Original article

Analysis of docosanol using GC/MS: Method development, validation, and application to *ex vivo* human skin permeation studies

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A B S T R A C T

Docosanol is the only US Food and Drug Administration (FDA) approved over-the-counter topical product for treating recurrent oral-facial herpes simplex labialis. Validated analytical methods for docosanol are required to demonstrate the bioequivalence of docosanol topical products. A gas chromatography/ selected ion monitoring mode mass spectrometry (GC/SIM-MS) method was developed and validated for docosanol determination in biological samples. Docosanol and isopropyl palmitate (internal standard) were separated on a high-polarity GC capillary column with (88% cyanoprop)aryl-polysiloxane employed as the stationary phase. The ions of *m/z* 83 and 256 were selected to monitor docosanol and isopropyl palmitate, respectively; the total run time was 20 min. The GC/SIM-MS method was validated in accordance with US FDA guidelines, and the results met the US FDA acceptance criteria. The docosanol calibration standards were linear in the 100—10000 ng/mL concentration range (R^2>0.994). The recoveries for docosanol from the receptor fluid and skin homogenates were >93.2% and >95.8%, respectively. The validated method was successfully applied to analyze ex vivo human cadaver skin permeation samples. On applying Abreva® cream tube and Abreva® cream pump, the amount of docosanol that penetrated human cadaver skin at 48 h was 21.5 ± 7.01 and 24.0 ± 6.95 ng/mg, respectively. Accordingly, we concluded that the validated GC/SIM-MS was sensitive, specific, and suitable for quantifying docosanol as a quality control tool. This method can be used for routine analysis as a cost-effective alternative to other techniques.

1. Introduction

Docosanol is used to treat recurrent oral-facial herpes simplex labialis (HSL), also known as cold sores or fever blisters [1,2]. HSL is caused by herpes simplex virus-1 (HSV-1) [2] and is primarily transmitted through contact with an infected person's oral or genital lesions and secretions. During 2015—2016, the prevalence of HSV-1 infection was reportedly 47.8% and 63.6% in the US and global populations, respectively [3]. Docosanol targets HSV-1 by inhibiting viral entry into host cells and preventing the development of drug-resistant mutant viral strains [4]. Topical docosanol is

the only US Food and Drug Administration (FDA) approved over-the-counter alternative to topical penciclovir and acyclovir available for treating HSL [4,5].

Various generic docosanol products are currently in developmental stages, and bioequivalence studies are critical for assessing these products. In order to establish the in vitro bioequivalence of generic docosanol creams, the tested products should demonstrate qualitative, quantitative, physicochemical, microstructural, and in vitro release characteristics similar to those of the reference listed drugs [6,7]. Several attempts have been made to establish a nonclinical bioequivalence assessment, such as in vitro release and ex vivo permeation tests, to obtain market approval for a topical product [6,8]. To demonstrate the bioequivalence of any drug test product, a sensitive, specific, and reproducible analytical method to quantify the drug in study samples is of utmost importance.

Docosanol is a 22-carbon saturated fatty alcohol that lacks chromophores or fluorophores. Hence, fatty alcohols are derivatized to introduce chromophores or fluorophores to facilitate fatty alcohol estimation using the high-performance liquid chromatography-ultraviolet (HPLC-UV)/fluorescence technique [9,10]. Reported HPLC-UV [9] and gas chromatography-mass spectrometry (GC-MS) [11] methods for determination of docosanol require a microwave-assisted and pentafluorobenzoyl derivatization technique for optimal sensitivity. However, the employed derivatization techniques to quantify docosanol in cream, biological, or in vitro samples are time-consuming. Furthermore, reducing the interference of other fatty alcohol contents in formulations remains challenging. Other reported analytical methods for quantifying docosanol without derivatization employ HPLC-evaporative light scattering detector (ELSD) [12] and HPLC-Thermo Scientific™ Dionex™ Corona™ charged aerosol detector (CAD) [13]. Both of these methods have a docosanol limit of detection (LOD) >0.45 µg/mL and require an analysis time >40 min [12,13].

To date, there are no sensitive and specific analytical methods for quantifying docosanol in creams, as well as in in vitro, ex vivo, or clinical study samples (Table S1) [9,12,13]. The present study was designed to develop and validate a simple, specific, and sensitive reproducible GC/MS in the selected ion monitoring (SIM) mode for quantifying docosanol in ex vivo study samples. The developed GC/SIM-MS method was applied to quantify docosanol in ex vivo human skin permeation receptor fluid and human skin homogenates.

2. Materials and methods

2.1. Chemicals and reagents

Docosanol pharmaceutical secondary standard and isopropyl palmitate United States Pharmacopeia reference standard (internal standard, IS) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Human cadaver skin was purchased from Science Care Inc. (Aurora, CO, USA). HPLC-grade solvents, including acetonitrile, methanol, isopropyl alcohol, and chloroform, were procured from Fisher Chemicals (Lenexa, KS, USA). Brij S20–SO-(MH) (Brij) was procured from Croda Inc. (Newark, NJ, USA).

2.2. GC/MS analysis

The method for quantifying docosanol was developed using an Agilent 7890 B GC system, equipped with a 5977A quadrupole mass spectrometer and a 7693 autosampler (Agilent Technologies, Santa Clara, CA, USA). Docosanol and isopropyl palmitate (IS) were separated on an HP-88 capillary column (Agilent J&W Scientific, Folsom, CA, USA), with dimensions of 60 m × 0.25 mm i.d., 0.20 µm film thickness. Helium was used as the carrier gas at a constant flow rate of 1.5 mL/min. The inlet temperature was set to 260 °C in split injection mode, with a split ratio of 5:1. The oven temperature program was initially set to 100 °C, maintained for 1 min, then increased to 240 °C at a rate of 10 °C/min, and isothermal for 5 min at 240 °C, with a total run time of 20 min. The mass selective detector was operated in both scan and SIM modes sequentially. The mass spectra for the scan were recorded at 70 eV from m/z 40–400 amu. Ions with m/z values of 83 and 256 were selected to monitor docosanol and IS, respectively. The MS transfer line was maintained at 280 °C. The MS source and quadrupole temperatures were set to 230 °C and 150 °C, respectively. Data acquisition was performed using the Agilent Mass-Hunter software (version B.07.06). The above instrumentation and chromatographic conditions were used for method development and validation of docosanol.

2.3. Preparation of calibration standards and quality control

For both docosanol and IS standards, 10 mg was accurately weighed and dissolved in chloroform to prepare the primary stock solutions. Working calibration standards of 2, 4, 10, 40, 80, 140, 180, and 200 µg/mL concentrations were prepared from the primary stock solutions of docosanol by subsequent dilutions in 95% isopropyl alcohol in Milli-Q water (diluent). Quality control (QC) working solutions of 2, 6, 100, and 160 µg/mL concentrations were prepared from primary stock solutions in diluent. The IS working solution (50 µg/mL) was prepared from primary working solutions in diluent. Then, 50 µL of working calibration standards/QCs was added to 900 µL of the diluent, followed by the addition of 50 µL of IS working solution. The above samples were vortexed for 2 min and transferred into vials for analysis.

2.4. Analytical method validation

The GC-MS method for docosanol was validated to quantify the docosanol content in human cadaver skin homogenates and receptor fluid used for ex vivo permeation studies.

2.4.1. Lower limit of quantitation (LLOQ) determination

The LLOQ of docosanol was calculated using the formula $10\alpha/S$, where α is the standard deviation of the y-intercepts of the regression lines and S is the slope of the docosanol calibration curve (ICH guideline) [14].

2.4.2. Extraction recovery

The percentage recovery of docosanol and IS from skin homogenates and receptor fluid was determined by comparing the docosanol and IS peak area ratio of QCs spiked in receptor fluid and skin homogenates ($n=6$) with the peak area ratio of QC spiked in diluent.

2.4.3. Specificity and selectivity

The specificity of the analytical method was assessed by injecting six blank matrices (diluent, extracted blank skin homogenates of different donors, and receptor fluid) and six LLOQ working solutions spiked into different matrices. The method specificity was accepted if at least 80% of the analyzed skin homogenate samples presented <20% peak area of LLOQ at the retention time of docosanol [15].

2.4.4. Standard calibration curve

The standard calibration curve was prepared with 100, 200, 500, 2000, 4000, 7000, 9000, and 10,000 ng/mL docosanol. The docosanol and IS peak area ratio of calibration standards versus nominal concentration in diluent were used to construct calibration curves. A weighting factor of $1/x^2$ was used for the linear regression analysis of calibration curves. Calibration curves with a correlation coefficient (R^2) of >0.99 were accepted. The calibration standards were accepted if the measured docosanol concentration at each level deviated from the nominal concentration $(100 \pm 15)\%$, except for the LLOQ, where a deviation of $(100 \pm 20)\%$ was accepted [15].

2.4.5. Precision and accuracy

The precision and accuracy of inter-day and intra-day analyses were evaluated by analyzing six replicates at LLOQ, low-quality control (LQC), middle-quality control (MQC), and high-quality control (HQC) levels of docosanol on four different days. The LLOQ, LQC, MQC, and HQC concentrations employed in the experiments were 100, 300, 5000, and 8000 ng/mL, respectively. The QCs were accepted if the measured docosanol concentration at each level deviated from the nominal concentration $(100 \pm 15)\%$, except

for the LLOQ, where a deviation of (100 ± 20)% was accepted [15]. At LQC, MQC, and HQC levels, a precision of <15% relative standard deviation (RSD) and at LLOQ level precision of <20% RSD were accepted [15].

2.4.6. Stability studies

The stability of docosanol was evaluated in diluent by spiking LQC and HQC concentrations and exposing these samples to different storage conditions. The stabilities of docosanol samples were assessed for their 24 h in autosampler, 8 h at benchtop, 72 h at (5 ± 3) °C, and 72 h at $-(70 \pm 5)$ °C. The concentrations of the stability samples were determined using standard calibration curves. For stability study samples, a (100 ± 15)% accuracy of assay value and precision of <15% RSD were accepted [15].

2.4.7. Dilution integrity

The dilution integrity of the samples was tested to study the effect of dilution on the final concentration of docosanol in receptor fluid or human skin homogenate samples. Six replicates of receptor fluid and skin homogenate samples containing 50,000 ng/mL of docosanol were diluted 10 and 50 times and analyzed. The accuracy of the dilution integrity sample concentration must be (100 ± 15)% of nominal concentrations with a precision of 15% RSD [15].

2.5. Ex vivo study

The docosanol permeability across human cadaver skin and docosanol penetration into human skin were evaluated using a vertical Franz diffusion cell with an active diffusion area of $0.64\ cm^2$. Cryo-preserved human skin was thawed at 32 °C and cut into circular sections. The thawed skin was thoroughly washed with phosphate-buffered saline (pH 7.4) and used for ex vivo permeation and penetration studies. Human cadaver skin was placed between the donor and receiver chambers of the Franz diffusion cell. The epidermal surface of the skin was exposed to donor chambers. The electrical resistance across the skin was measured to ensure skin integrity, and skin segments with $\geq 10\ k\Omega \cdot cm^2$ electrical resistance were used for ex vivo investigations. The receiver compartment was filled with 5 mL of 2% Brij in water (188 µg/mL of docosanol soluble in 2% Brij), which was constantly stirred using a magnetic stir bar at 600 r/min. The temperature of the Franz diffusion cell assembly was maintained at (32 ± 1) °C using a thermostatic water circulator. A dose of $15\ mg/cm^2$ of 10% docosanol cream (Abreva® cream pump and Abreva® cream tube) was loaded into the donor chamber. At predetermined time intervals (0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h), 200 µL of receptor fluid was withdrawn through the sampling port of Franz diffusion cells, and then replaced with an equal volume of fresh receptor fluid. After 48 h, the skin was removed and washed thoroughly to remove the formulation or drug from the skin surface. The skin was weighed, minced into smaller pieces, and homogenized using an ultraprobe sonicator. Docosanol contents in the receptor fluid and skin homogenate were determined using GC-MS.

2.6. Sample preparation

Human cadaver skin was snap-frozen and minced into smaller pieces. The minced skin was homogenized in 950 µL of isopropyl alcohol using an ultrasound probe sonicator, and 50 µL of IS working solution was added to this solution. Fifty microliters of receptor fluid was added to 900 µL of isopropyl alcohol, and 50 µL of the IS working solution was added to the above solution. The above samples were vortexed for 2 min and centrifuged at 13,000 r/min at 4 °C for 10 min. The supernatant was transferred to vials for GC/MS analysis to determine the docosanol content.

3. Results and discussion

3.1. GC/MS method

In a previously reported GC-MS method, docosanol in water samples was derivatized using pentafluorobenzoyl to increase sensitivity, and the run time for analysis was >20 min [11]. The derivatized product should be stable until the sample is analyzed, which is the most challenging aspect of analyzing a derivatized product [16]. In the current method, no derivatization technique was performed to analyze docosanol in samples, and the run time for analysis was 20 min. A reduced run time for analysis is essential for efficiently increasing the turnaround time [17]. In MS analysis, the IS is used to compensate for analyte extraction and signal intensity variation [18,19]. As shown in the representative spectra of docosanol and the IS (Figs. 1 and 2), ions at m/z 83 and 256 were the characteristic ion or base ion for docosanol and the IS, respectively.

These two ions were free of interference from the ions of other compounds or impurities in the matrix. Therefore, these two ions were selected for monitoring docosanol and the IS in the standards and samples. In addition, the GC/SIM-MS mode is more sensitive than the GC/MS method in scan mode [20].

3.2. Method validation

3.2.1. LLOQ determination

The HPLC-ELSD [12] and HPLC-CAD [13] methods for determining docosanol have reported LOD values of 0.45 and 1 µg/mL, respectively. The HPLC-UV [9] derivatized docosanol method reported LOD and LLOQ values of 0.078 and 0.236 µg/mL, respectively. Herein, the developed method was more sensitive than the previously reported methods (Table S1). The LLOQ of docosanol determined using the present analytical method was found to be 0.1 µg/mL. The linear regression equation with a weighting factor of $1/x^2$ was $y = 0.0001115x + 0.0019543$ ($y = mx + c$, where m is the slope and c is the y-axis intercept).

3.2.2. Extraction recovery

Analyte extraction from biological samples is the most crucial step in chromatographic analysis. The recovery of analytes from biological samples should be reproducible, and extraction should provide adequate sample cleanup to prevent the analytical interference of endogenous molecules. The criteria for selecting the extraction method depend on the physicochemical properties of the analyte, as well as the type of matrices analyzed [21,22]. Protein precipitation extraction methods are quick and economical when compared with liquid-liquid or solid-phase extraction methods [22].

Docosanol is a highly lipophilic molecule [23], allowing adequate recovery from matrices where highly lipophilic solvents are required. Docosanol and the IS were extracted from human skin homogenates and receptor fluid using the protein precipitation method. The recovery of docosanol from skin homogenates was lower with methanol than with acetonitrile and isopropyl alcohol. This finding could be attributed to the greater polarity of methanol when compared with that of acetonitrile and isopropyl alcohol [24]. The samples extracted with acetonitrile showed interference at the retention time of docosanol. The samples extracted with isopropyl alcohol provided good sample cleanup without interference at the retention time of docosanol and the IS.

The results of the extraction recovery of docosanol (Table 1) at LLOQ, LQC, MQC, and HQC levels indicated that the recovery of docosanol from human skin homogenate and receptor fluid was precise and reproducible across different concentrations. The recovery of docosanol from the receptor fluid and skin homogenates
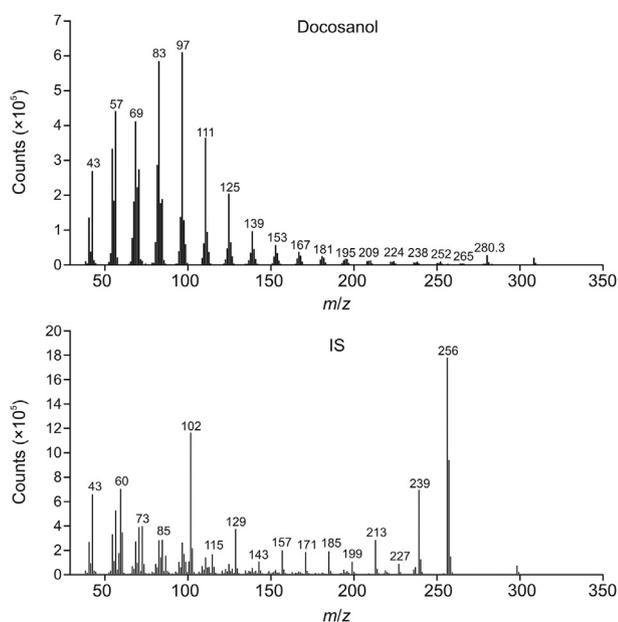
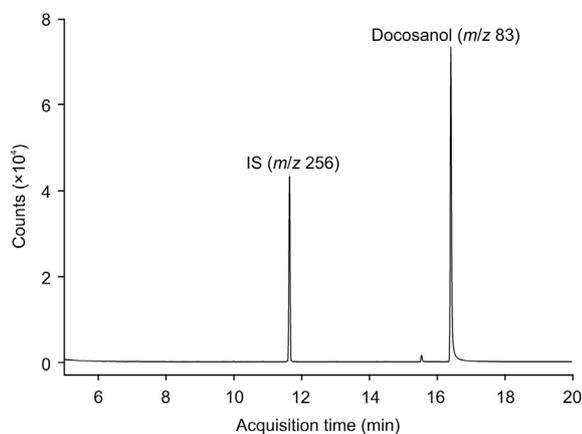**Fig. 1.** The standard spectra of docosanol and isopropyl palmitate (IS).

Fig. 2. The selected ion chromatogram of docosanol and the IS at *m/z* of 83 and 256, respectively.

Table 1
The recovery of docosanol at different concentration levels from receptor fluid and human skin homogenates (*n*=6).

Matrix	Quality control	Recovery (mean ± SD, %)
Receptor fluid	LLOQ	93.2 ± 3.80
	LQC	95.6 ± 1.89
	MQC	96.9 ± 3.41
	HQC	97.5 ± 2.04
Human skin homogenates	LLOQ	97.0 ± 2.50
	LQC	97.4 ± 1.12
	MQC	95.8 ± 2.61
	HQC	97.1 ± 2.46

SD: standard deviation; LLOQ: lower limit of quantitation; LQC: low quality control; MQC: middle quality control; HQC: high quality control.

3.2.4. Standard calibration curve

The docosanol calibration curve was reproducible in the 100–10000 ng/mL concentration range. A linear peak area ratio was observed with R^2>0.994 (range: 0.994–0.998) in the range of docosanol concentrations assessed. The percentage accuracy of calibration standards analyzed on four different days ranged between 98.2% and 103%, which was in accordance with the acceptance criteria. The docosanol method reported by Ahmed et al. [9] is the only method that has reported a calibration curve for docosanol analysis, and the calibration range is 2000 to 12,000 μg/mL, and the LLOQ is 20 times higher than that of the present method.

3.2.5. Precision and accuracy

The precision and accuracy of the developed method were evaluated by analyzing six replicates of QC samples (LLOQ, LQC, MQC, and HQC) on four different days (Table 2). The precisions of intra-day and inter-day analyzed QCs ranged between 2.51%–6.36% and 4.14%–10.4% RSD, respectively. The accuracies of intra-day and inter-day analyzed QCs were 99.2%–102% and 101%–104%, respectively. The intra-day and inter-day method precision and accuracy were in accordance with the acceptance criteria. The results demonstrated that the developed GC/SIM-MS method could be utilized to analyze samples without any intra-day or inter-day variabilities.

3.2.6. Stability studies

The stability of docosanol in receptor fluid was evaluated under possible sample storage and sample handling conditions. The stability of docosanol in receptor fluid was determined by comparing the nominal concentrations at LQC and HQC levels. In receptor fluid, docosanol was found to be stable under the following storage conditions: 24 h in autosampler, 48 h at 32 °C, 72 h at (5 ± 3) °C, and 72 h at −(70 ± 5) °C. The autosampler stability of docosanol samples ascertained that samples placed in the autosampler were stable for 24 h, and the data of the reanalyzed samples would be acceptable. The stability of the docosanol samples at 32 °C for 48 h showed that docosanol was stable in the receptor fluid during ex vivo permeation studies. The concentration of stability samples was within (100 ± 15)% of the actual concentrations (Table 3), which was in accordance with the acceptance criteria.

3.2.7. Dilution integrity

The receptor fluid spiked with higher known concentrations of docosanol diluted by 50 and 10 times presented accuracies in the range of 94.6%–113% and 91.0%–105%, respectively (Table S1). The precision of samples diluted by 50 and 10 times was 6.34% and 5.08%, respectively. Accordingly, the accuracy and precision of dilution integrity met the acceptance criteria. The dilution integrity

was >93.2% and >95.8%, respectively. The recovery of the IS from human skin homogenate and receptor fluid was (98.8 ± 7.65)% and (96.4 ± 3.15)%, respectively, at a concentration of 2500 ng/mL.

3.2.3. Specificity and selectivity

The peaks of docosanol and the IS demonstrated good resolution with the instrumentation and chromatographic conditions described in Section 2.2. The retention time of the IS and docosanol was 11.7 min and 16.5 min, respectively. The method developed was specific without any interference from human skin homogenates or receptor fluid at the retention time of the IS and docosanol in samples. The previously reported HPLC-UV method [9] was developed to quantify docosanol levels in cream samples. HPLC-ELSD [12], HPLC-CAD [13], and GC-MS [11] methods were developed to analyze docosanol in a mixture of 25 fatty acids, lipidomics, and water samples, respectively. The results of the current method indicated that the developed method can be utilized to determine docosanol levels in more complex samples, such as human skin homogenates, without any interference.
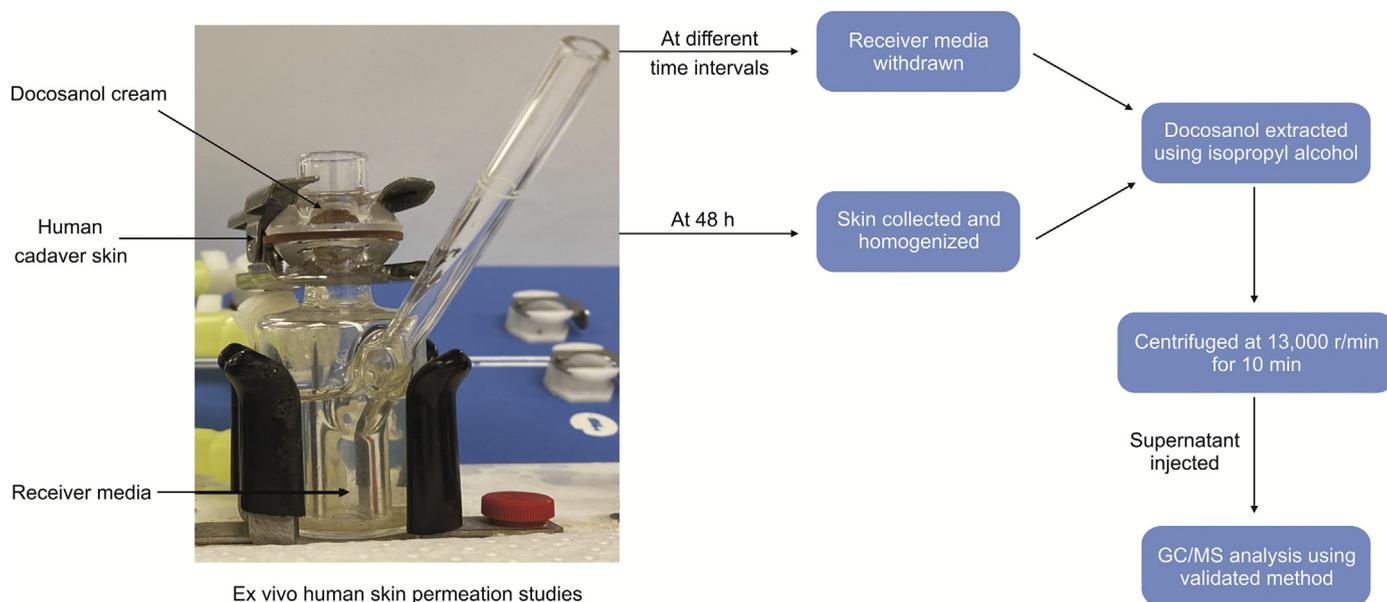
Table 2
The intra-day and inter-day precision and accuracy of the developed method for determination of docosanol.

Quality control	Measured concentration (mean ± SD, ng/mL)		RSD (%)		Accuracy (%)	
	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]
LLOQ	102 ± 6.52	104 ± 10.8	6.36	10.4	102	104
LQC	305 ± 13.3	311 ± 24.4	4.37	7.84	102	104
MQC	4987 ± 184	5058 ± 344	3.68	6.81	99.2	101
HQC	8176 ± 205	8065 ± 334	2.51	4.14	102	101

[a] Intra-day precision and accuracy with 4 replicates at each concentration.
[b] Inter-day precision and accuracy with 24 replicates at each concentration. RSD: relative standard deviation.

Table 3
The stability data of docosanol at LQC and HQC concentrations in receptor fluid (n=6).

Stability test	LQC			HQC		
	Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)	Mean ± SD (ng/mL)	RSD (%)	Accuracy (%)
0 h	287 ± 36.4	12.7	95.7	8414 ± 771	9.17	105
24 h in autosampler	309 ± 25.6	8.42	103	8067 ± 492	6.10	101
48 h at 32 °C	299 ± 18.4	6.16	99.8	7821 ± 392	5.01	97.8
72 h at (5 ± 3) °C	313 ± 10.9	3.50	104	8033 ± 494	6.15	100
72 h at −(70 ± 5) °C	333 ± 17.4	5.21	111	7653 ± 207	2.71	95.7

Fig. 3. A schematic diagram of the analytical procedure used to quantify docosanol in ex vivo permeation study samples.

study indicated that samples with docosanol concentrations above the upper limit of calibration standards can be accurately determined by appropriate sample dilution.

3.3. Ex vivo study

The method validation results revealed that the developed method was specific, sensitive, and reproducible with good extraction recovery for determining docosanol in receptor fluid and human skin homogenates. A schematic representation of the analytical procedure used to quantify docosanol in ex vivo permeation study samples is shown in Fig. 3. The ex vivo permeation of docosanol from both the Abreva® cream tube and Abreva® cream pump was evaluated for up to 48 h, and docosanol levels in receptor fluid were below the LOQ. The permeation of docosanol across the skin was markedly low; previously reported studies have shown that plasma docosanol levels in over 99% of subjects were below the LOQ (LLOQ=10 ng/mL) during the clinical use of 10% docosanol cream [25]. On applying Abreva® cream tube and Abreva® cream pump, the amount of docosanol that penetrated the human cadaver skin at 48 h was 21.5 ± 7.01 and 24.0 ± 6.95 ng/mg, respectively.

4. Conclusion

The GC/SIM-MS docosanol method was developed and validated in accordance with US FDA guidelines. The method was sensitive and reproducible for quantifying docosanol in the examined samples. A simple extraction method was employed to extract docosanol from the receptor fluid and human cadaver skin homogenates

with good extraction recovery. The developed GC/SIM-MS method was successfully employed to determine docosanol concentrations in ex vivo study samples of human cadaver skin homogenates. Furthermore, this method can be applied to determine the concentration of docosanol in formulations, in vitro release testing, and clinical samples to demonstrate the bioequivalence of docosanol products.

CRediT author statement

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2021.08.004.

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