# QUANTITATIVE DETERMINATION OF ISOCONAZOLE NITRATE AND DIFLUCORTOLONE VALERATE IN PHARMACEUTICAL CREAMS BY UPLC, HPLC AND IMPROVED DERIVATIVE UV SPECTROPHOTOMETRY

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Derivative ultraviolet spectrophotometry (DS), high performance liquid chromatography (HPLC), and ultra performance liquid chromatography (UPLC) were used to determine Isoconazole nitrate (ISO) and Diflucortolone valerate (DIF) simultaneously. In improved derivative spectrophotometric method, the spectral interference problem of unknown ingredients in this cream was solved by a new approach. Quantitative determinations were performed 289.2 nm (2nd derivative, n=5) for ISO and 262 nm (2nd derivative, n=9) for DIF. Linear calibration ranges and recoveries were 100-500 µg.mL^{-1}, 5-25 µg.mL^{-1} and 99.67%, 102.03% for ISO and DIF, respectively. In HPLC method, linear calibration ranges and recoveries were 15-240 µg.mL^{-1}, 6-60 µg.mL^{-1}, 98.46% 99.41%, for ISO and DIF, respectively. UPLC method was developed and linear calibrations ranges and recovery values were 1-200 µg.mL^{-1} for both ISO and DIF, and 98.37% - 96.49% for ISO and DIF, respectively. UPLC method reduced the analysis time significantly on the other hand organic solvent consumption decreased about 5 times with using less amount of sample.
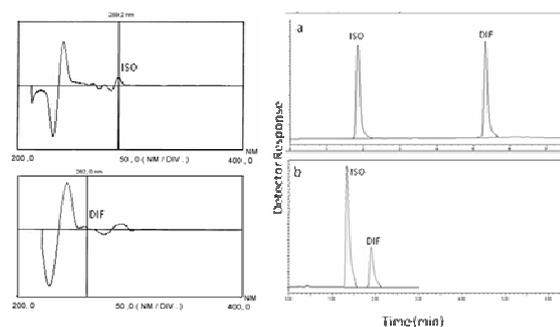
INTRODUCTION

Isoconazole nitrate (ISO), 1-[2-(2,4-dichlorophenyl)-2-[(2,6- dichlorophenyl)methoxy]ethyl]-1H-imidazole mononitrate, is an imidazole antifungal that is active against a broad spectrum of fungi, including Candida species and dermatophytes. It is also active against some gram positive bacteria. ISO is used topically in the treatment of vaginal and fungal skin infections. Diflucortolone valerate (DIF), 6,9-difluoro-11,21-dihydroxy-16-methylpregna-1,4-diene-3,20-dione-21-valerate, is a member of glucocorticoids class III having a high influence that belongs to a family of medications known as topical corticosteroids. DIF has an anti-inflammatory and anti-itching effect. It is used for the relief of inflammation and itching associated with skin conditions that caused by contact or allergic dermatitis, seborrheic dermatitis and psoriasis. Combination of these drugs is used for treatment of fungal infection and cutaneous eruption. The chemical structures of ISO and DIF were shown in Fig. 1.

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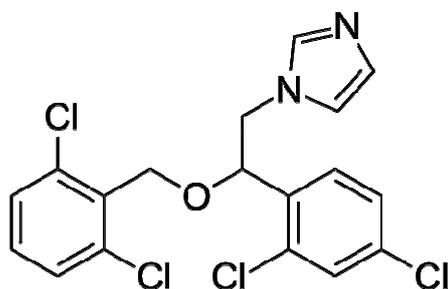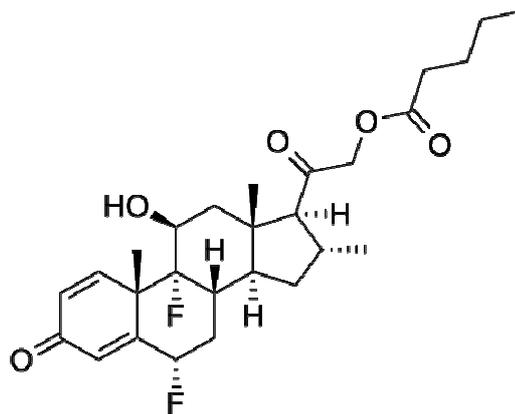
Isoconazolenitrate   **Diflucortolone valerate**

Fig. 1 – Chemical structure of ISO and DIF.

Very few methods appeared in the literature for the determination of ISO based on high performance liquid chromatography (HPLC),[1,2] high performance thin layer chromatography (HPTLC),[3] gas chromatography (GC),[4] and capillary zone electrophoresis.[5] The assay of ISO relies on potentiometric titration was present in British Pharmacopeia (BP) 2004[6] and European Pharmacopeia (EP).[7] In addition, degradation product of ISO in pharmaceutical forms was determined by HPLC.[8] There have been several reports on the determination of DIF by HPLC.[9-13] Also, in BP 2004, the liquid chromatographic method was suggested for the DIF.[6] There is only one literature about simultaneous determination of ISO and DIF by using both spectrophotometric and HPLC methods.[14]

Spectrophotometric methods are the most commonly used techniques and continue its popularity. The main advantages of this method are the simplicity, high precision, and accuracy. Also spectrophotometric methods more economic compared the chromatographic methods. The main problem of spectrophotometric multi-component analysis is the simultaneous determination of two or more active compounds in the same sample without any separation. On the other hand, preservatives are natural or synthetic substances that are added to pharmaceutical forms especially liquid or semi-liquid forms to prevent decomposition by microbial growth or by undesirable chemical changes. In addition to these compounds creams also include other additive substances used in production. Due to their absorption in the UV region, the presence of these compounds prevents a direct conventional spectrophotometric determination of DIF and ISO in pharmaceutical cream. Therefore derivative

spectrophotometry (DS) has been introduced for the resolution of overlapping peaks. In order to eliminate the interference effect coming from unknown ingredients in cream, an improved DS technique[15] was developed. DS method is an analytical technique which consists in the differentiating of normal spectrum by mathematical transformation of spectral curve into a derivative. This method improves resolution bands, eliminates the influence of background or matrix, and gives more detailed spectra due to its minor spectral details.

Liquid chromatography is a separation technique and is also most widely used for resolving mixtures especially in the pharmaceutical, biomedical, and agricultural fields. Chromatographic methods are considered to be best due to its ease of operation, selectivity, rapidity, and reproducibility. Additionally, the availability of a wide range of columns has made chromatography the most popular and acceptable technique for researchers. UPLC can be considered to be relatively new directions of liquid chromatography because of the smaller particles are used as packing material in analytical columns. It is well known that as particle size decreases to less than 2.5 μm, there is a significant gain in efficiency. By using smaller particles, speed and peak capacity can be extended to new limits which are known as ultra-performance. The use of sub-2 μm particles and suitable mobile phases at high linear velocities and instrumentation that operates at higher pressures than those used in HPLC, resulted in dramatic increases in resolution, efficiency, sensitivity and speed of analysis.[16, 17]

The purpose of the present study was to investigate the utility of simple, rapid, precise and fully validated simultaneous determination of ISO

and DIF in raw material and pharmaceutical preparations without the necessity of any time consuming sample pre-treatment step using improved DS, HPLC, and UPLC techniques. UPLC method was developed and applied first time for the determination of DIF and ISO in pharmaceutical forms.

The optimized methods had sufficiently good accuracy, precision and permitted a simple, time and solvent saving assay of this pharmaceutical combination. This proposed method is highly sensitive and specific and can also be used for routine analysis of pharmaceutical formulations consisting of ISO and DIF with a short preparation and analysis time. Analytical data is presented to illustrate the usefulness of the methods in dosage forms. The results of the analysis were fully validated and tabulated from the view point of statistics.[16-21]

RESULTS AND DISCUSSION

Derivative spectrophotometry

Before performing the quantitative determination of ISO and DIF in cream, we investigated the possibility of interference which would come from the additives which are petrolatum, polysorbate, cetostearyl alcohol, sorbitan stearate, EDTA and liquid vaseline present in pharmaceutical cream have absorbance at the UV region of spectrum because of their functional groups. In order to determine whether there was an interference effect coming from these additives, firstly four spectra were obtained: (a) 100 $\mu g.mL^{-1}$ ISO, (b) 10 $\mu g.mL^{-1}$ DIF, (c) synthetic solution contains 100 $\mu g.mL^{-1}$ ISO and 10 $\mu g.mL^{-1}$ DIF (d) cream solution contains 100 $\mu g.mL^{-1}$ ISO and 10 $\mu g.mL^{-1}$ DIF (Fig. 2). When (c) and (d) were compared, it was seen that there was a remarkable difference between cream and synthetic solution signals although including the same amount of ISO and DIF, so there is an interference effect caused by additives from cream. But we did not have any information which ingredients cause this interfering effect. In order to eliminate the interference effect coming from unknown ingredients, the first, second, third and fourth derivative spectra with varying n values (n=1 to 9) of the standard DIF, standard ISO and cream solution (contains the same and accurate quantity of DIF and ISO of standard DIF and ISO solutions) were recorded and overlapped with each other. Among the 36 (4x9) overlapping spectra for each active substance, 2 wavelengths were found to

be the best region without any interfering effect of unknown ingredients. The important point of this study is providing the same and accurate analyte quantities in standard solutions and cream solution. The chosen wavelength for quantitative determination of ISO is 289.2 nm (2nd derivative, n=5). At this wavelength, derivative absorbance value of ISO and derivative absorbance value of cream were precisely overlapped with each other, but derivative absorbance value of DIF is zero (Fig. 3). The chosen wavelength for quantitative determination of DIF is 262 nm (2nd derivative, n=9). At this wavelength, derivative absorbance value of DIF and derivative absorbance value of cream were precisely overlapped with each other, but derivative absorbance value of ISO is zero (Fig. 4).

Optimization of chromatographic conditions

The reversed-phase chromatographic methods were developed to provide a specific procedure suitable for the quality control analysis of ISO and DIF as a reference method for the developed derivative method. Both methods were used in a new, modified way. After optimization of a method for using in HPLC system, this was transferred to UPLC conditions with some modifications. For this reason various mobile phase systems were prepared with the combination of water, methanol and acetonitrile and used to provide an appropriate LC separation. Finally, the mobile phase selected as methanol:water (80:20, v/v) for HPLC and (69:31, v/v) for UPLC techniques which were found to be the most suitable carrier at the flow rates of 1 mL and 0.5 mL respectively. Three different stationary phase which are varied length and particle sizes were also tested in order to find precise and best resolution for both instruments. Phenomenex ODS (250x4.6 mm; 5 μm) and Acquity HSS C18 (50 x 2.1 mm; 1.8 μm) were selected as an analytical column for HPLC and UPLC respectively. The column temperature was set to 25 and 40 °C for both instruments and 25 °C was given a good separation in HPLC system. For UPLC analysis the temperature of the column was increased to 40 °C for reducing the back pressure. Injection volumes were optimized as 20 μl and 5 μl for HPLC and UPLC respectively, with the detection wavelength was chosen as 220 nm according to compounds absorption spectra. The isocratic system was used for the separation. Standard ISO and DIF solutions were eluted, forming well-shaped, symmetrical single peak and separated

from the solvent front. System suitability parameters were calculated to ascertain the suitability of the operating systems. The parameters tested for system suitability included retention time, tailing factor, resolution, theoretical plates, selectivity factor and RSD % of retention time.
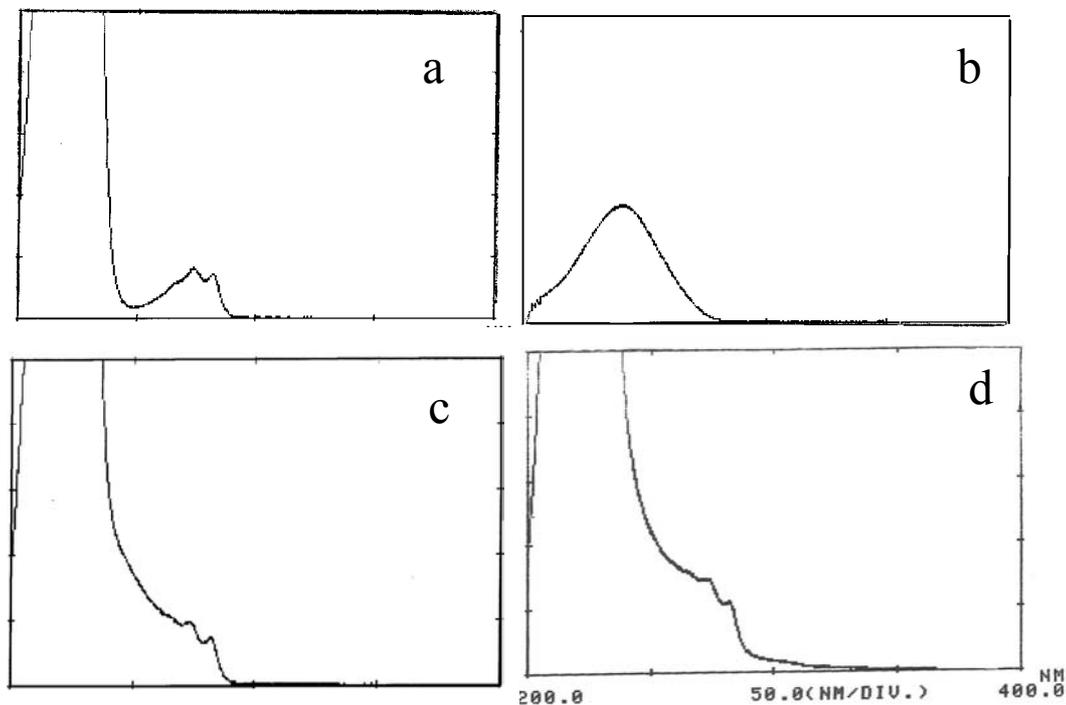

Fig. 2 – UV spectra of **(a)**100 µg.mL^{-1} ISO **(b)**10 µg.mL^{-1} DIF, **(c)** synthetic solution including 100 µg.mL^{-1} ISO and 10 µg.mL^{-1} DIF, **(d) c**ream solution including 100 µg.mL^{-1} ISO and 10 µg.mL^{-1} DIF.
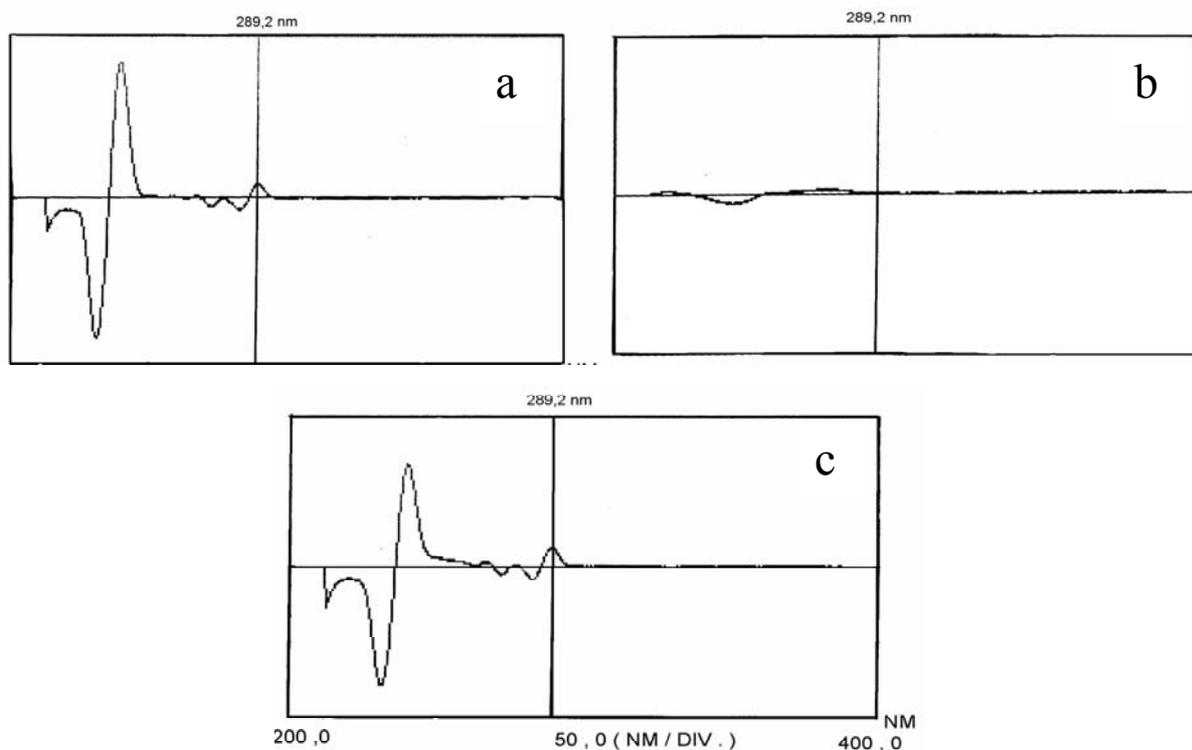
Fig. 3 – The second derivative spectra of **(a)**100 µg.mL^{-1} ISO **(b)**10 µg.mL^{-1} DIF **(c)** cream solution including 100 µg.mL^{-1} ISO and 10 µg.mL^{-1} DIF. The chosen wavelength for ISO: 289.2 nm.
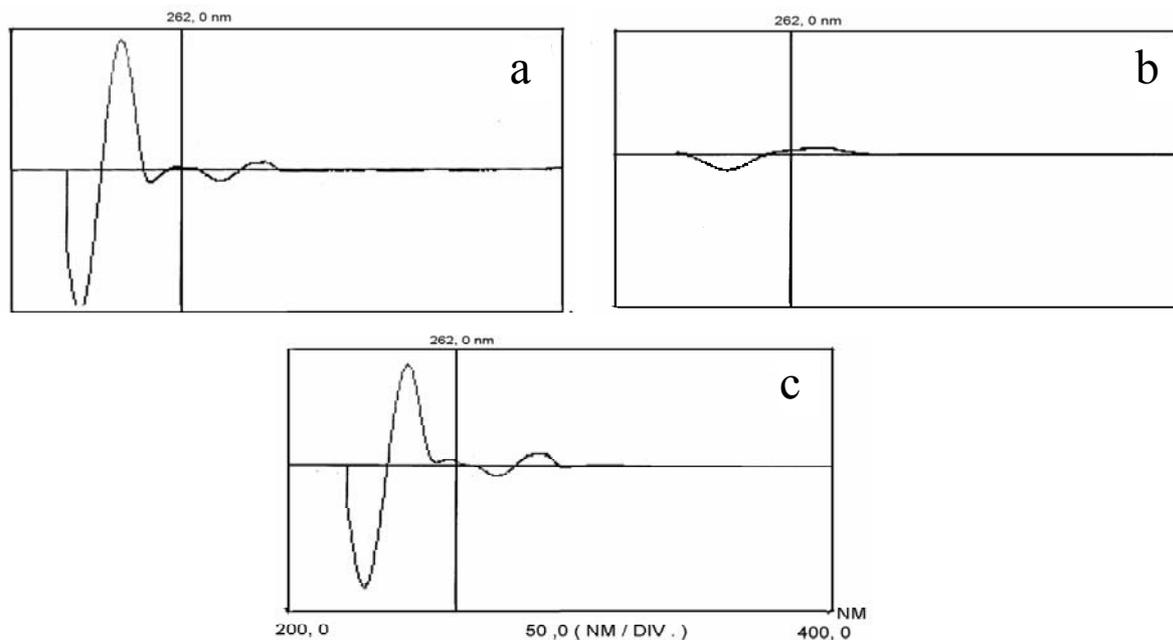
Fig. 4 – The second derivative spectra of **(a)**100 µg.mL^{-1} ISO **(b)**10 µg.mL^{-1} DIF **(c)** cream solution including 100 µg.mL^{-1} ISO and 10 µg.mL^{-1} DIF. The chosen wavelength for DIF: 262 nm.

Table 1

System suitability tests parameters

Technique	HPLC		UPLC	
Compounds	ISO	DIF	ISO	DIF
Retention time	1.84	5.39	1.45	1.95
Selectivity factor (α)	_	3.20	-	1.41
Resolution factor (Rs)	_	5.39	-	1.66
Theoretical plate numbers (N)	2177	5184	8220	38500

The results from system suitability tests are presented in Table 1 for each compound. Typically, at least two of these criteria are required to demonstrate system suitability for the proposed method. [18] As can be seen in Table 1, the presented chromatographic conditions ensure adequate retention of all compounds. By using these chromatographic conditions, retention times of ISO and DIF were found 1.84 and 5.39 min, 1.45 and 1.95 min for HPLC and UPLC respectively. According to these results UPLC method was faster and solvent saver. On the other hand it gives us the opportunity to work with smaller amount of samples. Therefore, the proposed method is safeguarding the environment when comparing with HPLC technique, analysis time reduced more than two times nearly 2.5 and consumption of organic waste was decreased more than five fold.

Determination of ISO and DIF

Simultaneous determination of ISO and DIF was performed simply by reading the $dA/d\lambda$ values at 289.2 nm (2nd derivative, n=5) and 262 nm (2nd derivative, n=9), respectively. Under established experimental conditions, the calibration graph was tested between 100-500 µg.mL^{-1} for ISO in the absence of DIF at 289.2 nm for second derivative spectra. Another calibration graph was also tested between 5-25 µg.mL^{-1} of DIF in the absence of ISO at 262 nm for the second derivative spectra. Typical chromatograms of ISO and DIF are shown in Fig. 5. The relationship between drug concentration and the response was linear, according to equation y= ax+b, where x was the drug concentration and y was the response value (peak area). The calibration curves were obtained using the linear least squares regression procedure.

Linearity ranges of drugs were 15-240 $\mu g.mL^{-1}$, 1-200 $\mu g.mL^{-1}$ for HPLC and 6-60 $\mu g.mL^{-1}$, 1-200 $\mu g.mL^{-1}$ for UPLC for ISO and DIF, respectively. Good linearity was observed in all cases (Table 2). The linearity of the calibration graphs and the adherence of the system to Beer's law are validated by the high values of correlation coefficient, 0.9998 for ISO and 0.9923 for DIF of the regression equations. For the chromatographic separations correlation coefficients were found as >0.999 for both compounds and techniques. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by using the 3.3 s/m and 10 s/m values, respectively, where s is the standard deviation of the signal for the lowest concentration of the calibration standard and m is the slope of calibration curve. [20, 21] The obtained LOD values of ISO and DIF were 2.63 $\mu g.mL^{-1}$ and 0.54 $\mu g.mL^{-1}$ and LOQ values were 8.78 $\mu g.mL^{-1}$ and 1.78 $\mu g.mL^{-1}$ respectively for the DS method. When compared to previous spectrophotometric study, developed method are more sensitive than the literature results.[14] The values corresponding to LOD and LOQ were determined 0.25 $\mu g.mL^{-1}$ and 0.82 $\mu g.mL^{-1}$ for ISO, 0.45 $\mu g.mL^{-1}$ and 1.51 $\mu g.mL^{-1}$ for DIF, respectively for HPLC and 0.11 $\mu g.mL^{-1}$ and 0.35 $\mu g.mL^{-1}$ for ISO, 0.09 $\mu g.mL^{-1}$ and

0.27 $\mu g.mL^{-1}$ for DIF, respectively for UPLC. Linear equations, correlation coefficient and other validation data were tabulated in Table 2.

After optimization and calibration results, the methods were applied to cream for quantification of ISO and DIF simultaneously (Fig. 6). It was found that the obtained results are in good agreement with the declared content (Table 3). The results of both proposed methods were statistically compared using Student's t-test and Fisher test. As shown in Table 3, the calculated t-values were less than the theoretical values, indicating no significant difference between the mean contents of ISO and DIF obtained by proposed methods. In similar way, the calculated F-values were less than the theoretical values, indicating no significant difference between precisions. In order to test the accuracy of the proposed method, recovery studies were used. The recovery studies were performed by spiking appropriate amount of stock solutions of the ISO and DIF into the cream sample. The results of the recovery analysis were shown in Table 3. The results concluded that the proposed methods are sufficiently accurate and precise in order to be applied to pharmaceutical dosage forms. High percentage recovery data show that the method is free from the interferences of the excipients used in the formulations.
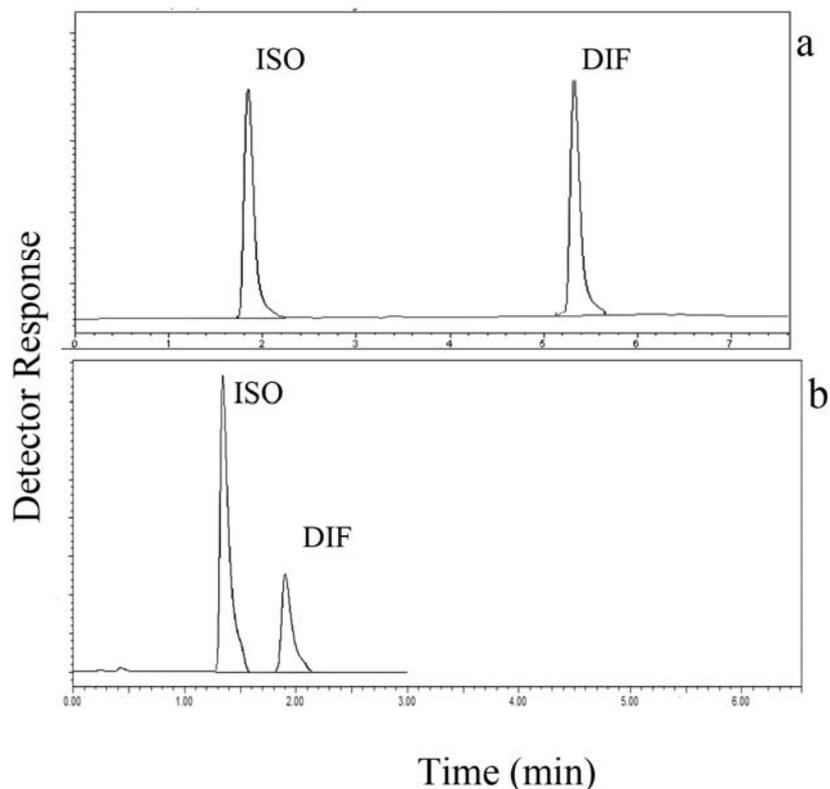

Fig. 5 – Chromatogram of synthetic mixture contains 100 $\mu g.mL^{-1}$ ISO and 100 $\mu g.mL^{-1}$ DIF; HPLC **(a)** and UPLC **(b).**

Table 2

Regression data of the calibration lines for quantitative determination of ISO and DIF by proposed techniques

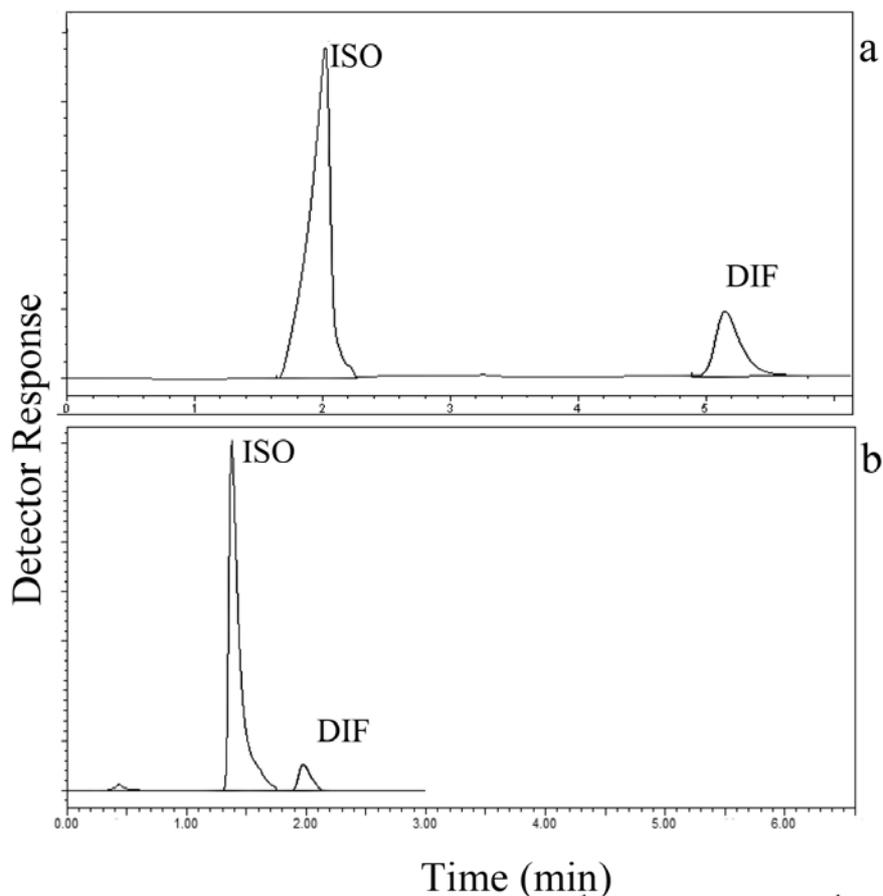| Compounds	UPLC		HPLC		UV-VIS	
	ISO	DIF	ISO	DIF	ISO	DIF
Linearity range ($\mu g.mL^{-1}$)	1-200	1-200	15-240	6-60	100-500	5-25
Slope	25519	8090	10.59	25.17	1.74×10^{-3}	5.62×10^{-3}
Intercept	-4348.9	-9.205	-31.41	3.18	-1.49×10^{-2}	-5.3×10^{-3}
Correlation coefficient	0.999	0.999	0.999	0.999	0.999	0.992
SE of slope	7.78×10^{1}	1.48×10^{1}	1.15	0.41	2.28×10^{-5}	4.03×10^{-4}
SE of intercept	5.82×10^{3}	1.150×10^{3}	19.19	15.15	7.58×10^{-3}	6.68×10^{-3}
Limit of detection ($\mu g.mL^{-1}$)	0.11	0.09	0.25	0.45	2.63	0.53
Limit of quantification ($\mu g.mL^{-1}$)	0.35	0.27	0.82	1.51	8.78	1.78
Within-day precision[a] (RSD%)	0.93	0.68	0.83	0.40	0.66	0.16
Between-day precision[b] (RSD%)	1.16	1.19	0.68	0.31	0.62	0.17

[a] Each value is obtained from five experiments.
[b] Between-day reproducibility is determined from five different runs over three days period.

Fig. 6 – Chromatogram of cream solution contains 100 $\mu g.mL^{-1}$ ISO and 10 $\mu g.mL^{-1}$ DIF, HPLC **(a)** and UPLC **(b)**.

Table 3

Assay results and mean recovery studies of ISO and DIF in pharmaceutical dosage forms

Compounds	UPLC		HPLC		UV-VIS	
	ISO	DIF	ISO	DIF	ISO	DIF
Labeled claim (mg/1 g)	10.00	1.00	10.00	1.00	10.00	1.00
Amount found (mg/1 g)[a]	9.77	0.97	10.11	1.03	9.82	1.00
RSD (%)[a]	0.26	0.91	2.80	1.63	2.03	3.67
Bias (%)	2.30	3.00	-1.10	-3.00	1.80	0.00
t_{value}	1.75	1.66	1.87	1.91	t_{theo}: 2.31	
F_{value}	1.16	1.82	2.01	5.86	F_{theo}: 6.39	
Recovery (%)	98.37	96.49	98.46	99.41	99.67	102.30
RSD% of recovery[a]	0.25	1.63	2.33	2.63	1.13	1.96
Bias (%)	1.63	3.51	1.54	0.59	0.33	-2.30

[a] Each value is the mean of five experiments.

EXPERIMENTAL

Apparatus

A Shimadzu UV-160 A double beam UV-visible spectrophotometer was used. The second derivative spectra of the standard and the sample solutions were recorded in 1 cm quartz cells against the blank solvent at 200-400 nm range. The spectral band width was 2 nm and the scan speed was set to 480 nm. Nuve Fuge CN 090 centrifuge and a Bondelin Sonarex type sonicator were used in entire study. The HPLC system consisted of model Agilent Technologies 1200 series solvent delivery system with a UV detector set to 220 nm. This wavelength was used for both chromatographic methods. The separation was performed on a Phenomenex ODS column (250 x 4.6 mm i.d., 5 μm). The UPLC system consisted of Waters Acquity system equipped with DAD detector. The chromatographic separation was performed using a Waters Acquity HSS C18 (50 x 2.1 mm, 1.8 μm) at 40 °C. Chromatographic grade water was obtained following distillation in glass and passage through a Milli-Q® system (Millipore, Milford, MA, USA) and was used to prepare all necessary solutions. The mobile phase consisted of a mixture of methanol:water (80:20, v/v) and (61:39, v/v) for HPLC and UPLC respectively with the injection volumes of 20 μL and 5 μL. The mobile phase was prepared daily, filtered, degassed before use and delivered at a 1 mL.min^{-1} for HPLC and 0.5 mL.min^{-1} for UPLC experiments.

Reagents and solutions

ISO and DIF were supplied by Bilim Drug Company (Ankara, Turkey) and dosage form Travazol® cream was purchased from the local market. All chemicals and solvents were analytical reagent grade. Chromatography grade, acetonitrile, methanol and analytical grade uracil, HCl, H_2O_2, H_3PO_4 (%85), NaOH, were obtained from Sigma-Aldrich (Munich, Germany). Doubly distilled water with conductivity

lower than 0.05 μScm^{-1} was used for preparing the mobile phase solutions.

For the preparation of standard stock solutions (1000 μg/mL), 25.0 mg ISO and 2.5 mg DIF were separately weighed, dissolved in ethanol and then adjusted to 25.0 mL with the same solvent. Standard solutions in the range of 100-500 μg·mL^{-1} and 5-25 μg·mL^{-1} were prepared by diluting the stock solution with ethanol for ISO and DIF respectively. For the chromatographic assays; in order to prepare ISO stock solution, 50.0 mg ISO was accurately weighed, dissolved in methanol and diluted to 25.0 mL. Standard solutions ranging from 15-240 μg·mL^{-1} and 1-200 μg·mL^{-1} for HPLC and UPLC respectively were prepared by adding mobile phase as solvent. For preparing stock solution of DIF, 24.0 mg DIF was weighed, dissolved in methanol and diluted to 25.0 mL. Standard solutions in the range of 6-60 μg·mL^{-1} and 1-200 μg·mL^{-1} were prepared by the addition of the mobile phases for HPLC and UPLC respectively. All of these solutions were kept in dark at the refrigerator at about +4°C.

Interference studies

Standard solutions containing 100 μg/mL ISO and 10 μg/mL DIF, cream solution containing 100 μg/mL ISO plus 10 μg/mL DIF and synthetic mixture solution containing 100 μg/mL ISO plus 10 μg/mL DIF were prepared. In addition to these solutions, the other substances in the cream such as petrolatum, polysorbate, cetostearyl alcohol, sorbitan stearate, EDTA and liquid vaseline were dissolved in ethanol and UV spectra were obtained in the 200-400 nm wavelength range.

Analysis of cream

Two and half grams of cream containing ISO and DIF were accurately weighed in a beaker and dissolved in ethanol. The solution was sonicated in an ultrasonic bath for 10 min, then transferred to a 25 mL volumetric flask and the volume was adjusted to the mark with ethanol for DS analysis. ISO

and DIF contents of the cream were calculated by referring to calibration curves obtained by using standard solutions of ISO and DIF. The second derivative amplitudes of ISO and DIF at 289.2 (n = 5) and 262 (n = 9) nm were used to create the calibration curves. On the other hand same amount of cream was taken then dissolved in 25.0 mL methanol and degassed in an ultrasonic sonicator for 10 min. One milliliter of this solution was then diluted 10.0 mL with mobile phase. In order to determine ISO and DIF contents of cream, standard solutions of ISO and DIF were injected and calibration curves were obtained as peak area versus concentration. By using calibration curve, quantitative determinations of ISO and DIF in cream were performed.

The proposed method was validated as to precision (reported as the relative standard deviation, RSD %), linearity (evaluated by regression equations), detection and determination limits and accuracy. The limit of detection (LOD) and limit of quantitation (LOQ) of the procedure are also obtained, which were calculated according to the 3 s/m and 10 s/m criterions,[20, 21] respectively, where s is the standard deviation of the response of the lowest amount of the linearity range and m is the slope of the corresponding calibration curve.

The precision studies were determined with different concentrations and are confirmed by RSDs % values. Based on these results, there was no significant difference for the assay, within-day and between-days. Repeatability (intra-day precision) was investigated by injecting five replicate run of the samples. Reproducibility (inter-day precision) was assessed by injecting the samples over three consecutive days for all methods. Precision data in terms of within-day and between-day variability are summarized in Table 2 and accuracy was determined by recovery studies (Table 3).

CONCLUSIONS

A new, rapid, sensitive, and validated improved derivative UV spectrophotometry, HPLC and UPLC methods were developed for the direct and simultaneous determination of ISO and DIF in pharmaceutical creams without any time-consuming extraction steps. Using described spectrophotometric and chromatographic conditions ISO and DIF were determined easily. Especially, interference effect coming from unknown ingredients in cream was eliminated by using improved derivative spectrophotometric study. This method did not require any time consuming sample treatment steps. When proposed methods were compared to previous study, LOD and LOQ values show that these methods were more sensitive. From the view of statistical point, there was no significant difference between spectrophotometric, HPLC, and UPLC methods. High percentage recovery values showed that the methods are free from the interferences of the additives in cream. According to the results UPLC method was faster and solvent saver on the other

hand it gives us the opportunity to work with smaller amount of samples. When comparing with HPLC technique, analysis time reduced more than two times and consumption of organic waste was decreased more than fivefold. In addition to these statements, proposed method can be called as environmental friendly when compared with published method.[14] The described methods give accurate, precise and reproducible results for determination of ISO and DIF mixtures and are easily applied for routine analysis.

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