

Simultaneous Determination of Prostaglandin E₁ and Prostaglandin E₁ Ethyl Ester in Hairless Mouse Skin Homogenate by High-Performance Liquid Chromatography

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ABSTRACT – A rapid and specific high-performance liquid chromatographic method was developed and validated for the simultaneous determination of prostaglandin E₁ (PGE₁) and prostaglandin E₁ ethyl ester (PGE₁-EE) in hairless mouse skin homogenate. The sample treatment procedure involved deproteination and precipitation by acetonitrile. PGE₁ and PGE₁-EE in supernatant were separated in a reversed-phase C18 column without being interfered by other components present in hairless mouse skin homogenate. 9-Anthracenecarboxylic acid was used as an internal standard. The retention times of PGE₁, 9-anthracenecarboxylic acid and PGE₁-EE were, 4.5, 9.5 and 18.0 min, respectively. The assay showed linearity from 1 to 40 µg/ml for both PGE₁ and PGE₁-EE. Precision expressed as RSD ranged from 2.3 to 14.1% for PGE₁ and 1.6 to 11.0% for PGE₁-EE. Accuracy ranged from 100.5 to 119.6 % for PGE₁ and from 98.0 to 103.7% for PGE₁-EE. This method was employed successfully to follow the time course of concentrations of PGE₁ and PGE₁-EE in hairless mouse skin homogenate for stability study.

Key words – Prostaglandin E₁ (PGE₁), Prostaglandin E₁ ethyl ester (PGE₁-EE), HPLC

Erectile dysfunction is the inability to achieve or maintain an erection sufficiently rigid for intercourse, ejaculation or both.¹⁾ Erectile dysfunction affects approximately 10 to 20 million men in United States and 10% of all men worldwide. It also affects an estimated 2 million males in Korea. The prevalence of the erectile dysfunction increases with age and the occurrence of other disease conditions such as diabetes and hypertension.²⁻⁴⁾ It is estimated that the incidence of impotence is 5% at 40 years of age and 15 to 25% at 65 years and above.¹⁾

Prostaglandin E₁ (PGE₁) (Figure 1) is a vasodilating agent and a platelet-aggregation inhibitor. It occurs naturally in the seminal vesicles and cavernous tissues of males. It has α -blocking properties mediated through a membrane receptor and relaxes the cavernous and arteriolar smooth muscle while causing restriction of venous outflow.^{5,6)}

PGE₁ is one of the most widely used drug for erectile dysfunction due to its efficacy and safety profile, long-term patient compliance and satisfaction.^{7,8)} Moreover, it is an officially approved drug by FDA in the treatment of men with erectile dysfunction.

Current pharmacotherapy for erectile dysfunction consists of oral, topical, intracavernous and transurethral drug therapy.⁹⁾ Among these medications, intracavernous injection such as Caverject[®] is the most common and extensively used treatment for patients with cardiovascular diseases.

However, alternative routes for administration of vasoactive substances have been investigated widely including topical way of application¹⁰⁾ since up to 50% of man using intracavernous injection therapy eventually discontinue treatment due to the pain on injection, lack of confidence in self-administration and loss of effectiveness.¹¹⁾

Transdermal delivery of PGE₁ in the treatment of erectile dysfunction has been also limited since PGE₁ is chemically labile and diffusion through skin is not rapid enough.^{12,13)} To overcome these drawbacks, new topical solutions containing prostaglandin E₁ ethyl ester (PGE₁-EE) (Figure 1), a prodrug of PGE₁ as a therapeutic agent for erectile dysfunction, could be developed for the better aqueous stability and enhanced percutaneous absorption. However, no methods have been reported for the simultaneous determination of PGE₁-EE and PGE₁ in hairless mouse skin which is employed for transdermal absorption study.

The present paper describes an isocratic reversed-phase HPLC method to simultaneously quantify PGE₁-EE and PGE₁ in hairless mouse skin homogenate using 9-anthracenecar-

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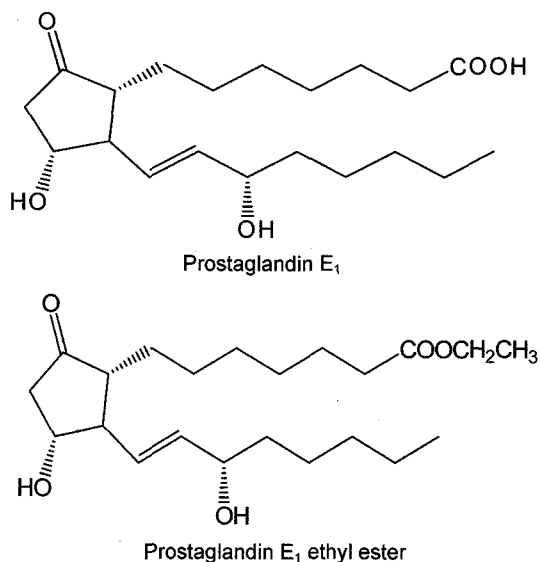


Figure 1—Chemical structures of prostaglandin E₁ and prostaglandin E₁-ethyl ester.

boxylic acid as an internal standard. This method was validated with respect to accuracy, precision, selectivity, and limits of quantitation (LOQ) and detection (LOD) according to Good Laboratory Practice Guidelines.^{14,15)}

Experimental

Materials and equipments

PGE₁-EE (Mw=382.53) and PGE₁ (Mw=354.48) were supplied by Cascade Biochem. Ltd. (U.K.). Acetonitrile and methanol were from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical grade and used as purchased without further purification. All the water used was purified by the Milli-Q-Grade water system (Millipore Bedford, MA, USA). The chromatographic system consisted of Jasco HPLC system utilizing Borwin program, Jasco PU-980 HPLC apparatus equipped with a Jasco UV detector (UV-975) and autosampler (MIDAS).

Chromatographic conditions

The mobile phase was a mixture of 0.01 M acetate buffer (pH 3.3) and acetonitrile (55:45, v/v), which was filtered through a 0.45- μ m HV filter (Millipore, Bedford, MA, USA), then deaerated ultrasonically prior to use. Elution rate and injection volume were 1.0 ml/min and 20 μ l, respectively. A guard column (Whatman column survival kit) containing identical packing material to that in the analytical column was used. All chromatographic operations were carried out at ambient temperature.

Table I—Materials for Topical Solution

Materials of topical solution	Portion (%)	Topical solution (100 mg)
PGE ₁ -EE	1.0	1000 μ g
Cineole	20.0	
Ethanol (Dehydrated)	39.5	
Propylene glycol	39.5	
Total	100	
10 % aq. sol. pH	pH 4.3	

Preparation of topical solution

As shown in Table I, topical solution containing PGE₁-EE was formulated, using ethanol/propylene glycol cosolvent system and cineole as an enhancer.

Preparation of standard solutions

Stock solutions of PGE₁ (0.2 mg/ml), PGE₁-EE (0.2 mg/ml), and 9-anthracenecarboxylic acid (0.2 mg/ml) were prepared in acetonitrile and stored at -80°C. Standard solutions of PGE₁ and PGE₁-EE in acetonitrile were prepared by spiking appropriate volume of the stock solution to give the final concentrations of 1, 2, 5, 10, 20 and 40 μ g/ml for both PGE₁ and PGE₁-EE. They were prepared extemporaneously and employed to prepare calibration standards and working solutions. Stock solution of 9-anthracenecarboxylic acid was diluted with acetonitrile to give the concentration of 10 μ g/ml.

Analytical procedure

The chromatographic system consisted of Jasco HPLC system utilizing Borwin program, Jasco PU-980 HPLC apparatus equipped with a Jasco UV detector and autosampler. HPLC separation was performed with a 20 μ l injection volume on a reversed-phase C18 column (Inertsil ODS-2 column, 5 μ m particle size, 4.6 \times 150 mm). The column eluate was monitored at 202 nm. All analyses were performed at room temperature. 9-Anthracenecarboxylic acid was used as an internal standard. The analytical samples were deproteinized by acetonitrile and the supernatant was evaporated in speed vacuum evaporator (Biotron co. Korea). The residue was then reconstituted with methanol. Calibration curve was constructed in the concentration range of 1–40 μ g/ml. The analytical procedure was validated before the implementation by examining its accuracy and precision.

Calibration curves and QC samples

A peak area ratio was used to calculate the concentration of PGE₁ and PGE₁-EE in reference to the internal standard. Standard solutions of PGE₁ and PGE₁-EE were used to spike

blank hairless mouse skin homogenate in order to obtain calibration standards at the concentrations of 1, 2, 5, 10, 20, and 40 µg/ml for both PGE₁ and PGE₁-EE. The calibration standards were prepared on a daily basis. Calibration curves were obtained by plotting the peak area ratios of either PGE₁ or PGE₁-EE to internal standard versus the nominal concentrations. Standard curves of PGE₁ and PGE₁-EE in skin homogenate samples were constructed on three different days to determine the between-run variability of the slopes and intercepts. The regression equations were obtained by the least-squares method using a regression analysis. QC samples for evaluating the accuracy and precision of the method were prepared at concentrations of 3, 8, 15, and 30 µg/ml in skin homogenate for both PGE₁ and PGE₁-EE and stored at -20°C until analysed. Quantification of QC sample concentrations was obtained by interpolation from the equations of the regression lines of the respective calibration curves.

Linearity

From recorded peak areas, the ratios of the drug to internal standard were calculated. Unweighted least-squares linear regression of the peak area ratio as a function of the theoretical concentrations was applied to each standard curve ($y = ax + b$; where x = concentration (µg/ml), y = peak area ratio, a = slope, and b = intercept). The equation parameters (slope and intercept) of each standard curve were used to obtain concentration values for unknown samples.

Concentrations were back-calculated and compared to the nominal concentrations, and the relative concentration residuals (RCRs) were calculated from Eq. 1, where RC is the interpolated concentration and NC is the nominal concentration.

$$\% \text{ RCR} = 100(\text{RC} - \text{NC})/\text{NC} \quad \text{Eq. 1}$$

Specificity

The specificity of the method was investigated by screening six different batches of blank hairless mouse skin homogenate to check if endogenous components were co-eluted with PGE₁, PGE₁-EE, and 9-anthracenecarboxylic acid.

Precision and accuracy

The precision and accuracy of this HPLC method were established by repetitive analyses of QC samples in skin homogenate against a calibration curve. Each QC sample with four concentration levels was analyzed six times consecutively within day ($n = 6$) to determine within-run precision and accuracy, and once a day for six successive days ($n = 6$) at four

concentration levels to determine between-run precision and accuracy. Accuracy was expressed as the recovery ($100 \times$ mean back-calculated concentrations/nominal concentrations), while the precision was given by the between- and within-run relative standard deviations (RSDs).

Determination of the limits of detection and quantitation

The lower limit of detection (LLOD) is the lowest concentration of analyte in the sample that can be detected but not quantified under the stated experimental conditions.¹⁴⁾ The lower limit of quantitation (LLOQ) was defined as the lowest concentration of the analyte in the sample which can be measured with acceptable accuracy and precision under the stated experimental conditions.^{14,16)} The measured concentration of the proposed LLOQ should lie between 80 and 120% of its theoretical concentration and the RSD should be less than 20 % on a day-to-day basis.^{17,18)} LLOD and LLOQ were derived from multiple measurements in the low concentration range and were determined based on the signal-to-noise approach.¹⁹⁾ The level of approximately three times greater than the noise level was defined as LLOD and the level of approximately ten times of the noise level was used as LLOQ,^{16,20)} which is the lowest concentration point in the calibration curve.²¹⁾

Hydrolysis of PGE₁-EE

The hydrolysis of PGE₁-EE in hairless mouse skin homogenate was carried out at 37°C in water bath. All operations to prepare hairless mouse skin homogenate were carried out at 4°C.²²⁾ After sacrificing the hairless mouse by decapitation, the fat and muscular tissues as well as capillaries adhering to the dermis were removed. The skin was minced, mixed with five volumes of cold Tris-buffer (pH7.4) containing 0.15 M KCl, and subjected to a tissue homogenizer (Ultra Turrax T25, Ika Labortechnik, Asia) at 19,000 rpm. There was a pause of 1 min between each 4 times burst to permit cooling of tissue. The whole homogenate was centrifuged at 10,000 × g for 20 min at 0°C. The supernatant was further centrifuged at 10,000 × g for 1 hr with a Centrikon T-1170 ultracentrifuge. The resulting supernatant was stored at -80°C until used in the hydrolysis experiments.

Hydrolysis experiment was initiated by addition of stock solution of PGE₁-EE in hairless mouse skin homogenate, which had been preincubated at 37°C for 10 min. At appropriate time intervals, 50 µl of aliquot was withdrawn and added to 250 µl of acetonitrile under ice bath to deproteinize and prevent further hydrolysis. After centrifugation, the clear supernatant was analyzed by HPLC.

Results and Discussion

This method was very specific and sensitive, and could be employed successfully to follow the time course of the concentration of PGE₁ and PGE₁-EE in the skin-homogenate of

hairless mouse following hydrolysis study.

Retention times and specificity

Typical chromatograms of blank hairless mouse skin homogenate, skin homogenate spiked with 20 µg/ml PGE₁ and PGE₁-EE, and skin homogenate sample obtained from stability study 30 min after starting the stability study in the presence of the internal standard are shown in Figure 2. Observed retention times were approximately 4.5, 9.5 and 18 min for PGE₁, 9-anthracenecarboxylic acid, and PGE₁-EE, respectively. There was no interference during the time intervals between the peaks of PGE₁ and PGE₁-EE, and internal standard.

Linearity

The calibration data and calibration curve parameters (Table III) demonstrate that the calibration curves were linear in the concentration range from 1 to 40 µg/ml for both PGE₁ and PGE₁-EE. The linear regression equation is $y = 0.0023x - 0.0004$ with determination coefficient of 0.9958 for PGE₁, and $y = 0.0049x + 0.0046$ with determination coefficient of 0.9992 for PGE₁-EE. Good linearities for both PGE₁ and PGE₁-EE were demonstrated in terms of peak area ratios as a function of analyte concentrations. The assay has the necessary sensitivity and linearity to cover the concentration range of PGE₁ and PGE₁-EE expected in the stability experiments in hairless mouse skin homogenates.

For each point of calibration standards, the concentrations

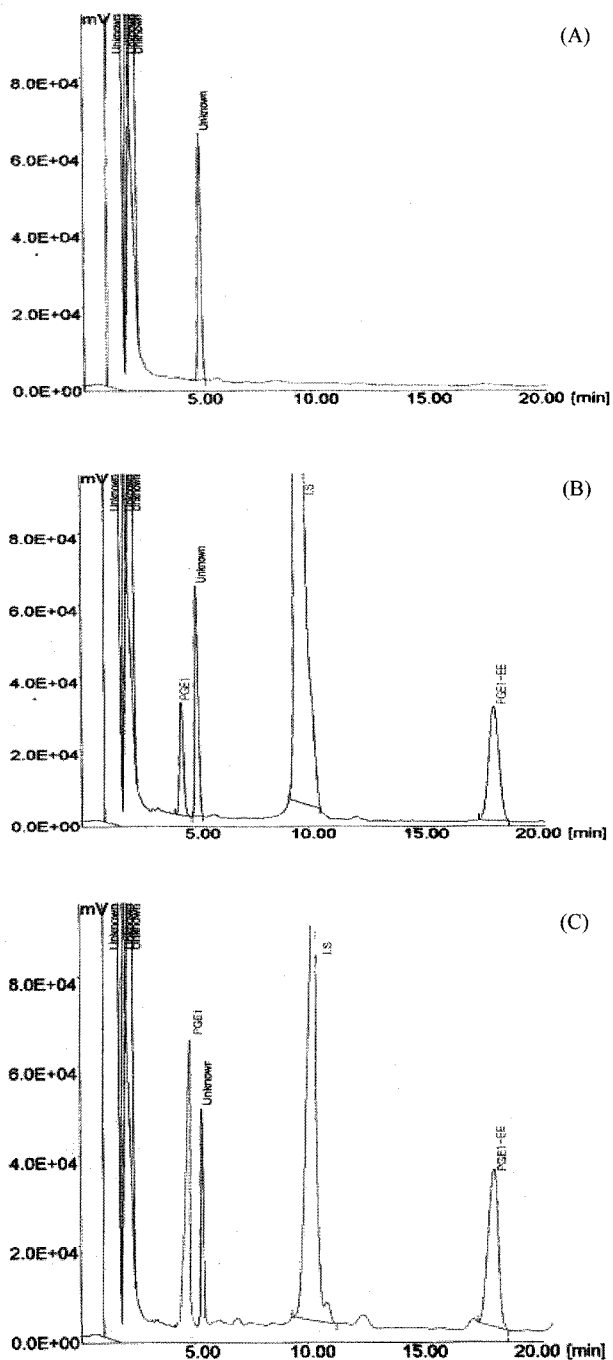


Figure 2—Chromatograms of blank skin homogenate (A), skin homogenate spiked with internal standard (10 µg/ml), PGE₁-EE (20 µg/ml), and PGE₁ (20 µg/ml) (B), and sample obtained 30 min after starting stability test (C) are shown.

Table II—Concentrations Found from Calibration Curve in Skin-homogenate by HPLC

Nominal concentration (µg/ml)	Concentration found (µg/ml)	RSD (%)	RCR (%)
PGE ₁ (n=6)			
1	1.1	11.2	107.3
2	2.1	4.4	106.3
5	4.9	6.8	97.6
10	10.0	6.6	100.3
20	19.7	3.8	98.4
40	40.9	2.8	102.2
PGE ₁ -EE (n=6)			
1	1.1	10.4	110.2
2	2.4	9.2	118.8
5	5.0	5.3	99.3
10	10.7	4.9	107.2
20	20.9	5.7	104.4
40	41.1	1.8	102.8

n, number of replicates. RSD, relative standard deviation. RCR, relative concentration residual.

Table III—Reproducibility of Daily Calibration Curves in Skin-homogenate by HPLC (n=3)

Analysis day	Slope	Intercept	Determination coefficient
PGE ₁			
1	0.002	0.001	0.997
2	0.0024	-0.001	0.9967
3	0.0024	0.0011	0.9938
Mean	0.0023	0.0004	0.9958
S.D. (±)	0.0002	0.0012	0.0018
PGE ₁ -EE			
1	0.0055	0.0054	0.9987
2	0.0046	0.0045	0.9993
3	0.0045	0.0039	0.9996
Mean	0.0049	0.0046	0.9992
S.D. (±)	0.0006	0.0008	0.0005

n, number of replicates.

were calculated from the equation of the linear regression curves and the RSDs were computed. As shown in Table II, from 1 to 40 µg/ml for both PGE₁ and PGE₁-EE, RSDs did not exceed 15% (2.8 to 11.2%); at the lowest concentrations (1 µg/ml for PGE₁; 1 µg/ml for PGE₁-EE), they were 11.2 and 10.4 %, respectively. Within-day variability of calibration standards is presented in Table III. All six validation curves had determination coefficients greater than 0.9938 for both PGE₁ and PGE₁-EE. The RSD of the slope was 8.7% for PGE₁ and 12.2% for PGE₁-EE, showing little day-to-day variability of the slopes. These data clearly demonstrated high reproducibility of the method with excellent linearity.

Precision and accuracy

In Table IV and Table V, the results for accuracy and within- and between-run precisions for QC samples are presented. The accuracy and precisions were within the acceptable criteria as discussed by Hartmann *et al.*²³⁾ and Hubert *et al.*²⁴⁾ The within-run precision was evaluated by replicate analyses of skin homogenate samples containing PGE₁ and PGE₁-EE at four different concentrations. The within-run precision showed RSDs of 2.3 to 4.2% for PGE₁ and 1.6 to 7.5% for PGE₁-EE. The between-run precision similarly evaluated over six consecutive days at four different concentrations varied from 2.4 to 14.1 % for PGE₁ and 2.4 to 11.0% for PGE₁-EE. Accuracy ranged from 100.5 to 119.6% for PGE₁ and from 98.0 to 103.7% for PGE₁-EE in the skin-homogenate of hairless-mouse.

Limit of detection and limit of quantitation

The LLODs were 0.5 µg/ml for both PGE₁ and PGE₁-EE in

Table IV—Coefficient of Variation and Accuracy for Determination of PGE₁ in Hairless Mouse Skin Homogenate by HPLC

Nominal concentration (µg/ml)	Concentration found (µg/ml)	RSD (%)	Recovery (%)
Within-run (n=6)			
3	3.12	4.2	104.0
8	8.04	2.3	100.5
15	15.14	3.1	100.9
30	30.90	3.3	103.0
Between-run (n=6)			
3	3.59	14.1	119.6
8	8.79	2.4	109.9
15	15.60	3.2	104.0
30	32.18	4.9	107.3

n, number of replicates. RSD, relative standard deviation.

Table V—Coefficient of Variation and Accuracy for Determination of PGE₁-EE in Hairless Mouse Skin Homogenate by HPLC

Nominal concentration (µg/ml)	Concentration found (µg/ml)	RSD (%)	Recovery (%)
Within-run (n=6)			
3	3.11	7.5	103.7
8	8.00	1.6	100.0
15	14.70	3.0	98.0
30	29.51	3.7	98.4
Between-run (n=6)			
3	3.09	11.0	103.1
8	8.03	2.7	100.4
15	15.05	2.4	100.4
30	30.42	2.6	101.4

n, number of replicates. RSD, relative standard deviation.

hairless mouse skin homogenate. The LLOQ for PGE₁ was 1 µg/ml and the precision (n=6) at this concentration was 11.2%. The LLOQ for PGE₁-EE was also 1 µg/ml and the precision (n=6) at this concentration was 10.4%, thus acceptable.^{17,18)}

Hydrolysis study

There were many attempt to improve transdermal absorption such as the use of chemical penetration enhancers, novel vehicle systems, *e.g.* microemulsion, liposomal-based delivery system and supersaturated formulations, or more complex physical enhancement strategies, *e.g.* iontophoresis, sonophoresis, and electroporation²⁵⁾ or prodrug approach. A prodrug modification is one of the molecular optimizing approach

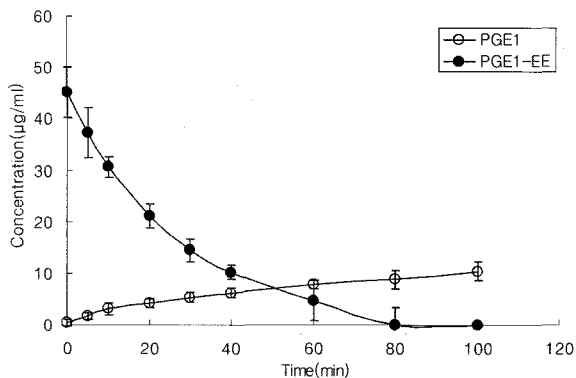


Figure 3—Hydrolysis of PGE₁-EE in hairless mouse skin homogenate. Symbols represent the mean±S.D. (n=3).

with the purpose of changing pharmaceutical and/or pharmacokinetic characteristics of the parent drug and thereby enhancing its skin penetration, efficacy and therapeutic value.²⁶⁾ An essential prerequisite for successful topical use of prodrugs is bioconversion of the prodrug in the skin and plasma.²⁷⁾ The hairless-mouse skin has been widely employed for testing of skin esterase capable of hydrolyzing the ester bond of prodrug.²⁸⁾ In this study, hydrolysis of PGE₁-EE in skin homogenate of hairless mouse was investigated. This method has found applications in assessing bioconversion of propranolol prodrugs for developing dermal delivery systems.^{22,28)} Sheu¹³⁾ suggested that principal mechanism could be attributed to detection of PGE₁ in the receiver solution during skin penetration; the formulation of PGE₁ from the PGE₁-EE during passage through the skin might occur by enzymatic hydrolysis.

Figure 3 showed conversion of PGE₁-EE to PGE₁ in the skin homogenate of hairless mouse by esterase. A constant of decomposition rate was found to be 0.148 min⁻¹ in the skin homogenate of hairless mouse. In this study, the sum of PGE₁ and PGE₁-EE was not consistent with the total amount of initial PGE₁-EE, suggesting that the intermediate might be occurred in the hydrolysis of PGE₁-EE.

Conclusions

A reversed-phase HPLC method with ultraviolet detection was developed and validated for the simultaneous quantitation of PGE₁ and PGE₁-EE in hairless mouse skin homogenate. The results of method validation demonstrated excellent precision and accuracy with acceptable specificity and chromatographic resolution. Good linearity in terms of peak area ratios as a function of analyte concentrations is also demonstrated by the high determination coefficients observed for

the regression lines.

The present method is relatively easy to perform and allows to determine simultaneously PGE₁ and PGE₁-EE in hairless mouse skin homogenate at the microgram level. Assay performance of the present method was assessed both on the basis of the statistical characteristics of individual calibration curves and from the results of QC samples. 9-Anthracenecarboxylic acid was regarded as an acceptable internal standard because it exhibits similar chromatographic properties as the analytes. The LLOQs were 1 µg/ml in hairless mouse skin homogenate for both PGE₁ and PGE₁-EE. The applicability of this newly developed and validated HPLC technique was proved to be satisfactory for stability tests and also can be applicable to absorption study using hairless mouse skin.

New topical formulations containing PGE₁-EE would be developed and tested in hairless mouse skin homogenate for its stability and percutaneous permeability employing this assay.

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