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Spectrophotometric determination of eflornithine hydrochloride as active pharmaceutical ingredient using sodium 1,2-naphthoquinone-4-sulfonate as the derivative chromogenic reagent

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ABSTRACT

A simple highly sensitive spectrophotometric method was developed for the quantification of eflornithine hydrochloride (2-difluoromethyl-DL-ornithine; DFMO). The method involves the reaction of the target compound with sodium 1,2-naphthoquinone-4-sulfonate (NQS) reagent at specific pH 5.6 to produce a wine red color chromogen. The derivative chromogen exhibits absorption maxima at 498nm. At the specific pH of the reaction where no degradation may occur with that medium the proposed method can be utilized as a stability indicating assay. The different experimental parameters affecting the derivatization reaction were carefully studied and incorporated into the procedure. Under the described conditions the proposed method is linear over the concentration range of 10-70mcg/ml and the coefficient of determination were >0.999(n=6) with a relative standard deviation of 1.98% (n=6). The average recovery of the target compound is 99.28% with a limit of quantification (LOQ) of 0.52mcg/ml and the limit of detection (LOD) 0.172mcg/ml. The mechanism of the derivatization reaction is proposed and advantages of the proposed method are discussed.

KEYWORDS

Eflornithine hydrochloride (DFMO);
Sodium 1, 2-naphthoquinone -4-sulfonate.

1. INTRODUCTION

The quality control of active pharmaceutical ingredients (API's) in the formulation is always a thrust area for the pharmaceutical industries which has a wide impact on the public health. So the development of reproducible, sensitive, simple and extremely inexpensive methods for the determination of API's in the formulation is always challenging and new methods are always welcomed.

Eflornithine (2-fluoromethyl-Dt-ornithine; MDL 71782A; DFMO) is a selective, irreversible inhibitor of ornithine decarboxylase enzyme, one of the key enzymes in the polyamine biosynthetic pathway[1,2]. The drug was originally developed for the use in cancer, and is in phase III clinical trials for its use in preventing recurrence of superficial bladder cancer. It has been used as antiprotozoan agent in the treatment of the meningoencephalic stage of trypanosomiasis caused by *Trypanosoma brucei gambienze* (African trypanosomiasis)[3-5]. It is now licensed for the use in sleeping sickness in the USA, Europe and twelve African countries[6]. In African trypanosomiasis, DFMO has been approved by the FDA, USA for the treatment of the meningoencephalic stage[7,8]. DFMO currently is in development and testing for its anti inflammatory activity[3]. DFMO 13.9% cream is used to inhibit growth and reduce the amount of facial hair in women[6]. The

drug development process of DFMO in these diseases is currently at a relatively early stage, and therefore the full pharmacokinetic characterization in patients, in conjunction with pharmacodynamics (clinical efficacy/safety) is essential for optimization of drug therapy.

DFMO is finding new dimensions of clinical importance. To support its investigation an appropriate analytical method (sensitive, selective, reproducible and simple) for quantification of DFMO is essential.

A number of analytical methods have been reported for measuring DFMO in biological fluids and tissue extracts. These methods involved HPLC techniques[9-11]. The HPLC techniques currently available for the quantification of DFMO in biological fluids involve either pre or post column derivatization with UV or fluorescence detection[10-12] and LC carried out by evaporative light scattering detection.

A reverse HPLC method utilizing pre-column dansylation was described for the analysis of DFMO in serum[11]. Derivatization for at least 04hrs was necessary for maximum derivative formation. All the above mentioned methods are either long procedures or require sophisticated sample preparation or chromatographic procedures[9-12].

For the drugs that obey the beer Lambert's law, spectrophometric methods of analysis of single component in solution are usually rapid, sensitive and economical[13].

The aim of this work was to develop a sensitive and simple spectrophotometric method for the quantification of DFMO using a specific derivative chromogenic reagent (NQS) that constitute an alternative to other analytical methods previously proposed for the analysis of the target compound in pharmaceutical preparations without any further separation required.

2. EXPERIMENTAL

2.1 Apparatus

A model Shimadzu UV-1601 double beam spectrophotometer with a fixed slit width of 2nm using a pair of 1cm matched quartz cells was used for spectrophotometric analysis of derivative chromogen.

All pH measurements were made with Digisun D1-707 digital pH meter.

2.2 Materials

All the chemicals were of analytical reagent grade, and the solvents were of spectroscopic grades. A 1000mcg/ml eflornithine hydrochloride (2-difluoro methyl-DL-ornithine; DFMO) (Wintac Limited, Bangalore, Karnataka State, India) was prepared by dissolving 2.0g in distilled water and diluted it to 200ml.A stock solution of 1%w/v sodium 1,2-naphthoquinone-4-sulfonate (NQS) (s. d. fine-chem. Limited, Mumbai, India) was prepared by dissolving 2.5g in distilled water and diluting it to 250ml. Acid phthalate buffer pH 5.6 was prepared by mixing 50ml of 0.2M potassium hydrogen phthalate and 38.8ml of 0.2M NaOH solution and diluted to 200ml.

2.3 Spectrophometric method

2.3.1. Effect of pH and volume of buffer solution on the DFMO and NQS reaction

DFMO samples were prepared in the buffer solutions (pH 2.6-6.0) at concentration of 40, 60 and 80mcg/ml. Assay samples were mixed with freshly prepared 1%w/v NQS and heated on a boiling water bath at100^{0}C for 1hr. The solutions were then cooled to room temperature. The UV visible spectra over a wavelength range of 400 to 800nm were measured (**Figure** 1.) using the mixture of 1%w/v NQS reagent and respective buffer solution of the appropriate concentration as the blank.

2.3.2 Effect of the NQS concentration on the NQS-DFMO reaction

The optimum NQS-DFMO ratio for the DFMO-NQS reaction was determined by adding varying volumes of NQS solutions to a known constant concentration of DFMO.

A DFMO solution of 500mcg/ml was prepared in acid phthalate buffer of pH 5.6. Aliquots of this DFMO

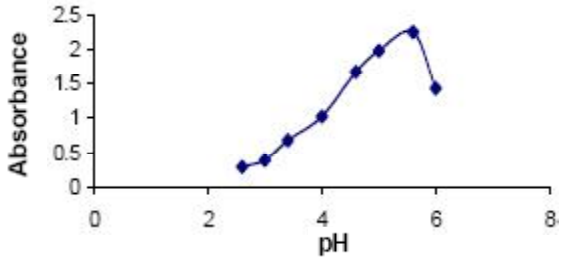

Figure 1: Effect of pH on absorbance of DFMO-NQS derivative chromogen. DFMO 40mcg/ml: NQS 1%w/v: 3.5ml; reaction time 60 min

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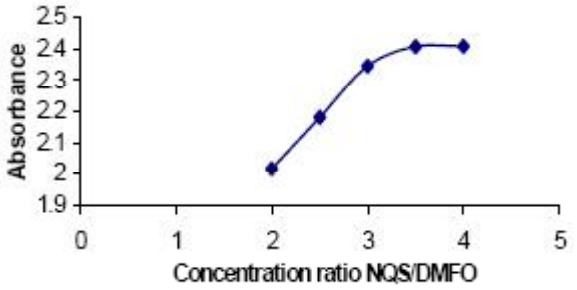

Figure 2: Effect of amount of sodium 1,2-naphthoquinone-4-sulfonate. DFMO 500 mcg/ml: 1.2 ml; NQS 1% w/v; acid phthalate buffer pH 5.6; Reaction time: 60 min

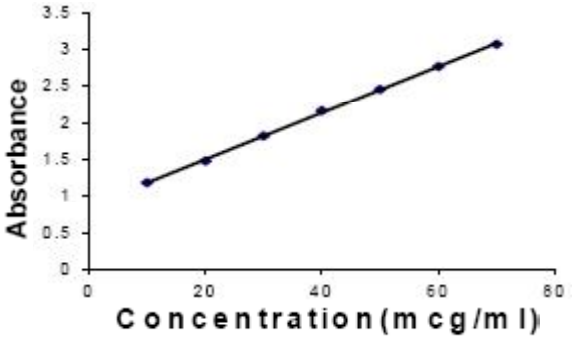

Figure 3 : Calibration curve. Acid phthalate buffer solution (pH 5.6): 10.0 ml; NQS (1%): 3.5.00 ml; reaction time: 60 min

solution were mixed with different volume of the NQS reagent and different volume of pH 5.6 acid phthalate buffer to obtain a sample final volume (40 60 mcg/ml). The blanks were obtained in a similar manner except that pH 5.6 acid phthalate buffers were added in the place of the aliquots of the DFMO solution. The samples were heated and cooled as described above and the UV visible absorption spectra was measure over the wavelength range of 200-800nm (Figure 2).

2.3.3. Effect of heating time on the DFMO-NQS reaction

A DFMO solution of 500mcg/ml was prepared in the pH 5.6 acid phthalate buffer. Aliquot of this DFMO solution was mixed with 10ml acid phthalate buffer of pH 5.6 and 3.5ml of NQS reagent 1%w/v and heated on a boiling water bath at 100^{O}C for different times (15,30,45,60 and 75min). The samples were then cooled to room temperature and different volume of buffer of pH 5.6 were added to obtain a final volume (40 and 50mcg/ml). The UV visible absorption spectra were measured over the wavelength range of 200-800nm and absorbance were measured at 498nm

TABLE 1: The absorbance of solution is measured under different temperature. DFMO 50mcg/ml: NQS 1% w/v; acid phthalate buffer pH 5.6; Reaction time 60 min.

A	T (^{O}C)
1.305	40
1.695	60
1.931	80
2.306	100

(TABLE 1)

2.4. Calibration graph of DFMO

Under the selected conditions, a linear relationship between the absorbance A of DFMO-NQS derivative chromogen and the concentration C of DFMO is obtained in the range of 10-70 mcg/ml. The linear regression equation obtained from the calibration graph is $A = 0.8691 + 0.0316\ C$ (mg l^{-1}) with a correlation coefficient of 0.9995 and a molar absorption coefficient of 6.377×10^{2} l mol^{-1} cm^{-1}. The absorbance has been plotted as a function of the concentration of DFMO (Figure 3). The linear range of DFMO is 10-70 mcg/ml.

3. RESULT AND DISCUSSIONS

The analytical applications of NQS in the quantification, assay and characterization of primary amines have been established by Sullivan[14]. The reagent had been used to quantitate primary aliphatic and aromatic amines. The NQS derivative chromogenic reagent reacts with primary aliphatic or aromatic amines in medium to form wine red colored product. The use of NQS for the detection of primary aliphatic or aromatic amines initiated the present study.

Experimental conditions for the derivatization reaction

The experimental conditions affecting the development and stability of the colored chromogens produced were carefully studied. It was found that stable colored chromogen was obtained at a definite pH (TABLE 1). The colored chromogens are stable for at least 15min which permits the convenient application of the proposed method.

Different experimental conditions, specially pH and NQS concentration should be carefully selected as they could greatly affect the quantification of the target compound.

TABLE 2: Evaluation of the accuracy and precision of the proposed method

Added[a]	Found±S.D.[b]	R.S.D.%	S.A.E.[c]	Confidence limit[d]
04	4.04±0.034	0.027	1.4×10^2	0.0313
06	5.38 ±0.041	0.030	1.5×10^2	0.0156
08	7.43 ±0.019	0.013	0.87×10^2	0.0625

[a]concentration in mcg/ml, [b]Mean ± S.D. for six determinations, [c]S.A.E., standard analytical error, [d]Confidence limits at P=0.05 and five degrees of freedom.

TABLE 3: optical characteristics of proposed method

Sl. no.	Parameter	Results
1	Aborption maxima (nm)	498
2	Beer's law limits (mcg/ml)	10-70
3	Molar extinction coefficient (mole^{-1} cm^{-1})	6.377×10^2
4	Sandell's sensitivity (mcg/cm^2/ 0.001 absorbance units)	0.0173857
5	Regression equation (y)	0.9995
	Slope (b)	0.8691
	Intercept (a)	0.0316
6	Coefficient of variance	1.9819429
7	Standard deviation	0.0454420
8	Limit of detection (mcg/ml)	0.1723852
9	Limit of quantitation (mcg/ml)	0.5223795

Since it was indicated by Silva and Strojny[15] the reactivity of compound possessing a primary amino group is pH specific. So attempts were made to improve pH control in the target reaction. It has been carried out by several assays of solution containing 40, 60 and 80 mcg/ml of DFMO and 10ml of different buffer solutions that covered a wide pH range

2.6 to 6.0. Figure 1, illustrates the absorbance of derivative chromogen Vs pH. As it can be observed in this fig., when the pH of the final solution is adjusted to 5.6, maximum absorbance was achieved. At pH6.0.the wine red color disappears and solution changes to brownish-black indicating the degradation of the Chromogen and exhibits the absorbance of 1.434 at 449 nm (λ_{max}).

As a result of this experience, it is necessary to maintain a pH of 5.6 as optimum to obtain the derivative chromogen. Several buffers of different composition could be used but the best results were obtained with an acid phthalate buffer solution of 0.2M of pH 5.6. The volume of buffer necessary to obtain the highest and most stable absorbance was determined and established as 10ml.

The effect of NQS concentration on the derivative chromogen formation was observed by measuring the absorbance at different NQS-DFMO concentration ratios, while all other experimental conditions were kept constant at the optimum values. Figure 2 shows that maximum response was obtained when the NQS-DFMO concentration ratio was within 160-650 fold concentration excess in subsequent work, a ratio of 600:1 was employed as the minimum for the determination of DFMO throughout the rest of the experimental work. The order of mixing of the analyte, buffer, NQS and water to obtain the derivative chromogen was examined. No appreciative changes were observed, so the sequences analyte, buffer, NQS was chosen for the present study.

In order to obtain optimum derivative chromogen with highest and most stable absorbance, the effect of the reaction time and heating temperature on the absorbance of the reaction product was studied. The reaction was carried out at different temperature (60^O, 80^O and 100^{O}C) using a thermostated water bath for periods ranging from 10 to 90min. Maximum and constant absorbance was obtained at 100^{O}C after 60min. the colored product was stable for at least 15min.

3.1. Calibration, sensitivity and precision

From the results obtained in the experimental section, the absorbance of the eflornithine hydrochloride derivatized with NQS was proportional to the concentration of the DFMO over the range 10-70 mcg/ml (Fig. 2) and the total concentration of DFMO can be calculated using the corresponding correlation equation with a correlation coefficient (r) = 0.999 for n=6 with the detection limit of 0.172mcg/ml.

The precision of the proposed method was studied by determination of the drug in six replicates, individually derivatized with NQS at concentration of 40 mcg/ml obtaining relative standard deviations of 1.98% (TABLE 2).

3.2 Optical Characteristics

The optical characteristics of the proposed method have been calculated. The values are given in TABLE 3.

4. Discussion of reaction mechanism

It was reported[14] that sodium 1, 2-naphthoquinone-4-sulfonate could react with the amino group of primary amino derivative. Alpha amino group of

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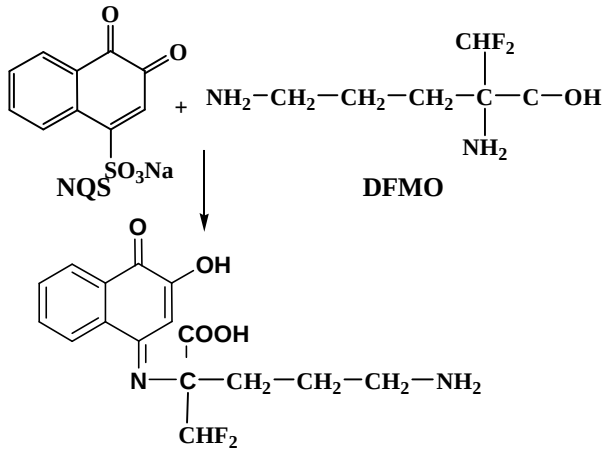

DFMO-NQS derivative chromogen.

Figure 4: Reaction mechanism

DFMO displays nucleophilicity due to the fact that its lone pairs of electron of nitrogen can attack the electron deficiency center. The 4-C of sodium 1, 2-naphthoquinone-4-sulfonate becomes an electron deficiency center. Three 4-C=C conjugate with 2-C=O. So, DFMO can react with NQS in a condensation reaction[3]. According to the literature[3], the reaction equation is as follows (Figure 4.)

5. CONCLUSIONS

In contrast with previous methods, the described method has many advantages: it does not need expensive apparatus; it is simple and quick; its linear range is relatively wide; it has good selectivity. Furthermore, the proposed method can be successfully used to determine DFMO in pharmaceutical formulations. Accordingly, the method is practical and valuable.

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