Synthesis A Reagent [3-Hydroxy 4- (1-Azo-2,7-Dihydroxy) Naphthalene Sulfonic Acid] and Used it for Determination of Flurometholone in Bulk and Pharmaceutical Formulations by Spectrophotometric Method

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Abstract
A Reagent [3-hydroxy 4- (1-azo-2,7-dihydroxy) naphthalene Sulfonic acid] (AAN) has been synthesized for the determination of flurometholone (FLU) in pure form and in ophthalmic suspensions (drops) by a simple, sensitive and extraction-free spectrophotometric method. The method is based on the formation of yellow colored complex between FLU and ANN maximum at 416 nm. The stoichiometry of the complex in either form was found to be (1:1). Reaction conditions were optimized to obtain the maximum color intensity. Beer’s law was obeyed in the concentration ranges of 0.5-17.0 µg/mL. The limit of quantification (LOQ) was 0.14 µg/mL and molar absorptivity (ɛ) values was 38555 L/moL·cm⁻¹. The proposed method has been applied successfully to the analysis of FLU in pure form and in its dosage forms and no interference was observed from common excipients present in pharmaceutical formulations. Statistical comparison of the results with the reference method showed excellent agreement and indicated no significant difference in accuracy and precision.

Keywords: Spectrophotometry; Synthesis; Flurometholone

Introduction
Flurometholone (FLU) Systematic name is 9-Fluoro-11β, 17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione (Figure 1). Flurometholone is an ophthalmic suspension 0.1% which is a topical anti-inflammatory agent for ophthalmic use. Flurometholone is indicated for the treatment of corticosteroid-responsive inflammation of the palpebral and bulbar conjunctiva, cornea and anterior segment of the globe [1].

The assay of FLU in pure and dosage forms requires more investigation. The different analytical methods that have been reported for its determination include HPLC [2], with UV by using 1,4-Dihydrazinophthalazine as reagent [3], derivative spectrophotometry and HPLC [4], UV spectrophotometric by using methanol and sulfonic acid Buffer pH=3 [5], HPTLC [6].

The aim of this work is to synthesize organic reagents and used it in spectroscopic analytical study for the determination of Flurometholone (FLU) through complexation with new complex dye [3-hydroxyl 4-(1-azo-2,7-dihydroxyl) naphthalene Sulfonic acid] (AAN) (Figure 2) in dichloromethane medium. The colored product was quantified spectrophotometrically using absorption bands at 416 nm for complex of (FLU-AAN) and at 223 nm medium. The colored product was quantified spectrophotometrically using absorption bands at 416 nm for complex of (FLU-AAN) and 363 nm medium. The colored product was quantified spectrophotometrically using absorption bands at 416 nm for complex of (FLU-AAN) and 363 nm medium.

A dye [3-hydroxy 4-(1-azo-2,7-dihydroxy) naphthalene Sulfonic acid] (AAN) (1 × 10⁻⁴ M) prepared by shaking 10.42 mg of Fumeron Fort (Rama Pharma Company for pharmaceutical industry, China) with dichloromethane in a 250 mL calibrated flask. A dye [3-hydroxy 4-(1-azo-2,7-dihydroxy) naphthalene Sulfonic acid] (AAN) (1 × 10⁻⁴ M) prepared by shaking 10.42 mg of Fumeron Fort (Rama Pharma Company for pharmaceutical industry, China) with dichloromethane in a 250 mL calibrated flask.

Experimental
Apparatus
Infrared Spectrometer (FTIR) from company Bruker (Germany) model ALPHA, (LC-MS) from company (Shimadzu) UFLC Shimadzu model LC MS-2010 EV. Melting point KRUS (Germany) model CE-KSP1, spectrophotometric measurements were made in Jasco company model LC MS-2010 EV. Melting point KRUSS (Germany) model CE-120. A stock solution of FLU (2.0 × 10⁻⁴ M) were prepared by dissolving the appropriate weight of FLU in 70 mL dichloromethane and the volume were diluted to the mark 100 mL in calibrated flask with dichloromethane and take from the last solution 1 mL to the calibrated flask 10 mL too with the same solvent. Working standard solutions were prepared from suitable dilution of the standard stock solution.

Working standard solution was prepared daily by added different volumes of stock solutions to 2 mL of reagent BCG (1 × 10⁻⁴ M) diluting to 10 mL with dichloromethane.

The concentration of FLU (0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 9.0, 12.0, 15.0, 17.0 µg/mL ) were used for the analysis of FLU by the spectrophotometric method. The method was based on forming complex between Synthesis AAN dyes and FLU in dichloromethane medium. The colored product was quantified spectrophotometrically using absorption bands at 416 nm for complex of (FLU-AAN) and at 416 nm for (FLU-AAN).

A dye [3-hydroxy 4-(1-azo-2,7-dihydroxy) naphthalene Sulfonic acid] (AAN) (1 × 10⁻⁴ M) prepared by shaking 10.42 mg of AAN dye in 100 mL dichloromethane to dissolve and made up to mark with dichloromethane in a 250 mL calibrated flask.

Flurometholone ophthalmic suspensions brand name is Fumeron, Fumeron Fort (Rama Pharma Company for pharmaceutical industry,

Reagents and solutions
Pharmaceutical form of Flurometholone (FLU 99.88%) was received from Univision Pharmaceutical Co. Ltd. (China). A stock solutions of FLU (2.0 × 10⁻⁴ M) were prepared by dissolving the appropriate weight of FLU in 70 mL dichloromethane and the volume were diluted to the mark 100 mL in calibrated flask with dichloromethane and take from the last solution 1 mL to the calibrated flask 10 mL too with the same solvent. Working standard solutions were prepared from suitable dilution of the standard stock solution.

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Received February 29, 2016; Accepted March 23, 2016; Published March 28, 2016

Citation: Sakur AA, Okdeh M, Al Fares B (2016) Synthesis A Reagent [3-Hydroxy 4-(1-Azo-2,7-Dihydroxy) Naphthalene Sulfonic Acid] and Used it for Determination of Flurometholone in Bulk and Pharmaceutical Formulations by Spectrophotometric Method. Mod Chem appl 4: 177. doi:10.4172/2329-6798.1000177

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Citation: Sakur AA, Okdeh M, Al Fares B (2016) Synthesis A Reagent [3-Hydroxy 4- (1-Azo-2,7-Dihydroxy) Naphthalene Sulfonic Acid] and Used it for Determination of Flurometholone in Bulk and Pharmaceutical Formulations by Spectrophotometric Method. Mod Chem appl 4: 177. doi:10.4172/2329-6798.1000177

Syria) containing 100 mg and 250 mg in 1 mL, Fludrop, Fludrop Fort (Obary Company, Syria) containing 100 mg and 250 mg in 1 mL, Methoflor (Dyamond pharmaceutical industry, Syria) containing 100 µg in 1 mL. And also there is FLORA-T (Medico pharmaceutical industry, Syria) containing 100 µg in 1 mL from local medical stores. All reagents and solvents were of analytical grade.

Synthesis reagent AAN

The synthesis Reagent is AAN according the following diazotization reaction (Figure 3) [7].

Spectrophotometric procedure

Increasing volumes of FLU working standard solution were transferred into series of 10 mL volumetric flasks that contain 2 mL of AAN reagent (1 × 10^-4 M). Solutions were mixed gently and allowed to stand at room temperature. Volumes were made up to mark with dichloromethane and mixed before the spectra were recorded at 416 nm against reagent blank that had been treated similarly.

Determination of FLU/Dye stoichiometric relationship

The composition ratio of drug FLU to dye (AAN) of the colored complex was determined using the molar ratio and continuous variation methods.

Procedure for pharmaceutical samples

An accurately volume amount of the sterile ophthalmic suspensions (drops) equivalent to 75.29 µg of FLU was transferred into 10 mL volumetric flask and added 2 mL of AAN, and diluted with dichloromethane up to the mark. After then the spectra was recorded at 416 nm against reagent blank that had been treated similarly.

Results and Discussion

Dye structure identification (AAN)

Physical properties: Weight: 410 g/mol. Appearance: the dye (AAN) appears red color powder. The solutions in water are stable. Solubility: The dye (AAN) free Soluble in water, alcohol, dimethylformamide; insoluble in acetone. Melting point: 111-116°C.

UV-Visible spectrophotometry: The Figure 4 shows the spectra of solution (0.01%) of dye AAN in ethanol at λ_max = 498 nm.

IR Spectrophotometry: The Figure 5 shows the FTIR spectra of potassium bromide disk the Distinctive peak

ν =1513 cm^-1 (N=N )
ν =3411 cm^-1 (O=H )
ν =1620 cm^-1 (C=C )

LC-MS: The Figure 6 shows the separation chromatogram of AAN by using mobile phase (Water: Methanol) (30:70); after separation MS achieved after applying negative and positive volt (Figure 7). The result shows that the weight of reagent is 410 g/mol.
Optimization of reaction conditions for (FLU-AAN)

**Solvent effect:** In order to select a suitable solvent for preparation of the reagent solutions used in the study, the reagents were prepared separately in different solvents such as, chloroform methanol, dichloromethane and dichloromethane, and the reaction of FLU with AAN was followed. Dichloromethane was suited for the complete forming with AAN. Similarly, the effect of the diluting solvent was studied for the method and the results showed that none of the solvents except dichloromethane formed sensitive and stable colored in method. Therefore, dichloromethane was used for dilution throughout the investigation. Dichloromethane was preferred as the most suitable solvent because in this medium, the reagent blank gave negligible blank absorbance and the formed ion-pair complex was found to exhibit higher sensitivity and stability. In other solvents, the reagent blank yielded high absorbance values.

**Effect of reaction time and stability:** The optimum reaction time for the development of color at ambient temperature (25 ± 2°C) was studied and it was found that the complex forming after added the reagent and no time necessary for the complete formation of ion-pair complexes in a method giving yellow colored solutions have maximum absorbance at λmax. The formed color was stable for more than 24 h in method.

**Effect of dye concentration:** The influence of the concentration of AAN on the intensity of the color developed at the selected wavelength and constant drug concentration was studied. As shown in Figure 8, the constant absorbance readings were obtained between (0.25-5 mL) of (1 × 10⁻⁴ M) of AAN, 2 mL of each AAN was used for methods A and B, respectively.

**Stoichiometric ratio:** Molar ratio method [8]: The stoichiometry of (FLU: Dye) complex by molar ratio method according to following equation: 
\[
\lambda_{\text{max}} = \frac{[\text{FLU}]}{[\text{Dye}]}
\]
confirms that the ratio of complex FLU:AAN is equal to 1:1 (Figure 9).  
Job’s method [9]: In order to establish the stoichiometry of FLU and dye (AAN) complex by Job’s method of continuous variations was applied. The plot reached a maximum value at a mole fraction of 0.5 which indicated the formation of 1:1 (FLU: Dye) complex (Figure 10) between FLU and AAN.

**Validation of the proposed method**

Under the optimum experimental conditions, standard calibration curve was constructed at ten concentration levels (n=5) (Figure 11). The correlation coefficient was 0.9999 for method A and 0.9998 indicating very good linearity, over the concentration range of 0.5-17.0 µg/mL. The intercept, slope, limit of detection (LOD), and limit of quantitation (LOQ) are summarized in Table 1. LOD and LOQ values were calculated as 3.3Sb/m and 10Sb/m, respectively where molar absorptivity of regression (Table 1).

The results obtained are summarized in Table 2. The low values of Relative Standard Deviation (RSD) indicate good precision and reproducibility of the method. The average percent recoveries obtained were 98.70-101.53% for AAN, indicating good accuracy of the method.

The repeatability of proposed methods were estimated by measuring five replicate samples of each concentration of fluorometholone prepared in one laboratory on the same day. The precision expressed as the Relative Standard Deviation (RSD%) ranged from 0.45% to 3.66% for the smallest concentration, indicating good precision (Table 2).

**Application to ophthalmic suspension (eye drops)**

The proposed method was applied to the determination of FLU in eye drops. The results in Table 3 showed that the methods are successful for the determination of ZMT and that the excipients in the dosage forms do not interfere. A statistical comparison of the results for determination of ZMT from the same batch of material by the proposed and reference method is shown in Table 4. The results agreed
Accuracy is judged by comparing the results obtained from the reference method to the results obtained by the proposed method using Student’s t-test for accuracy and F-test for precision. The results were statistically compared with each other Table 4 using t- and F-tests. With respect to t- and F-tests, no significant differences were found between the calculated values of both the proposed and the reported methods at 95% confidence level.

Conclusion

The proposed method for the estimation of FLU using the reagent AAN is advantageous over many of the reported methods. The proposed method is rapid, simple, and has good sensitivity and accuracy. Proposed method makes use of simple reagent, which an ordinary analytical laboratory can afford. The high recovery percentage and low relative standard deviation reflect the high accuracy and precision of the proposed method. The method is easy, applicable to a wide range of concentrations, besides being less time-consuming and depends on simple reagents which are available, thus offering economical and acceptable methods for the routine determination of FLU in its formulations.

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Table 1: Statistics and analytical parameters of FLU determination by AAN.

| Parameter | Result |
|-----------|--------|
| λ_max (nm) | 418 |
| Linear range (µg/mL) | 0.5-17.0 |
| Molar absorptivity (Ɛ) | 3.8555 × 10^4 |
| Intercept | 0.0061 |
| Slope | 0.9999 |
| Limit of detection (µg/mL) | 0.14 |
| Limit of quantification (µg/mL) | 0.45 |

Table 2: Precision of determination of FLU in pure form using proposed method.

| Product | Taken FLU µg / 1 mL | Found FLU µg / 1 mL | SD (µg/mL) | RSD (%) | Confidence limit | Recovery % |
|---------|---------------------|---------------------|------------|---------|-----------------|------------|
| Fumeron 100 µg / 1 mL | 100.63 ± 0.05 | 100.30 ± 0.05 | 100.34 ± 0.76 |
| Fumeron Fort 250 µg / 1 mL | 101.58 ± 0.07 | 102.17 ± 0.05 | 101.21 ± 0.54 |
| Fludrop 100 µg / 1 mL | 100.30 ± 0.05 | 100.16 ± 0.06 | 100.16 ± 0.06 |
| Fludrop Fort 250 µg / 1 mL | 101.58 ± 0.07 | 101.11 ± 0.45 | 101.21 ± 0.54 |
| Methoflor 100 µg / 1 mL | 101.58 ± 0.07 | 101.11 ± 0.45 | 101.21 ± 0.54 |
| Flora-T 100 µg / 1 mL | 101.58 ± 0.07 | 101.11 ± 0.45 | 101.21 ± 0.54 |

*Average of five determinations ± Confidence limit.

Table 3: Results of the estimation of FLU in eye drops

| Parameter | Result |
|-----------|--------|
| Limit of detection (µg/mL) | 0.14 |
| Limit of quantification (µg/mL) | 0.45 |

*Average and standard deviation of five determinations for the proposed method.