RESEARCH ARTICLE

Analytical Method Development and Validation of Denaverine Hydrochloride in Bulk and Injectable Pharmaceutical Dosage Form by HPLC Method

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Received: 25th February, 2022; Revised: 09th April, 2022; Accepted: 26th May, 2022; Available Online: 25th June, 2022

ABSTRACT

An accurate, precise, and rapid reverse-phase high-performance liquid chromatographic (RP-HPLC) method is developed and validated to estimate Denaverine HCl in bulk and injection dosage form is used in the treatment of antispasmodic drugs in Veterinary medicine. This method is based on the drug separation in reversed-phase mode using Symmetry C_{18} Column (4.6 x 150 mm, 5 µm, Make : XTerra) The optimized mobile phase was disodium hydrogen phosphate buffer (pH 3.5): Acetonitrile (30:70 %v/v). The flow rate was 0.6 mL/min and UV detection was 306 nm. The retention time was 3.2 min for Denaverine HCl. Under ICH guidelines validation, it was accurate and reproducible. Linearity was in the concentration range of 10–50 µg/mL for Denaverine HCl. The mean percent recovery of samples at each level for both drugs was 101.3 %v/v for denaverine HCl. It may be used for quality control of bulk and injectable pharmaceutical dosage forms.

Keywords: Denaverine hydrochloride, Estimation, HPLC, Validation.

International Journal of Pharmaceutical Quality Assurance (2022); DOI: 10.25258/ijpqa.13.2.14

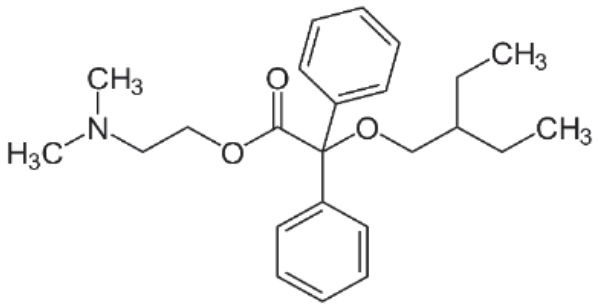
How to cite this article: Krishnamoorthy G, Ahamed JI, Senthamarai R, Ismail AM. Analytical Method Development and Validation of Denaverine Hydrochloride in Bulk and Injectable Pharmaceutical Dosage Form by HPLC Method . International Journal of Pharmaceutical Quality Assurance. 2022;13(2):164-168.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Denaverine hydrochloride[1-6] is a muscle relaxant. It was developed and patented in Germany in 1974. Under the brand name Sensiblex, denaverine hydrochloride has an indication in veterinary medicine as a muscle relaxant for the myometrium of cows and dogs during parturition. Now, the drug is in trial with human plasma to treat urogenital and gastrointestinal spasms under the brand Spasmalgan (Scheme 1).



Scheme 1: Chemical Structure of Denaverine hydrochloride 2-dimethylaminoethyl 2-(2-ethylbutoxy)-2,2 diphenylacetate hydrochloride

Denaverine hydrochloride, is a neurotropic–musculotropic spasmolytic with an analgesic effect. It treats gastrointestinal and urogenital smooth muscle spasms, postoperative abdominal pain and obstetrics. Although denaverine hydrochloride has been successful for over 30 years, there was little information on its biotransformation in humans.

Mechanisms of Action

Denaverine inhibits the enzyme phosphodiesterase as it inhibits the inactivation of the intracellular second messengers cyclic Adenosine MonoPhosphate (cAMP) and cyclic Guanosine MonoPhosphate (cGMP) by one or more of the five subtypes of the enzyme PhosphoDiEsterase (PDE). It has anticholinergic properties. Anticholinergics (anticholinergic agents) are drugs that prevent the neurotransmitter acetylcholine (ACh) from acting at synapses in the central and peripheral nervous systems.

EXPERIMENTAL

Materials and Methods

Instrumentation: A Shimadzu Prominence HPLC iLc2030

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Materials and Reagents

All the chemicals used were of analytical grade. An analytically pure sample of Denaverine hydrochloride was procured as gift sample from Nebulae Hitech Laboratory, Chennai.

RP-HPLC Method

Preparation of Phosphate Buffer

7.0 grams of Potassium di hydrogen Phosphate was weighed and transferred into a 1000 mL beaker, dissolved and diluted to 1000 mL with HPLC water. The pH to 3.5 was adjusted with ortho phosphoric acid.

Preparation of mobile phase

Phosphate buffer 300 mL (30%) and 700 mL of Acetonitrile HPLC (70%) was mixed well and degassed in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Chromatographic Conditions

Mode of operation	: Isocratic
Instrument	: HPLC Waters
Detector	: UV detector
Column	: Symmetry C_{18} (4.6 x 150mm, 5 µm, Make: XTerra)
Temperature	: Ambient
Flow rate	: 0.6 mL/min
Wave length	: 306 nm
Runtime	: 5 min
Sample size	: 20 µL
Mobile Phase	: Phosphate buffer: Acetonitrile (30:70 %v/v)

Standard Solution Preparation

Accurately weigh and transfer 10 mg of denaverine working standard into a 10 mL volumetric flask add about 7 mL of mobile phase and sonicate to dissolve it completely and make volume up to the mark with the same solvent (1000 µg/mL). Further 0.3 mL was pipette out (1-mg/mL) of the above stock solution into a 10 mL volumetric flask and diluted up to the mark with mobile phase (30 µg/mL). Then the solution was mixed well and filter through 0.45 µm filter.

Sample Solution Preparation

A 0.25 mL of injection (Sensiblex 40 mg/mL) was measured accurately, and the sample (equivalent to 10 mg of Denaverine hydrochloride) was put into a 10 mL volumetric flask. A 7 mL of mobile phase was added and sonicated to dissolve the same, keeping volume up to the mark with mobile phase (1000 µg/mL). The solution was mixed well and filter through 0.45 µm filter. Further 0.3 mL was pipette out of the above stock solution into a 10 mL volumetric flask and diluted up to the mark with mobile phase (30 µg/mL). Mix well and filter through 0.45 µm filter.

Method Validation

The parameters such as linearity, accuracy, precision, specificity, assay, limit of detection (LoD), limit of quantification (LoQ), robustness, ruggedness and stability of the solution were assessed as per ICH guidelines.[7-9]

RESULTS AND DISCUSSION

Chromatographic Conditions

System Suitability

These studies are conducted under ICH and USP guidelines. Parameters like tailing factor, number of theoretical plates, etc. were obtained.

Linearity

From the stock standard (1000 µg/mL), the aliquots (0.1 to 0.5 mL of 1000 µg/mL) solution of 0.1, 0.2, 0.3, 0.4, 0.5 mL were taken in 10 mL volumetric flasks and made till 10 mL with mobile phase (10–50 µg/mL). It is added to the chromatographic system and chromatogram readings should be noted. A calibration graph with peak area (Y-axis) and standard solution concentration (X-axis) is drafted (Figures 1 to 10 and Tables 1 and 2).

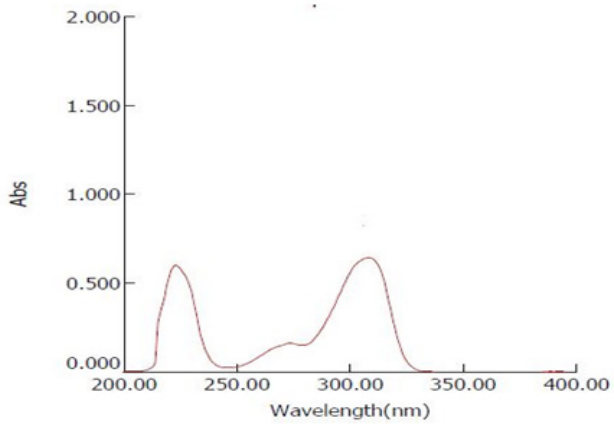
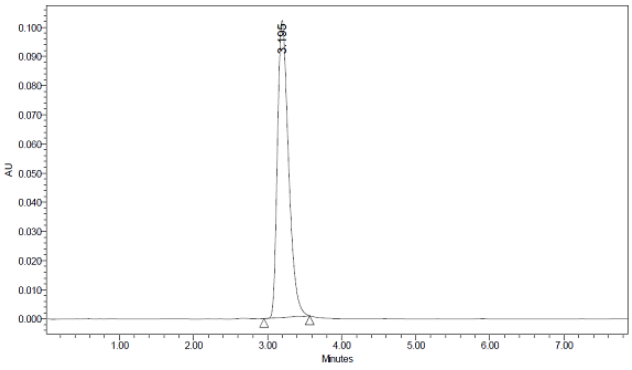


Figure 1: UV spectrum of denaverine in phosphate buffer pH 3.5:



	RT	Area	Height	USP Plate Count	USP Tailing
1	3.195	1058299	102124	2178.3	1.4

Figure 2: Optimized chromatogram phosphate buffer pH3.5: acetonitrile (30: 70%V/V)

Table 1: System suitability

Parameter	*Denaverine*
Tailing factor	1.6
No of Theoretical plate	2951
Retention time	3.2

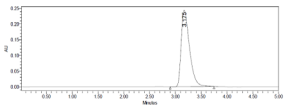


Figure 3: Linearity chromatogram 10 µg/mL

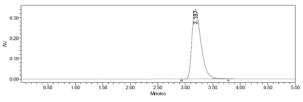


Figure 4: Linearity Chromatogram 20 µg/mL

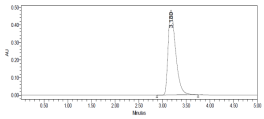


Figure 5: Linearity Chromatogram 30 µg/mL

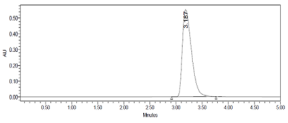
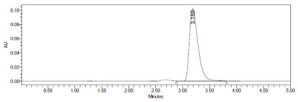


Figure 6: Linearity Chromatogram 40 µg/mL



RT	Area	Height (µV)
3.189	1113634	102352
3.175	2712792	244484
3.187	3908404	342239
3.180	5328851	484112
3.187	6652686	555333

Figure 7: Linearity Chromatogram 50 µg/mL linearity -Data

Table 2: Results of Linearity

S.No	*Concentration (µg/ml)*	*Peak Area*	*LoD*	*LoQ*
1	10	1323634		
2	20	2712792		
3	30	3998490	0.014	0.0465
4	40	5328851		
5	50	6652686		

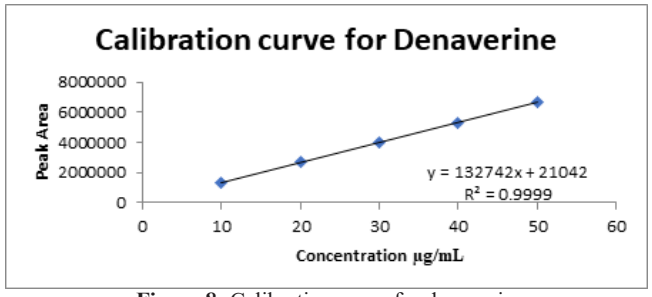
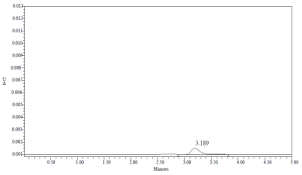


Figure 8: Calibration curve for denaverine

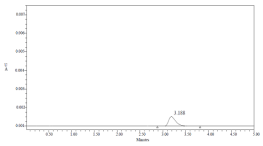
LoD



Retention Time (min)	Area (µV*sec)	Height (µV)
3.189	1545	142

Figure 9: LoD chromatogram

LoQ



Retention Time (min)	Area (µV*sec)	Height (µV)
3.188	5277	485

Figure 10: LoQ Chromatogram

Quantification of Formulation

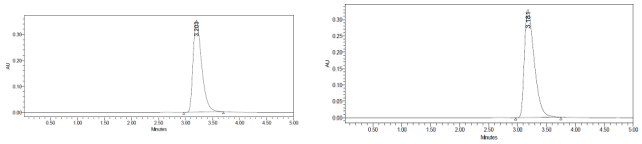
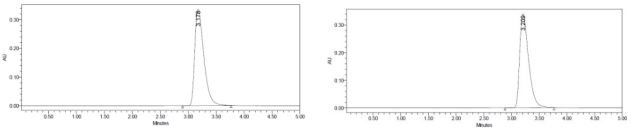


Figure 11: Sample Chromatogram



RT	Injection	Area
3.178	1	3775295
3.209	2	3778402

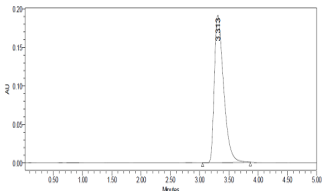
Figure 12: Standard Chromatogram

Table 3: Results of Assay

S.No	*Standard Peak area*	*Sample Peak Area*	*Percentage purity (%)*	*Average percentage (%)*	*SD*	*%RSD*
1	3775295	3912105	101.65	101.52	0.1767	0.1740
2	3778402	3907203	101.40			

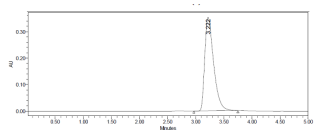
Recovery:

0.25 mL of injection (Sensiblex 40mg/mL) was measured accurately and transferred the sample (equivalent to 10 mg of Denaverine hydrochloride) into three separate 10 mL volumetric flask. Then 5, 10 and 15 mg (50, 100, 150%) of standard were accurately weighed and added. 7 mL of mobile phase was added and sonicate to dissolve it completely and the solution was kept to same volume. 0.3 mL was pipette out from each flask and transferred to a separate 10 mL volumetric flask. Then the solution was made volume till the same. A 20 **µ**L solution was injected into chromatographic system and the chromatogram was recorded (Figures 11 to 15 and Table 3 and 4).



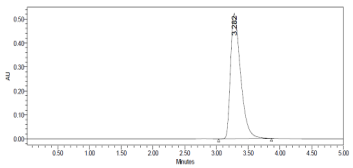
RT	Injection	Area
3.313	1	2108215
3.184	2	2109521
3.235	3	2114103

Figure 13: Recovery-50%



RT	Injection	Area
3.222	1	3875174
3.191	2	3888449
3.217	3	3893469

Figure 14: Recovery -100%



RT	Injection	Area
3.282	1	5781825
3.189	2	5779001
3.238	3	5773680

Figure 15: Recovery-150%

Table. 4: Results of Recovery

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	2110613	5.5	5.57	101.3%	
100%	3885698	10.1	10.2	101.5%	101.3%
150%	5778169	15.1	15.2	101.0%	

Precision

Repeatability and intermediate precision studies were done to the precision of the method. Repeatability studies were done by consequently measuring the absorbance of standard solution. These solutions were prepared in duplicate and absorbances were measured at 306 nm against blank and calculate the %RSD (Figures 16 to 24 and Tables 5 and 6).

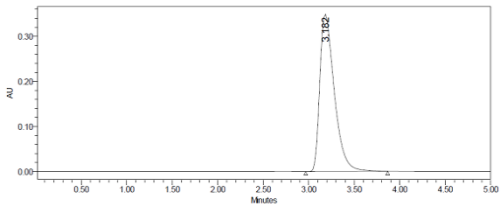


Figure 16: Precision study -1

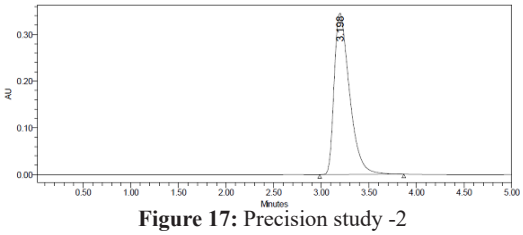


Figure 17: Precision study -2

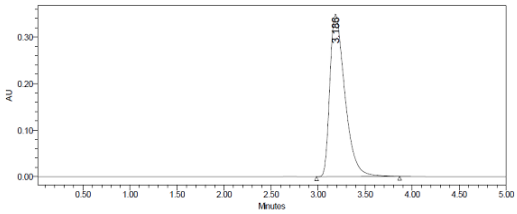


Figure 18: Precision study -3

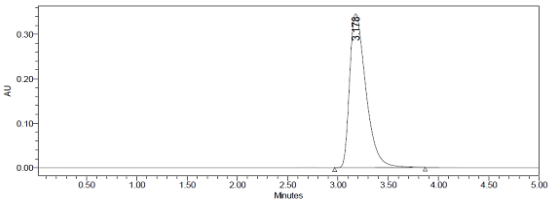
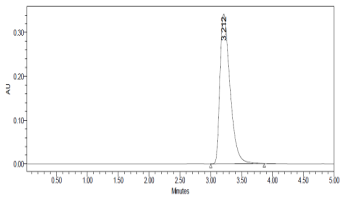


Figure 19: Precision study -4



RT	Injection	Area
3.182	1	3855508
3.198	2	3865126
3.186	3	3871273
3.178	4	3878408
3.212	5	3882797

Figure 20: Precision study -5

Table. 5: Results of Precision

S.No	*Concentration (µg/ml)*	*Peak Area*	*Average*	*SD*	*%RSD*
1		3855508			
2		3865126			
3	30	3871273	3870622	10815.8	0.28
4		3878408			
5		3882797			

Intermediate precision

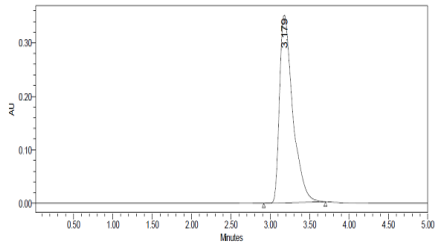


Figure 21: Intermediate precision-1

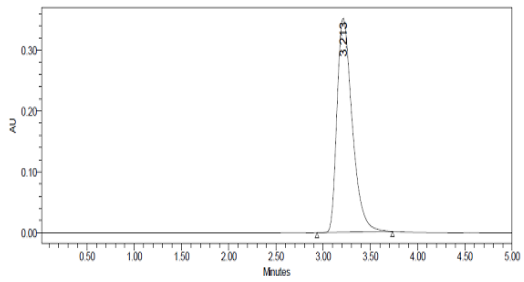


Figure 22: Intermediate precision-2

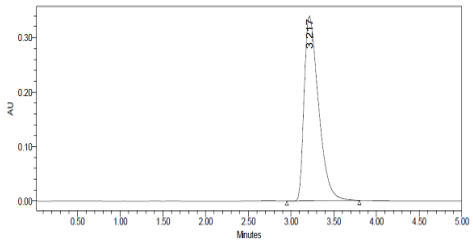


Fig23: Intermediate precision-3

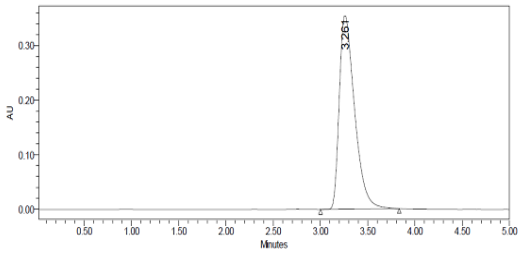
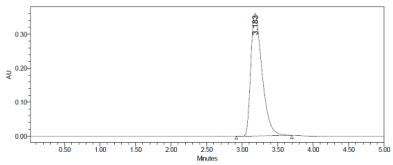


Figure 24: Intermediate precision-4



RT	Injection	Area
3.179	1	4095410
3.213	2	3935121
3.217	3	3963812
3.261	4	3990300
3.183	5	3976949

Figure 25: Intermediate precision-5

Table. 6: Results of Intermediate Precision

S.No	*Concentration (µg/ml)*	*Peak Area*	*Average*	*SD*	*%RSD*
1		4095410			
2		3935121			
3	30	3963812	3992318	61140.1	1.53
4		3990300			
5		3976949			

Robustness

Experimental conditions were intentionally altered and reviewed to present the robustness of the developed method. For this study, mobile phase and flow rate have slightly changed and the assay was checked (Figures 25 to 29 and Table 7).

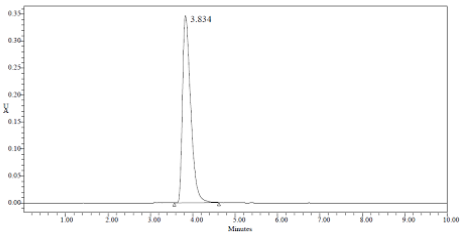
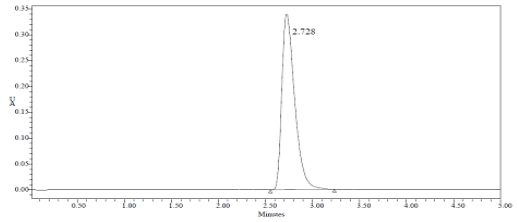
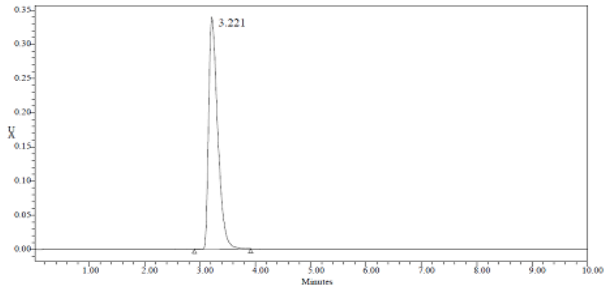


Figure 26: Robustness-Less Flow



Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
3.834	4694313	2889.4	1.6

Figure 27: Robustness-More Flow



Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
2.728	3231666	2961.0	1.5

Figure 28: Robustness- Less Organic

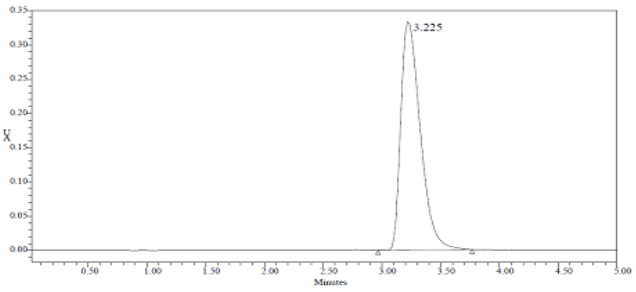


Figure 29: Robustness-More Organic

Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
3.225	3828751	2856.9	1.5

Table. 7: Results of Robustness

Parameters	*Theoretical plate*	*Tailing factor*
Less flow (0.5 mL/min)	2889	1.6
More flow (0.7 mL/min)	2961	1.5
Less organic phase (60%)	2874	1.6
More organic phase (80%)	2856	1.5

CONCLUSIONS

It concludes that the developed method was robust, sensitive, accurate, and reproducible. It has the ability to perform routine quality control analysis of Denaverine Hcl in bulk and injectable formulations.

ACKNOWLEDGEMENTS

The authors thank the Founder Chairperson Dr. K. Veeramani of Periyar College Pharmaceutical Sciences, Trichy for providing necessary facilities. Authors also thankful to our belonged Advisor Mr.V. Anburaj for his constant encouragement and inspiration.

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