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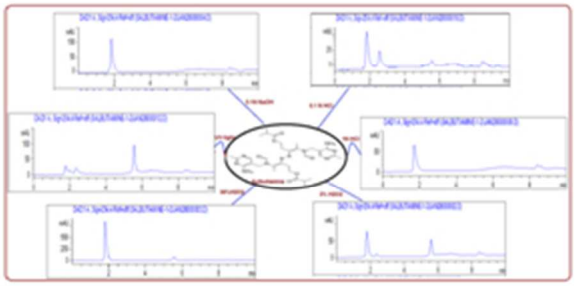

A novel stability indicating HPLC-DAD method for stability study of sulbutiamine with great accuracy, precision, sensitivity and short analysis time.

Title Page

Validated HPLC-DAD Method for Stability Study of Sulbutiamine HCl

Short title: HPLC-DAD for Stability Study of Sulbutiamine HCl

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ABSTRACT

Sulbutiamine (SUL) is a widely used synthetic thiamine derivative for treatment of memory disorders. In this study, a newly developed gradient HPLC-DAD method demonstrating no interference from SUL different degradation products has been optimized and validated. The drug was subjected to variable stress conditions including hydrolysis (at different pH values), oxidation, photolysis and dry heat. The drug was found to be labile to hydrolysis, oxidation and photolysis but stable in thermal and neutral hydrolytic degradation.

Successful chromatographic separation of SUL from all degradation products with significantly different t_R values was achieved on ZORBAX Eclipse Plus C18 column using a mobile phase containing a gradient mixture of solvent A (50mM KH_2PO_4 (pH 3.6±0.2)) and solvent B (methanol). UV detection was performed at 254nm using photodiode array detector (DAD). The reliability of the method was assessed by evaluation of accuracy, precision, specificity, robustness and ruggedness according to USP guidelines. The linear regression analysis data for the calibration curve showed good relationship in the range of 2-40µg/mL. System suitability tests were performed, selectivity (α) and resolution (Rs) factors were found to be greater than 1.5 and 2, respectively. The assay method was successfully used to estimate SUL in arcalion forte® tablets and good percentage recoveries were obtained. The developed method compared favorably with the reported spectrophotometric one.

Keywords: Sulbutiamine, HPLC-DAD, gradient elution, stability indicating, forced degradation.

1. INTRODUCTION

Sulbutiamine HCl (SUL), is a non pharmacopoeial drug which is chemically known as NN′-{Dithiobis[2-(2-isobutyryloxyethyl)-1-methylvinylene]}bis[N-(4-amino2methyl pyrimidin-5ylmethyl)formamide] [1]. It is a more efficient version of vitamin B_1 that mimics the effect of thiamine at more drastic level. It is more efficient than thiamine at crossing the blood brain barrier and can be used alone or stacked with other nootropics. It is used to treat asthenia, improve memory and improve erectile dysfunction [2]. In addition, many athletes were using SUL as a legal performance to enhance their sport performance [3].

Only two methods have been found in the literature for determination of SUL in biological fluids and pharmaceutical dosage form. The first method was a kinetic spectrophotometric method which depended on the catalytic effect on the reaction between sodium azide and iodine in aqueous solution and then measuring the decrease in absorbance of iodine at 348 nm [4]. While the second reported one was HPLC with fluorescence detection method for analysis of SUL along with other thiamine disulphides [5]. It depended on gradient elution using methanol and 0.011 M phosphate buffer, pH 4.5 (from 10% methanol to 62 %) with analysis time of 50 minutes) using λ_{ex} = 365 nm and λ_{em}= 433 nm).

During manufacturing, storage and distribution, many drugs are susceptible to different environmental factors such as light, temperature and humidity, so force degradation studies are necessary for providing information about chemical and physical factors that result in drug instability and hence selecting suitable packing and storage conditions. Also results of stability studies are necessary for setting expiration dates for the API (active pharmaceutical ingredients) or drug products [6-10]. According to ICH guidelines Q1AR2 [10] the stability testing of active ingredient should be performed under variable accelerated conditions (hydrolysis under different pH values, oxidation, photolysis and thermal degradation). Stability-indicating method is the method that can selectively analyze the parent constituent (API) from the pharmaceutical product, it is developed to separate and determine the active drug in presence of its impurities and degradation products [11].

On reviewing the literature in hand, none of the known pharmacopoeias described any method of analysis for SUL, moreover the two published methods are not stability indicating ones and time consuming.

Due to the importance of drug stability studies and absence of information about SUL stability, the work in this manuscript aimed to perform a stability study for SUL according to ICH guidelines [10] through a validated stability indicating gradient HPLC-DAD method. The new developed method has the advantages of being the first stability indicating one for SUL with high sensitivity, precision and accuracy. On the other hand, minimum sample preparation is required and the analysis was performed within ten minutes.

2. EXPERIMENTAL

2. 1. Instruments:

HPLC (Agilent 1260 Infinity, Germany) instrument was equipped with Agilent 1260 Infinity preparative pump (G1361A), Agilent 1260 Infinity Diode array detector VL (G131SD), Agilent 1260 Infinity Thermostated column compartment (G1316A) and Agilent 1260 Infinity preparative Autosampler (G2260A). Separation and quantitation was performed on ZORBAX Eclipse Plus C18 column (250×4.6 mm i.d, 5µm particle size) (USA).

2. 2. Samples:

2. 2. 1. Pure samples:

Pharmaceutical grade of SUL was provided as a gift from SIGMA Pharmaceutical Industries- Quesna City-Egypt- S.A.E.. Its purity was checked and found to be 99.90% ± 1.404 according to the reported method [4].

2. 2. 2. Pharmaceutical preparation:

Arcalion forte® tablets (Batch No. 18255) were manufactured by SIGMA Pharmaceutical Industries- Quesna City-Egypt- S.A.E. and labeled to contain 400mg per tablets.

2. 3. Chemicals and solvents:

- Methanol [(Sigma-Aldrich, Chromasolv®, Germany), and (Fisher Scientific, UK)].
- Deionized water (SEDICO Pharmaceuticals Co., Cairo, Egypt).

• Potassium dihydrogen phosphate, sodium hydroxide orthophosphoric acid, hydrochloric acid and hydrogen peroxide were of analytical grade and were purchased from El- NASR Pharmaceutical Chemicals Co., Abu- Zabaal, Cairo, Egypt.

2. 4. Solutions:

Stock standard solution of Sulbutiamine (1mg/mL) was prepared by accurately weighing 0.1 gm SUL in 100-mL volumetric flask and dissolving in methanol.

50mM KH_2PO_4 buffer (pH=3.6) was prepared by dissolving 6.8 g KH_2PO_4 in 1L deionized water and then adjusting pH to 3.6±0.2 with aqueous phosphoric acid.

Working standard solution of Sulbutiamine (0.1mg/mL) was prepared by transferring 10 mL from SUL stock standard solution (1 mg/mL) into 100-mL volumetric flask and then diluting with methanol: 50mM KH_2PO_4 buffer (pH=3.6) (50:50, v/v).

Pharmaceutical dosage form solution

Ten arcalion forte® tablets were crushed and triturated well in a mortar. An accurately weighed amount of the powdered tablets equivalent to 100 mg of SUL was transferred into 100-mL volumetric flask. 75 mL methanol was added and the solution was ultra-sonicated for 15 minutes, filtered and then the volume was completed with methanol.

3. PROCEDURE

3.1. Chromatographic Conditions

Chromatographic separation was performed on ZORBAX Eclipse Plus C18 column using gradient mixture of methanol: 50mM KH_2PO_4 buffer (pH=3.6±0.2) as a mobile phase. Gradient elution program is given in **Table (1).** Injection volume was 50 µL and detection has been carried out at 254 nm using DAD maintaining the column temperature at 25^0C. The run time was 10 min and the peak area ratio (using area of 20µg/mL SUL as an external standard) was used to quantify SUL.

3.2. Method Validation

Method validation was performed with respect to USP guidelines [12].

-Linearity

Linearity test solutions for the assay method were prepared from SUL working standard solution (0.1mg/mL) at different concentration levels (2-40 µg/mL). Triplicate 50µL of each solution was injected into the HPLC system. The integrated relative peak area (using area of 20 µg/mL SUL as an external standard) of SUL was obtained and the regression equation was computed.

-Accuracy

Accuracy of the method was evaluated by analyzing nine concentrations of pure SUL in its linearity range. Relative peak area for each concentration was obtained and the mean % recoveries were then calculated.

-Precision

It was expressed as percentage relative standard deviation (%RSD) of percentage assay and it was evaluated by testing intraday and interday variations.

-Repeatability (intraday variation) was checked by analyzing three concentrations of pure SUL (16, 20 and 38 µg/mL) three times within the same day using the previously mentioned procedure under chromatographic conditions. % RSD values were then calculated.

- *Intermediate precision* was verified on three different days using the previously chosen concentrations in the same laboratory using the specifications under chromatographic conditions. % RSD values were then calculated.

-Limits of detection and quantitation

In order to determine detection and quantification limits, SUL concentrations in the lower part of the linear range of the calibration curve (0.5, 1 and 2 µg/mL) and the equations LOD =

3.3 × N/B and LOQ = 10 × N/B were used, where N is the standard deviation of the response and B is the slope of the corresponding calibration curve.

-Specificity

It was determined by exposing SUL samples to different stress conditions and then calculating the resolution factors (Rs) of the drug peak from the nearest peak. Specificity was also established through determination of SUL in arcalion forte® tablets and comparing t_R value of SUL in the sample with that of pure SUL. Moreover, the peak purity was checked by using DAD detector.

-Robustness

It is expressed as %RSD and it was checked by small deliberate alternation in experimental conditions, the relative peaks areas of SUL were calculated from which percentage recoveries and %RSD values were obtained. The altered parameters were changing in mobile phase composition (±2% methanol) and pH of the buffer (±0.2 pH).

-Ruggedness

Ruggedness evaluates the degree of reproducibility of the results obtained under variety of conditions, such as performing the analysis by two different analysts or using methanol from different manufactures [(Sigma-Aldrich, Chromasolv®, Germany), and (Fisher Scientific, UK)]. In each variation the relative peaks areas of SUL and %RSD values were calculated.

3.3. System suitability testing parameters

In all chromatographic systems, system suitability testing parameters should be checked before starting sample analysis. The peak a symmetry, capacity, selectivity and resolution factors, number of theoretical plates and height equivalent to theoretical plates were calculated for the principle peak.

3.4. Assay of Pharmaceutical Preparation

Concentration of 16µg/mL of SUL sample was prepared from arcalion forte® working solution (0.1mg/mL), injected in triplicates following the procedure under chromatographic

conditions. The concentration of SUL in the prepared solution was then calculated from the constructed regression equation. Standard addition technique was carried out to prove the accuracy of the suggested method, it was performed by spiking the pre-analyzed SUL sample (16µg/mL) with extra 80, 100 and 120% of standard SUL.

3.5. Forced Degradation Studies

Sulbutiamine stock standard solution (1mg/mL) was used during forced degradation studies and concentration of 25µg/mL of each degraded sample was prepared in mixture of methanol: 50mM KH_2PO_4 buffer (pH=3.6) (50:50, v/v). Then the procedure under chromatographic conditions was followed. From the relative peak area of SUL in each chromatographed sample, SUL % degradation was then calculated.

a- Hydrolytic degradation

Acidic hydrolysis was carried out at 80^0C for 3 hours by using solutions of 0.1 and 1N HCl while basic hydrolysis was performed at room temperature for half an hour using 0.1N NaOH. For neutral hydrolysis deionized water was used at 80^0C for 5 hours.

Separate 5-mL of SUL stock standard solution (1mg/mL) was transferred to four separate 25-mL volumetric flasks and then mixed with 5 mL of either 0.1N HCl, 1N HCl, 0.1N NaOH and deionized water. The prepared solutions were kept away from light to exclude the possible photodegradation, at 80^0C except for 0.1N NaOH which was kept at room temperature. The samples were cooled and neutralized with an amount of acid or base equivalent to that of the previously added amount and then the volume was completed to the mark with a mixture of methanol: 50mM KH_2PO_4 buffer (pH=3.6) (50:50, v/v) to prepare samples working solutions of 200µg/mL each.

b- Oxidative degradation

3% and 30% H_2O_2 was used to carry out oxidative degradation of SUL. By mixing 5 mL of SUL stock standard solution (1mg/mL) with 5 mL of either 3% or 30% H_2O_2 in two separate 25-mL volumetric flasks, the solutions were kept at 80^0C for 5 hours away from light to prohibit the possible effect of light. Samples were then evaporated on water bath to expel the

remaining H_2O_2, and the volume was adjusted using a mixture of methanol: 50 mM KH_2PO_4 buffer (pH=3.6) (50:50, v/v) to prepare samples working solutions of 200 µg/mL each.

c- Photolytic degradation

The effect of light was studied on SUL solid and liquid samples. 5 mL from SUL stock standard solution (1 mg/mL) and 5mg SUL powder was transferred separately to two 25-mL volumetric flasks. Samples were subjected to UV light for 3 hours (liquid sample) or 5 hours (solid sample). 5 mL methanol was added to each flask and the volume was then adjusted with methanol: 50 mM KH_2PO_4 buffer (pH=3.6) (50:50, v/v) to prepare samples working solutions of 200 µg/mL each.

d- Thermal degradation

Sulbutiamine 5 mg was stored at 80°C for 3 hours in an oven. The powder was transferred to 25-mL volumetric flask, dissolving in 5-mL methanol and then the volume was completed with methanol: 50mM KH_2PO_4 buffer (pH=3.6) (50:50, v/v) to obtain samples solutions of 200 µg/mL.

4. RESULTS AND DISCUSSION

Because of complex nature of separation of multiple components during analysis of stability samples, chromatographic methods have taken priority over the conventional methods of analysis [13-15]. They possess greater accuracy and sensitivity for even small quantities of degradation products produced. The popularity of HPLC in stability studies is due to its high-resolution capacity, sensitivity and specificity [16]. Stability-indicating methods are traditionally performed using gradient elution, in order to ensure that degradants of various chemical compositions are all detected [17].

Up to date, no stability indicating method was found in the literature for determination of SUL. In this manuscript we aimed to perform a stability study for SUL and to develop a novel stability indicating HPLC-DAD method for its analysis. Complete separation of the analyte was accomplished in less than 10 minutes and the method can be successfully applicable to perform long-term and accelerate stability studies of SUL.

-Method development and optimization

The initial method development was conducted on pure SUL sample and samples obtained from different degradation conditions in order to select conditions that achieve good resolution between the drug and the degradation products. The suitability of the mobile phase was decided on the basis of suitability for stability studies, time required for analysis and SUL peak broadening.

-Optimization of mobile phase

Initially samples were analyzed using isocratic elution of a mobile phase consisting of water: acetonitrile (30:70, v/v) at a flow rate of 1.0 mL/min. Under these conditions, SUL peak was very broad. Replacing acetonitrile with methanol slightly improved the peak broadening. Several trials were done using water: methanol (30:70, v/v) system in order to enhance the chromatographic resolution and decease SUL peak broadening such as addition of 0.25% triethyl amine (pH=5), 0.5% trifluoroacetic acid (pH=5) and 0.15mM heptanes sulfonic acid. Unfortunately, there was no improving in either resolution or peak shape.

The second step was to replace water with 50 mM KH_2PO_4 buffer at different pH values (3-8). It was noticed that changing the buffer pH greatly affected the t_R of SUL and hence the resolution between SUL and the closest eluted degradation product. Extremely acidic pH resulted in decreasing SUL t_R leading to bad chromatographic resolution while alkaline pH increased SUL t_R giving rise to broader SUL peak and increasing analysis time.

After extensive trials, gradient elution was tested by using gradient solvent mixture consisted of methanol: 50 mM KH_2PO_4 (pH 3.6±0.2). Different gradient elution programs were tried until optimum system suitability testing (SST) parameters were obtained with symmetric SUL peak**.** Details of the used gradient elution program are given in **Table (1).**

-Selection of stationary phase

Different stationary phases were also tried such as ZORBAX Eclipse Plus C18 and C8 columns. Both stationary phases gave almost the same result.

-Selection of detection wavelength

The photodiode array detector was set at different wavelengths including 230, 254 and 365 nm. Using 254nm as a detection wavelength gave the best results with respect to sensitivity and peak shape.

-Optimization of column temperature

The thermostated column compartment was adjusted at different temperatures (20, 25 and 30^0C). The column temperature neither affected the chromatographic separation nor the peak shape.

-Application of the method

After optimization of all factors affecting method selectivity and sensitivity, it was applied for determination of SUL in its pharmaceutical formulation. Firstly calibration curve relating the integrated relative peak area of SUL (using 20 µg/mL SUL as external standard) to its corresponding concentration was constructed. Good linear calibration fit in the range of 2–40 µg/mL and the calibration equation was:

$$A=0.0520C+ 0.0265 \qquad r=0.9999$$

Where A is the integrated relative peak area, C is the concentration in µg/mL and r is the correlation coefficient. Calibration curve parameters are given in **Table (2)**.

Secondly and in order to evaluate the suitability of the method, it was applied to arcalion forte® tablets. A single peak at t_R= 5.55 ± 0.03 was observed in the chromatogram of the drug samples extracted from tablets indicating no interference from the excipients which routinely occur in tablets. The mean % recovery of the drug content was found to be 102.75± 0.799 as shown in **Table (3).** The recovery studies were executed out at 80%, 100%, and 120% of the test concentration. The % recovery of SUL at all the three levels was found to be acceptable, **Table (3).**

The suggested method compared favorably with the reported spectrophotometric [4] one as shown from the values of the calculated student's-t and F-ratio, confirming that there was no significant difference within probability of 95% between the two methods, **Table (4).**

Method validation

-Linearity

Linearity of the developed method was estimated, the linear regression data for the calibration curve (n = 9) showed a good linearity (r = 0.9999) over the concentration range of 2–40 µg/mL with respect to relative peak area, **Table (2).**

-Accuracy

It was calculated as % recovery of pure SUL and was found to be 100.76 as shown in **Table (2)**. Also when the proposed method was applied for estimation of SUL in its pharmaceutical dosage form after spiking with 80, 100, and 120% of additional pure SUL, good mean % recovery was resulted and listed in **Table (3).**

-Precision

The precision of the developed method was checked by testing intra (repeatability) and interday (intermediate precision) variations and it was represented in terms of % relative standard deviation (% RSD). The obtained values of RSD%, **Table (2)** were < 2% verifying the high precision of the developed method.

-Limits of detection and quantitation

Limits of detection and quantitation for SUL was found to be 0.5 and 1.51 µg/mL, respectively. This showed the adequate sensitivity of the developed method.

-Specificity

Specificity of the developed method was assessed by its ability to resolve the major compound from possible degradation products as shown from the chromatograms in **Figs. (1,2).** The results revealed that the proposed method was able to completely discriminate the SUL from all degradation products, confirming the selectivity of the method. Also the acceptable results obtained on applying the method to arcalion forte® tablets, **Table (3)** assessed the method specificity and that tablets excepients did not interfere. On the other hand, when the peak purity was checked using DAD detector the purity factor was found to

be 650.17 and purity threshold was 146.59. The purity factor was more than the purity threshold, indicating that no additional peaks were co-eluted with the parent drug and thus confirming the ability of the method to determine the analyte of interest in the presence of different degradation products.

-Robustness

Percentage relative standard deviation (%RSD) of peak areas was calculated for each studied parameter. It was found to be 0.467% for varying in mobile phase composition and 1.458 for varying pH of the used buffer. The low values of % RSD, **Table (5)** ascertain robustness of the method.

-Ruggedness

It was evaluated by applying the method using two different analysts and methanol from different manufactures. The values of resulted %RSD were reasonably low (<2.0%) confirming the good reproducibility of the suggested method, **Table (5).**

System suitability testing parameters

System suitability testing was carried out during method development and optimization as well as through the validation procedure [18]. The resolution (Rs) and selectivity (α) factors were calculated between SUL and the nearest eluted peak and were found to be >1.5 and 2, respectively in all degradation conditions. Also the symmetry factor was calculated for the basic component and was equal to 1. Other parameters such as capacity factor, number of theoretical plates and height equivalent to theoretical plates were calculated and their values were within the acceptable limits, **Table (6)**.

Results of forced degradation studies

Results of SUL stability studies are given in **Table (7)**

a- Hydrolytic degradation

The drug was found to be very sensitive to hydrolysis by 0.1N NaOH and 1N HCl and was completely degraded with additional peak at t_R=1.83 minutes for basic degraded sample and

at $t_{R=}$ 1.71 for acid degraded sample, **Fig. (1).** On subjecting SUL to hydrolysis with 0.1N HCl, the peak height of the parent drug was reduced and new peaks at 1.81, 2.55 minutes were produced, **Fig. (1).** On the other hand, the drug was not affected by hydrolysis under neutral conditions as seen in the chromatogram in **Fig (1).**

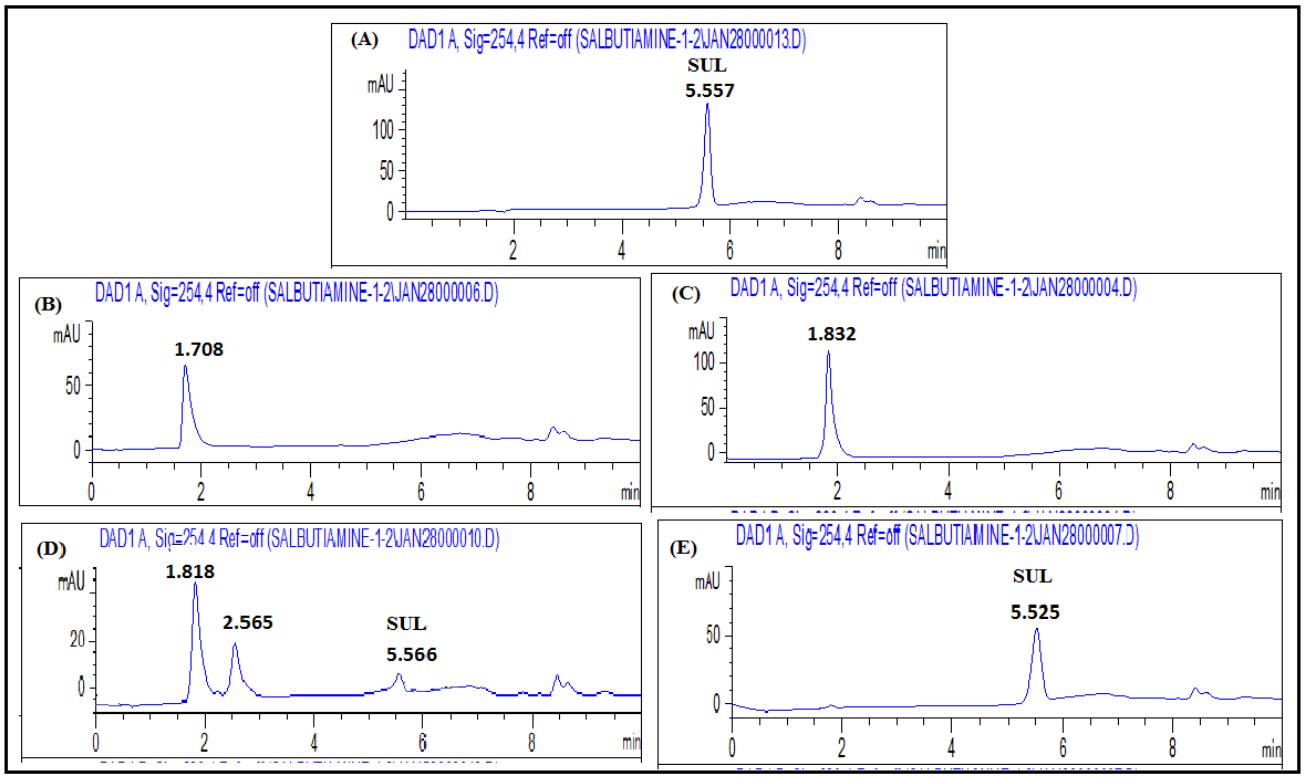

Fig. 1: HPLC Chromatograms of (A) Sulbutiamine, and its hydrolytic degradation by (B) 1N HCl, (C) 0.1N NaOH, (D) 0.1N HCl and (E) neutral hydrolysis.

b- Oxidative degradation

Oxidative degradation was tested using 3 and 30% H_2O_2. The height of SUL peak was significantly reduced when treated with 30% H_2O_2 with the appearance of a degradation product peak at 1.78 minutes. While oxidation with 3% H_2O_2 resulted in appearance of two degradation products at 1.78, 2.35, **Fig (2).**

c- Photolytic degradation

The chromatogram in **Fig (2)** showed that SUL is photolabile and degraded by UV light producing three different degradation products at R_t= 1.78, 2.01,2.34 when it was in solution form while it was found to be photo-stable when it was in solid state.

d- Thermal degradation

Sulbutiamine was found to be thermally stable as no additional peaks were observed when the drug was subjected to dry heat as shown in **Fig (2).**

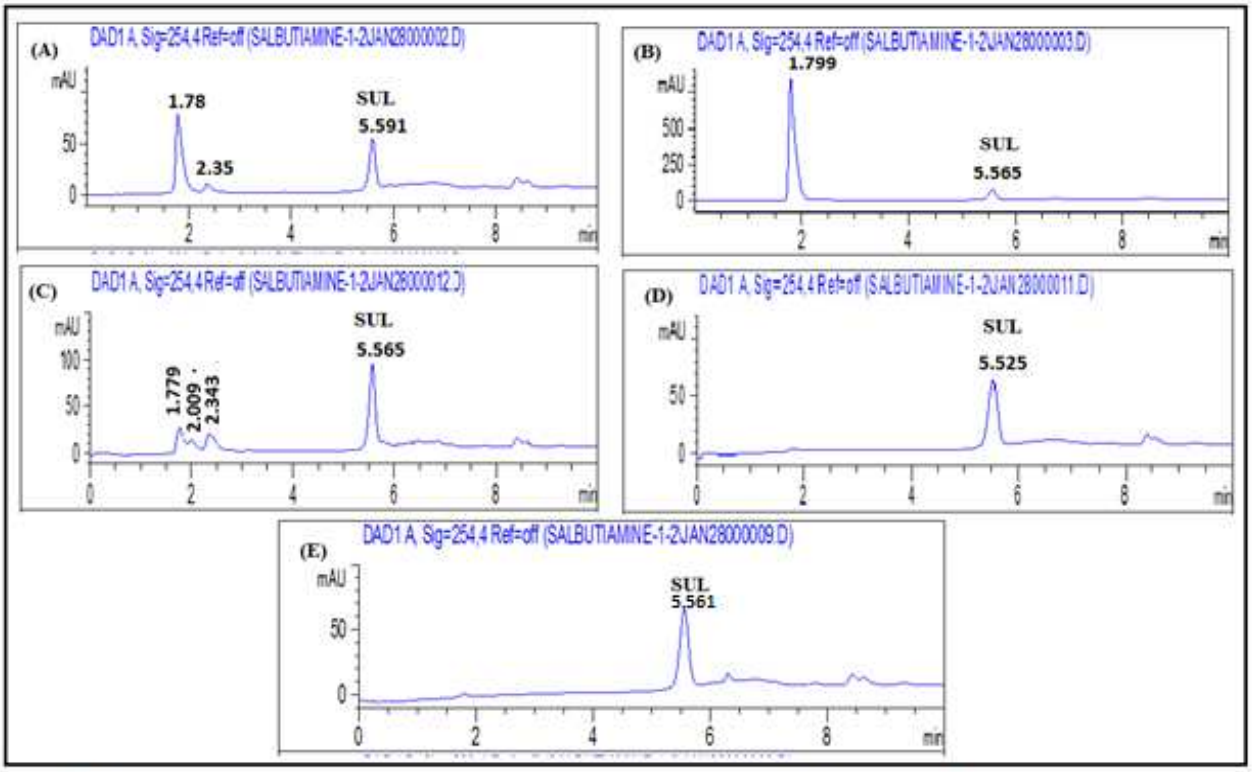

Fig. 2. HPLC Chromatograms showing (A)oxidative degradation of sulbutiamine using 3% hydrogen peroxide and (B) 30% hydrogen peroxide, (C) Photolysis of liquid sample, (D) Photolysis of solid sample and (E) Thermal degradation.

The chromatograms **(Figs. 1,2)**, verified the stability indicating properties of the proposed method and assessed its ability to resolve the peak of the studied drug from all degradation products.

5. CONCLUSION

An accurate and reproducible HPLC-DAD method has been developed and validated for determination of SUL in pure form and marketed tablets. It is the first developed stability indicating method, where all SUL degradation products were completely resolved from the parent drug. The short chromatographic run time of only 10 minutes, makes this method suitable for processing of many samples in limited time which is very important in quality control analysis of any drug.

Acknowledgment

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Figure Captions

Fig. 1: HPLC Chromatograms of (A) Sulbutiamine, and its hydrolysis by (B) 1N HCl, (C) 0.1N NaOH, (D) 0.1N HCl and (E) neutral hydrolysis.

Fig. 2: HPLC Chromatograms showing (A)oxidative degradation of sulbutiamine using 3% hydrogen peroxide and (B) 30% hydrogen peroxide, (C) Photolysis of liquid sample, (D) Photolysis of solid sample and (E) Thermal degradation.

Table Captions

Table.1. Details of gradient elution program.

Table 2: Regression and analytical parameters of the proposed HPLC-DAD method for determination of Sulbutiamine (SUL).

Table 3: Determination of Sulbutiamine (SUL) in Arcalion forte® tablets by the proposed HPLC-DAD method and results of standard addition technique.

Table 4: Statistical comparison of the results obtained by applying the proposed HPLC-DAD method and the reported spectrophotometric for determination of Sulbutiamine (SUL) in pure form.

Table 5: Robustness and ruggedness studies of the developed HPLC-DAD method.

Table 6. System suitability testing parameters of the developed HPLC-DAD method.

Table 7. Summary of forced degradation studies**.**

Tables

Table.1. Details of gradient elution program

Time (minutes)	% methanol	Flow rate (minute)
0	55	1.5
2	55	1.5
4	85	2
8	85	2
9	55	1.5
10	55	1.5

Table 2: Regression and analytical parameters of the proposed HPLC-DAD method for determination of Sulbutiamine (SUL).

Parameters	SUL
Linearity	
Range	2 - 40 µg/mL
Slope	0.0520
Intercept	0.0265
Correlation coefficient (r)	0.9999
Accuracy (mean ± %RSD)	100.76± 0.997
Precision (%RSD)	
Repeatability*	0.533
Intermediate precision**	1.831
LOD	0.50 µg/mL
LOQ	1.51 µg/mL

* The intraday (n = 9), average of three different concentrations (16, 20 and 38 µg/mL) repeated three times within day.

** The interday (n = 9), average of three different concentrations (16, 20 and 38 µg/mL) repeated three times in three successive days.

Table 3: Determination of Sulbutiamine (SUL) in Arcalion forte® tablets by the proposed HPLC-DAD method and results of standard addition technique.

Pharmaceutical formulation	Taken	Found	% Found* ±%RSD	Standard addition technique	
				Pure added (µg/mL)	% Found**
Arcalion Forte® tablets (B. N. 18255) claimed to contain 400 mg SUL/tablet	**16.00**	**16.44**	**102.75± 0.799**	12.00	96.88
				16.00	99.65
				20.00	99.25
		Mean ± %RSD			**98.59± 1.497**

*Average of 6 determinations.

** Average of 3 determinations.

Table 4: Statistical comparison of the results obtained by applying the proposed HPLC-DAD method and the reported spectrophotometric for determination of Sulbutiamine (SUL) in pure form.

Items	HPLC method	Reported method** [4]
Mean	100.76	99.90
%RSD	0.997	1.404
Variance	3.683	1.968
n	9	7
Student's t- test	1.411 (2.145)*	
F- value	1.953 (3.581)*	

* Figures between parenthesis represent the corresponding tabulated values of t and F at P = 0.05.

**Spectrophotomeetric method that depended on measuring the decrease in absorbance of I_2 at 348nm.

Table 5: Robustness and ruggedness studies of the developed HPLC-DAD method.

Factor	**Robustness (%RSD)**	
	1- Mobile phase composition (±2% methanol)	0.467
	2-pH of phosphate buffer (±0.2 pH)	1.458
	Ruggedness (%RSD)	
	1-Different methanol manufacturer	0.914
	2-Two analysts	1.975

Table 6. System suitability testing parameters of the developed HPLC-DAD method.

Parameters		SUL	Reference value [13]
t_R		5.55±0.03 min	
Peak a symmetry		1	<1.5-2 or < 2
K' (capacity factor)		2.36	1-10 acceptable
N (number of theoretical plates)		9417.44	Increases with increasing the efficiency of separation
H (in cm) (height equivalent to theoretical plates)		2.65×10^{-3}	The smaller the value the higher the column efficiency
Resolution (R_s)	*0.1N NaOH*	14.25	R> 2
	0.1N HCl	8.67	
	1N HCl	14.25	
	3 %H_2O_2	11.27	
	30 %H_2O_2	14.22	
	Light	10.77	
Selectivity (α)	*0.1N NaOH*	3.04	α> 1.5
	0.1N HCl	4.16	
	1N HCl	3.04	
	3% H_2O_2	5.70	
	30% H_2O_2	27.93	
	Light	5.75	

Table 7. Summary of forced degradation studies

Stress conditions		Time of degradation (hrs)	Number of degradates (t_R)	%Degradation
0.1 N NaOH at room temperature		1/2	1-(1.83)	100%
0.1HCl at 80^0C		3	2-(1.81, 2.55)	93.6%
1HCl at 80^0C		3	1-(1.71)	100%
H_2O at 80^0C		5	No degradation	Zero%
3% H2O2 at 80^0C		5	2-(1.78, 2.35)	38.14%
30%H2O2 at at 80^0C		5	1-(1.78)	93.13%
Photolysis	*On liquid sample*	3	3-(1.78, 2.01,2.34)	26.8%
	On solid sample	5	No degradation	Zero%
Dry heat at 80^0C		3	No degradation	Zero%