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Simultaneous Analytical Method Development for Dosulepin Hydrochloride (CNS Drug) and Methylcobalamin by UV and HPLC

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Abstract Nowadays, new analytical method development is a need for a routine exercise for the analysis purpose due to its advantages over the non-instrumental methods. The objective of research work is to develop simple, accurate and precise analytical methods and validation of analytical methods for the determination of selected drugs acting on CNS and its combination in bulk and pharmaceutical formulations as well as degradation studies of the same drugs. The developed and optimized HPLC method was validated with respect to linearity, ranges, precision, accuracy, robustness, LOD, and LOQ as per ICH Q2R1 guidelines and can be applied for the estimation of DOS and MCA in the combined formulation. The method is also found to be specific as specificity is the ability to assess the analyte unequivocally in the presence of components like impurities, degradants, matrix, etc. as per ICH Q2R1 guidelines. It was possible to separate the drug from its degradation product effectively; hence, it was employed as a stability-indicating method for estimation of DOS and MCA in their tablet dosage form. So, it can be applied for the routine analysis.

Keywords: Dosulepin hydrochloride, Methylcobalamin UV, HPLC

Introduction

A 'regulatory analytical procedure' is used to analyse a defining characteristic of the raw materials, active pharmaceutical ingredients and pharmaceutical formulations in pharmaceutical sectors. Now a day, methods of interest for quantification or estimation are sophisticated analytical methods, i.e. HPLC, GLC, and HPTLC, which are generally used for routine or laboratory purpose. Chromatographic methods are mainly used for the qualitative and quantitative estimation of drug substances, drug products, raw materials throughout the drug development, from the initial stage of research to release of drug products. The sophisticated analytical methods are simple, effective, and robust for the estimation of raw materials, active pharmaceutical ingredients and pharmaceutical formulations [1]. Initially, the methods were based on simple titrations and different qualitative reactions characteristic of the analyte, but these ways, human errors were always there, and the efforts were continued to minimize these errors. Resultant outputs of these efforts are different analytical instruments having fewer chances of human error as the human eyes are replaced by the highly automated detectors [2]. Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. There are different modes of separation in HPLC. They are normal phase mode, reversed-phase, reverse phase ion pair chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography) [3-5]. Validation of analytical methods means activity or procedures under pre- established criteria which provide

documented evidence that a method developed will produce a consistent result, i.e. linearity, accuracy, precision, robustness, ruggedness, etc. Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose [6-7]. A successful Validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled. Method validation builds a degree of confidence, not only for the developer but also to the user. Validation appears costly and time-consuming, but it results in inexpensive, eliminates frustrating repetitions and leads to better time management at the end.Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons, but the method validation absorbs the shock of such conditions and pays for more than invested in the process [8]. Various drugs acting on CNS were selected for the development and validation of stability-indicating assay methods in bulk and formulations. Dosulepin hydrochloride (DOS) also known as Dothiepin hydrochloride, is a tricyclic antidepressant with anxiolytic properties that is used in the treatment of depression. Dosulepin inhibits the reuptake of biogenic amines, increasing available neurotransmitter levels at the synaptic cleft. Dosulepin is a thio derivative of Amitriptyline with similar efficacy to that of Amitriptyline, and also exhibits anticholinergic, antihistamine and central sedative properties. Its hydrochloride form is a common active ingredient in different drug formulations. Methylcobalamin (MCA) also called as mecobalamin, which is a form of vitamin B12. It differs from cyanocobalamin in that the cyano group is replaced with a methyl group. Methylcobalamin can be used to prevent or treat pathology arising from a lack of Vitamin B12 intake (Vitamin B12 deficiency) [9]. It is also used in the treatment of peripheral neuropathy, diabetic neuropathy, and as a preliminary treatment for amyotrophic lateral sclerosis. As per literature review, various individual analytical methods are available for estimation of above mentioned drugs acting on CNS in their individual dosage forms or in combination with other dosage forms. Any stability indicating HPLC method for simultaneous estimation of above mentioned drugs acting on CNS in pharmaceutical dosage form [10]. The proposed work is to develop simple, selective, sensitive, less expensive and stability-indicating methods for the analysis of selected drugs acting on CNS in their bulk and formulations. The outlines of the objectives are to development and validation of the spectroscopic method for analysis of selected CNS drugs and its combination in bulk and pharmaceutical formulations. Development and validation of HPLC method for analysis of selected CNS drugs and its combination in bulk and pharmaceutical formulations.

Materials

Dosulepin hydrochloride and Methylcobalamin API were received as gift samples from Elite Pharmaceutical Pvt. Ltd., Ahmedabad. The tablet formulation containing Dosulepin hydrochloride 50 mg and Methylcobalamin 1500 mcg was purchased from local Pharmacy. Methanol (Merck) was used.

Methods

Preparation of Stock Solutions: The stock solutions of Dosulepin hydrochloride and Methylcobalamin were prepared by transferring 50 mg of DOS and 60 mg of DOS in 100 ml of volumetric flask and made up to the mark to get the strength of 500 µg/ml of DOS and 600 µg/ml of MCA. Working standards were prepared by diluting 2 ml of standard stock of each drug solutions in 100 ml volumetric flask with methanol to get 10 µg/ml strength of DOS and 12 µg/ml strength of MCA as MCA is light sensitive, all solutions prepared in ambered coloured volumetric flask wrapped with aluminium foil.

Preparation of Calibration curves for Dosulepin hydrochloride and Methylcobalamin: For calibration curve of Dosulepin hydrochloride 3, 4, 5, 6 and 7 ml of Dosulepin hydrochloride working standard solution were taken and made up the volume up to 10 ml with methanol in 10ml volumetric flasks (3-7 µg/ml) and for Methylcobalamin 3, 4, 5, 6 and 7 ml of Methylcobalamin working standard solution were taken and made up the volume up to 10 ml with methanol in 10ml volumetric flasks (3.6-8.4 µg/ml).

Preparation of Sample Solution: Twenty tablets were weighed and finely powdered. The powder equivalent to 50 mg of DOS and 1.5 mg of MCA was weighed accurately. Using standard addition method accurately weighed 58.5

mg of standard MCA added to above tablet powder and transferred into 100 ml volumetric flask and make up the volume up to 100 ml using methanol to get 100 µg/ml of DOS and 120 µg/ml of MCA. From the resulting solution 2 ml was transferred to 100 ml volumetric flask and dilute up to 100 ml with the same solvent to get a final concentration 10 µg/ml of DOS and 12 µg/ml of MCA.

Stability Indicating RP-HPLC Method Development and Validation for DOS and MCA in Combined Tablet Formulation [11]:

Optimization of Mobile Phase: On the basis of physiochemical properties of drugs and literature regarding the mobile phase development for the same class of drugs, various solvent systems were used to get the better separation, i.e. resolution and selectivity. The selection was also based on parameters like separation of a peak, peak shape, theoretical plate and resolution.

Preparation of Mobile Phase: The mobile phase was prepared by mixing 60 ml of Acetonitrile and 40 ml of 0.03 M Phosphate buffer. (Phosphate Buffer: 0.03 M KH_2PO_4 with 0.2% Triethylamine and 0.2% Hexane sulfonic acid, pH adjusted to 3 with O- Phosphoric acid). Triethylamine used to reduce peak tailing Hexane sulfonic acid used to retain the peak.

Preparation of Stock solutions: DOS 330 mg and MCA 10 mg were weighed accurately, mixed with mobile phase (15 ml), ultrasonicated for 10 min and diluted to 100 ml with mobile phase to get stock solution of 3300 µg/ml and 100 µg/ml for DOS and MCA, respectively. Different aliquots of the stock solution were diluted in such a way to get concentrations in a range of 165-495 µg/ml and 5-15 µg/ml for DOS and MCA, respectively.

Preparation of sample solution: Twenty tablets were accurately weighed and finely powdered. The powder equivalent to 330 mg of DOS and 10 mg of MCA was taken in 100 ml volumetric flask, mixed with mobile phase (15 ml), ultrasonicated for 10 min and diluted to 50 ml with the mobile phase. The solution was filtered through 0.45 µm cellulose nitrate membrane filter paper and dilute to 100 ml with mobile phase to get a sample solution containing 3300 µg/ml of DOS and 100 µg/ml of MCA.

Method Validation: Method validation involved various validation parameters; Linearity, Precision, Recovery, LOD and LOQ, Robustness. All the validation parameters were successfully determined as per ICH Q2 (R1).

Estimation of Pharmaceutical Dosage Form of DOS and MCA by RP-HPLC: The sample solution was analysed by RP-HPLC, and the content of DOS and MCA from marketed formulation was calculated from the calibration curve.

Results and Discussion

Preliminary Study: The melting point of both API was taken and compared with the reported melting point (107°C, 108°C); the melting points were found to be in the range of reported melting point. The MP of drug Sample DOS is 216-220 °C and MCA >300 °C. The IR spectrum of DOS was taken by KBr pellets technique and compared with the reference spectrum; the transmittance peaks were found to be identical in API spectra and observed frequency are -C-H(Aromatic) 3063, -C-S 593, -N (quat) 2360, -C=C (Aromatic) 1469 cm^{-1}. The IR spectrum of MCA was taken by KBr pellets technique and compared with the reference spectrum; the transmittance peaks were found to be identical in API spectra and observed frequency are -OH s 3329, -CH 3010, -C=O 1792, C=N 2220 and -C=O (Amide) 1663. The stability indicating assay method for estimation of DOS and MCA in Pharmaceutical Formulation by RP-HPLC for the selection of detection wavelength was optimized. The standard solutions were scanned in the UV spectrometer at 200–400 nm and the spectrums were recorded. From UV spectrum 285 nm wavelengths were selected for the estimation of both drugs in RP-HPLC method. The selection and optimization of chromatographic conditions based on the physiochemical properties of selected drugs and literature regarding the analytical methods for the same class of drugs, various solvent systems were used to get the better separation i.e. resolution and selectivity. The different mobile phases tried as shown in Table 1. The optimized chromatographic conditions for DOS and MCA was shown in Table 2. The linearity range for DOS and MCA were found to be 3-7 and 3.6-8.4µg/ml, respectively. The RSD values for DOS and MCA was found to be 0.55 and 0.94, respectively. As the % RSD is ≤2.0, which indicates that the developed method is repeatable. The RSD values of inter day and intraday variations for DOS and MCA reveal that the developed method was precise. The recovery experiments were performed by the standard addition in pre-analyzed concentration. The results show that the percentage recoveries for DOS were 99.26–99.98%**,** while for MCA,

it was found to be in range of 99.96–100.56%. The low value of standard deviation indicates that the developed method was accurate. The standard deviation of the retention time and peak area were calculated for each parameter and the % RSD was found to be less than 2% for DOS and MCA. The column efficiency, resolution and theoretical plates, tailing factor were calculated for the standard solutions. RP-HPLC method was used to determine DOS and MCA in pharmaceutical formulation. There was no interference of the excipients in the estimation of active ingredient; hence the developed method was applicable for the routine analysis of DOS and MCA in pharmaceutical formulation. The present work involves the development and validation of the simple, accurate and precise spectroscopic method and stability-indicating RP-HPLC method for the estimation of various drugs acting on CNS in their combined tablet formulation. Both the methods were validated as per ICH Q2R1guideline.

Table 1: Selection of Mobile Phase for DOS and MCA

Trial No.	Mobile Phase	DOS RT (min)	MCA RT (min)	Remarks
1	Water: Methanol (50:50)	-	-	No peak observed in run time of 30 min
2	Water: Methanol (30:70)	-	-	No peak observed in run time of 30 min
3	Methanol: 1% Ammonium	-	-	No peak observed in run time of 30 min
4	Water: Acetonitrile (50:50)	22.24	1.69	Broad peak of DOS observes with tailing of 2.23. MCA peak observed before solvent front.
5	Tetrahydrofuran: Acetonitrile: Phosphate Buffer (0.5% KH2PO4 pH adjusted to 3 with O-Phosphoric acid) (10:40:50)	10.737	1.761	DOS peak observed with tailing (3.35). MCA peak observed before solvent front.
6	Acetonitrile: Phosphate Buffer (0.4% KH2PO4 + 0.2% TEA + 0.1% Hexane sulfonic acid pH adjusted to 3 with with O- Phosphoric acid) (70:30)	2.976	1.333	MCA peak observed before solvent front.
7	Acetonitrile: Phosphate Buffer (0.03 M KH2PO4 + 0.2% TEA + 0.2% Hexane sulfonic acid, pH adjusted to 3.0 with O- Phosphoric acid) (60:40)	8.263	3.183	Two peaks were observed with proper separation and resolution

Table 2: Optimized Chromatographic Conditions for DOS and MCA

S. No.	Parameters	Specification
1	Stationary phase	Kromasil C_{18} (250 x 4.6 mm, 5 µm)
2	Mobile phase	Acetonitrile: 0.03 M Phosphate Buffer (60:40)
3	Flow rate	1 ml/min
4	Run time	30 min
5	Injection volume	10 µl
6	Detection wavelength	285 nm
7	Retention time	DOS: 8.263 min MCA: 3.183 min

Table 3: Linearity Data for DOS and MCA

DOS	MCA

Conc. (µg/ml)	Peak Area±SD (n=3)	% RSD	Conc. (µg/ml)	Peak Area±SD (n=3)	% RSD
165	1480786±584.55	0.04	5	77616.8±125.23	0.16
247.5	2317891 ±1809.54	0.08	7.5	125952±237.4	0.19
330	3086490 ±3003.19	0.1	10	157295.8 ±286.89	0.18
412.5	3995101 ±2498.37	0.06	12.5	203225.2 ±200.16	0.1
495	4706800 ±3355.07	0.07	15	254290±200.42	0.08

Table 4: Regression Analysis Data for RP-HPLC Method

Parameters	DOS	MCA
Wavelength (nm)	285	285
Linearity range(µg/ml)	165–495	May-15
Regression equation	y=9853.6x-134282	y=17225x-8572.1
Regression Coefficient (R2)	0.999	0.9945

Table 5: Repeatability Data for DOS and MCA

DOS		MCA	
Concentration (µg/ml)	**Peak Area**	**Concentration (µg/ml)**	**Peak Area**
330	3086495	10	157257
330	3069307	10	158156
330	3079539	10	157265
330	3067547	10	157451
330	3052616	10	156996
330	3075748	10	157682
Mean	3071875	**Mean**	157467
SD	11693.73	**SD**	406.92
%RSD	0.38	**%RSD**	0.25

Table 6: Intraday Precision Data for DOS and MCA

Drug	Concentration (µg/ml)	Mean Peak Area ±S.D. (n=3)	%RSD
	165	1477758 ±4592	0.31
DOS	330	3075762 ±14929	0.48
	495	4714408 ±19769	0.41
	5	77230±645	0.84
MCA	10	157826±978	0.62
	15	253929±1337	0.52

Table 7: Inter day Precision Data for DOS and MCA

Drug	Concentration (µg/ml)	Mean Peak Area ±S.D. (n=3)	%RSD
	165	1499656 ±20976	1.39
DOS	330	3124357 ±43180	1.37
	495	4731260±57825	1.22
	5	77273±1183	1.53
MCA	10	156792±2612	1.66
	15	254524±4647	1.82

Table 8: LOD and LOQ of DOS and MCA

Parameter	DOS	MCA
LOD(µg/ml)	0.75	0.04

LOQ (µg/ml)	2.28	0.121

Table 9: Accuracy Data for DOS and MCA

Drug	Recovery Level	Amount of sample taken(µg/ml)	Amount of standard spiked (µg/ml)	Amount recovered (µg/ml)	% recovery ±SD (n=3)
DOS	50%	165	82.5	246.95	99.78±0.58
	100%	165	165	327.55	99.26±0.41
	150%	165	247.5	412.41	99.98±0.28
MCA	50%	5	2.5	7.497	99.96±0.71
	100%	5	5	10.02	100.21±0.83
	150%	5	7.5	12.57	100.56±0.68

Table 10: Robustness study of DOS and MCA

Parameter	Method Condition	DOS		MCA	
		Peak Area ±SD	% RSD	Peak Area ±SD	% RSD
Flow rate (ml/min)	0.8	3030254±11211	0.37	154856±805	0.52
	1	3065303±11728	0.38	157257±349	0.22
	1.2	3091163±13601	0.44	159635±622	0.39
Mobile phase Ratio (%V/V)	59:41:00	3046672±14624	0.48	153563±476	0.31
	60:40:00	3065303±11728	0.38	157257±349	0.22
	61:39:00	3093753±15778	0.51	155209±682	0.44
pH	2.8	3092528±10823	0.35	156843±956	0.61
	3	3065303±11728	0.38	157257±349	0.22
	3.2	3074510±14450	0.47	154621±757	0.49

Table 11: Validation and System Suitability Parameters of DOS and MCA

Validation Parameters		
Parameters	**DOS**	**MCA**
Linearity	165-495 µg/ml	5-15µg/ml
Accuracy (n=3)	99.26-99.98%	99.96-100.56%
Precision (%RSD)		
Repeatability (n=6)	0.38	0.25
Intraday (n=3)	0.31-0.48	0.52-0.84
Interday (n=3)	1.22-1.39	1.53-1.82
Robustness (%RSD)	0.37-0.51	0.31-0.61
LOD (µg/ml)	0.75	0.04
LOQ (µg/ml)	2.28	0.121
System Suitability Parameters		
Retention time (Rt)	8.263min	3.183min
Theoretical plates (N)	8413	7474
Tailing factor (T)	1.364	1.368
Resolution (Rs)	25.99	

Table 12: Estimation of DOS and MCA in Tablet Dosage Form

Drug	Conc. of Dosage Form	Conc. Found ±SD	% Assay ±SD (n= 3)	%RSD
DOS	50mg	49.255±0.14	98.51±0.28	0.28
MCA	1.5mg	1.933±0.011	128.89±0.77	0.77

Table 13: Comparison of assay results of two proposed methods of DOS and MCA by Student's paired 't'-Test (d.f.=4)

Drugs	**DOS**		**MCA**	
Methods	**UV**	**HPLC**	**UV**	**HPLC**
% Assay	99.18	98.21	114.66	129.66
	99.56	98.54	112	128.12
	100.06	98.78	113.1	128.89
One tailed t-test	$T_{0.05}$(Cal)	0.0038	$T_{0.05}$(Cal)	0.0002
	$T_{0.05}$(Tab)	2.13	$T_{0.05}$(Tab)	2.13
Two tailed t- test	$T_{0.05}$(Cal)	0.0077	$T_{0.05}$(Cal)	0.0004
	$T_{0.05}$(Tab)	2.78	$T_{0.05}$(Tab)	2.78

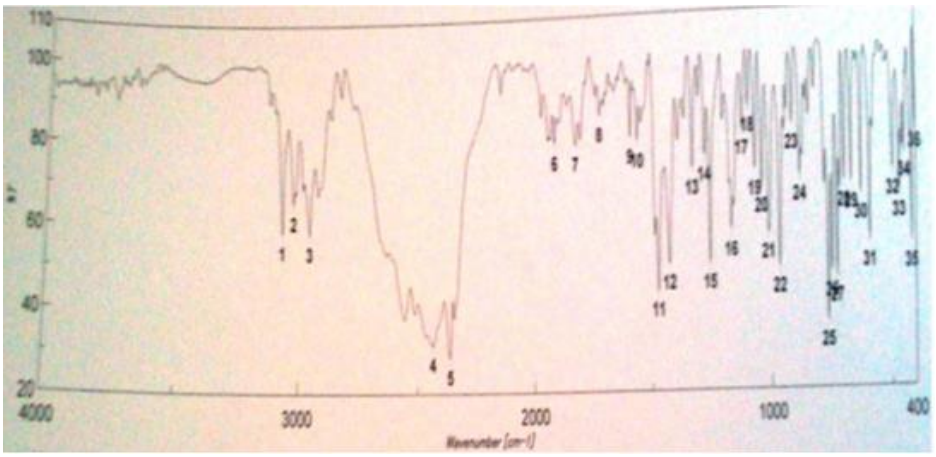

Figure 1: IR Spectra of DOS

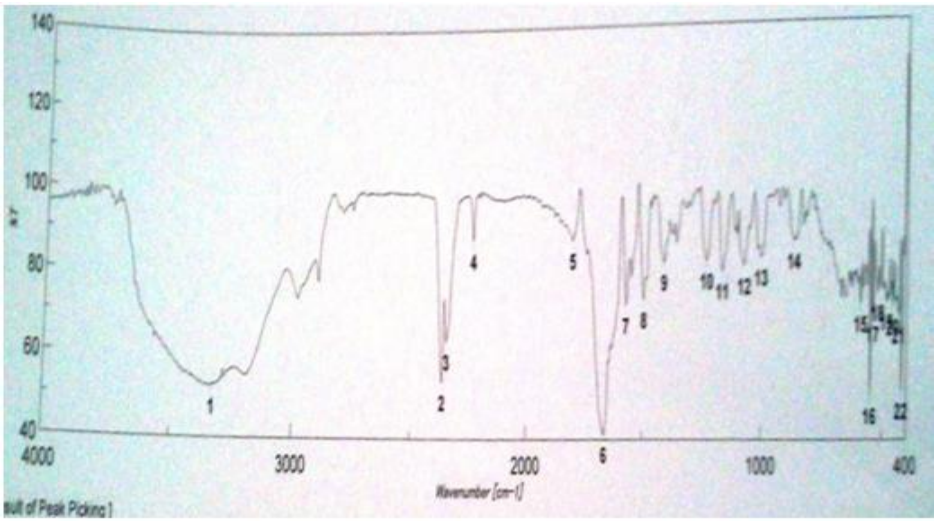

Figure 2: IR Spectra of MCA

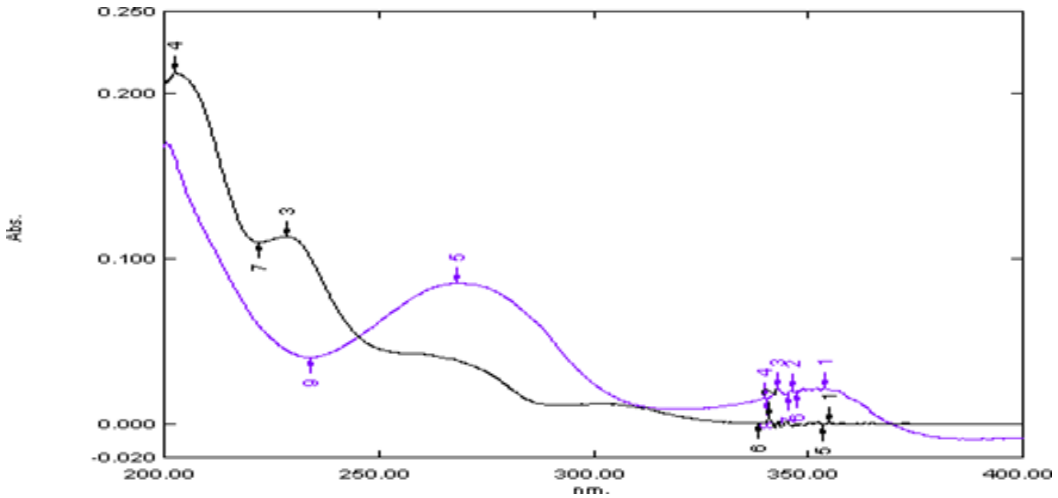

Figure 3: Overlain Spectra of DOS and MCA

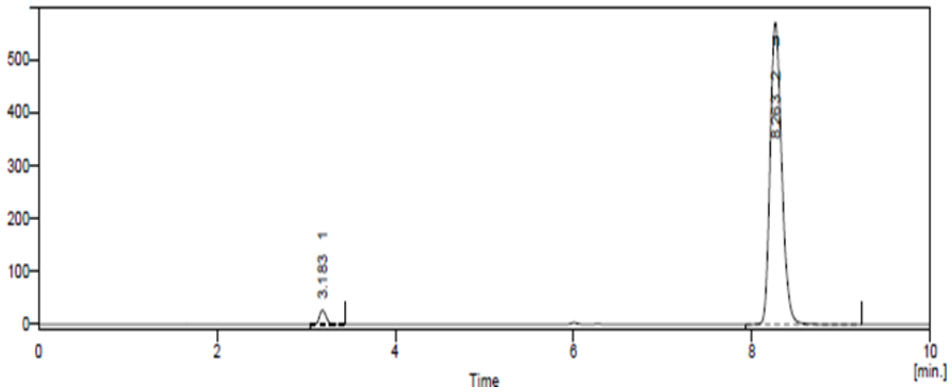

Figure 4: Chromatogram of standard mixture of DOS and MCA

Conclusion

DOS and MCA were separated and estimated using newly developed Stability Indicating RP-HPLC method in combined tablet formulation. The separation was achieved on Kromasil C18 (250x4.6mm,5µm particle size) using acetonitrile: phosphate buffer with pH 3.0 adjusted using orthophosphoric acid in the ratio of 60:40 v/v as optimized mobile phase and flow rate of 1 ml/min. A wavelength of 285 nm was chosen as a detection wavelength because both the drugs had sufficient absorption at this wave length. The retention time for DOS and MCA was obtained 8.263 min and 3.183 min, respectively. The linearity of the developed method was in the range of 165-495 µg/ml and 5-15 µg/ml with regression coefficient 0.999 and 0.9945 for DOS and MCA, respectively

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