RESEARCH ARTICLE

Development and Validation of Stability Indicating HPLC Method for the Determination of Related Substances in Droxidopa Drug Substance

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ABSTRACT

Droxidopa, a synthetic amino acid precursor to norepinephrine, was licensed by the FDA in 2014 to treat neurogenic OH. The enzyme L-amino acid decarboxylase in sympathetic neurons converts Droxidopa to norepinephrine, which raises the amount of norepinephrine in the blood.[1] Because noradrenaline crosses the blood-brain barrier poorly. Droxidopa is used to raise the concentration of noradrenaline in the brain instead of noradrenaline itself. It is recommended for the treatment of dopamine beta-hydroxylase deficiency, non-diabetic autonomic neuropathy, orthostatic dizziness, and lightheadedness in adult patients with symptomatic neurogenic orthostatic hypotension resulting from primary autonomic failure (Parkinson's disease, multiple system atrophy, and pure autonomic failure.[2-5] It is a synthetic analog of catecholamine acid that raises norepinephrine and epinephrine levels throughout the body by being immediately metabolized by dopa-decarboxylase. In the CNS, it also penetrates the blood-brain barrier to carry out its pharmacological actions. Through the induction of peripheral arterial and venous vasoconstriction, it raises blood pressure peripherally and causes slight, momentary increases in plasma norepinephrin.[6-9]

Keywords: Droxidopa, method development, validation

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INTRODUCTION

Droxidopa prepared by using an immobilized enzyme (Threonine Aldolase) reaction involves 3,4 dihydoxy benzaldehyde treated with glycine in the presence of immobilized enzyme forms Droxidopa. Crude droxidopa isolate by using column chromatography with SP 700 resin gives 99.5% pure compound. This column purification eliminates the unreacted raw material (3,4-dihydoxy benzaldehyde, glycine) and bi-products (Erythro Droxidopa). In synthetic organic chemistry, threonine aldolase is a potent catalyst that can generate carbon-carbon bonds, allowing for the selective synthesis of beta-hydroxy-alpha-amino acids in both enantio and diastereo forms.

MATERIALS AND METHODS

Method development for determination and validation of Droxidopa and its related substances[10-12]

After various trials, changes were done to the buffer, mobile phases A&B, and columns in order to develop a method for determining and validating the stability of droxidopa and its related substances. The development results were included in Table 7.4.

Instruments

This is a High Performance Liquid Chromatograph system that includes a separations module, 2489 Ultra violet and visible detector (Waters or comparable), data processing software (Lab solution or equivalent) and column (30 mm long, 4.6 mm internal diameter) for gradient elution in reverse phase HPLC.

Chromatographic Conditions

An AQ-C18 (250 x 4.6 mm) was utilized, operating at 25°C temperature and 1.0 mL/mi flow rate at λmax 220 nm by injection volume of 10 µL, packed with Octadecyl silane chemically bonded to porous silica particles of 5 μm.

Preparation of Solutions

System suitability solution

Dissolve around 20 mg of droxidopa enhanced with Erythro droxidopa, 3,4-dihydroxy benzaldehyde, and vanillin/reference sample in a 10 mL dry volumetric flask. added 7 mL of diluent, dissolved with a Sonicate, and adjusted volume

Preparation of buffer

Melted 1000 mL of water containing 1.36 g of potassium dihydrogen phosphate and 1.0 g of sodium salt of 1-hexane sulfonic acid. Orthophosporic acid was used to bring the

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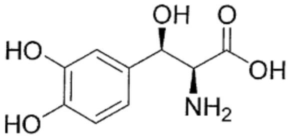


Figure 1: Droxidopa structure

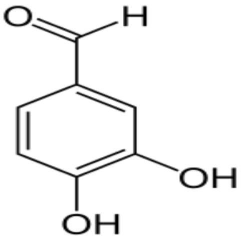


Figure 2: 3,4-Dihydroxy benzaldehyde

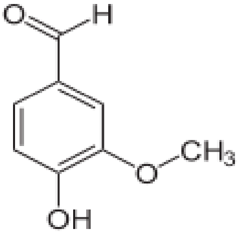


Figure 3: Vanillin

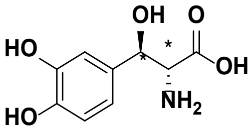


Figure 4: Erythro droxidopa

pH down to 2.0. ran the buffer via a membrane filter with a 0.45 µ porosity.

Eluent A

Prepared Eluent-A using a buffer solution

Eluent B

Utilized 100% acetonitrile as Eluent-B

Diluent (0.1M HCI)

1000 mL of water were used to dilute 8.5 mL of strong hydrochloric acid.

Impurity stock solution

diluted in a 50 mL dry volumetric flask, 3.0 mg of vanillin, 3,4-dihydroxybenzaldehyde, and Erythro droxidopa were weighed and added. After adding 30 mL of diluent and sonicating it to dissolve it, more diluent was added to get the desired volume.

Sample solution

About 200 mg of the material was precisely weighed and then put into a 100 mL dry volumetric flask. Dissolved with a sonicator after adding 70 mL of diluent, and then added more diluent to reach volume made a new solution ready.

Chromatographic conditions

The procedure designed to identify Droxidopa entails the following particular circumstances, where Inert-sustain AQ-C18 (250 × 4.6) mm 5 µm¸ or similar was utilized as a Gradient Pump Operation Setting Chromatography Column where the initial volume of eluent -B was 5% till 15 minutes, then 16% to increase at 28 minutes and then hold up to 43 minutes. The injection volume of 10 µL of sample was used to set the liquid rate flow to 1.0 mL/minute. at 25°C of column Oven and 5°C of sample tray temperature with a runtime of 80 min.

Validation[13-19]

Specificity

To verify the retention period, individual injections of solutions containing all known associated compounds were made. The drug substance droxidopa (control sample), the drug ingredient preparing and injecting droxidopa into the HPLC in compliance with the protocol, and the sample was spiked at a certain level with known related compounds. Using the waters Empower software, peak purity was determined.

System suitability

Six duplicates of the sensitivity solution, diluent, system suitability solution, and standard solution were injected into the chromatograph one at a time to conduct this test. and recording the chromatograms. By recording the standard deviation and relative mean, every day, the system's appropriateness and stability were assessed.

Limit of quantification and detection

Applying the approach of signal to noise ratio, the LoD and LoQ were ascertained. Droxidopa and similar compounds have detection and quantification limits that were computed using the peak reaction of the analyte (known concentration). For LoD, At least 3:1 for the S/N ratio and at least 10:1 for the LoQ are required.. By preparing the solutions containing droxidopa and its related substances at approximately these predicted concentrations and injecting each solution into HPLC six times for droxidopa and the associated compounds, the anticipated concentrations of the limits of quantification and detection were confirmed with accuracy.

Precision

Following protocol, a test technique was used to generate a standard solution that was then repeatedly injected into the HPLC. To assess the procedure's accuracy, six sample solutions were created by mixing known related chemicals into a single batch of the medication Droxidopa at the specified level. The sample solutions were then injected one at a time. Furthermore, sample solutions of identical sets to identify the intermediate precision (inter and intraday precision), (from the same batch that the precision approach uses) prepared independently on a different day by a different analyst using a different system

Table 1: Development of methods for droxidopa and its related substances

Adsorbent	*Buffer*	*Eluent*	*Conclusion*
AQ-C18	1000 mL of water with 0.5% perchloric acid	eluent A: 100% Buffer. eluent B: Acetonitrile	Peaks are separated but blank interference is observed.
YMC Triart C18	1.36g of potassium dihydrogen phosphate plus 1000 ml of water.Ph-2.0 conforms to OPA	eluent A: Buffer eluent B: Acetonitrile	Peaks not separated
Inersustain AQ C18 (250 x 4.6, 5 micron)	1000 mL of water with 1.0 g of heptane 1-suphonic acid and 10 mm of potassium dihydrogen orthophosphate.	eluent A: 100% Buffer. eluent B: Acetonitrile	Every peak was clearly distinguished. However, Imp-A Blank interference was noted.
Inersustain AQ C18 (250 x 4.6, 5 micron)	1000 mL of water with 1.0 g of heptane 1-suphonic acid and 10 mm of potassium dihydrogen orthophosphate. Change the pH to 2.0.	eluent A: 100% Buffer. eluent B: Acetonitrile	all peaks were well separated.
Inersustain AQ C18 (250 x 4.6, 5 micron)	1000 mL of water with 1.0 g of heptane 1-suphonic acid and 10 mm of potassium dihydrogen orthophosphate. Change the pH to 2.0.	eluent A: 100 % Buffer. eluent B: Acetonitrile	all peaks were well separated.

and different column in accordance with the methodology described under method precision.

Linearity

The use of droxidopa and its allied substances to create a range of solutions, each of which was injected into an HPLC at concentrations between 5 and 120% of the specification level. Once the LoQ levels for the associated medications, including droxidopa, were established, the linearity was computed from this data, and is shown below, ranging from LoQ levels to 120% of specification levels.

Accuracy

Droxidopa drug ingredient was used to generate sample solutions in triplicate, and each solution was injected into an HPLC at LoQ, 50, 100, and 120% of the level of specifications. A recognized chemical that was linked was added to the samples. Utilising the calibration curve formula and the recovered concentration data, the recovery % was computed.

Solution stability

The eluent, standard, and sample solutions are among the solutions, were produced, analyzed & first and later time-reviewed at room temperature and then refrigerated (5 ± 3°C) spiked at the specification level with recognized related compounds.

Robustness

The preparation of the standard solutions was done using the test technique to assess the method's and the system's suitability for capacity to remain unaffected, sensitivity, and system suitability. A sample solution was also generated, which had been injected into the HPLC under a range of deliberately altered conditions and spiked with known related compounds at all specified levels. The circumstances that vary are as follows: deviations from the methodology values in terms of flow rate (± 10%), temperature (± 5°C) of the column oven, wavelength (± 2 nm), pH variation (± 0.2 unit) of the buffer, organic fluctuation (± 2% absolute) in the column, and batch/lot variation in mobile phase A.

Forced degradation

To demonstrate the established method's specificity and to demonstrate that it is stability indicating, forced degradation was used. Conditions of heat, humidity, oxidation, photolysis, and hydrolysis (acid and base) were studied in relation to the stress degradation of droxidopa. The medication underwent three different treatments: acid degradation (150 minutes at 85°C) using 1M HCl, base degradation (15 minutes at 85°C) using 0.2M NaOH, and peroxide degradation (60 minutes at 85°C) using 6.0 % H_2O_2. Moreover, heat degradation was investigated by heating the normal medication solution to for 120 hours, at 105°C. The medication was subjected to 200 watts hour's/meter square of UV radiation and 1.2 million lux hours of white fluorescent light in order to study photolytic degradation. For 120 hours at room temperature and 92.5% relative humidity, the deterioration of humidity was also reviewed by the droxidopa peak purity data from each degradation sample. As a result, the test procedure is deemed to be precise and stable. Indicating that it may be used to determine related compounds that are either specified or not in the drug ingredient droxidopa.

RESULTS AND DISCUSSION

Specificity

The spiked sample and the chromatograms depicting related chemicals amply illustrate the efficient separation of related substances from closely eluting peaks of droxidopa and other related drugs. The spiked sample, the droxidopa peak in the control sample/diluted, and the spiked sample/diluted all exhibit homogeneous peaks without any co-eluting peaks when the peak purity data for known related chemicals are analyzed (Table 2). With confidence, one may conclude from the information supplied that The test procedure is designed

Table 2: RT of the droxidopa and its relative substances

Compound name	*Retention time (min.)*
Erythro droxidopa	0.90
Droxidopa	1.00
3 ,4-Dihydroxybenzaldehyde	2.15
Vanillin	3.05

specifically to find similar chemicals in the droxidopa. The standard's retention time and that of droxidopa are nearly identical. Table 2 provides specific information about the threshold of droxidopa and its related substances, as well as the retention times (RT) of the control and spiked samples, relative retention times (RRT), purity angles, and both. The accuracy and specificity of the test procedure for the precise identification of related compounds in droxidopa are highlighted by these findings taken together (Figures 5 & 6).

Limits of detection (LoD) and quantification (LoQ)

Droxidopa acceptance requirements for signal to noise ratio are met by the data and droxidopa-related substances in LoD and In terms of LoD and LoQ, %RSD satisfies the acceptability requirements for droxidopa and related compounds. Consequently, the method of testing for determining related compounds in the active ingredient Droxidopa is accurate. Additionally, droxidopa and its related compounds have LoQ values that are much below the 50% specified level. Table 3 presents the statistical information for six duplicates of the Droxidopa and relevant compounds.

Linearity

It was found that, over 0.990 is the correlation coefficient for droxidopa and droxidopa known related substances. Hence, the response of droxidopa and each of the related substances of droxidopa are linear between LoQ and 120% of the specified amount. Table 4 contains a mention of the findings. Figures 7(a-d) depict the linearity plot (concentration vs area) of droxidopa and relative substances.

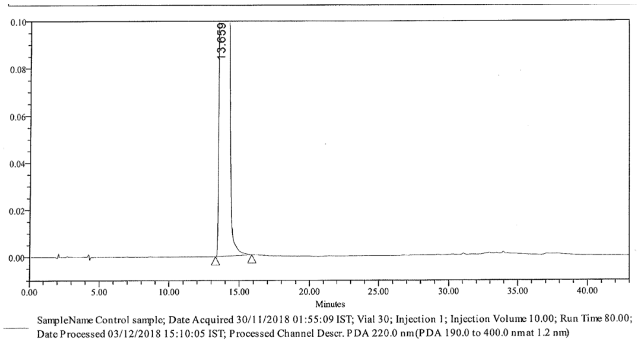


Figure 5: Chromatogram displaying the control samples' retention duration for droxidopa and related compounds

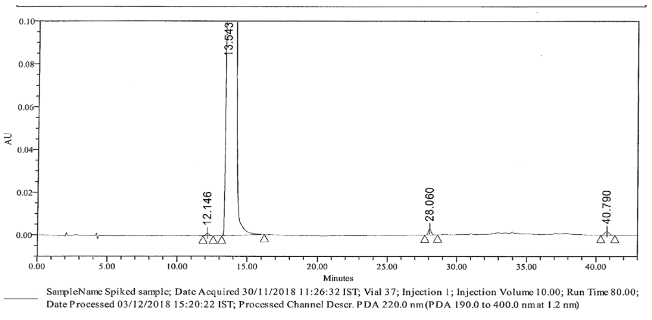


Figure 6: Chromatogram displaying the spiked samples' retention duration for droxidopa and related compounds

Precession

With regard to analyst-to-analyst, daily, system-to-system, and column-to-column variations, the method is robust for the determination of related compounds in droxidopa drug material, according to the results (System precession, Method precision, and Intermediate precision). Results are shown in Table 5.

Accuracy

The recovery findings demonstrated that the test method possesses a satisfactory level of precision in determining the relevant components in the droxidopa drug substance, ranging from the limit of quantification (LoQ) to 120% of the specified value. The results of accuracy at different levels are displayed in Tables 6 and 7.

Robustness

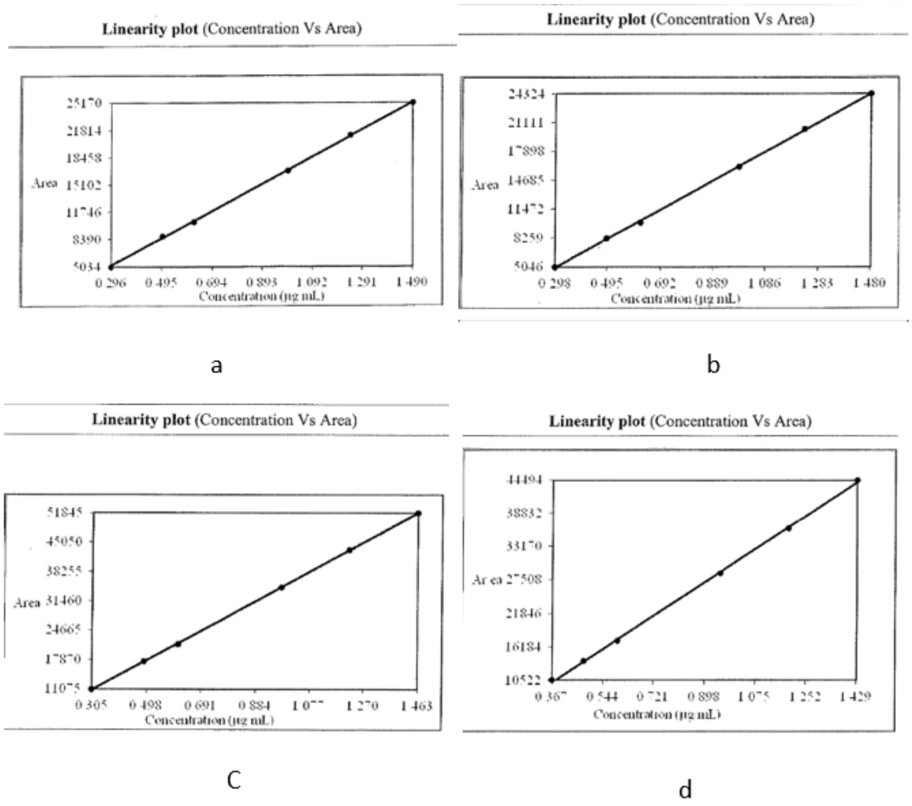
Additionally, chromatograms of samples tampered with using known related compounds from other sources revealed this. The circumstances mentioned above, wherein the RRTs of related chemicals obtained under various conditions do not significantly differ from those obtained under STP settings. Therefore, if the test procedure is appropriate for identifying related drugs, it can be said. in the medicinal substance droxidopa within the change values investigated for each of the previously indicated attributes. Tables 8 & 9 show the results for robustness.

Table 3: Droxidopa LoD and LoQ results

Name of the compound	*%RSD*	*Conc. (μg/mL)*		*Conc (% w/w)*		*S/N ratio*
	LoQ	*LoD*	*LoQ*	*LoD*	*LoQ*	*LoD*
Droxidopa	0.9	0.12	0.31	0.006	0.0155	12
Erythro droxidopa	1.4	0.12	0.31	0.006	0.0154	12
3,4-Dihydroxybenzaldehyde	2.6	0.12	0.31	0.006	0.0156	26
Vanillin	6.4	0.18	0.36	0.009	0.0181	16

Table 4: Linearity of the droxidopa and its related substances

Name of the Compound	*Slope*	*Intercept*	*Residual sum of squares*	*Correlation coefficient*	*Response factor (or) Correction factor*
Droxidopa	16816	170	94453	0.9998	
Erythro droxidopa	16391	51	66707	0.9998	1.03
3,4-Dihydroxybenzaldehyde	35343	132	112926	0.9999	0.48
Vanillin	31926	1555	476800	0.9997	0.53



a

b

C

d

Figure 7a-d: Linearity plot of droxidopa

Table 5: Droxidopa and its related compound precision data

Name of the sample	*System precision*					
	%w/w (Mean ± SD)		*%RSD*	*95% Confidence interval*		
Droxidopa	20625 ± 313		1.5	329		
Compound name	*Method precision*			*Intermediate precision (Overall precision)*		
	%w/w (Mean ± SD)	*%RSD*	*95% Confidence interval*	*%w/w (Mean ± SD*	*%RSD*	*95% Confidence interval*
Erythro droxidopa	0.060 ± 0.001	1.7	0.001	0.058 ± 0.00	0.0	0.0
3,4-Dihydroxybenzaldehyde	0.061 ± 0.000	0.0	0.000	0.060 ± 0.00	0.0	0.0
Vanillin	0.057 ± 0.001	1.8	0.001	0.064 ± 0.00	0.0	0.0

Solution Stability

System appropriateness, A sample solution that had been tampered with at the specification level using known related compounds was prepared together with standard solutions prepared according to procedure. Subsequently, the solutions were examined at ambient temperature (25°C) and in a refrigerator (between 5 and 3°C) for different times. The stability of the mobile phase has been demonstrated by utilizing standard solution stability at room temperature (~ 25°C) to assess the applicability of the system. Based on the data presented above, it can be said that the mobile phase remains stable at room temperature (~25°C) for at least 48 hours, while the sample solution is not stable (Table 9).

Table 6: Accuracy of the droxidopa and its compounds at LoQ level

	LoQ level		
Name of the sample	*LoQ Level*		
	%w/w (Mean ± SD)	*%RSD*	*95%Confidence interval(+)*
Erythro droxidopa	101.9 ± 0.65	0.6	1.6
3,4-Dihydroxybenzaldehyde	97.4 ± 0.65	0.7	1.6
Vanillin	88.9 ± o.95	1.1	2.4

Table 7: Accuracy of the droxidopa and its compounds at different specification level

Name of the sample	*At a level of 50%*		*At a level of 100%*		*At a level of 120%*		*Overall accuracy*	
	%w/w Average	*% of RSD*	*%w/w Average*	*% of RSD*	*%w/w Average ± SD*	*% of RSD*	*%w/w Average ± SD*	*% of RSD*
Erythro droxidopa	103.4 ± 0.00	0.0	103.4 ± 0.00	0.0	104.3 ± 0.00	0.0	103.7 ± 0.45	0.3
3,4-Dihydroxybenzaldehyde	98.9 ± 1.91	1.9	99.5 ± 0.92	0.9	99.1 ± 0.81	0.8	99.1 ± 1.16	1.2
Vanillin	86.7 ± 0.00	0.0	95.6 ± 0.92	1.0	98.2 ± 0.75	0.8	93.5 ± 5.25	5.6

Table 8: Sturdiness of the compounds related to Droxidopa at various specification levels

A parameter	*Difference*	*System suitability*		
		USP plate count	*USP resolution*	*%of RSD*
STP	-	6287	2.5	0.8
Flow rate	-10%	6426	2.6	1.4
	+10%	6212	2.5	0.9
STP*	-	5365	2.5	1.6
Column lot variation STP**	-	6215	2.5	1.0
STP	-	6523	2.4	2.4
Column oven temperature	-5°C	6312	2.4	2.9
	+5°C	6816	2.2	0.7
STP	-	5430	2.8	1.0
Wave length variation	-2 nm	5429	2.4	1.8
	+2 nm	5429	2.4	2.9
STP	-	5430	2.2	0.4
Buffer pH variation	-0.2 units	5791	2.4	1.3
	+0.2 units	6180	2.2	1.2

*1st Column (Lot No. RAQ5-4814, ID No.: AUZ 118)
**2nd Column (Lot No. RAQ5-4814, ID No.: AUZ 121)

Table 9: Robustness of the spiked Droxidopa related substances

Spiked sample				
Parameter	*variation*	*RRT of*		
		Erythro droxidopa	*3,4-Dihydroxy-benzaldehyde*	*Vanillin*
STP		0.90	2.20	3.20
Flow rate	-10%	0.90	2.11	3.07
	+10%	0.90	2.30	3.35
STP		0.90	2.21	3.23
Column oven temperature	-2 °C	0.90	2.18	3.17
	+2 °C	0.90	2.24	3/29
STP		o.89	2.13	3.08
pH of Buffer	-0.2 Units	0.90	2.08	3.05
	+0.2 Units	0.88	2.30	3.34
Wave length	-2 nm	o.89	2.13	3.08
	+2 nm	o.89	2.13	3.08
STP*		0.90	2.25	3.26
STP**	Column lot variation	0.90	2.09	3.04

Forced Degradation

In alkaline settings (0.2M NaOH/85°C /15 minutes), the drug material Droxidopa is prone to degradation; nevertheless, in acidic conditions (IM HCI/85°C/150 minutes), oxidative conditions (6.0% H2O2/85°C/ 60 minutes), and humidity, thermal, and photolytic degradation conditions, it remains stable, according to the results of the application of various stress conditions to the drug substance's degradation. Droxidopa carboxamide is a possible degradation impurity, according to the forced degradation data that was produced.

Furthermore, the uniformity of the peak and the absence of co-eluting peaks were shown by the Droxidopa peak purity data from each degradation sample. As a result, the test procedure is deemed to be precise and stable, indicating that it may be

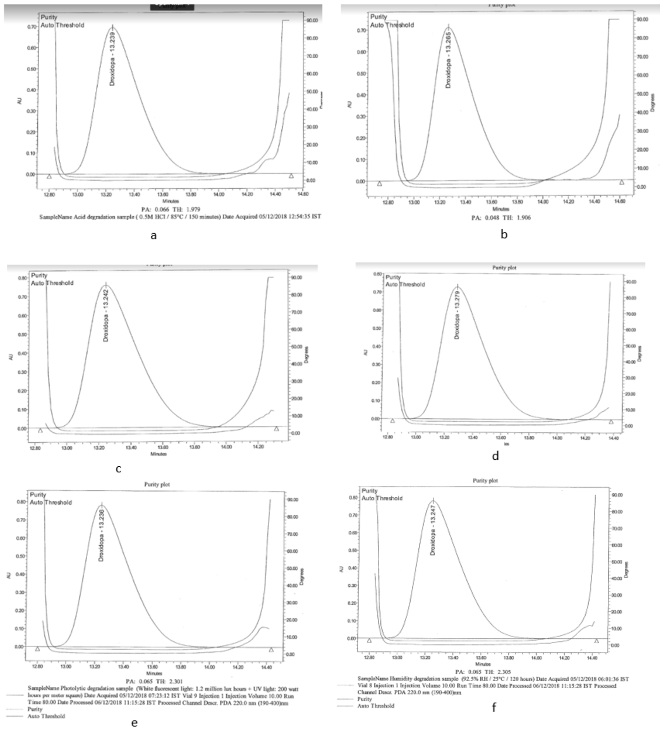


Figure 8a-f: Purity plots of droxidopa in different degradation methods

Table 10: Stability of droxidopa and related sub stances

Standard solution - At room temperature (~25°C)		
Time	*Droxidopa*	
	Area	*% Difference*
Initial	20350	-
After 24 hours	20120	1.1
After 48 hours	19850	1.3

Sample solution - At ambient temperature (~25°C)				
Time	*Erythro droxidopa*		*3,4-Dihydroxy-benzaldehyde*	
	Area	*%Difference*	*Area*	*%Difference*
Initial	18810	-	44560	-
After 24 hours	19200	2.0	46767	2.6
After 48 hours	19328	2.8	49164	3.7

Sample solution - At ambient temperature (~25°C)		
Time	*Vanillin*	
	Area	*% Difference*
Initial	40500	-
After 24 hours	40800	0.7
After 48 hours	40750	0.6

Stability of eluent			
Time	*System suitability*		
	System suitability solution at room temperature (~ 25°C)		
	USP resolution	*USP plate count*	*%RSD*
Initial	2.6	6097	0.9
After 24 hours	2.6	6100	1.2
After 48 hours	2.5	5990	0.9

Table 11: Forced degradation droxidopa

Degradation condition	*Purty angle*	*Purity threshold*
Acid degradation (1M HCL/85°C/150 minutes)	0.066	1.979
Base degradation (0.2M NaOH/85°C/15 minutes)	0.048	1.906
Peroxide degradation (6.0% H2O2/85°C/ 60 minutes)	0.080	1.990
Thermal degradation (105°C/120 hours)	0.069	2.143
Photolytic Degradation (White fluorescent light with 1.2 million lux hours and UV light with 200 watt hours per square meter)	0.065	2.301
Humidity Degradation (25°C /92.5% RH/ 120 hours)	0.065	2.305

used to determine related compounds that are either specified or not in the drug ingredient droxidopa (Table 10).

CONCLUSION

Specificity, detection and quantification limits, linearity, precision, accuracy, standard and sample solutions, and system stability are all confirmed for the developed method. Based on the aforementioned statistics, it can be inferred that the method is characterized by specificity, selectivity, and accuracy. This method is applicable for regular analysis to quantitatively determine the associated chemicals in the droxidopa drug substance.

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AUTHOR CONTRIBUTIONS

The writers each contributed significantly to this paper, taking an active part in the editing and review process, and gave their consent for the publication of the completed draft.

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