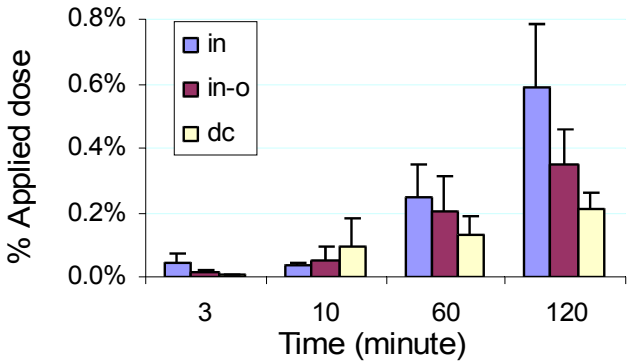
6.1 Time-point data

Amount of caffeine collected in the receptor fluid per time-point.

Calculated as percentage of the applied dose (mean ± standard error)

Sample	Time (minute)			
	3	10	60	120
in	0.041% ± 0.029%	0.033% ± 0.014%	0.244% ± 0.105%	0.588% ± 0.196%
in-o	0.014% ± 0.010%	0.050% ± 0.043%	0.207% ± 0.105%	0.351% ± 0.110%
dc	0.005% ± 0.002%	0.096% ± 0.083%	0.131% ± 0.059%	0.210% ± 0.049%

- Caffeine penetrated through the epidermis into the receptor chamber within the first 3 minutes, increasing in the amount at each time point.
- One of six replicates of "dc" application has a higher penetration rate at the 10-minute time-point, which can be considered an anomaly of the biological membranes.



- Application of "in" and "in-o", except for the 10-minute time-point yields higher amount of caffeine penetrating the epidermis than the application of "dc".
- Occlusion is shown to reduce the penetration of caffeine through the epidermis from the same formulation, i.e. Fuse Science Enhanced Encapsulation Formula ("in").

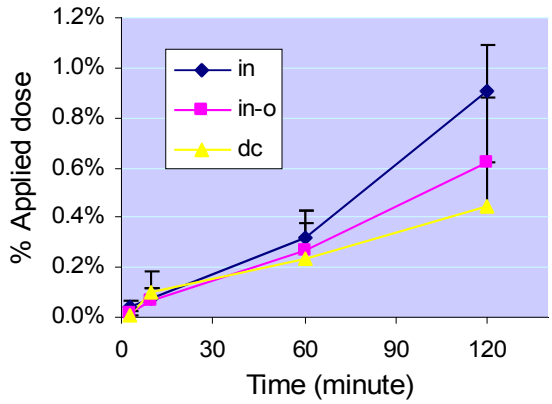
6.2 Cumulative data

Cumulative amount of caffeine collected in the receptor fluid at each time-point.

Calculated as percentage of the applied dose (mean ± standard error)

Sample	Time (minute)			
	3	10	60	120
in	0.041% ± 0.029%	0.074% ± 0.042%	0.317% ± 0.110%	0.905% ± 0.184%
in-o	0.014% ± 0.010%	0.064% ± 0.052%	0.271% ± 0.156%	0.623% ± 0.259%
dc	0.005% ± 0.002%	0.101% ± 0.085%	0.232% ± 0.142%	0.442% ± 0.182%

- At the 120-minute (2-hour) time-point, the cumulative amount of caffeine penetrating from "in" application was the highest, followed by "in-o" and the least was "dc".
- The cumulative amount of caffeine from "in" application is twice as much as the one from "dc" application.



- As already observed from the time-point data, occlusion reduced the penetration of caffeine through the epidermis from the “in” application.

7. CONCLUSION

- Measureable penetration of caffeine across human epidermis is observed already in the first 3 minutes after the application of formulations. This is the first reported *in vitro* study to get caffeine through the epidermis in three minutes.
- Fuse Science Enhanced Encapsulation Formula (no occlusion) yields the highest amount of caffeine penetrating through the epidermis, whereas Fuse Science Reduced Encapsulation Formula (no occlusion) yields the lowest.
- Enhanced Encapsulation Formula with higher carrier content is related to higher caffeine penetration across the epidermis.
- Occlusion with parafilm reduced the caffeine penetration across the epidermis.

End of report.

APPENDIX A: Study Protocol

Comparison of Formulations Using In Vitro Penetration Study Through Human Epidermis

1. INTRODUCTION

Pre-clinical selection of topical formulations is performed using an *in-vitro* penetration study through human epidermal membrane, which will assess the percutaneous absorption profiles of the applied drugs. The method is based on the use of excised human skin as a membrane in a diffusion system that simulates *in-vivo* skin conditions. By analyzing the amount of the drug reaching the receptor solution, penetration profile of the drug in the skin can be assessed and used as a tool to determine the feasibility of formulations for subsequent development. The method using full thickness skin has historic precedent for accurately predicting *in-vivo* percutaneous absorption kinetics.[1]

2. STUDY OBJECTIVE

To assess the percutaneous absorption profile of active ingredient(s) from various topical formulations using an *in-vitro* penetration study through human epidermis.

3. STUDY DESIGN

An open label, within donor, comparison study of several topical formulations/test condition. Each formulation/test condition will be tested in six replicates by a finite dose application. The receptor solution fills up the receptor chamber of the cell continuously, bathing the lower surface of the skin, and is collected after a certain time interval.

4. MATERIALS AND METHODS

4.1. Study Formulations

The drug candidate(s) formulations will be used as delivered, without any modification or analysis prior to the study, except for storage and acclimatization.

4.2. Reagents

All reagents used in this study will be of USP reagent grade or better. Source of each reagent will be noted after the first mention of each chemical within the text.

[1] Franz TJ (1978) The finite dose technique as a valid in vitro model for the study of percutaneous absorption in man. Curr. Probl. Dermatol., vol. 7, Karger, Basel. pp. 58-68.

4.3. Skin donor criteria:

- Excised skin will be obtained from human donor, fresh (within 24 hour after harvest) or frozen (stored within 24 hour after harvest, in temperature < 4°C for not more than 3 days, or in temperature <-20°C for not more than 1 week)
- The donor should be not younger than 16 years and not older than 70 years old and do not have diseases affecting the normal appearance of skin.
- The skin will be harvested mainly but not exclusively from the abdomen, back or thigh. The body part origin will be noted.
- During transport the harvested skin should be placed in the essential media, such as MEM (Eagle's Minimum Essential Media) or equivalents. The media will be noted.

4.4. Skin preparation

Excised human skin without obvious signs of damage or disease will be used in this study. After the removal of subcutaneous fat, the surface of the skin is wipe clean with tissue paper soaked with purified water. The skin was placed, dermal side down, on hot plate set at 60°C for approximately 2 minutes. Using tweezer, the epidermis was separated from dermis. The epidermis sheet is washed twice in purified water, then air-dried and stored at room temperature prior to use.

Epidermis sheet from a single donor is cut into multiple smaller membrane sections to fit on the Franz-type diffusion cells (Permegear Inc., Bethlehem, PA). The receptor solution of Phosphate Buffered Solution (PBS pH 7.4, 1 mM) fills the receptor chamber of the diffusion cell, free from any air bubbles and continuously bathing the inner surface of the membrane. The donor chamber of the cell is left open to ambient laboratory environment. Each cell is equipped with water jacket connected to a circulating water bath maintained at 33.0 ± 0.2°C.

4.5. Drug application

Each formulation will be applied to triplicate sections of the same donor skin at a target dose of 5-20 µL/cell (or 7-35 mg/cm^2). The formulation will be applied and distributed to the skin using a positive displacement pipetter.

4.6. Diffusion study

The receptor fluid is collected in a vial at the specified time-points from the receptor chamber of the diffusion cell. Fresh receptor fluid refills the receptor chamber. The vial will be weighed and saved for sample assay. The receptor fluid will be collected up to the specified end-of-study time.

4.7. Sample analysis

All samples will be analyzed for active ingredient(s) content by the designated analytical method according to the validated assay.

5. STATISTICAL METHODS

5.1. Parameters

The total absorption in the receptor fluid will be calculated:

5.2. Statistical evaluation

A mean and standard deviation will be calculated per formulation for all parameters based on the replicate data obtained from each donor. Subsequently, a grand mean and standard error will be calculated per formulation across all donors.

APPENDIX B: Raw Data

1. Fuse Science Enhanced Encapsulation Formula (no occlusion)

Timepoint Amount

Sample	Time (minute)			
	3	10	60	120
A	0.011%	0.004%	0.174%	1.478%
F	0.000%	0.041%	0.081%	0.365%
G	0.046%	0.023%	0.726%	0.288%
L	0.006%	0.013%	0.331%	0.680%
A	0.000%	0.021%	0.029%	0.594%
G	0.181%	0.097%	0.121%	0.122%
Average	0.041%	0.033%	0.244%	0.588%
S.E.M	0.029%	0.014%	0.105%	0.196%
n	6	6	6	6

Cumulative Amount

Sample	Time (minute)			
	3	10	60	120
A	0.011%	0.015%	0.189%	1.667%
F	0.000%	0.041%	0.121%	0.486%
G	0.046%	0.069%	0.795%	1.083%
L	0.006%	0.019%	0.350%	1.030%
A	0.000%	0.021%	0.050%	0.645%
G	0.181%	0.278%	0.399%	0.521%
Average	0.041%	0.074%	0.317%	0.905%
S.E.M	0.029%	0.042%	0.110%	0.184%
n	6	6	6	6

APPENDIX B (continued)

2. Fuse Science Enhanced Encapsulation Formula; epidermis surface occlusion with parafilm

Timepoint Amount

Sample	Time (minute)			
	3	10	60	120
C	0.000%	0.010%	0.041%	0.185%
I	0.004%	0.000%	0.127%	0.461%
J	0.001%	0.002%	0.027%	0.106%
C	0.000%	0.001%	0.090%	0.106%
D	0.060%	0.263%	0.704%	0.789%
J	0.016%	0.027%	0.253%	0.461%
Average	0.014%	0.050%	0.207%	0.351%
S.E.M	0.010%	0.043%	0.105%	0.110%
n	6	6	6	6

Cumulative Amount

Sample	Time (minute)			
	3	10	60	120
C	0.000%	0.010%	0.052%	0.237%
I	0.004%	0.004%	0.131%	0.592%
J	0.001%	0.003%	0.030%	0.136%
C	0.000%	0.001%	0.092%	0.197%
D	0.060%	0.323%	1.027%	1.816%
J	0.016%	0.043%	0.296%	0.757%
Average	0.014%	0.064%	0.271%	0.623%
S.E.M	0.010%	0.052%	0.156%	0.259%
n	6	6	6	6

APPENDIX B (continued)

3. Fuse Science Reduced Encapsulation Formula (no occlusion)

Timepoint Amount

Sample	Time (minute)			
	3	10	60	120
B	0.000%	0.010%	0.046%	0.148%
H	0.005%	0.004%	0.030%	0.315%
K	0.004%	0.006%	0.136%	0.113%
B	0.011%	0.512%	0.413%	0.406%
I	0.007%	0.030%	0.105%	0.161%
K	0.001%	0.014%	0.056%	0.119%
Average	0.005%	0.096%	0.131%	0.210%
S.E.M	0.002%	0.083%	0.059%	0.049%
n	6	6	6	6

Cumulative Amount

Sample	Time (minute)			
	3	10	60	120
B	0.000%	0.010%	0.056%	0.204%
H	0.005%	0.008%	0.039%	0.354%
K	0.004%	0.010%	0.146%	0.259%
B	0.011%	0.523%	0.936%	1.342%
I	0.007%	0.037%	0.142%	0.303%
K	0.001%	0.016%	0.071%	0.190%
Average	0.005%	0.101%	0.232%	0.442%
S.E.M	0.002%	0.085%	0.142%	0.182%
n	6	6	6	6