A validated RP-HPLC method to investigate finasteride in human skin after *in vitro* topically applying vesicular nanocarrier

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Abstract: The pharmacotherapeutic efficiency of topical drug delivery systems is mainly dominated by the skin distribution of therapeutic agents. In this work, a sensitive, rapid and fully-validated reversed-phase high performance liquid chromatography (RP-HPLC) method was developed to determine finasteride in human cadaver skin after different vesicular formulations were applied. Drug in different depth of skin layers were measured with an EclipseXDB-C18 column. The mobile phase consisted of 75% (v/v) methanol containing 0.2% phosphoric acid buffered to pH 3.0 with triethylamine under isocratic conditions. The system was operated at 40°C and the mobile phase flow rate was set at 1 mL/min. The standard-calibration curve was linear within range of 5 to 200 ng/ml with correlation coefficient 0.9996. The intra-assay precision was less than 3.9% while the inter-assay precision was less than 7.1% with the bias range of -8.6 to 4.1%. This method was found to be specific, accurate, and sensitive and was successfully used to determine the accumulation of finasteride after *in-vitro* percutaneous delivery by liposomal or ethosomal drug delivery nanocarriers.

Keywords: Finasteride; reverse-phase high-performance liquid chromatography; skin accumulation; ethosomes; liposomes; determination.

INTRODUCTION

Finasteride (FINA), a 5α-reductase inhibitor, which belongs to a 4-aza-3-oxosteroid compound, has been extensively studied and clinically used for the treatment of benign prostatic hyperplasia (BPH) and androgenetic alopecia (AGA). The pharmacological mechanism of FINA is to block the conversion of testosterone to stronger androgenic dihydrotestosterone (Finn and Beadles-Bohling *et al.,* 2006; Boyapati and Sinclair, 2012). 5α-reductase is mainly located in the hair follicle and the prostate (Leavitt and Perez-Meza *et al.,* 2005; Ryu and Kim *et al.,* 2006). Due to the first pass elimination (>20%) and systemic side-effects, it is more desirable to use percutaneous approach to delivery FINA directly into the targeted tissues, in AGA treatment, at least into the basal epidermis or the pilosebaceous unit (Javadzadeh and Shokri *et al.,* 2010).

However, the limitation of common topical formulations of FINA for BPH or AGA therapy was attributed not only to the inadequate drug percutaneous penetration, but also to the heterogeneous distribution of drug in deeper skin tissues (Madheswaran and Baskaran *et al.,* 2012). Better therapeutic results could be achieved using barrier-altering penetration enhancers or the modified vesicular preparations, such as liposome or ethosome (Tabbakhian and Tavakoli *et al.,* 2006; Elsayed and Abdallah *et al.,* 2007; Kumar and Singh *et al.,* 2007; Sinico and Fadda, 2009). In our previous study (Rao and Zheng *et al.,* 2008), a transdermal drug delivery system of FINA-loaded ethosomes was developed and the influence of vesicular nanocarriers was studied. The RP-HPLC methods were reported for quantitative assay of FINA in skin tissue or transdermal receptor medium from *in-vitro* Franz's diffusion experiments. However, the previous methods were not fully validated for quantitative assay of FINA in human cadaver skin slices. And also there were various other analytical methods cited in published studies used for quantitative analysis for FINA for formulation or pharmaceutical purposes (Almeida and Almeida *et al.,* 2005; Chen and Gardner *et al.,* 2008; Brun and Torres *et al.,* 2010; Patel and Patel, 2010; Phapale and Lee *et al.,* 2010). But none of the HPLC methodologies were validated for percutaneous studies. Furthermore, the issue of assay sensitivity for the topical drug delivery becomes more complicated for the reason of low FINA concentration in different depth of skin layers. Considering the heterogeneity and complexity of different skin layers, any analysis of the FINA distribution in skin tissue needs a reliable and specific method. In this study, we present a fully validated RP-HPLC method to investigate FINA at different skin depths after *in-vitro* topically applying vesicular nanocarrier. And the skin accumulation of FINA from either ethosomes or traditional liposomes was successfully compared using this validated method.

MATERIALS AND METHOD

Chemicals and reagents

Human abdominal skin (supplied by Anatomy Department of ZheJiang University, China, and approved by Institutional Review Board of College of Medicine, Zhejiang University). Micronized FINA was obtained

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from Xian Ju Pharmaceutical Co., Ltd, China. Soybean phospholipid (SPL) was obtained from Lipoid GmbH, Germany). Methanol (Merck, Germany) was of HPLC grade without further purification. Purified water was always used (Molecular, PR China).

RP-HPLC conditions

The RP-HPLC method applied for drug concentration assay is comprised of Agilent 1100 modules (Wilmington, USA), with a quaternary pump, mobile phase degasser, auto-sampler with thermostat and a column heater unit. The chromatographic condition was set up as follows: an EclipseXDB-C18 column (150×4.6mm, 5μm, Agilent); mobile phase, 75% (v/v) methanol containing 0.2% phosphoric acid buffered to pH 3.0 with triethylamine; flow-rate set at 1.0 ml/min; detection wavelength set at 230 nm; injection volume of 100 μL; temperature set at 40°C. The FINA content in skin tissue sample was analyzed applying an external standard control.

Sample preparation

The quantitative assay of FINA at different depth of skin layers was carried out on horizontal slices of the skin tissue. When the *in-vitro* Franz's diffusion experiment was terminated, the skin samples, after rinsed three times with normal saline, were then punch biopsied ($2.8cm^2$), fixed in the tissue freezing medium and settled on the cryostat. Horizontal skin layer with thickness of 50 μm was sliced at ambient temperature, and gathered in Eppendorf tube. From each slice, FINA was extracted with 200 μl of 75% (v/v) methanol at 60°C for 15 min under continuously stirring condition. Following the extraction, 200 μl of 1mol/L $HClO_4$ was added to remove proteins by centrifugation at 5000 g for 10 min. The supernatant was filtered with 0.45μm filter membrane and assayed by the RP-HPLC.

To validate the extraction efficiency, a series of blank skin slices was applied in recovery and specificity studies. Some slices were submitted to the measure process and the resolution of FINA was investigated with endogenous components. A series of known amounts of FINA were added to the blank samples and submitted to the above-mentioned extraction and analysis procedures.

Calibration procedures

To prepare calibration curve, six different calibration standards of FINA were fabricated with mobile phase. The concentration ranged from 5.0 to 200.0 ng/ml. The linear calibration curve was obtained by plotting the individual peak area as a function of drug concentration. The calibration curve was applied to assay the recovery of the drug from skin slice samples.

Method validation

(Tesoro and Novakovic *et al.*,, 2005; Collier and Shah *et al.,* 2011)**.** Detection limit and quantitation limit were measured based signal-to-noise (S/N) ratio of 1:3 and 1:10, respectively. The quantitation limit was duplicated for five times. The intraday precision was determined by applying three different concentrations of the FINA, 5, 100 and 200 ng/ml (each in five replicates). The interday precision was tested and the accuracy was examined by the relative standard deviation (RSD).

The efficiency of FINA extraction from skin slices was measured at 5, 100 and 200 ng/ml, in three replicates. The stability study of FINA in skin slice was carried out under two different depository conditions. The test condition included the analysis of quality control sample storage at room temperature for 12 h and at -80°C for 1 month. All samples in the stability study were tested in three replicates. The agent is considered to be stable in skin slice when the 85% to 115% of the initial content was measured.

STATISTICAL ANALYSIS

Statistical analysis was performed by applying Student's t-test. The difference was considered to be significant for the p value of less than 0.05.

RESULTS

Accuracy and precision were established across the analytical range for FINA. Based on the standard calibration curve, the accuracy and the precision were measured and listed in table 1. The results indicated that the intra-assay was less than 3.9% while the inter-assay precision was less than 7.1% with the bias range of -8.6 to 4.1%.

The linearity of this RP-HPLC method was determined by preparing FINA standard calibration curve for the concentration range from 5.0 to 200.0 ng/mL. The regression equation for the calibration curve was y=13.4361x+1.0523. The correlation index between analyte peak areas and concentrations of the FINA was examined with correlation coefficient 0.9996.

The quantitation limit for FINA was calculated to be 5.0 ng/mL. An estimate of the detection limit based signal-to-noise (S/N) ratio of 1:3 was 1.5 ng/mL for FINA. Although FINA was present at a relatively low concentration in skin slices, the quantitation and detection were successfully achieved.

There was no detectable decomposition measured in skin slice spiked with different amounts of FINA at ambient temperature within 12hr or under -80°C for 1month (table 2).

The systemic suitability study ensured the validity of the analytical process as well as confirms the appropriate resolution for the interest analyte. Adequate resolution of

Table 1: Precision and accuracy of RP-HPLC analysis of FINA in human skin slices (n=3, Mean ±S.D.)

Conc. norminal (ng/ml)	Intraday			Interday		
	Measured conc(ng/ml)	Precision (RSD%)	Accuracy (bias%)	Measured conc(ng/ml)	Precision (RSD %)	Accuracy (bias %)
5.0	4.57±0.18	3.9	-8.6	4.67±0.33	7.1	-6.6
100.0	102.5±2.2	2.1	2.5	104.1±4.7	4.5	4.1
200.0	201.1±5.9	2.9	0.55	204.8±11.0	5.4	2.4

Table 2: Stability of FINA in human skin slice by HPLC assay (n=3, Mean ±S.D.)

Added concentration (ng/ml)	Detected concentration (%)		
	RT, 0hr	RT, 12hr	-80□, 1month
5.0	99.1±4.2	98.2±7.1	95.7±6.1
100.0	102.3±3.3	99.9±4.0	97.6±4.5
200.0	100.7±4.4	101.3±5.2	96.9±3.9

Table 3: Profiles of vesicular FINA delivering drug into the strata of human cadaver skin using a Franz diffusion cell after 12 h (Mean ± SD, n=3)

Skin layer	Hydroethanolic (30%,v/v)		Liposomes		Ethosomes	
	Drug content (μg)	Percentage	Drug content (μg)	Percentage	Drug content (μg)	Percentage
Epidermis	31.6±3.7	48.5%	19.3±2.9	47.9%	17.1±2.8	15.4%
Dermis	14.8±2.5	22.8%	7.8±1.1	19.4%	51.0±4.8	46.0%
Receptor fluid	18.7±2.1	28.7%	13.2±1.1	32.7%	42.7±3.2	38.6%

>10 between FINA and the skin extract peak guaranteed the specificity of HPLC method. The mean (n=5) peak area RSD% was 2.4% and the column efficiency was more than 7000 plates/m. All of the key parameters measured were in the acceptable range on all days.

DISCUSSION

In this study, the main purpose was to effectively analysize FINA in percutaneous drug delivery process via the RP-HPLC. The chromatogram of FINA was shown in fig. 2. The chromatographic condition, especially the mobile phase composition was adjusted to obtain appropriate resolution of FINA and extract component in skin slice. There was no interfering peak co-eluting with FINA. The protein precipitation process with 1 N $HClO_4$ and the acidification of mobile phase were found necessary to obtain relatively clear elution chromatogram and to ensure the symmetry the peak shape. The best peak performance was obtained when the mobile phase was optimized to 75% (v/v) methanol containing 0.2% phosphoric acid buffered to pH 3.0 with triethylamine.

Using this validated RP-HPLC, we investigated the *in-vitro* distribution of FINA in human cadaver skin after percutaneous delivery by two different vesicular carriers, ethosomes and liposomes. Experiments were done with modified Franz diffusion cells applying, as donor, vesicular suspensions of FINA ethosomes, liposomes or 30% (v/v) hydroethanolic solution. Fig. 4 showed distribution profiles in dermis slices after 12 hr diffusion experiment of ethosomes, liposomes and the hydroethanolic. It can be clearly seen that ethosomes achieved significant highly homogeneous accumulation of FINA in dermis compared with liposomes or the hydroethanolic. Liposomes, however, showed a gradient distribution of drug in the dermis, which was more obvious in the deep dermis layers.

Twelve-hour of *in-vitro* percutaneous permeation experiments showed an accumulation of FINA in the whole skin ranging from 9.7 to 24.3μg/cm^2. The hydroethanolic solution showed significantly high epidermis drug accumulation, while the drug in the epidermis for the ethosomes and liposomes showed no significant difference (19.3±2.9 vs. 17.1±2.8). However, the ethosomes showed the highest drug amount in skin accumulation and in receptor fluid, especially in deeper dermis layers, in which the drug concentration was 6.5 and 3.4 times higher than that of FINA from liposomes and hydroethanolic solution, respectively. The study also showed, after 12 hr *in-vitro* penetration, 84.6% FINA (46.0% in dermis and 38.6% in receptor fluid) from ethosomes was absorbed, while nearly half of FINA was confined in external layer of the skin for liposomes (47.9%) or for hydroethanolic solution (48.5%). This

distinct reverse distribution profile of ethosomes was definitely favorable for the pharmacological effect exertion of FINA.

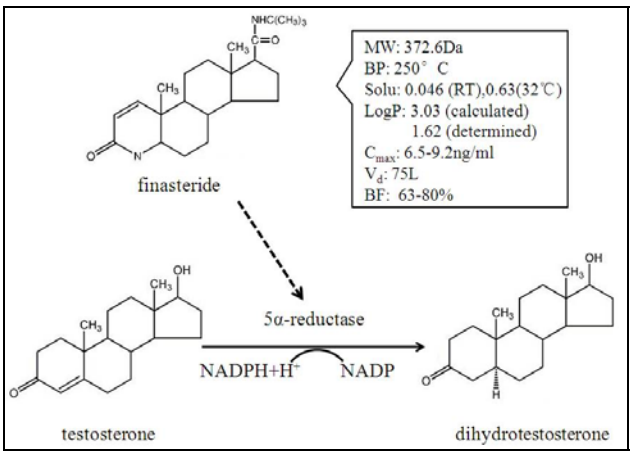

Fig. 1: The action mechanism, Principal Pharmacokinetic parameters and physicochemical properties of FINA.

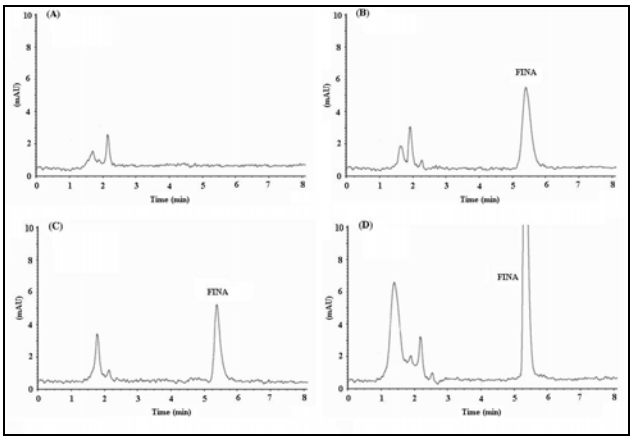

Fig. 2: Typical chromatograms: (A) blank skin slice extract: (B) blank skin slice extract spiked with 20ng/ml FINA: (C) FINA accumulated in skin layers after 12 hr: (D) and a receptor cell sample of Franz diffusion experiments.

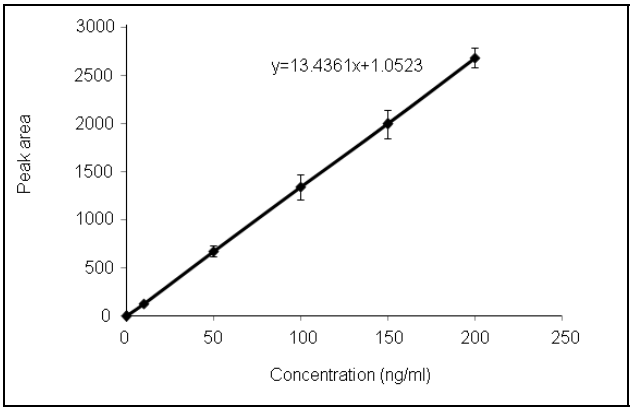

Fig. 3: Calibration curve of finasteride.

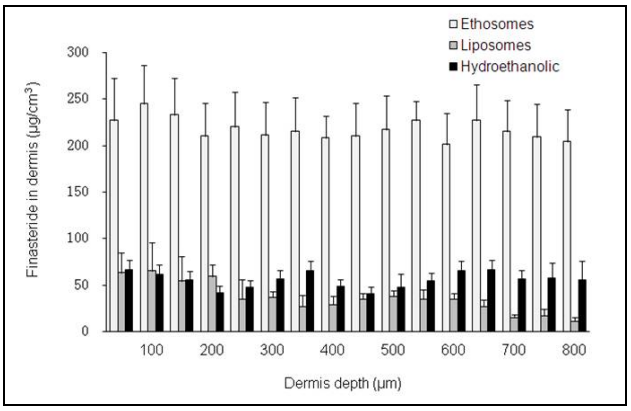

Fig. 4: Distribution of FINA in human skin layers following 12 hr of passive Franz's diffusion experiments (n=3).

CONCLUSIONS

A rapid and sensitive RP-HPLC method for finasteride assay in percutaneous drug delivery studies was established and fully validated for the analytical characteristics such as specificity, linearity, range, precision, accuracy and stability. This method can be concluded to be simple, accurate, reliable and economical. The usefulness of this method is demonstrated by successful application for the assay of finasteride in deferent depth of human skin layers after *in-vitro* applying topical vesicular nanocarrier formulations.

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