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Diffusion and Lipolysis Test on Forskolin Microemulsion Gel

Rahma NAFI'AH [1,4], Yeyet Cahyati SUMIRTAPURA [2], Sasanti Tarini DARIJANTO [2], Maria Immaculata IWO [3*]

1 Doctoral Program of Pharmaceutic, School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia.
2 Department of Pharmaceutic, School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia.
3 Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia.
4 Department of Pharmacy, YPIB University, Cirebon, Indonesia
* Corresponding Author. E-mail: maria1428@itb.ac.id (M.I.I.); Tel. +62-8562202445.

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ABSTRACT: Forskolin is a labdane diterpene that acts as lipolysis, while microemulsion is a delivery system that works as a penetration enhancer at topical application. This study aims to determine the diffusion profile, lipolysis test and skin histology of FSK microemulsion gel applied topically. The diffusion test used Franz cell, while lipolysis activity was carried out on 42 mice which were divided into 7 groups consisting of 6 groups given high fat-fed and 1 group given standard fed as normal control. The six groups were: A1 gel, A1 gel used twice a day, A1 gel base, emulgel, slimming gel product, and control with high fed-fat. The results showed that the A1 microemulsion formula and A1 gel were able to diffuse around 36.81±2.08% and 18.05±0.32% for 6 hours of observation, respectively. Statistical analysis using the least significant difference in body weight changes gave the result that gel A1 twice a day reduced body weight by -3.81 ± 3.17% with $p < 0.01$ against the control, whereas in the ex vivo lipolysis test it released glycerol levels of 15.99±1.55 nmol/hour with $p < 0.05$ to control. Histological observations showed that topical application of A1 gel formula showed lysis of lipids in the hypodermis. From the results of this study, microemulsions can deliver forskolin to adipose tissue, thereby forskolin can act as a lipolytic agent.

KEYWORDS: Microemulsion; forskolin; diffusion; lipolysis.

1. INTRODUCTION

Forskolin (FSK) is labdane diterpene that commonly found in *Coleus Forskohlii* plant root. This plant grows in subtropical areas such as: India, Nepal, Burma, Thailand, Egypt, and China [1].

One of the pharmacological effects of forskolin as lipolytic agent is shown through its ability to stimulate the enzyme Adenylate Cyclase (AC) [2] thereby increasing the amount of cyclic adenosine mono phosphate (cAMP), where cAMP is useful for activating protein kinase A (PKA) enzyme. PKA enzyme can activate phosphorylated Hormone Sensitive Lipase (HSL) to stimulate hydrolysis of triglycerides in adipose into fatty acids and glycerol [3,4]. In vitro lipolysis test showed forskolin can increase cAMP which is needed for triglyceride hydrolysis reaction [4–6].

In several studies, FSK has shown lipolytic activity, for example: there was an equal amount of free glycerol levels in formula containing 0.1% FSK to 5% caffeine [7]. In vitro and ex vivo results show that forskolin has potential as lipolysis agent, so a delivery system is needed to increase its usefulness and effectiveness. FSK is poorly soluble in water, where the solubility in water is 0.2 mM [8]. There are several developments of delivery systems, including: emulsion [9], nanoemulsion [10] and nanostructured lipid carrier (NLC) [11]. The three delivery systems contained 0.075% w/v FSK. To increase the dose of FSK as a lipolysis agent, a delivery system is needed to increase the solubility.

Microemulsion is drug delivery system that can be used to increase the solubility, penetration, and stability of active pharmaceutical ingredients. Microemulsion spontaneously formed monophase system composed of oil, water and a surfactant-cosurfactant mixture (Smix). The use of microemulsions can increase

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the penetration of active pharmaceutical ingredients into the hypodermis layer. This has been done by Bolzinger et al, who stated that caffeine in microemulsions can be delivered to the hypodermis layer [12].

Based on the things above, this study aims to obtain forskolin microemulsion formula as well as testing for lipolysis both ex vivo and in vivo as well as histology on the skin.

2. RESULT

2.1. Formulation and solubility of FSK in microemulsions

FSK can be formulated in microemulsions consisting of Maisine CC, Tween 20, polyethylene glycol (PEG 400) and/or propylene glycol (PG) as oil, surfactant and cosurfactant. The addition of oil content can affect the success of the formulation, so that the ratio of oil:surfactant:cosurfactant that can be used for the FSK formulation is 4:25:5. The solubility of FSK in formula A using PEG 400 was higher than formula B using PG, with FSK solubility around 2.19 mg/mL in formula A and 1.42 mg/mL in formula B. The formulation and solubility can be seen in Table 1 and Table 2, respectively.

Table 1. Formulation for FSK microemulsion

Materials (w/v %)	**Formula**					
	A1	**A2**	**A3**	**B1**	**B2**	**B3**
FSK	0.1	0.1	0.1	0.1	0.1	0.1
Maisine CC	4	5	6	4	5	6
Tween 20	25	25	25	25	25	25
PEG 400	5	5	5	-	-	-
PG	-	-	-	5	5	5
Phosphate buffer pH 6 ad	100	100	100	100	100	100
Result						
Appearance	Transparent	Bluish	Cloudy	Transparent	Cloudy	Cloudy
Globule size (Mean ± SD nm)	28.80 ± 2.85	30.67 ± 4.68	N. A.	18.23 ± 0.31	N. A.	N. A.
PI (Mean ± SD)	0.31 ± 0.17	0.41 ± 0.02	N. A.	0.19 ± 0.01	N. A.	N. A.

PI: Polydispersity index, N.A.: Not applicable, n= 3

Table 2. Microemulsion FSK solubility

Formula	**Solubility (Mean ± SD mg/mL**
A1	2.19 ± 0.03
B1	1.42 ± 0.02

n= 3

2.2. Method validation

Validation of the method gives the results that FSK appears at the retention time of about 6.5 minutes, linearity with a correlation coefficient of r^2 = 0.9997, limit of detection (LOD) = 1.96 ppm, limit of quantitation (LOQ) = 6.53 ppm. The bias value in the recovery test is in the range of -1.19 – 0.39%, while the relative standard deviation (RSD) value in the intraday and interday tests is in the range of 0.26 – 1.73% and the lowest precision test is 7.31%.

Table 3. ~~Determination of limit of detection (LOD) and limit of quantification (LOQ)~~

Table 3. Statistical analysis of LOD and LOQ

FSK (ppm)	Area (Yi)	Yii	(Yi-Yii)²	$S(y/x)^2$	$S(y/x)$	LOD (ppm)	LOQ (ppm)
10	74236.3	75568.6	1775157	$S(y/x)^2 = \frac{\sum(yi-yii)^2}{n-2}$	√22570649.67	LOD=3x S(y/x)/b	LOQ=10x S(y/x)/b
20	150113.5	148566.6	2392900				
30	222879.0	221564.6	1727647				
40	295486.5	294562.6	853591.2				
50	362232.8	367560.6	28385453				
60	443427.0	440558.6	8227719				
Total			90282598.67	22570649.67	4750.86	1.96	6.53

Yi: area obtained from measurement
Yii: area obtained from calculation using calibration curve equation, n= 6

Table 4. FSK recovery in microemulsion formula

FSK (ppm)	Concentration of calculation (Mean ± SD ppm)	Recovery (Mean ± SD%)	RSD (%)	Bias (%)
40	40.14 ± 0.45	100.36 ± 1.12	1.12	0.36
50	49.40 ± 0.19	98.81 ± 0.37	0.38	-1.19
60	60.08 ± 1.08	100.13 ± 1.80	1.80	0.13

SD = standard deviation, RSD = relative standard deviation, n = 3

2.3. Formulation gel microemulsion, micellar, emulgel and its n vitro diffusion test

The characteristics of the formula and the results of the diffusion test can be seen in Table 7 and Figure 2, respectively. In vitro diffusion test using Franz cell diffusion with Spangler synthetic membrane gave the results that A1 microemulsion formula could diffuse about 36.81±2.08%, A1 gel formula and Tween 20 micellar formula were around 18.50±0.32% and 19.58±0.47 % respectively, while the emulgel formula did not diffuse. Meanwhile, the kinetics release that occur in each formula follow the Higuchi model.

Table 5. FSK precision measurement

FSK (ppm)		Interday			Intraday
		Day 1st	Day 3rd	Day 7th	
40	Mean AUC	296426	293409	285636.3	291823.8
	SD	774.70	1866.71	3649.37	5566.77
	RSD (%)	0.26	0.64	1.28	1.91
50	Mean AUC	367392	362510	365145	365015.7
	SD	4908.34	413.61	4682.88	2443.72
	RSD (%)	1.34	1.06	1.28	0.67
60	Mean AUC	448780	445620	452145	448848.3
	SD	1571.06	4742.32	7793.18	3263.04
	RSD (%)	0.35	1.06	1.73	0.73

AUC = area under curve, n= 3

Table 6. Lowest concentration precision

FSK (ppm)	AUC
2	10383
	11038
	10385
	11094
	10470
	11158
	10412
	10885
	11854
	12355
	10875
	12888
Mean AUC	11149.75
SD	815.22
RSD (%)	7.31

2.4. Lipolysis test

The lipolysis test observed in this study consisted of changes body weight of mice, free glycerol levels released from lipid samples and histology of mice skin. Observation of changes in body weight showed that only the animal group that was given the A1 gel formula twice a day gave weight loss with significant value of $p < 0.01$ to the control group and normal group. As for the ex vivo lipolysis test, the groups that gave low glycerol release results with p value < 0.05 gave histological results of the lysis of hypodermic lipids. The data of body weight changes, ex vivo lipolysis tests and histology can be seen in Figure 3, Figure 4 and Figure 5, respectively.

Table 7. Formula and character of microemulson, microemulsion gel, micellar and emulgel

Materials (w/v%)	Formula			
	A1	**A1 gel**	**Micellar**	**Emulgel**
FSK	0.2	0.2	0.2	0.2
Maisine CC	4	4	-	20
Tween 20	25	25	25	8.5
PEG 400	5	5	-	-
Span 20	-	-	-	0.75
HPMC	-	1	-	1
Phosphate buffer pH 6 ad	100	100	100	100
Result				
Globule size (Mean±SD nm)	21.30±1.10	21.03±0.77	12.05±0.89	872.30±5.5
PI (Mean±SD)	0.13±0.04	0.31±0.03	0.10±0.07	0.37±0.02
pH	6.4	6.38	6.39	5.89
Viscosity (cPs)	104.45	4485	46.9	17400

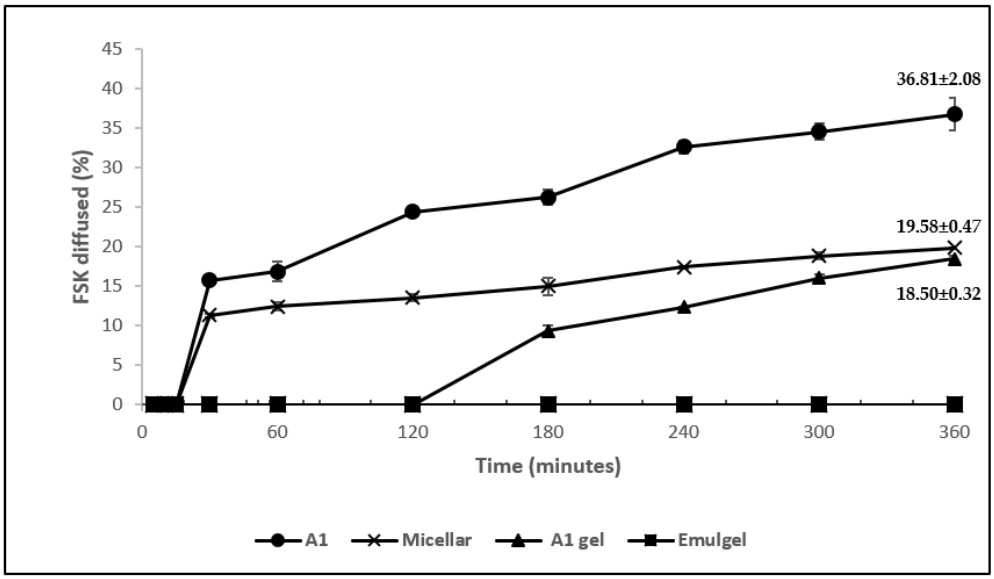

Figure 2. Diffusion of FSK from several drug delivery systems, n=2

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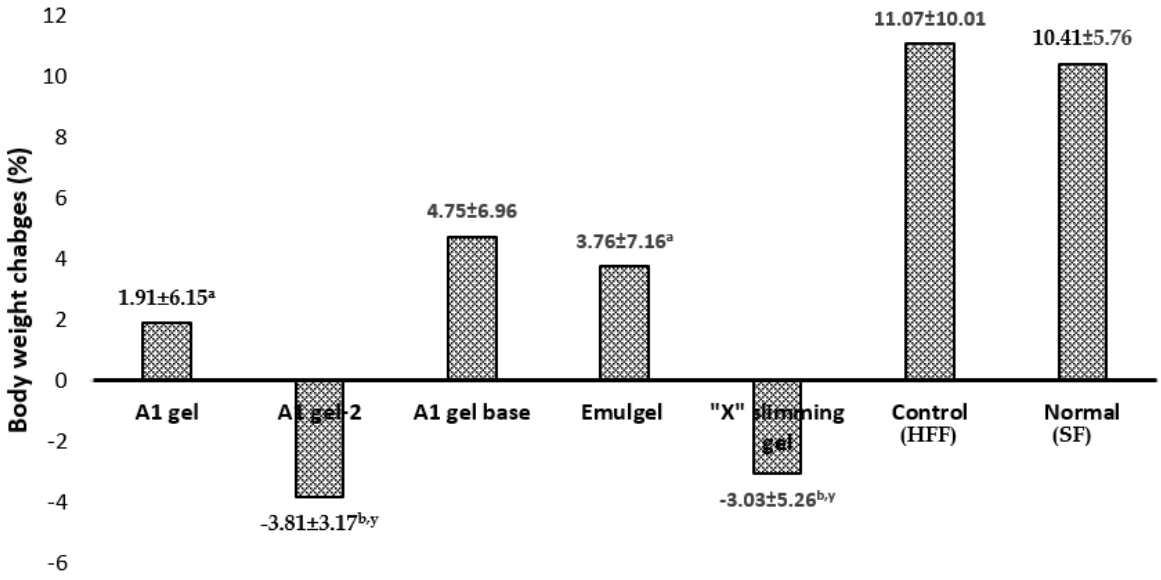

Figure 3. Body weight changes of mice (%) during 56 days of treatment.
The statistical significance value is indicated by the p value are a = $p < 0.05$, b = $p < 0.01$ compared to the control; x = $p < 0.05$, y = $p < 0.01$ compared to normal, n= 6

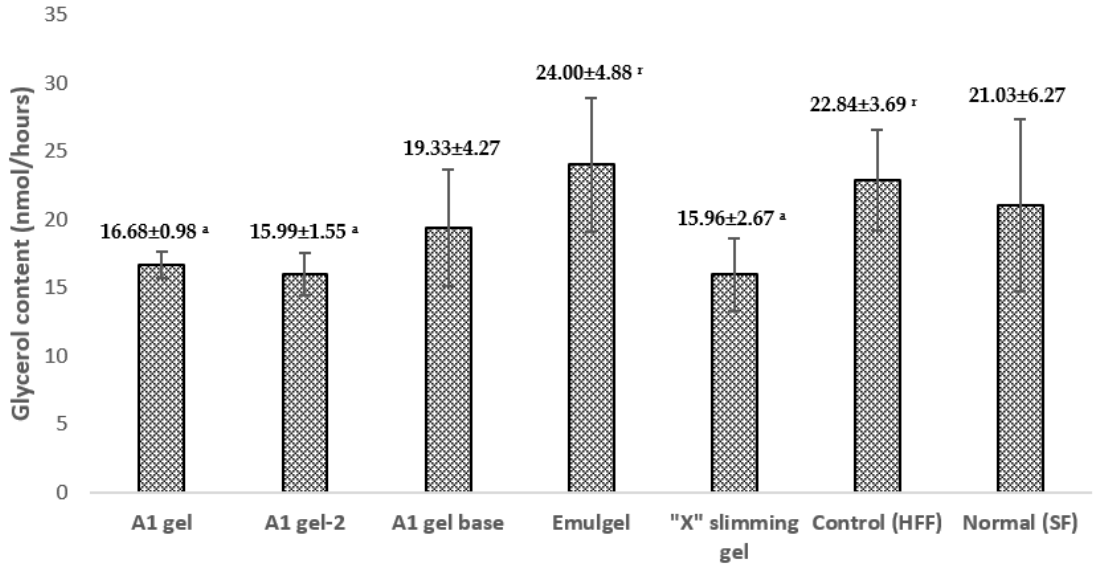

Figure 4. Free glycerol content of mice adipose fat. The value of statistical significance is indicated by the p value, namely: a = $p < 0.05$, b = $p < 0.01$ against the control (HFF); q = $p < 0.05$, r = $p < 0.01$ against the "X" slimming gel, n= 4.

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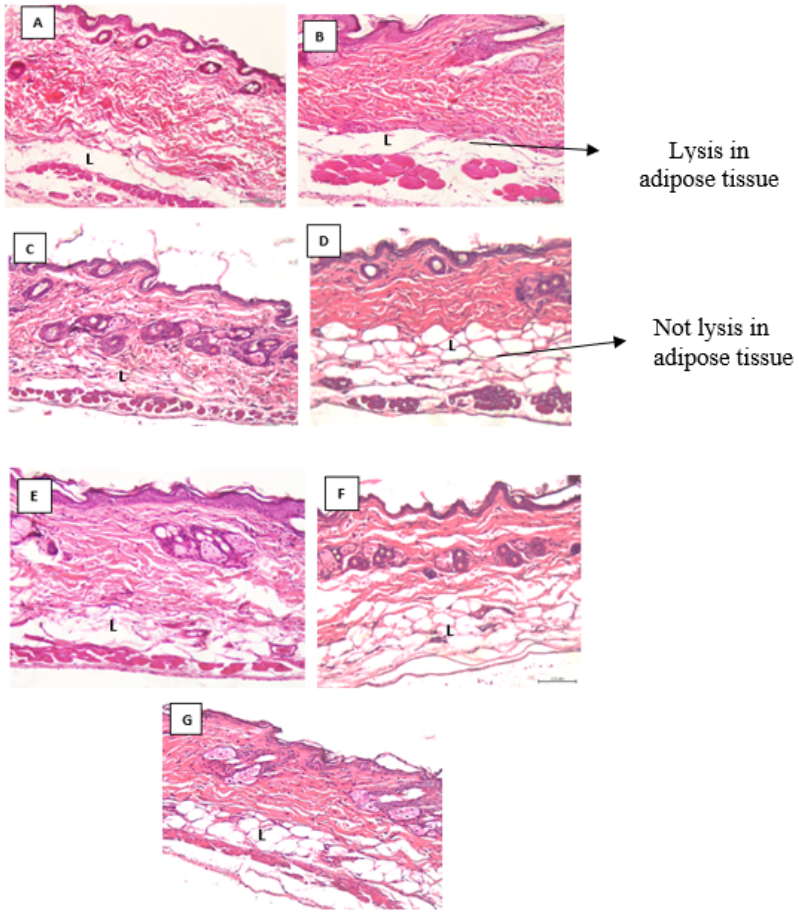

Figure 5. Histology of the skin that has been applied: A. A1 gel, B. A1 gel twice a day, C. A1 gel base, D. Emulgel, E. "X" slimming gel, F. Control (HFF), G. Standard feed (SF) . Using Hematoxylin–Eosin staining with magnification = 200x, L = lipid.

3. DISCUSSION

Forskolin has solubility about 20 mg/mL in nonionic surfactants with HLB about 15 [13], Tween 20 has HLB of 16.7. Table 1 shows the results of the microemulsion formulation using the Smix concentration of 30%. The use of PEG 400 as a cosurfactant in the formulation of microemulsions gave better results than PG in terms of appearance and globule size. The addition of oil tends to cause an increase in globule size because surfactants cannot reduce the surface tension [14]. The oil concentration that can be used in formula A and formula B are 4%. The solubility test FSK in microemulsion, formula A1 solubilized FSK was 2.19 mg/mL, while formula B1 was 1.42 mg/mL. The solubility test of the microemulsion formula can be seen in Table 2. The difference in the solubility of FSK in formula A1 to formula B1 is probably caused by the role of PEG 400 in dissolving FSK, where PEG 400 has polyethylene group which can form more hydrogen bonds than PG when interacting with FSK in microemulsion. Furthermore, the formula used was formula A1 which contains FSK 2 mg/mL or 0.2%

Method validation was carried out for the determination of FSK levels. These tests included: linearity test, determination of LOD and LOQ, recovery test and precision test. Figure 1 shows the linearity test of the correlation coefficient r = 0.9997 and the calibration curve equation y = 7327.3x + 1377.1. The determination of LOD and LOQ was calculated statistically using linear regression from the calibration curve. The results of the LOD and LOQ calculations can be seen in Table 3, where the LOD value is 1.96 ppm and the LOQ is 6.53 ppm.

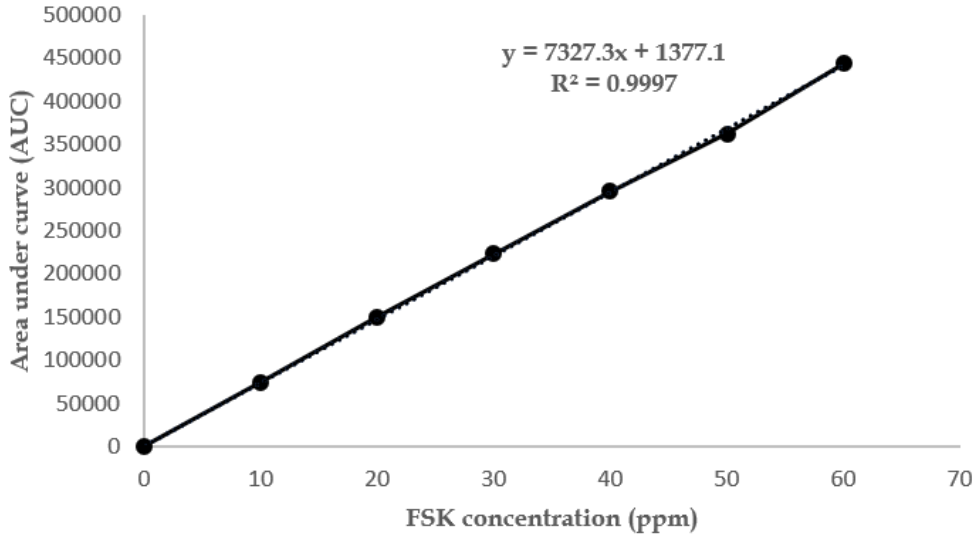

Figure 1. FSK calibration curve, n = 4

The recovery analysis was determined by the spiked method, in which FSK was added to the microemulsion matrix. The recovery result can be seen in Table 4, where the recovery value is in the range of 98.81 – 100.36% with bias value of <± 2. Meanwhile, results of the precision analysis determined on inter-day and intraday can be seen in Table 5. Interday precision shows intermediate precision data, while intraday precision represents repeatability on the same day. Inter-day and intraday precision data analysis gives RSD ≤ 2.0% results. The analysis of the lowest concentration precision data contained in Table 6 gives an RSD value = 7.31%, where the acceptance criteria for precision RSD in the diffusion test was ≤ 20% [15]. From the results of this validation, the method can be used to determine FSK levels for diffusion test.

In vitro diffusion test was carried out on the formulas listed in Table 7. In this test, the comparison were made of the levels of FSK that penetrated through the Spangler membrane contained in the microemulsion, microemulsion gel, micellar and emulgel formulas. The microemulsion gel formula uses high viscosity HPMC K100M type which was mixed with the microemulsion formula. The micellar formula consists of water and Tween 20, while the emulgel contains HPMC and an emulsion base.

This test intended to compare diffusion profiles formulas based on type of delivery system. The diffusion test was carried out using Franz diffusion cell and Spangler membrane. This membrane was made of cellulose membrane impregnated in Spangler's sebum synthetic, which consists of lipid, oils, and cholesterol similar found in human stratum corneum [9]. The results of the diffusion test can be seen in Figure 2. The diffusion results showed that the A1 formula gave flux value of 36.81±2.08%, while the A1 gel and micellar formula was 18.50±0.32% and 19.58±0.47 % respecively, while the emulgel formula does not diffuse.

The comparison of the diffusion test between the A1 microemulsion formula and the micellar formula showed that A1 microemulsion formula was easier to diffuse than micellar formula [16]. Both formulas have the same surfactant, namely Tween 20 which nonionic surfactant with lipophilic chain length of C12. The ability of Tween 20 to diffuse there are several possibilities, thereby Tween 20 can interact with lipid bilayer membranes in the stratum corneum intercellular pathway, causing the stratum corneum to become more permeable [17], and due to the difference in chain length with lipids in membrane which are generally C18, it causes the hydrophobic bond energy to decrease so that Tween 20 easily diffuses [18]. Meanwhile, constituents of Maisine CC oil in formula A1 can change the membrane lipid conformation and increase permeability membrane [19]. The comparison of the diffusion test of the A1 microemulsion formula to the A1 gel form, there is a lag time produced by the gel form. This can be caused by the FSK in the microemulsion can diffuse directly without passing through the matrix, but the FSK in the microemulsion gel must pass through the gelling agent matrix, resulting in a lag time [20]. The FSK in the emulgel could not diffuse, this was probably due to the large globule size which was around 872 nm and the solubility of FSK in oil. It was known that FSK has a log P = 3.89 which indicates that FSK tends to dissolve easily in oil [21]. With a fairly high concentration of Maisine CC oil in the emulgel (i.e 20%) FSK tends soluble in oil so that difficult to partition.

From the above diffusion results, the FSK diffusion results are related to the carrier in the delivery system. These results agreed with the release kinetics calculated which can be seen in Table 8. FSK diffusion in the A1, A1 gel and micellar formulas showed pattern of release following the Higuchi model. This mathematical model shows that the diffusion flux is directly proportional to the square root of the time and drug solubility in the carrier. This indicates that the FSK diffusion rate occurs controlled by the carrier in the delivery system [22,23].

Table 8. FSK kinetics release in formula

Formula	Zero order		First Order		Higuchi	
	r^2	k ($\mu g.min^{-1}$)	r^2	k (min^{-1})	r^2	k ($\mu g.min^{-1/2}$)
A1	0.966	0.304	0.928	0.0027	0.980	7.573
A1 gel	0.874	1.591	0.981	0.0039	0.995	7.667
Micellar	0.973	0.120	0.9439	0.0017	0.990	2.993

The microemulsion gel form has good adhesion and kinetically has the same release constant as microemulsion k = 7.67 $\mu g.min^{-1/2}$, thus this form is used for in vivo tests on animal skin. The results of the body weight changes can be seen in Figure 3. In principle, this study observed changes in body weight of mice that were given topical lipolysis dosage forms and simultaneously fed high fat fed (HFF). From the observations and statistical analysis using the least significant difference (LSD), there appear that the group of animals given the A1 gel formula twice a day gave difference in body weight by -3.81±3.17 % with p value < 0.01 against control. According to America's Finest Inc. (AFI), the dose of FSK to provide topical lipolysis effect was 0.1 – 0.5% [24]. In the A1 gel formula, the FSK dose is 0.2%, so that the use twice a day will give weight loss significant against control.

The ex vivo lipolysis test can be seen in Figure 4. These results indicate levels of free glycerol resulting from the lipolysis reaction of lipid tissue from mice after being treated for 56 days. Lipid tissue consisting of triglycerides will undergo hydrolysis reaction after being incubated in the medium. The results of the determination of glycerol levels in the ex vivo test showed the hydrolysis reaction of triglycerides remaining from the results of the in vivo test, so that high levels of glycerol indicated a high amount of triglycerides.

In the group of animals that were given the formula A1 gel, A1 gel-2 , and the "X" slimming gel there were significant difference in the lipolysis reaction with p value < 0.05 compared to the control with the glycerol content value of 16.68±0.98, 15.99±1.55 and 15.96±2.67 nmol/hour, repectively. From the results of the ex vivo test, it appears that there was lipolysis effect resulting from the A1 gel and market gel formulas. This test proves that FSK can provide a lipolysis reaction in vivo which was proven ex vivo. As for observation of ex vivo lipolysis on differences in dosage forms, it shows that dosage form can affect results of lipolysis reaction. FSK microemulsion preparations can give lipolysis reaction, but in emulgel dosage form there was no lipolysis reaction. This has been proven in in vitro diffusion tests, where FSK in emulgel did not diffuse.

Figure 5 shows the histology of lipid tissue in hypodermis layer of mice skins. The figure shows that application of formula A1 gel, A1 gel base and "X" slimming gel gave lysis on the lipid. Meanwhile, in the group that was given the emulgel dosage form, normal and control, lipolysis did not occur, so that the lipid tissue membrane appeared clear and intact. On these observations, the occurrence of lipolysis was characterized by changes in the shape of the lipids in the tissue [4,25,26].

The occurrence of lipolysis in hypodermic layer could be due to the ability of the delivery system to bring FSK to lipid tissue. In study conducted by Bolzinger et. al. on release of caffeine in microemulsions in vitro, it was shown that microemulsions can deliver caffeine to hypodermis layer [12]. Likewise in this study, it was shown that the microemulsion formula could deliver FSK to hypodermis. Through the intercellular route in stratum corneum, forskolin in free form can pass through the epidermal layer to the dermis and hypodermis layers, while through the hair follicle pathway, forskolin can permeate directly to the dermis and hypodermis layers [27,28].

4. CONCLUSION

Accumulation of fat under the skin will give change in the surface contour of the skin that is uneven, making it less aesthetically appealing. Administration of lipolytic agents can help with this condition. Microemulsions for topical FSK delivery systems have been successfully developed and have shown lipolysis effects in vitro, ex vivo, in vivo and histology. The results of the in vitro diffusion test showed that the FSK in the microemulsion formula and gel microemulsion were able to diffuse around 36.81±2.08% and 18.05±0.32%, respectively. The result of changes in body weight showed that the application of microemulsion gel topically twice a day could reduce body weight by -3.81±3.17% with $p < 0.01$ to the control. The ex vivo test showed that the application of the microemulsion gel twice a day gave a glycerol level of 15.99±1.55 nmol/hour with $p < 0.05$ to the control. The results of histological observations showed that there was a lipolytic effect on the hypodermis fat tissue as indicated by changes in the shape of the lipids in the tissue.From the results of this study, FSK can be developed into topical delivery system as lipolysis agent.

5. MATERIALS AND METHODS

5.1. Microemulsion Formulation

The ingredients used in the formulation were Forskolin (BOC Sciences, USA), Maisine CC oil (Gattefosse, France), Tween 20 (Croda, Singapore), PEG 400 (Indokemika, Indonesia), propylene glycol (Dow, Singapore) and HPMC (Kingstone Chemical, China).

The FSK microemulsions were made by dissolving the FSK in the oil phase, followed by the addition of surfactant, cosurfactant, and water which was stirred using a magnetic stirrer at a speed of 300 rpm for 5 minutes at room temperature. Formula optimization is done by varying the concentration of the ingredients. According to Rhein, the concentration of non-ionic surfactants with polyoxyethylene groups that do not cause irritation is 1-30% (18), so that the maximum optimization of Smix concentration in this study was 30% and the oil concentration was 4-6%. The selected formula is globule size formula of less than 50 nm and have polydispersity index value of less than 0.5.

5.2. Method Validation

Forskolin levels were determined using HPLC Waters 2487, with Cap Cell C18 Soda column. The eluent used was water: acetonitrile (35: 65) with flow at 1mL/minute, measured at a wavelength of 210 nm and an injection volume of 50µl. Method validation parameters include: specificity, linearity, LOD-LOQ, accuracy and precision. Variations in FSK concentration used for linearity testing were 10, 20, 30, 40, 50 and 60 ppm. As for the accuracy and precision testing using concentrations of 40, 50 and 60 ppm, the lowest level of precision was carried out at a concentration FSK of 2 ppm.

5.3. Formulation gel microemulsion, micellar, emulgel and its n vitro diffusion test

Microemulsion gels were prepared by mixing formula A1 into HPMC K100M as a gel base, both of which were stirred using a stirrer at 200 rpm for 1 hour. Micellar was prepared by dissolving FSK in Tween 20, then slowly adding water and stirring with a magnetic stirrer at 300 rpm for 10 minutes. Emulgel preparation was carried out by first making a forskolin emulsion using homogenizer for 5 minutes at speed of 4000 rpm, then adding HPMC as gelling agent.

The diffusion test was carried out using Franz cell diffusion with synthetic membrane Spangler's. The membrane were made of cellulose membrane impregnated with Spangler's liquid synthetic sebum. The Spangler fluid was composed of palmitic acid, stearic acid, coconut oil, liquid paraffin, spermaceti, olive oil, squalene, cholesterol, oleic acid, and linoleic acid [30,31]. Next, the membrane was arranged on the Franz cell diffusion. The test was carried out 36°C, stirred at 100 rpm of speed and volume of receptor compartment was 45 ml of phosphate buffer solution pH 6 [32]. Sampling was carried out on the receptor compartment as much as 700 µl at minute: 5, 15, 30, 60, 120, 180, 240, 300, and 360. Then the samples were filtered and injected into HPLC column. Furthermore, the kinetic analysis of the diffuse formula was carried out.

5.4. Lipolysis Test

In vivo lipolysis test was carried out on mice aged 8-9 weeks with weight ranging from 35-40 grams (33). Mice were divided into 7 groups (n = 6), of which 6 groups were fed with high fat fed (HFF) and 1 group was given standard fed (SF). The six groups were each given: A1 gel formula, A1 gel formula twice a day, A1 gel base formula, emulgel formula, slimming gel product containing green tea extract ("X" slimming gel) and HFF control. Each mice were shaved on the abdomen with area of 3x4 cm^2, then the preparation was applied

daily as much as 0.25 grams for 56 days. Every week the animals were weighed [34]. Lipolysis test observations included: changes in body weight, ex vivo and histology.

5.4.1. Ex vivo lipolysis test

The materials used for the ex vivo lipolysis test were: DMEM media (Thermo Scientific-Gibco, USA), BSA free fatty acid (Sigma, USA), Free Glycerol reagent (Sigma, USA), Glycerol (Sigma, USA), BCA protein assay kit (Thermo Scientific-Pierce, USA), BSA (Thermo Scientific, USA), sodium dodesil sulphate, chloroform, acetic glacial, sodium hydroxyde and methanol (Merck, USA)

Mice that have been sacrificed are taken for lipids from abdomen. Furthermore, the lipid tissues were weighed about 50 mg and stored in refrigerator at -20 °C. Prior to use, lipid samples were left at room temperature, then 200µl DMEM containing 2% fatty acid-free BSA was added, incubated for 60 minutes, at 37 °C [4]. Furthermore, the incubated lipid (IL) was separated from the incubation medium (IM) and stored at -80°C. IM samples were used for determination of free glycerol levels while IL samples were used for determination of protein content [35,36]

IM samples were removed from the refrigerator and left at room temperature. The free glycerol levels of samples were determined by adding 5 µl of media with 100 µl of Free Glycerol reagent (FG) in 96 well plate, left for 10 minutes and measured using Tecan NanoQuant at wavelength of 540 nm. The absorbance readings were extrapolated to glycerol calibration curve made using standard glycerol at various concentrations of 0, 1, 1.25, 1.5 and 1.75 nanomol using FG reagent.

The IL samples were removed from the refrigerator and left at room temperature before use. The protein content of the samples were determined using Bicinchoninic acid (BCA) reagent Protein Assay Kit. The IL sample was dissolved in 1 ml of solution chloroform: methanol (2:1) and 1% glacial acetic acid, incubated in shaker for 60 minutes at 37 °C at 100 rpm. The fat part of the extraction was removed, while the existing tissue was transferred to tube containing 500 µl of lysis solution (0.3 N NaOH + 0.1% SDS), then the solution was incubated in shaker for 6 hours at 55 °C and 100 rpm in speed.

Determination of protein levels in tissue was determined using the BCA protein assay kit, bovine serum albumin (BSA) as standard, and measured using spectrophotometer at wavelength of 562 nm.

5.4.2. Histology

On the 56th day the mice were sacrificed, the intact skin was taken with an area of 1 cm^2 then stored in 10% formaldehyde solution for 24 hours. Skin samples stained with hematoxylin and eosin each for viewing under an Olympus CKX41 microscope with a magnification of 200 times [25,37].

5.5. Ethical approval

This research received ethical approval number: 03/KEPHP/-ITB/10-2021 released by Ethical Commission for the Use of Experimental Animals, School of Pharmacy, Institute of Technology Bandung for in vivo lipolysis test of forskolin microemulsion.

5.6. Statistical analysis

The results of difference test are expressed as mean ± standard deviation (SD). Data were analyzed by analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparisons using SPSS version 25.

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