IJBPAS, December, 2023, 12(12): 5767-5784

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF RELATED SUBSTANCES IN KETOBEMIDONE.HCL BULK FORM

GANDI ANUSHA*

Asst. Prof, Department of Pharmaceutical Analysis, Vignan Institute of Pharmaceutical Technology, Besides VSEZ, Duvvada, Visakhapatnam, Andhra Pradesh-530049, India

***Corresponding Author: Ms. Gandi Anusha: E Mail: gandianusha11@gmail.com**

Received 19th Oct. 2022; Revised 16th Nov. 2022; Accepted 13th April 2023; Available online 1st Dec. 2023

https://doi.org/10.31032/IJBPAS/2023/12.12.7650

ABSTRACT

Objective: The study of present work is to develop and validate a method which is new rapid, simple, sensitive, accurate, economical and specific isocratic RP-HPLC for the determination of related substances in bulk form of Ketobemidone.HCl which is used as Opioid analgesic for various class of ascetic pains such as post-operative, stones in kidney, cancer and fractures.

Results: Chromatographic separation was carried out isocratically with Waters Alliance 2695 model pump with 2998 PDA detector with wavelength of 280nm, luna phenyl hexyl 250x4.6mm. Mobile phase is prepared by using buffer: acetonitrile in the ration of 80:20. Rate of flow was adjusted to 1.5ml/min which gave well separated peaks with good time intervals. The developed method is then validated for precision, linearity accuracy, Limit of Quantification and Limit of detection, robustness. The Limit of detection values of Ketobemidone.HCl. Impurity A, B, C, D are 0.001, 0.001, 0.001, 0.001, 0.003. The Limit of Quantification values of Ketobemidone.HCl, Impurity A, B, C, D are 0.003, 0.003, 0.002, 0.004, 0.01.The correlation coefficient value of Ketobemidone.HCl is (R) = ≥ 0.95 and its impurities was found to 0.9988,0.9938,0.9958,0.9938.The accuracy of Ketobemidone. HCL was found to be % Relative

standard deviation (%RSD < 10.0. The precision and robustness of Ketobemidone.HCl was found to be %RSD = ≥ 10.0 and %RSD = ≥ 5.0.

Conclusion: The proposed new method is a good approach for obtaining reliable results and which is suitable for doing analysis on Ketobemidone.HCl in research institutions, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies, quality control department in industries, and in clinical pharmacokinetic studies.

Keywords: Ketobemidone. HCL, RP-HPLC, Materials & Methods, Method development, Method validation

INTRODUCTION:

The goal of the current study is to create and verify a brand-new, quick, easy, sensitive, accurate, affordable, and specific isocratic RP-HPLC technique for the detection of related compounds in bulk ketobemidone. HCL. It is an opioid analgesic which is used to treat all class of ascetic pains, such as postoperative cancer, stones in kidney and fractures **[1]**. The IUPAC name of this compound is 1-[4-(3-hydroxyphenyl)-1-methylpiperidin-4-yl] propan-1-one hydrochloride. The molecular formula of Ketobemidone.HCl is $C_{15}H_{22}ClNO_2$ **[2]** with a molecular weight of 283.8g/mol. It is available in the form of white crystalline powder. The melting point of compound is 156.5^0C. It is freely soluble in water, alcohol, partially soluble in dichloromethane. Structure of ketobemidone hydrochloride was shown in **Figure 1**.

Ketobemidone.HCl is available under the brand names of Ketogan, Ketorax, Cetobemidone. Its effectiveness against pain is in the same range as morphine, and it also has some NMDA-antagonist properties. It opens K+ channels (mainly through mu and delta receptors) by reducing intracellular cAMP formation. These actions result in neuronal hyper polarization.

The literature survey reveals that there is only few methods on Ketobemidone.HCl **[3]**. The present work is to develop, validate for the determination of related substances in bulk form of Ketobemidone.HCl.

MATERIALS AND METHODS:

Drug and chemicals

Drug Ketobemidone.HCl is accomplished as a freebie sample from Varanous labs. Pvt. Ltd. Nacharam, Hyderabad. Acetonitrile, ammonia and ammonium acetate are of AR grade purchased from the Merck.

Equipments used

1. Analytical balance with the model name Sartorius.

2. pH meter with model name of Eutech.

3.HPLC Water alliance system 2695 model pump with 2998 PDA detector and 2489 UV detector.

Composition of Buffer

Weight and transfer about 1.54 grams of ammonium acetate in 1000 ml of water and sonicate to dissolve. Adapt the pH to 8.0(±0.05) using with dilute ammonia solution. Filter through 0.45µ porosity membrane and degas.

Mobile phase: Buffer (solution A) and acetonitrile in the ration of 80:20(v/v)

Diluent: Buffer solution (solution A)

Composition of stock solutions:

Stock solution-1: Weigh and transfer about 10.0 mg of standard sample into a 100.0ml volumetric flask, add 20ml diluent sonicate to dissolve and make up to the mark with diluent

Stock solution-2: Weigh and transfer about each 10.0 mg of Impurity B and Impurity C standards into 100ml volumetric flask add 20 ml of diluent and make upto mark with diluent.

Composition of standard solution: Measure 2.0 ml of the above stock solution-1 into 100 ml volumetric flask and make up to the mark with diluent

Stock solution-3: Weigh and transfer about each 10.0 mg of Impurity A and Impurity D standards into 100ml volumetric flask add 20 ml of diluent and make upto mark with diluent.

Composition of test solution: Weigh and transfer about 50.0 mg of test substance into a 25.0 ml volumetric flask ,add 5.0ml of diluent, sonicate to dissolve and make up to the mark with solution A.

METHODOLOGY

Method development

During trial runs, **[4, 5]** for the separation of related impurities in Ketobemidone.HCl, mobile phase with different proportions (Buffer : Acetonitrile (50:50) and (60:40) were tired on Luna phenyl hexyl (250X4.6mm) column but three impurities are separated in trail 1 and peaks are separated but not with good resolution in trial 2. In the optimised conditions are listed in **(Table 1)** buffer and acetonitrile composition in the ratio of 80:20 using luna phenyl hexyl column with the flowrate 1.5ml/min and wavelength 280nm **[6]**. Thereby peaks are well separated with good time intervals are exhibit in **(Figure 2)**.

Method Validation

1 System suitability:

Composition of System suitability solution:

Transfer the above 2.0 ml of stock solution 2 into a 50.0 ml volumetric flask and makeup the volume with diluent **[7]**. The results of system suitability are mentioned in **Table 2** and chromatogram in **Figure 3**.

2. Specificity:

Individual sample solutions of impurity A, B, C, D and Ketobemidone.HCl in diluent.

Prepared a spiked sample solution by spiking of impurity A,B,C,D and Ketobemidone.HCl sample solution. Table 3 lists the specificity results, and Figure 4 shows the chromatogram (a,b,c,).

3.Linearity

The linearity of Ketobemidone.HCl and its impurities solutions was prepared ranging from LOQ to 150.0% of the specification limit and injected into HPLC system **[8]**. The results of linearity are mentioned in **Table 4, 5, 6, 7, 8** and plot in **Figure 5** (a, b, c, d, e).

Composition of stock solution:

Accurately weigh and transfer about 10.1 mg of Impurity A, B, C, D and Ketobemidone.HCl into 100 ml volumetric flask and add 25ml of diluent and sonicate 5mins and make up mark with diluent.

4.Precision:

Composition of Sample solution:

Weigh and transfer about 50.0mg of sample into a 25ml volumetric flask, dissolve in diluent and make up to the mark with diluent. Inject 6 replicate volumes of 20 µl of this solution and record the chromatograms. Calculate % RSD for the chromatograms **[9]**. **Table 9** contains the results of the precision calculations.

5.Accuracy [12]

The accuracy was performed on samples spiked with known amounts of each specified impurities were prepared for 50%, 100%, 150% concentration solutions in order to determine the percentage recovery. The accuracy results were compiled in **Tables 10, 11, 12, and 13**.

6. Limit of Detection:

The limit of detection **[13]** is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. The results of LOD are mentioned in **Table 14** and chromatogram in **Figure 6 (a, b, c)**.

7. Limit of Quantification:

The limit of quantification is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations (approx. 3.3 folds to limit of detection) of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably quantified. The results of LOQ are mentioned in **Table 15** and chromatogram in **Figure 7 (a, b, c)**.

8. Robustness

Robustness of this developed method was determined by changing in flow rate

(±0.2ml/min) and buffer pH (±1) range. The % RSD of peak areas was calculated. The results of robustness was tabulated in **Table 16**.

RESULTS:

Table 1: Optimised Chromatogram Conditions

Parameters	Optimised chromatogram conditions
Stationary Phase	Luna phenyl hexyl (250X4.6mm), 5 µ or its equivalent.
Mobile Phase	Buffer:Acetonitrile (80:20)
P^H	Adjust the P^H to 8.0 (± 0.05) with dilute ammonia solution
Elution mode	Isocratic
Column oven temperature	40^0C
Injection Volume	20µl
Flow rate	1.5 ml/min
Detection Wavelength	UV at 280nm
Run time	60 min
Observation	Peaks are well-separated with good time intervals.

Table 2: Results of System Suitability Data

	Name	RT	Area	%Area	Resolution
1.	ImpurityB	6.373	55203	52.71	
2.	ImpurityC	7.380	49522	47.29	4.0

Table 3: Specificity Data of Ketobemidone.HCl

Peak name	Retention time (min)	RT Ratio	Peak purity		Resolution
			Purity angle	Purity Threshold	
Impurity-A (Isomer-1)	3.937	0.352	5.263	6.935	---
Impurity-A (Isomer-2)	4.728	0.423	3.439	4.774	5.1
Impurity-B	6.416	0.574	4.063	5.349	8.3
Impurity-C	7.519	0.673	7.458	9.244	4.4
Ketobemidone.HCL	11.173	1.000	0.099	1.097	4.0
Impurity-D	44.365	3.971	16.419	19.550	24.65

Table 4: Linearity for Ketobemidone.HCl

Level	Actual strength (%)	Conc. (mg/mL)	Avg. peak areas
LOQ	0.02	0.000061	2051
30%	30.30	0.00756	29093
50%	50.50	0.01260	46788
100%	101.00	0.02520	93792
120%	121.20	0.03024	112622
150%	151.50	0.03780	144507
Slope			3813538.2612
Intercept			-1242.3187
Correlation coefficient (R)			0.9997
R^2			0.9994
Intercept response of 100 % standard solution			-1.29

Table 5: Linearity for Impurity-A

Level	Actual strength (%)	Conc. (mg/mL)	Avg. peak areas
LOQ	0.02	0.000061	1596
30%	30.30	0.00756	24751
50%	50.50	0.01260	42540
100%	101.00	0.02520	87659
120%	121.20	0.03024	110078
150%	151.50	0.03780	139608
Slope			3124628.2436
Intercept			-1186.4257
Correlation coefficient (R)			0.9994
R^2			0.9988
Intercept response of 100 % standard solution			-2.59

Table 6: Linearity for Impurity-B

Level	Actual strength (%)	Conc. (mg/mL)	Avg. peak areas
LOQ	0.02	0.000041	2289
30%	30.30	0.00756	31097
50%	50.50	0.01260	50899
100%	101.00	0.02520	97821
120%	121.20	0.03024	117524
150%	151.50	0.03780	149618
Slope			2434619.1652
Intercept			-1089.5672
Correlation coefficient (R)			0.9969
R^2			0.9938
Intercept response of 100 % standard solution			-3.32

Table 7: Linearity for Impurity-C

Level	Actual strength (%)	Conc. (mg/mL)	Avg. peak areas
LOQ	0.02	0.000081	1854
30%	30.30	0.00756	26740
50%	50.50	0.01260	47798
100%	101.00	0.02520	96748
120%	121.20	0.03024	119632
150%	151.50	0.03780	150590
Slope			5246129.1152
Intercept			-1262.3642
Correlation coefficient (R)			0.9997
R^2			0.9985
Intercept response of 100 % standard solution			-1.16

Table 8: Linearity for Impurity-D

Level	Actual strength (%)	Conc. (mg/mL)	Avg. peak areas
LOQ	0.02	0.000205	2698
30%	30.30	0.00750	34189
50%	50.50	0.01250	53957
100%	101.00	0.02500	98864
120%	121.20	0.03000	119982
150%	151.50	0.03750	147927
Slope			4652127.1317
Intercept			-1841.2568
Correlation coefficient (R)			0.9969
R^2			0.9938
Intercept response of 100 % standard solution			-2.82

Table 9: Results of Precision Data

Injection No	Peak areas				
	Impurity A (1&2)	Impurity B	Impurity C	Ketobemidone.HCl	Impurity D
1	477	319	581	646	1478
2	488	306	585	720	1502
3	466	314	604	608	1462
4	490	317	593	700	1480
5	490	310	600	690	1485
6	486	306	585	737	1498
Avg. peak area	483	312	591	684	1484
SD	9.5586	5.5498	9.2232	48.1985	14.5385
%RSD	1.98	1.78	1.56	7.05	0.98

Table 10: Accuracy % Recovery Results for Impurity A

Level	Theoretical conc.(mg/ml)	Measured conc.(mg/ml)	% Recovery	Average	SD	%RSD
50%	0.01260	0.01242	98.57	98.64	0.124	0.12
	0.01260	0.01238	98.25			
	0.01260	0.01249	99.12			
100%	0.02520	0.02498	99.18	99.34	0.178	0.18
	0.02520	0.02502	99.28			
	0.02520	0.02509	99.56			
150%	0.03780	0.03820	101.05	101.67	0.263	0.26
	0.03780	0.03842	101.64			
	0.03780	0.03868	102.32			

Table 11: Accuracy % Recovery Results for Impurity B

Level	Theoretical conc.(mg/ml)	Measured conc.(mg/ml)	% Recovery	Average	SD	% RSD
50.0%	0.01260	0.01187	94.20	94.80	0.147	0.15
	0.01260	0.01215	96.42			
	0.01260	0.01182	93.80			
100.0 %	0.02520	0.02750	109.12	107.72	0.287	0.29
	0.02520	0.02689	106.70			
	0.02520	0.02705	107.34			
150.0 %	0.03780	0.03692	97.67	97.78	0.341	0.34
	0.03780	0.03719	98.38			
	0.03780	0.03681	97.31			

Table 12: Accuracy % Recovery Results for Impurity C

Level	Theoretical conc.(mg/mL)	Measured conc.(mg/mL)	% Recovery	Average	SD	% RSD
50.0%	0.01260	0.01251	99.28	99.36	0.125	0.13
	0.01260	0.01248	99.04			
	0.01260	0.01257	99.76			
100.0 %	0.02520	0.02609	103.53	100.28	0.168	0.17
	0.02520	0.02498	99.12			
	0.02520	0.02475	98.21			
150.0 %	0.03780	0.03734	98.78	99.03	0.323	0.32
	0.03780	0.03758	99.41			
	0.03780	0.03739	98.91			

Table 13: Accuracy % Recovery Results for Impurity D

Level	Theoretical conc.(mg/mL)	Measured conc.(mg/mL)	% Recovery	Average	SD	% RSD
50.0%	0.01250	0.01259	100.72	100.21	0.134	0.13
	0.01250	0.01251	100.08			
	0.01250	0.01248	99.84			
100.0 %	0.02500	0.02389	95.56	98.21	0.189	0.19
	0.02500	0.02498	99.92			
	0.02500	0.02479	99.16			
150.0 %	0.03750	0.03572	95.25	96.05	0.232	0.23
	0.03750	0.03593	95.81			
	0.03750	0.03685	98.26			

Table14: Limit of Detection for Ketobemidone.HCl and Its Impurities

Component name	Impurity-A (I^{st} Isomer)	Impurity-A (2^{nd} Isomer)	Impurity-A (I^{st}& 2^{nd} Isomer)
Weight taken (mg)	10.121		
Conc. (mg/mL)	0.00002		
LOD w.r. to sample conc. (%)	0.001		
S/N ratio	4.3:1	5.1:1	9.4:1
Reported LOD (%)	0.001		

Component name	Impurity-B	Impurity-C	Ketobemidone.HCL hydrochloride	Impurity-D
Weight taken (mg)	10.135	10.145	10.159	10.235
Conc. (mg/mL)	0.000014	0.000027	0.000020	0.000068
LOD w.r. to sample conc. (%)	0.0007	0.0014	0.0010	0.0034
S/N ratio	4.3:1	3.6:1	4.9:1	4.0:1
Reported LOD (%)	0.001	0.001	0.001	0.003

Table 15: Limit Of Quantification for Ketobemidone.HCl and Each Specified Impurities

Component name	Impurity-A (Ist Isomer)	Impurity-A (2nd Isomer)	Impurity-A (Ist& 2nd Isomers)
Weight taken (mg)	10.121		
Conc. (mg/mL)	0.000061		
LOQ w.r. to sample conc. (%)	0.0031		
S/N ratio	10.0:1	14.9:1	24.9:1
Reported LOQ (%)	0.003		

Component name	Impurity-B	Impurity-C	Ketobemidone.HCL hydrochloride	Impurity-D
Weight taken (mg)	10.135	10.145	10.159	10.235
Conc. (mg/mL)	0.000041	0.000081	0.000061	0.000205
LOQ w.r. to sample conc. (%)	0.0021	0.0041	0.0031	0.0103
S/N ratio	13.1:1	15.0:1	10.2:1	11.1:1
Reported LOQ (%)	0.002	0.004	0.003	0.01

Table 16: Data for Robustness Parameters

Robustness parameter	%RSD peak area of kbh.HCL in standard solution (NMT 5.0)	%RSD peak area of Impurity A in standard solution (NMT 5.0)	%RSD peak area of Impurity B in standard solution (NMT 5.0)	%RSD peak area of Impurity C in standard solution (NMT 5.0)	%RSD peak area of Impurity D in standard solution (NMT 5.0)
Actual	0.53	0.59	0.36	0.78	0.82
Flow:1.7ml/min (high)	0.41	0.62	0.59	0.71	0.29
Flow:1.3ml/min (low)	0.59	0.40	0.27	0.62	0.36
Buffer pH 8.20 (high)	0.74	0.82	0.41	0.92	1.24
Buffer pH 7.80 (low)	0.32	0.53	0.56	0.58	0.54

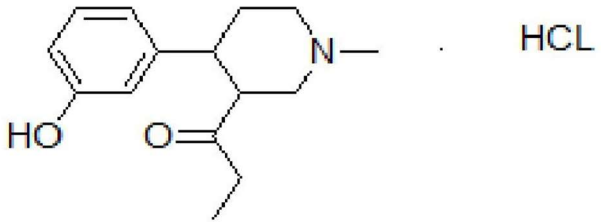

Fig. 1: Structure of Ketobemidone hydrochloride

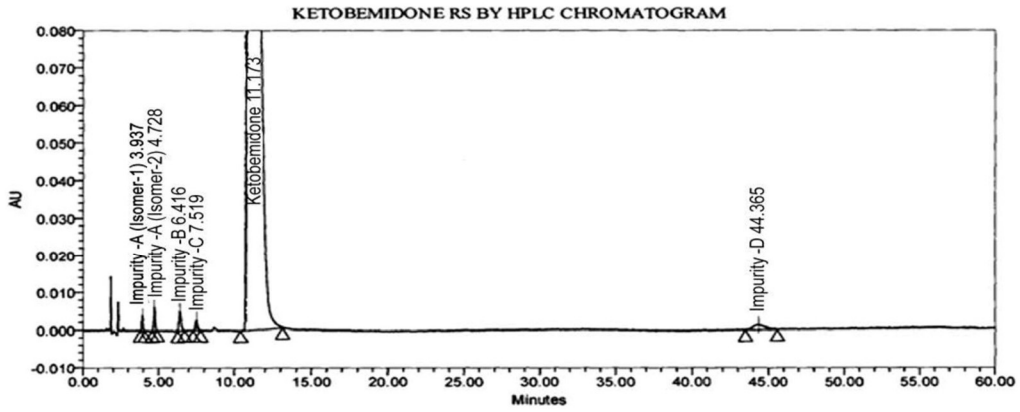

Fig.2: Optimised chromatogram of Ketobemidone.HCL.

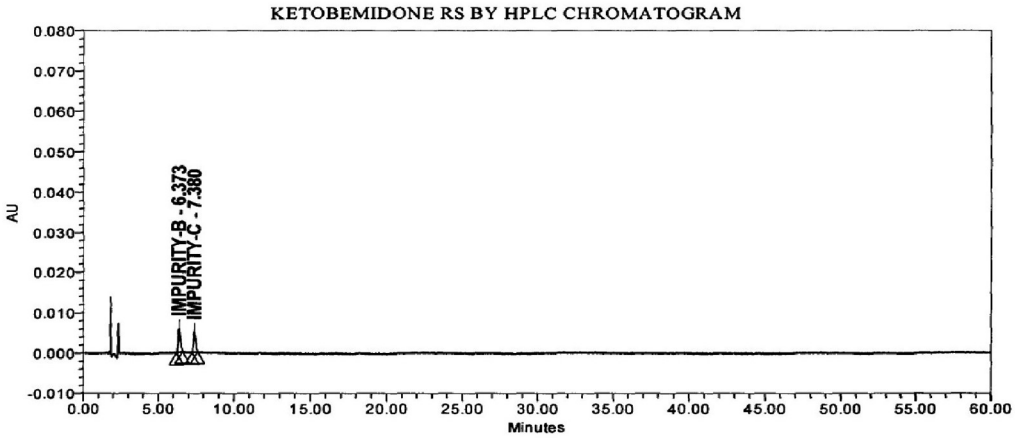

Fig. 3: System suitability Chromatogram

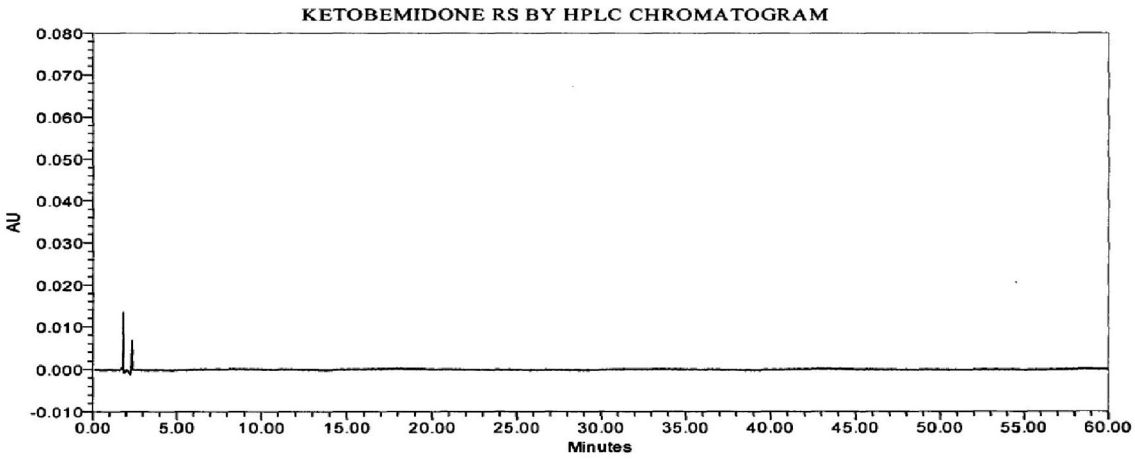

Fig. 4: (a) Blank Chromatogram

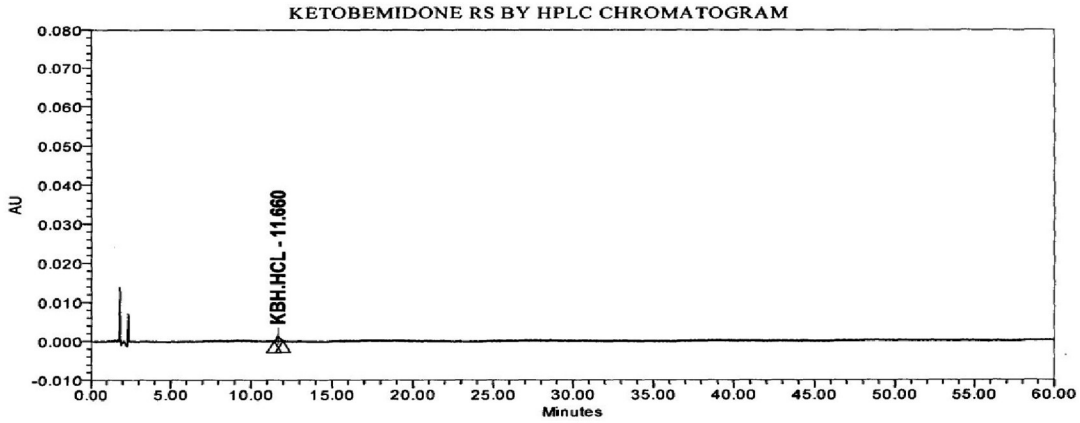

Fig. 4: (b) Specificity of standard solution Chromatogram

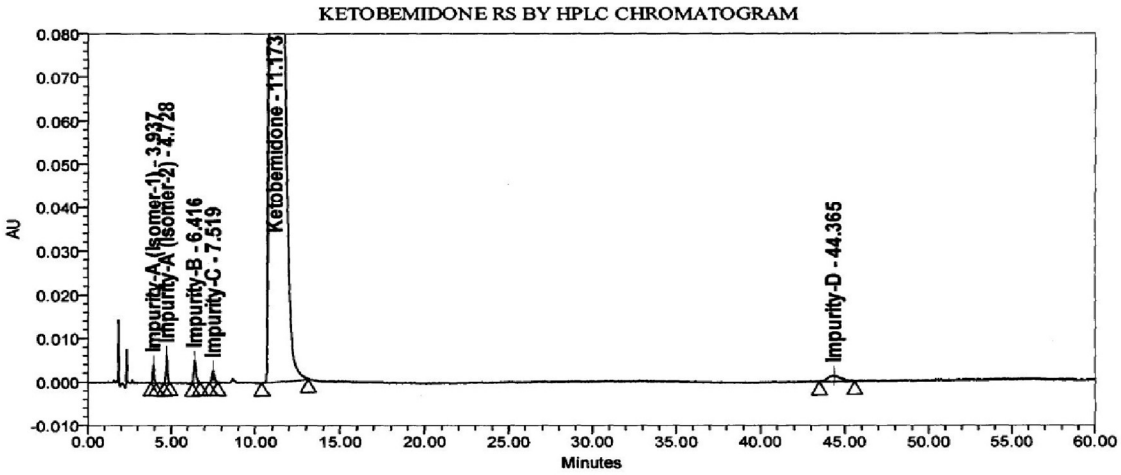

Fig .4(c): Specificity of spiked sample solution Chromatogram

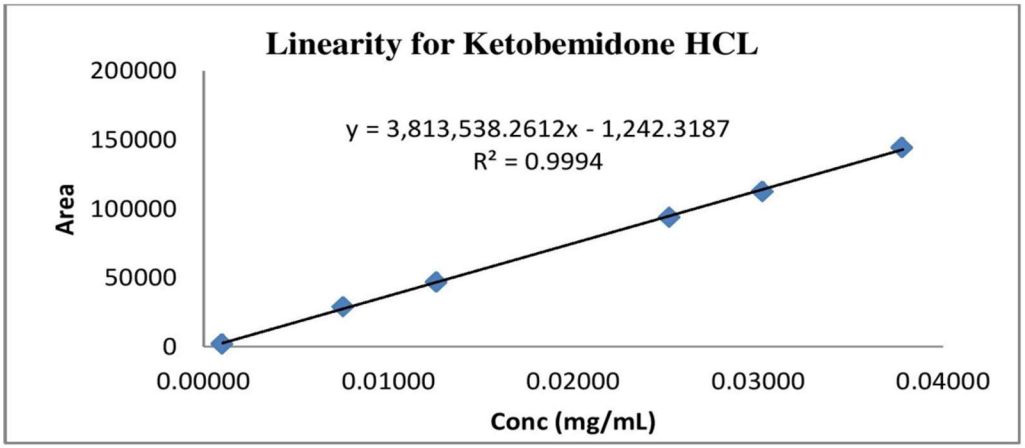

Fig. 5:(a)Linearity plot for Ketobemidone.HCL

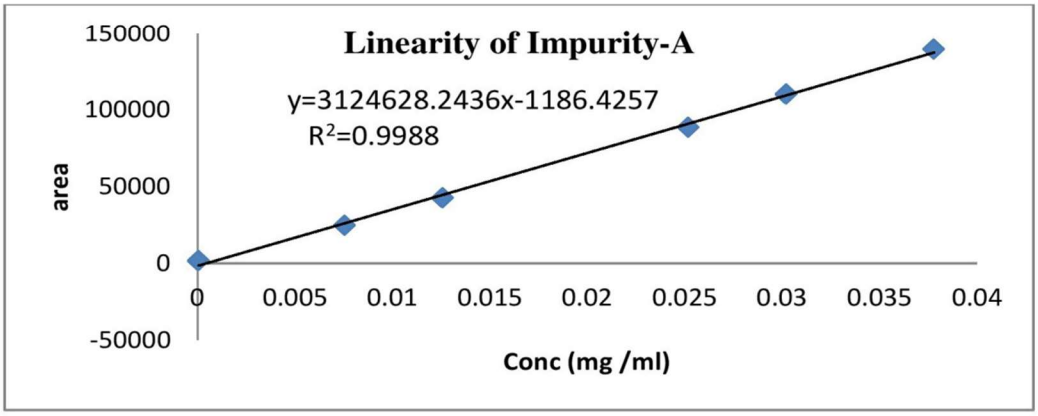

Fig. 5: (b) Linearity plot for Impurity-A

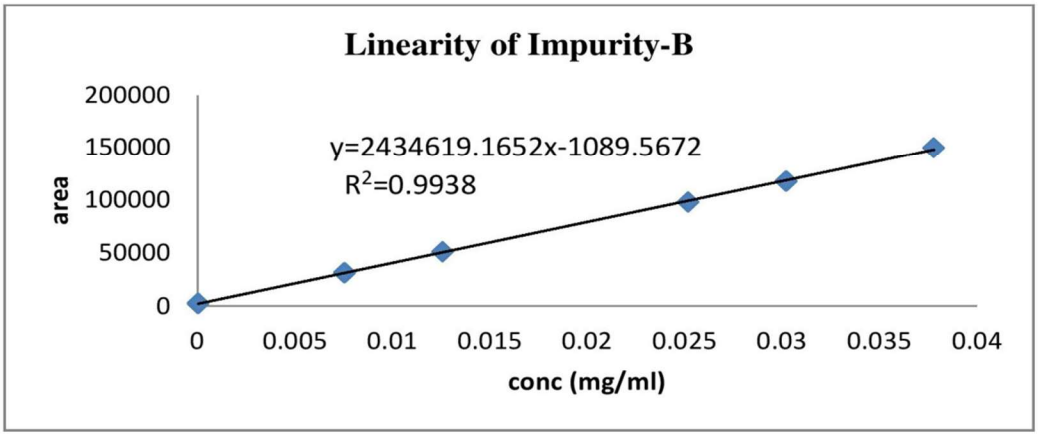

Fig.5:(c) Linearity plot for Impurity-B

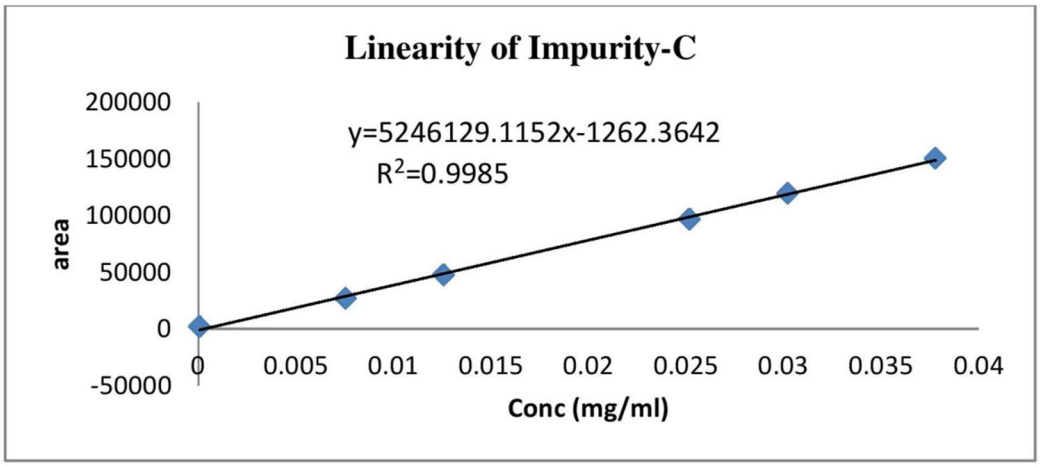

Fig. 5:(d) Linearity plot for Impurity-C

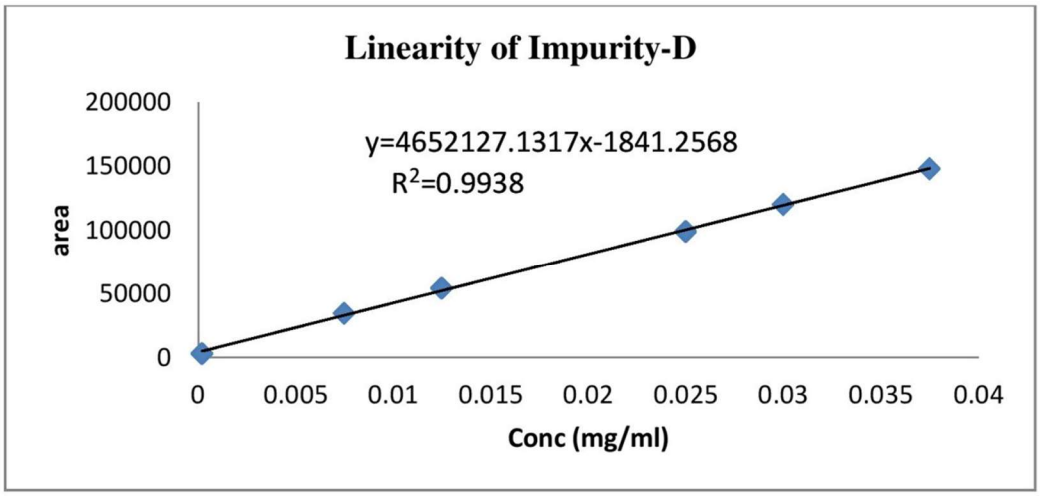

Fig. 5:(e) Linearity plot for Impurity-D

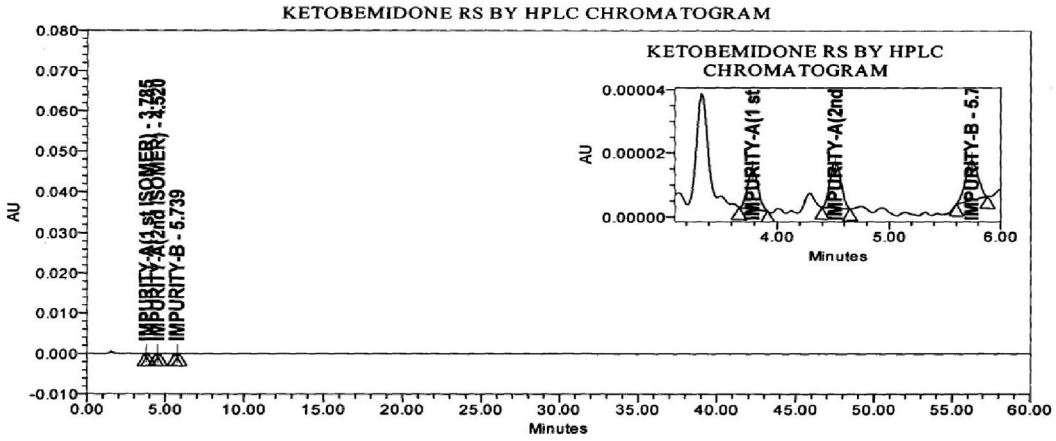

Fig. 6: (a) Chromatogram of LOD Solution of Impurity A & B

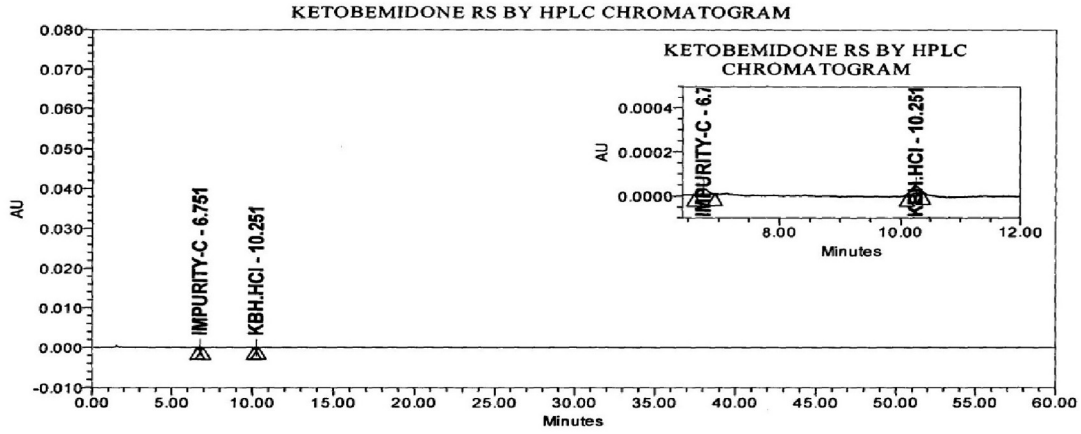

Fig. 6: (b) Chromatogram of LOD Solution of Impurity C & KBH.HCL

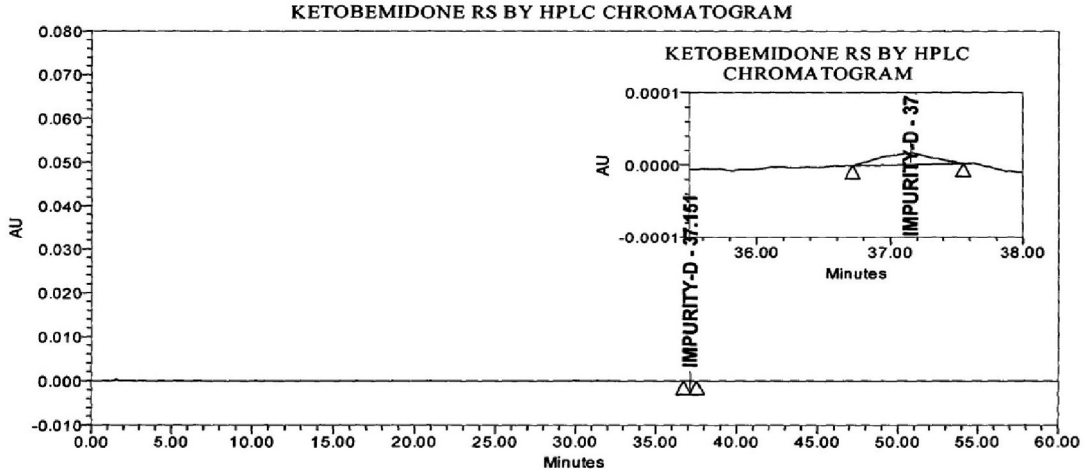

Fig. 6:(c) Chromatogram of LOD Solution of Impurity D

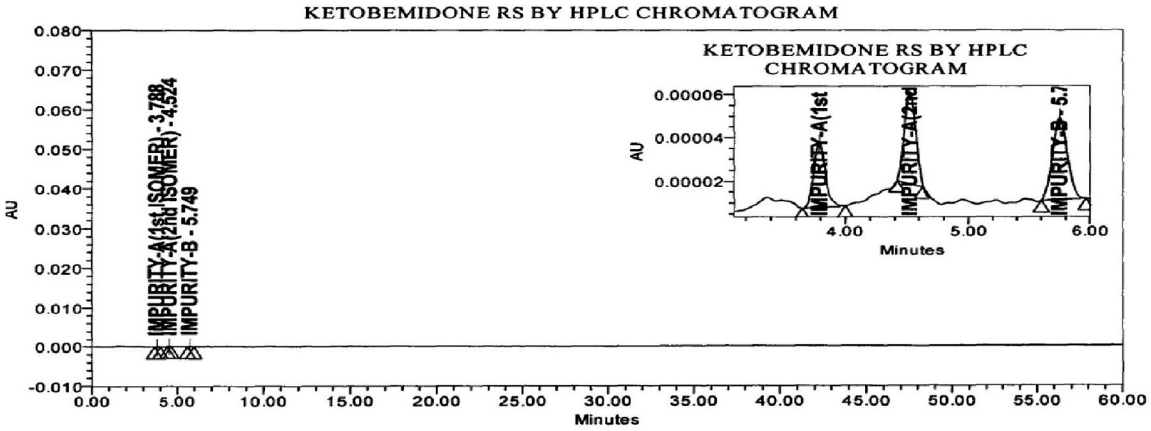

Fig. 7:(a) Chromatogram of LOQ Solution of Impurity A & B

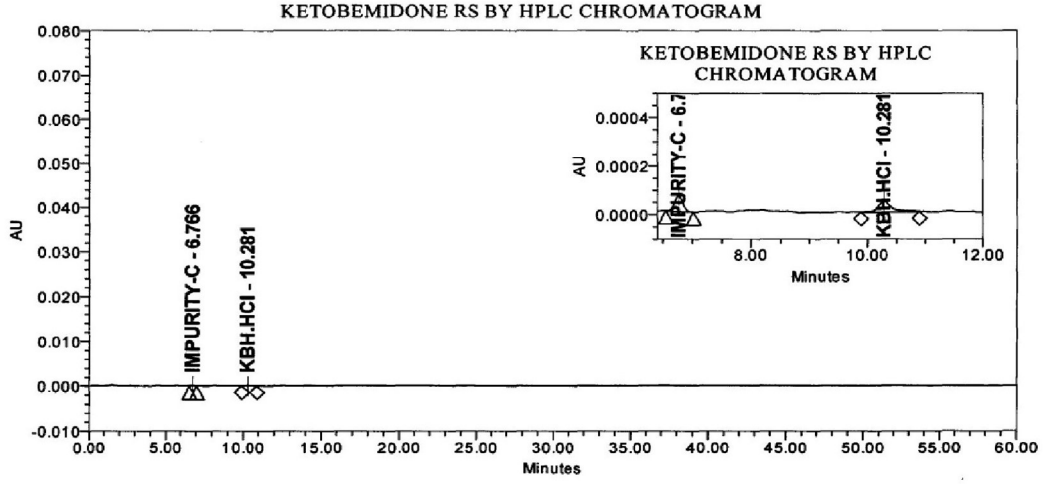

Fig. 7: (b) Chromatogram of LOQ Solution of Impurity C & KBH.HCL

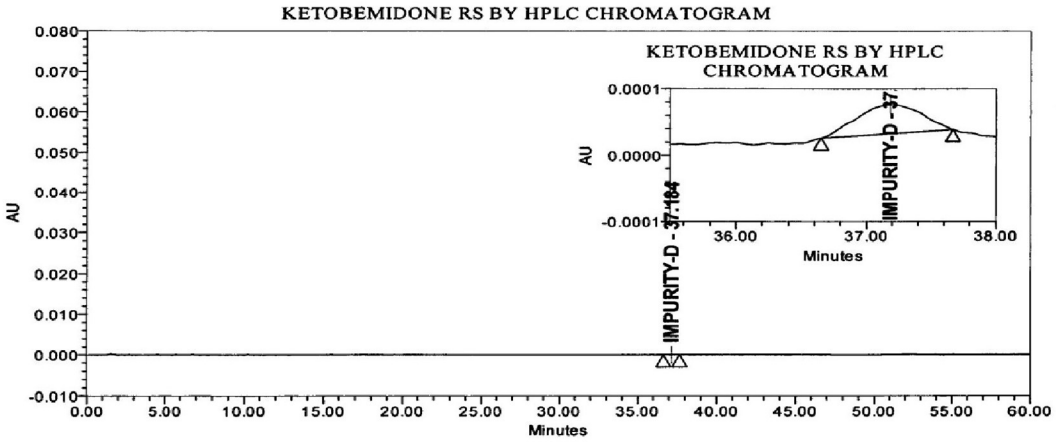

Fig. 7: (c) Chromatogram of LOQ Solution of Impurity D

DISCUSSION:

The resolution between Ketobemidone.HCl impurity-B and impurity-C is not less than 4.0 in system suitability solution **(Table 2)**. For the specificity, there is no blank interference at the RT of Ketobemidone.HCl, Impurity-A, Impurity-B, Impurity-C and Impurity-D. Peak was homogeneous and there is no co-eluting peaks. The Peak purity of analyte(s) was passes because purity angle is less than purity threshold **(Table 3)**. Signal to Noise ratio for LOD should be ≥3:1.As shown in the **(Table 14)**, the S/N ratio values are obtained as about 9.4:1 for impurity-A (0.001%), 4.3:1 for impurity-B(0.001%), 3.6:1 for impurity-C (0.001%), 4.9:1 for Ketobemidone hydrochloride (0.001%)and 4.0:1 for impurity-D (0.003%).Signal to Noise ratio for LOQ should be ≥ 10:1 and the quantification limit should be less than level of specification, preferably much less. As shown in the **(Table 15)**, the S/N ratio values are obtained as about 24.9:1 for impurity-A (0.003%), 13.1:1 for impurity-B (0.002%), 15.0:1 for impurity-B (0.004%), 10.2:1 for Ketobemidone hydrochloride (0.003%) and 11.1:1 for impurity-D (0.01%). Hence the acceptance criteria is met for both LOD and LOQ.The correlation coefficient (R) should not be less than 0.9900. Intercept should not be more than ± 10.00 % of response of 100 % standard solution.As shown in **(Figure 5(a,b,c,d,e))**, the linearity results for all the each specified impurities and Ketobemidone.HCl. the specified concentration range were found to be satisfactory, with a Correlation coefficient (R) greater than 0.9900. The percentage relative standard deviation for the peak areas of Ketobemidone hydrochloride and each specified impurity in six replicate injections at LOQ level should be less than 10.00%. The percentage relative standard deviation for the peak areas of Ketobemidone hydrochloride and each specified impurity at LOQ level are in the range of 0.98% to 7.05% are shown in **(Table 9)**. The % recovery is in the range of 94.80 to 107.72 are shown in **(Table 10,11,12,13)** & the method was found to be accurate. The % RSD for the peak areas of Ketobemidone hydrochloride & its specified impurities were should not be more than 5.00 % are shown in **(Table 16)**. Hence, all the parameters' results were obtained within the acceptance criteria. Therefore, the proposed RP-HPLC method was found to be linear, precise, accurate and robust.

CONCLUSION:

In conclusion, this method is validated as per ICH guidelines. A simple, rapid, accurate, economical isocratic reverse phase high performance liquid chromatography method developed for the determination of related substance in bulk form of Ketobemidone.HCl.

Acknowledgements:

We would thankful to Varanous labs. Pvt. Ltd. Nacharam, Hyderabad for providing Ketobemidone.HCl as a freebie sample and also providing all required facilities to carry out research work.

REFERENCES

[1] Ketobemidone.HCl. Drug profile. www.drugbank.com /drugs/ DB06738.

[2] European Pharmacoepia 7.0 monographs I- L Pg no.2316-2317

[3] Torben *et al*; High-performance thin-layer chromatography (TLC) with visual detection (post-chromatographic derivatization) was used in screening for the drug Ketobemidone.HCl in human urine samples., Journal Of Liquid Chromatography & Related Technologies Volume 38, Issue 9, 2014.

[4] 4.Sharma BK. Instrumental methods of chemical analysis, Introduction to Analytical chemistry, 23thed.Goel Publishing House Meerut, 2004, P12-23.

[5] D. A. Skoog. J. Holler, T.A. Nieman. Principle of Instrumental Analysis, 5th edition, Saunder College Publishing, 1998, P.778-787.

[6] R.Snyder, J. Kirkland, L. Glajch. Practical HPLC method development, II Ed, A Wiley International publication, 1997, P.235, 266-268,351-353.653-600.686-695.

[7] Method validation guidelines International Conference on harmonization; GENEVA; 1996

[8] Skoog, Holler, Nieman. Principals of Instrumental Analysis, 5th Edition, Harcourt Publishers International Company, 2001, P.543-554.

[9] P.D. Sethi. HPLC: Quantitative Analysis Pharmaceutical Formulations, CBS Publishers and distributors, New Delhi (India), 2001, P.3-137.

[10] Spectroscopy by B.K Sharma GOEL publishing house, Meerut, Delhi.

[11] Tangri Pranshu, Rawat Prakash Singh, Jakhmola Vikash: Validation: A Critical Parameter for Quality Control of Pharmaceuticals. Journal of Drug Delivery& Therapeutics 2012; 2(3):34-40.

[12] Validation of Analytical Procedure Methodology "ICH Harmonized Tripartite Guidelines,"1996: 1-8.

[13] ICH- Guidelines Q2 (R1), Validation of Analytical Procedure: Text and Methodology.

[14] ICH Guidelines Q2B, Technical Requirements for Registration of Pharmaceutical for Human Use

Guideline on Validation of Analytical Procedures-Methodology,Geneva, Switzerland.

[15] R. Snyder, Toxicol. Sci., 2000, 58, 3–4.G. G. Gibson and P. Skett, in Introduction to Drug Metabolism, Nelson Thornes Publishers,Cheltenham,3rd edn, 2001, pp. 1–34, 64–66,171-202.

[16] Bondesson U, Danielsson B, Lindberg C. Synthesis and mass spectra of deuterium-labelled ketobemidone and of some potential ketobemidone metabolites. Acta Pharm Suec 51980;17:1–11.

[17] Bondesson U, Hartvig P, Abrahamsson L, et al. Simultaneous de- termination of ketobemidone and its N-demethylated metabolite in patient plasma samples by gas chromatography mass spectrometry with selected ion monitoring. Biomed Mass Spectrom 1983;510: 283–286.

[18] J. W. Grimm and R. E. See, Neuroscience, 2000, 100, 507–514.H. Benveniste and P. C. Prog. Neurobiol., 1990, 35, 195

[19] Paxinos and C. Watson, The Rat Brain in Stereotaxic Coordinates, 4th edn, Academic Press, San Diego, 1998.R. Klintenberg and P. E. Andrén, J. Mass Spectrom., 2005, 40, 261–270.R. D. Mellon and B. M. Bayer, Drug Alcohol Depend., 2001, 62, 141–147.

[20] M. R. Emmett and R. M. Caprioli, J. Am. Soc. Mass Spectrom., 1994,5, 605–613.A. Mansour, C. A. Fox, H. Akil and S. J. Watson, Trends Neurosci., 1995, 18, 22.

[21] A. M. Graybiel, T. Aosaki, A. W. Flaherty and M. Kimura, Science (New York, N.Y), 1994, 265, 1826–1831.T. W. Robbins and B. J. Everitt, Curr. Opin. Neurobiol., 1996, 6, 228–236.

[22] H. W. Strobel, C. M. Thompson and L. Antonovic, Curr. Drug Metab., 2001, 199214.U Yasar, A. Annas, J.-O. Svensson, L. Lazorova, P. Artursson and Al-Shurbaji, Xenobiotica, 2005, 35, 785–796.

[23] S. Higa, T. Suzuki, A. Hayashi, I. Tsuge and Y. Yamamura, Anal.Biochem., 1977, 77, 18–24.M. Tugnait, F. Y. K. Ghauri, I. D. Wilson and J. K. Nicholson,Pharm. Biomed. Anal., 1991, 9, 895–899.