ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 627–639

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

LC method for the determination of assay and purity of sibutramine hydrochloride and its enantiomers by chiral chromatography☆

T. Radhakrishna, Ch. Lakshmi Narayana, D. Sreenivas Rao, K. Vyas, G. Om Reddy *

Department of Analytical R&D., Dr Reddy's Research Foundation, Miyapur, Hyderabad 500 050, India

Received 26 November 1998; received in revised form 15 June 1999; accepted 25 June 1999

Abstract

Two isocratic liquid chromatography (LC) methods have been developed for the purity estimation and quantitative determination of sibutramine HCl, using 4-chloro aniline and lovastatin as internal standards, respectively. The precision has been checked in terms of *F-test* variance ratio using latter method as reference. The ratio of variances of the two methods is close to unity, confirming their good precision. The correlation coefficient for linear regression is more than 0.999. The inter and intra-day precision is found to be $< 1.3\%$ RSD. The accuracy determined as relative mean error (RME) for the intra-day assay is $\pm 1.7\%$. The enantiomeric separation of sibutramine by chiral chromatography method has been described also. This method is capable of separating the two enantiomers with a selectivity of 1.4 and a resolution of 4.0. Both methods are found to be stability indicating and useful in the quality control of the bulk material.

Keywords: Sibutramine hydrochloride; Assay; Purity; Validation; *F-test*; Enantiomeric separation

1. Introduction

Sibutramine (**I**) belongs to a series of novel cyclobutyl alkyl amine derivatives which have been synthesized as anti depressant agents [1]. Sibutramine HCl also belongs to the first class of compounds used for the treatment of obesity [2]. It is a serotonin and noradrenaline re-uptake inhibitor (SNRI), with a dual action, enhancing both satiety and metabolism [3]. Sibutramine (**I**) is an optically active drug, having as a chemical name *N*-(1-(4-chloro phenyl cyclobutyl)-3-methyl-butyl)-*N*-*N*-dimethyl amine (Fig. 1). Both enantiomers are individually potent drugs for the treatment of the above diseases, but the administration of the racemic mixture shows adverse effects. As a result, these compounds should be administered as single isomer [4,5].

Hitherto, there are no publications dealing with the LC analysis of sibutramine HCl either for determination of the purity, quantitative determi-

☆ This is publication No. 54 from DRF.

* Corresponding author. Fax: +91-40-3045438.

0731-7085/00/$ - see front matter
PII: S0731-7085(99)00303-9

nation or enantiomeric separation. Two isocratic reversed-phase LC methods namely method A and method B were developed for the purity estimation and quantitative determination of sibutramine HCl using 4-chloro aniline and lovastatin as internal standards, respectively. These methods offer a rapid and reliable analysis of sibutramine HCl as bulk or in dosage forms.

Method A was validated thoroughly for the determination of sibutramine HCl. The *F-test* was carried out using method B as reference, to check the precision of method A.

In addition, a chiral LC method was also described. The resolution between two enantiomers of sibutramine HCl was found to be more than 4.0 with a selectivity of 1.4, making this method ideal for determination of enantiomeric excess.

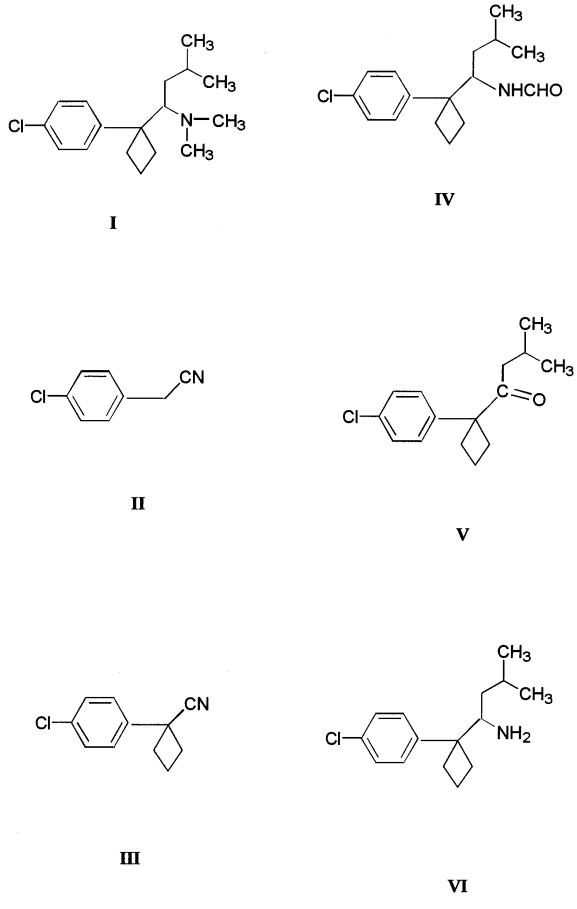

Fig. 1. Structures of sibutramine and its related substances.

2. Experimental

2.1. Chemicals

Sibutramine HCl and all related substances of sibutramine HCl were received from process research and development division of Dr Reddy's Research Foundation, Hyderabad, India. HPLC grade acetonitrile was obtained from Merck, USA. Diammonium hydrogen phosphate was obtained from Sigma, St Louis. 4-chloro aniline (Art. 802613) came from Zur Synthesis, Merk-Schuchardt. Triethylamine and trifluoroacetic acid were purchased from Spectrochem, Bombay, India. Lovastatin came from Bio-Tech. research and development division of Dr Reddy's research foundation, Hyderabad, India. Hexane and isopropyl alcohol were purchased from Merck, E-Merck India, India. High purity water was prepared by a Waters Milli Q plus purification system.

2.2. Equipment

The isocratic LC system consisted of a Perkin–Elmer Series 200 lc pump, a Rheodyne injector fitted with a 20 μl sample loop and a Waters 996 Photodiode detector (Waters make) and was used in laboratory A. The Waters LC system LC Module I Plus, consisted of a pump and a UV-visible tunable variable wavelength detector and was used in laboratory B. The analysis were carried out at temperature of 30°C, obtained by using a Waters temperature control module, Millipore. A Hypersil BDS C18, 4.6×250 mm, 5 μm particle size RP column (Shandon) or Partisphere C18, 5μm, 4.6 x 250 mm RP column (Whatman) was used. A chiralcel OD 250×4.6 mm, 10 μm, column (Diacel make) with a 5 cm guard column and chiralcel OD-H 150×4.6 mm, 5μm, column without guard column (Daicel make) were used for chiral separation of the enantiomers. The output signal was monitored and integrated using Millennium 2010 chromatography manager software (Waters) on Pentium Computer (Digital Equipment) in both the laboratories.

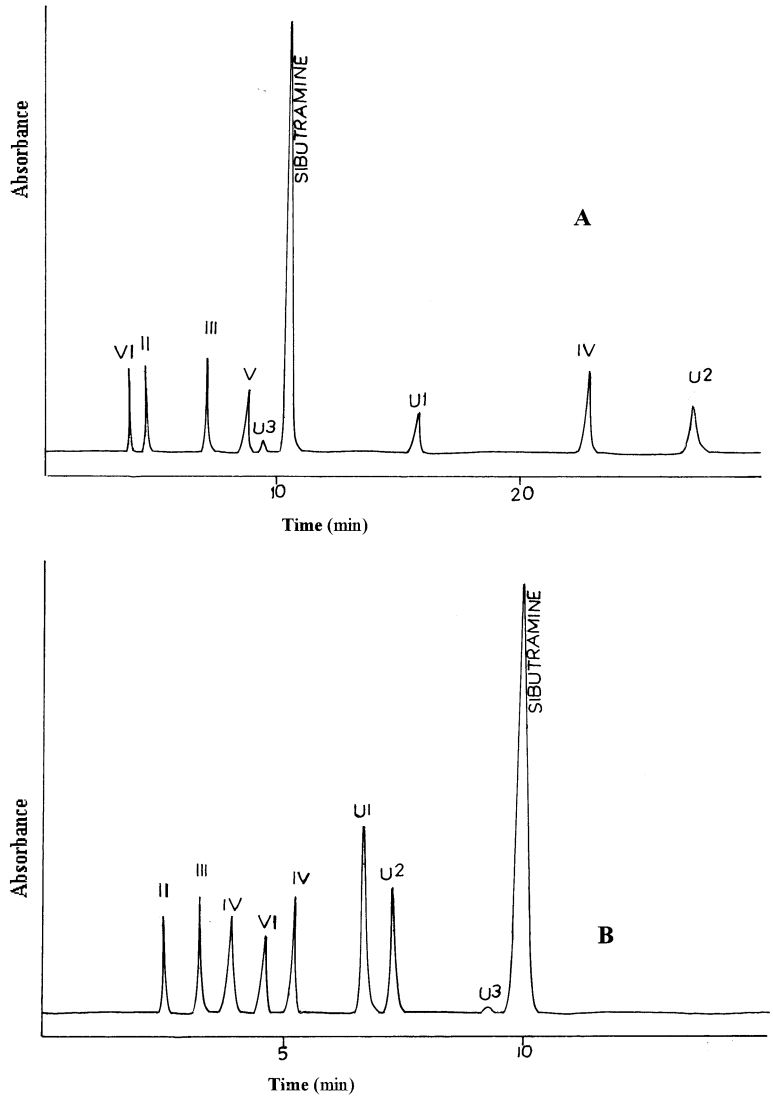

Fig. 2. Chromatograms of crude sibutramine HCl spiked with all the related substances; (A) Using the conditions of method A; (B) Using the conditions of method B.

2.3. Sample preparation

Sample solutions of related substances (Fig. 1) and sibutramine HCl were prepared with mobile phase preparation. The internal standard concentration was 0.3 mg/ml in each sample solution of sibutramine HCl and was used for validation studies. Samples for chiral chromatography were prepared by dissolving sibutramine in the minimum amount of ethanol and make up to the mark with mobile phase of chiral LC.

2.4. Chromatographic conditions

2.4.1. For purity and assay determination of sibutramine hydrochloride

The LC conditions listed below were used for both purity control and quantitative determina-

tion of sibutramine HCl using UV detection at a wavelength of 225 nm with a concentration of 0.3 mg/ml and an injection volume of 10 μl. The column temperature was maintained at 30°C for both the methods. In method A chromatographic separations were performed using a Hypersil C18 BDS, 5μm, 250 × 4.6 mm I.D. column and using a mobile phase consisting of 35% buffer (0.05 M diammonium hydrogen phosphate, pH adjusted to 6.0 with concentrated orthophosphoric acid) and 65% acetonitrile, with a flow rate of 1.0 ml/min. In method B, chromatographic separations were performed using a Partisphere C18, 5μm, 250 × 4.6 mm I.D. column and using a mobile phase consisting of 30% buffer (1.0% triethylamine, pH adjusted to 6.0 with concentrated orthophosphoric acid) and 70% acetonitrile, with a flow rate of 1.5 ml/min.

2.4.2. Conditions for enantiomeric separation

The separation of enantiomers was performed using a chiralcel OD 10μm, 250 × 4.6 mm column with guard column and using a mobile phase consisting of 930 ml of hexane, 70 ml of ethanol

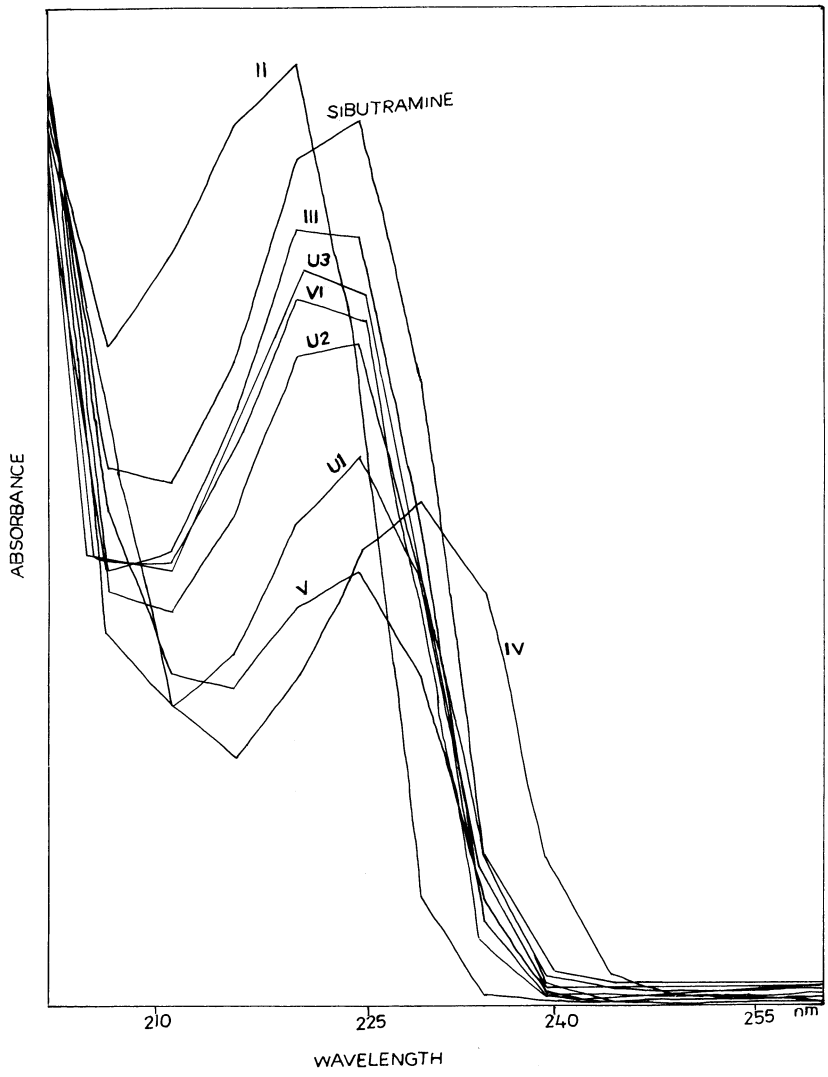

Fig. 3. Absorption spectra of sibutramine HCl and its related substances.

Table 1
System suitability reports for method A and method B[a]

Compound ($n = 3$)	k'	α	R	N	T
I	3.6	1.2	3.26	12654	1.05
	(3.41)	(1.2)	(1.79)	(10553)	(0.98)
II	0.992	1.4	4.18	16407	1.13
	(0.2)	(1.2)	(1.5)	(4704)	(1.37)
III	2.11	2.13	14.7	20489	1.022
	(0.43)	(4.62)	(4.83)	(6419)	(1.15)
IV	8.89	1.52	13.65	23157	1.035
	(1.29)	(1.3)	(2.66)	(9338)	(1.26)
V	2.84	1.35	6.66	15010	0.93
	(0.71)	(1.65)	(2.8)	(4497)	(0.92)
VI	0.72	1.57	3.8	10967	1.22
	(0.99)	(1.4)	(1.4)	(4299)	(1.26)
U1	5.84	1.62	12.1	22780	1.035
	(1.95)	(1.52)	(6.34)	(11838)	(1.05)
U2	10.79	1.22	6.5	22903	1.01
	(2.21)	(1.13)	(2.26)	(12065)	(1.21)
U3	3.12	1.1	2.31	24588	1.05
	(3.13)	(1.42)	(7.18)	(14790)	(0.95)

[a] The values entered in brackets pertaining to method B; k', capacity factor; α, selectivity; R, resolution (USP); N, no. of plates(USP); T, tailing factor.

and 0.5 ml of trifloroacetic acid, with a flow rate of 1.0 ml/min. The eluent was monitored with a wavelength of 225 nm. The volume of injection is 10 μl of concentration 1.0 mg/ml.

3. Results and discussion

3.1. Purity and assay determination

Sibutramine and all its process related substances (**II**, **III**, **IV**, **V** and **VI**) are shown in Fig. 1. The LC conditions were optimized to obtain the best separation and resolution between these compounds and also for the purity estimation and the quantitative determination of sibutramine. Two isocratic reversed-phase LC (RP-LC) methods namely method A and method B were developed and were found to be suitable for the above analysis. The chromatograms of crude sibutramine HCl spiked with all the related substances recorded using both the methods are shown in Fig. 2. The impurities **U1**, **U2** and **U3** have not been identified. UV maxima for unknown impurities **U1**, **U2** and **U3** were found to be at 224.3, 224.3 and 219.6 nm, respectively. The absorption spectra for the above said impurities was given in Fig. 3. In both the methods, the separation between all compounds was found to be satisfactory and results are presented in the Table 1. In the presented methods the selectivity was found to be more than 1.0 with a resolution more than 1.4 for the case of all the compounds. Sibutramine and its impurities show significant UV absorbance at λ 225 nm. Hence, this wavelength has been chosen as detection for the analysis of sibutramine. The absorption spectra of sibutramine HCl and its impurities were shown in Fig. 3.

The purity values were found to be reproducible when a crude sibutramine sample was analysed in both the methods. The chromatograms of the sample using these methods are shown in Fig. 4. During development stage, it was observed that there was a significant increase in retention of sibutramine HCl, even when there was a small increase in pH of mobile phase or in the temperature of the column. Hence pH and column temperature were fixed at 6.0 and 30°C, respectively.

For the assay determination of sibutramine HCl, 4-chloro aniline was used as internal standard in method A. In method B, lovastatin, a cholesterol-lowering agent was used as internal standard. Method A was selected for the validation owing to the best resolution (>2.0) between impurities. Validation of method A is presented in the following sections for the determination of assay.

3.1.1. Specificity

The specificity of the method was checked by adding all possible potential impurities to pure sibutramine sample and analysed. The resultant assay results were compared with the results of pure sample. There was a good match between the results (Table 2).

Selectivity of the method was checked by using four different columns of different manufacturers,

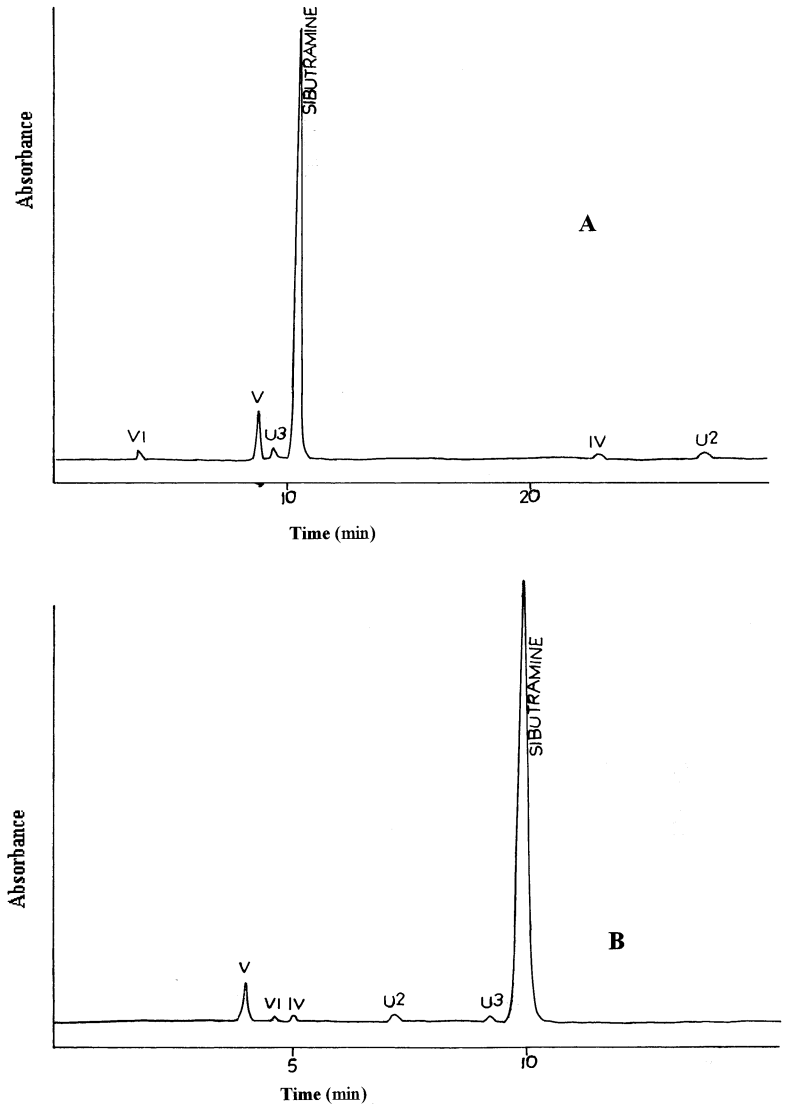

Fig. 4. Chromatograms of crude sibutramine HCl (0.3 mg/ml); (A) Using the conditions of method A; (B) Using the conditions of method B.

Table 2
Test for specificity of the method

Sample spiked with all impurities	Pure sample
99.28	99.72
98.89	99.53
99.12	99.41
Mean: 99.10	Mean: 99.55
SD: 0.196	SD: 0.156
RSD: 0.198	RSD: 0.157

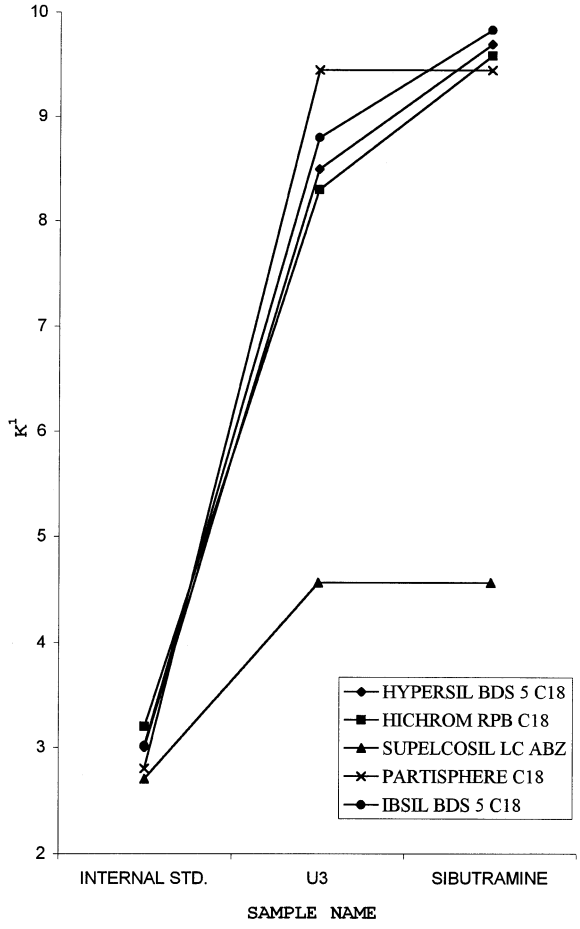

Fig. 5. Method selectivity using different columns.

which are equivalent to hypersil column. The columns used for study were: (1) Phenomenex IB Sil BDS C18 250 mm; (2) Hichrom Hichrom RPB C18 250 mm; (3) supelco supelcosil LC ABZ plus 250 mm; and (4) Whatman Partisphere C18 250 mm. Since the separation between **U3** and sibutramine was very critical, hence a mixture containing the above two and internal standard was selected to check the selectivity. The retention behavior of sibutramine, internal standard and the related compound on each column is shown in Fig. 5. In supelcosil LC ABZ (+) and in partisphere C18 the related compound co-elute with sibutramine peak. The separation was good both in hichrom RPB C18 and IB Sil C18 columns. Hence those two columns can be used as alternative columns to hypersil BDS C18.

3.1.2. Linearity

Six sample solutions at different concentration levels ranging from 0.1 to 0.75 mg/ml were prepared and checked for linearity. A calibration curve was drawn between response ratio (area of sibutramine sample/area of internal standard) and concentration of sibutramine. The equation for calibration curve is $y = 3.1x + 0.0042$. The RSD values (%) for the slope and intercept are 0.87 and 48.8, respectively. The correlation coefficient of the plot found to be more than 0.999, indicating good linearity.

3.1.3. Accuracy

Accuracy of the method was checked at three concentration levels, i.e. at 0.25, 0.4 and 0.6 mg/ml, each in triplicate for 3 successive days. On each day samples were prepared a fresh from the three stock solutions. Solutions for the standard curves also were prepared a fresh from the stock on each day. The accuracy of assay variation shown in terms of relative mean error (RME) and total error (TE). The relevant details are presented in the Table 3. The RME and TE values obtained for intra-day assay calculations were below $\pm$ 1.7%.

Furthermore, the precision of the method A using *F-test* with method B as reference. The obtained values are presented in Table 4. The calculated value of *F-test* of variance ratio, close to 1.0, implies that the two developed methods (method A and method B) have equal precision. *F-Test* indicates that there is no difference between Methods A and B at 5% significant level.

3.1.4. Precision

Precision of the method was studied for repeatability and intermediate precision. Assay values were obtained with different concentration levels (0.2, 0.3 and 0.4 mg/ml) in triplicate for three consecutive days. Intra-day variations were expressed in terms of RSD values calculated from the each day data. RSD values were found to be well below 2.0%, indicating a good repeatability (Table 5). The inter-day variations calculated for each concentration level from the 3 days data and expressed in terms of RSD values. At each concentration level the RSD values were well below 1.3%, indicating a good intermediate precision (Table 5).

3.1.5. Ruggedness

The ruggedness of an assay method is defined as degree of reproducibility of assay results obtained by analysis of the same sample under variety of normal test conditions such as different labs, different analysts, different instruments and different lots of reagents. The second day's samples were analysed at laboratory B with different instrument (LC Module I plus HPLC system containing Pump and an UV-Visible detector), different mobile phase and different column of the same make by different analyst. The data obtained was compared with parent laboratory. RSD values calculated from the data of two laboratories were within 2.0%, indicating the ruggedness of the developed method.

Table 3
Accuracy in the assay determination of sibutramine HCl[a]

Day of analysis	Taken (mg)	Recovery (mg) ($n=3$)	% Recovery	RME (%)	TE (%)
0 day	0.1962	0.1976	100.7	0.7	−0.3
	0.2704	0.2733	101.1	1.1	1.6
	0.384	0.3857	100.4	0.4	1.1
1 day	0.1962	0.1949	99.3	−0.7	−0.2
	0.2704	0.2677	99.0	−1.0	−0.6
	0.384	0.3883	101.1	1.1	1.6
2 day	0.1962	0.1948	99.3	−0.7	−0.3
	0.2704	0.2725	100.8	0.8	1.4
	0.384	0.3827	99.7	−0.3	0.2

[a] % recovery of sibutramine from the sample against taken; RME, relative mean error; RME, {(MEAN−TCONC)/TCONC}*100; TE, Total error; TE, (2 SD+(MEAN−TCONC)/TCONC)*100, in which SD is the S.D. of the mean of triplicate of each concentration level and TCONC the theoretical concentration of the analyte of each level.

Table 4
F-Test for comparison of two methods

Assay results from method A	Assay results from method B	$f\text{-test} = s_A^2/s_B^2$	Value from table ($F_{(8,8)}$)
101.73	101.0	1.13	4.43
100.71	101.42		
99.64	99.90		
101.71	99.87		
101.62	100.34		
99.88	101.05		
100.07	99.05		
101.58	99.89		
99.66	100.22		
Mean: 100.73	Mean: 100.19		
SD: 0.9	SD: 0.9		

Table 5
Inter and intra-day assay variation of sibutramine

Intra-day			
0 day			
Mean of concentration (mg/ml) $n=3$	0.1976	0.2733	0.3857
SD	0.00205	0.00277	0.0034
RSD(%)	1.0	1.0	1.0
1 day			
Mean of concentration ($n=3$)	0.1949	0.2677	0.3883
SD	0.00236	0.00182	0.00223
RSD (%)	1.2	0.7	0.6
2 day			
Mean of concentration ($n=3$)	0.1948	0.2725	0.3827
SD	0.002084	0.00147	0.005
RSD (%)	1.1	0.5	1.3
Inter-day			
Mean (of mean concentration of three days)	0.1956	0.2711	0.3855
SD	0.00159	0.0030	0.0028
RSD (%)	0.8	1.1	0.7

3.1.6. Robustness

The robustness of a method is its ability to remain unaffected by small changes in parameters such as percentage organic content, pH of the mobile phase and temperature, etc.

To determine the robustness of the method, experimental conditions were purposely altered and evaluated its effects on chromatographic characteristics. The effect of change in buffer pH (5.6–6.2) on retention time (capacity factor k') of sibutramine HCl, related substance **U3** and internal standard is shown in Fig. 6. The pH change was insignificant on the internal standard, but there is not only significant increase in the retention of sibutramine and **U3**, but also separation between these two was increased with increase of pH. In another experiment, column temperature varied from 25 to 40°C in steps of 5°C each time. Variation of temperature, resulted in anomalous behavior in terms of increase in retention of sibutramine HCl and **U3**. The effect of change in temperature on retention is shown in Fig. 7. The increase in temperature resulted in a decrease in retention of internal standard. Despite the above changes the robustness was demonstrated by the assay results having $< 2.0\%$ RSD.

3.1.7. Stability

The stability of sibutramine HCl in solution containing mobile phase and the internal standard have been determined by keeping one sample in refrigerator and other in a tightly capped volumetric flask placed at ambient temperature under normal lighting conditions. The samples were checked for assay in three successive days of storage and compared with freshly prepared sample. The RSD values of assay were found to be below 2.0% in both the cases. This indicates that the sibutramine HCl is stable in the solution and compatible with internal standard.

3.2. Enantiomeric separation by chiral chromatography

To resolve the enantiomers of sibutramine HCl, different chiral columns namely chiralcel-OJ, chiralcel-OD, chiralcel OD-H of Daicel manufacturers and whelk O-1 of E. Merck were employed without derivatisation. Various experiments were conducted, to select the best stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers. There is an indication of separation of enantiomers on chiralcel-OD and chiralcel OD-H columns but no separation was found on chiralcel OJ and whelk O-1 columns. An optimal separation was reached by varying the amount of ethanol and hexane in the mobile phase system, using chiralcel od column. The mobile phase system hexane:ethanol (98:2 v/v) gave baseline separation of enantiomers of sibutramine HCl on the above column with resolution and tailing factors around 2.0 and 3.5, respectively. Introduction of isopropanol instead of ethanol, further reduced the resolution. To improve peak symmetry, 0.05% trifluoro acetic acid (TFA) was added to mobile phase system. The presence of TFA in the mobile phase plays an important role on the retention of the two enantiomers, chromatographic efficiency and the resolution of the isomers. Addition of TFA enhanced

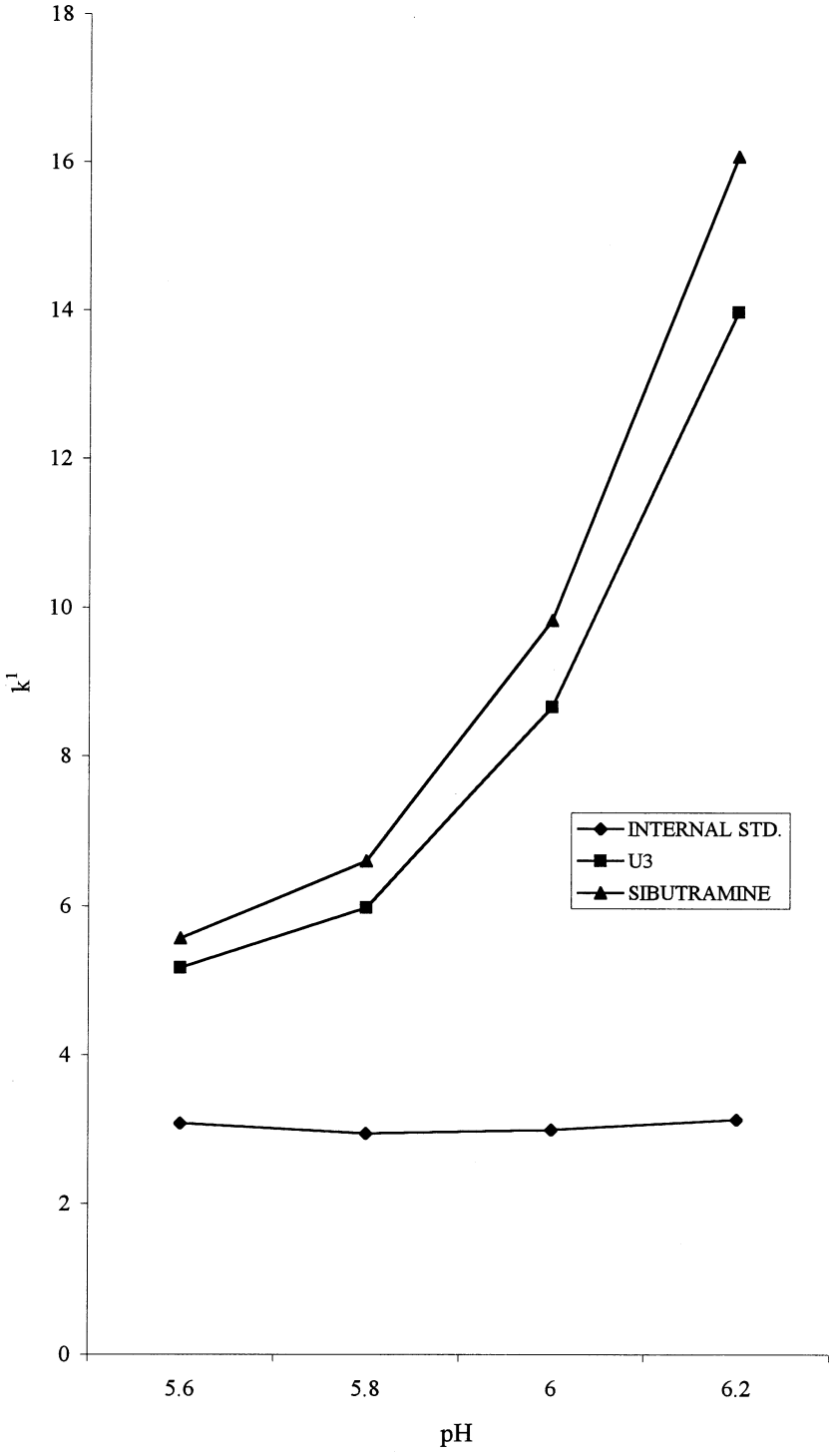

Fig. 6. Effect of change in buffer pH on retention.

the retention and resolution of enantiomers of sibutramine HCl with good peak shape and efficiency. chiralcel OD column with the mobile phase hexane:ethanol: TFA (93:7:0.05, v/v/v) found to be best conditions for the separation of enantiomers of sibutramine HCl. In the above mentioned conditions the (−) sibutramine eluted prior to (+) sibutramine. The typical chromatogram is presented in Fig. 8, which shows that (±) enantiomers of sibutramine were adequately resolved from each other. chiralcel OD-H column was also investigated with final conditions. Good

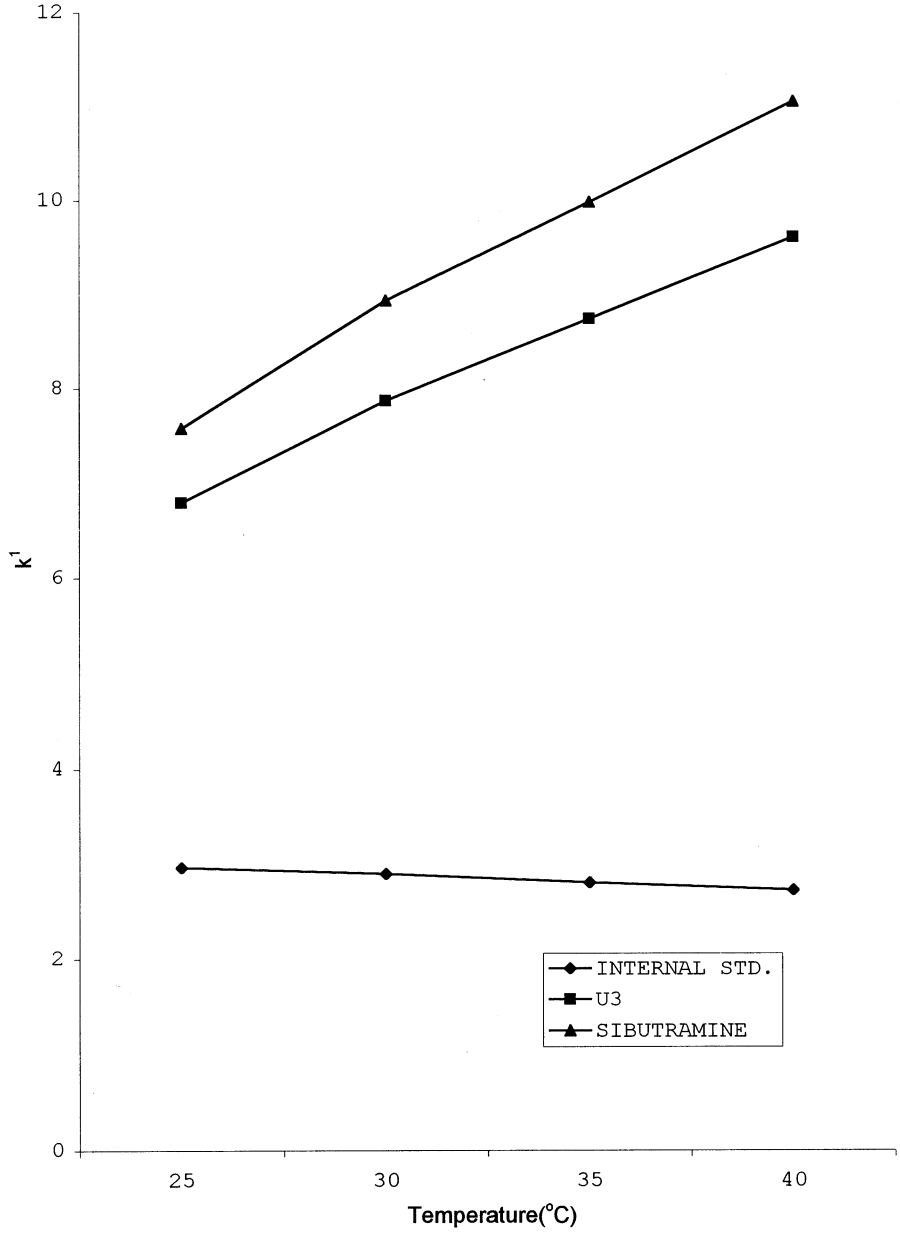

Fig. 7. Effect of change in column temperature on retention.

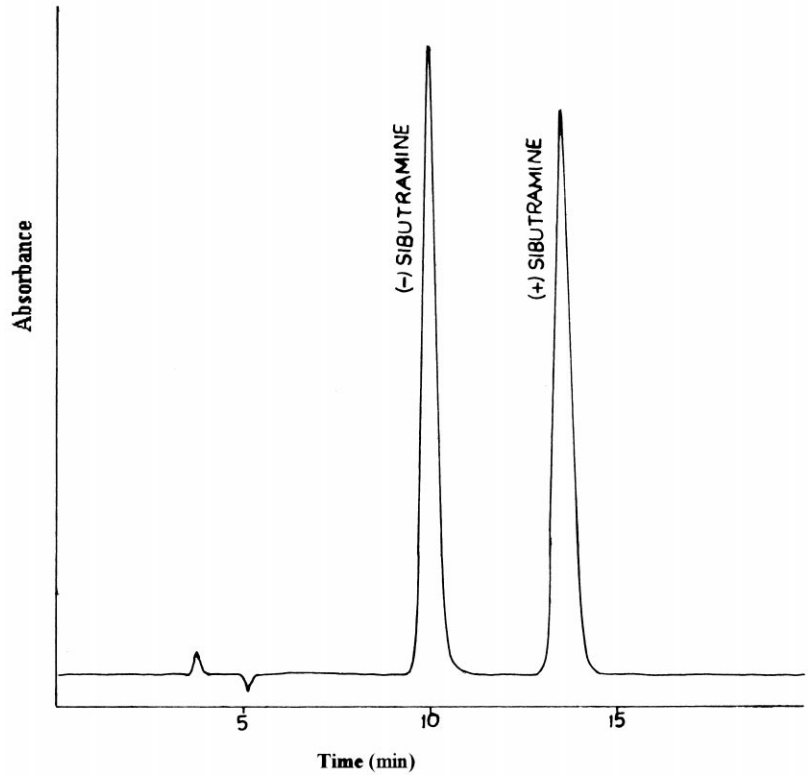

Fig. 8. Enantiomeric separation of (±) sibutramine HCl (1.0 mg/ml) using chiral LC.

resolution with more tailing was observed. Comparison of system suitability results obtained with chiralcel-OD and with chiralcel OD-H columns are present in the Table 6. The results indicate that chiralcel OD-H column can also be used as an alternative for the separation of enantiomers.

4. Conclusion

An analytical reversed-phase HPLC method was developed and validated thoroughly for the determination of purity and assay of sibutramine HCl. A chiral chromatography method was also described for the enantiomeric separation of sibutramine HCl. The developed methods are sensitive, simple and rugged and they can be employed for monitoring the purity in bulk manufacturing. The methods are proved to be selective and stability indicating.

Table 6
System suitability report for the columns Chiralcel OD and OD-H[a]

Column	k'	α	R	N	T
Chiralcel OD	8.95	1.4	4.48	3850	2.02
	12.58			4150	1.97
Chiralcel OD-H	2.32	1.75	5.08	2751	2.53
	4.05			2639	2.37

[a] k', capacity factor; α, selectivity. R, resolution (USP); N, no. of plates(USP); T, tailing factor (capacity factors calculated using dead volume = 1.0).

Acknowledgements

The authors wish to thank the management of Dr Reddy's group for supporting this work and

Dr A.Venkateswarlu, President, Dr Reddy's Research Foundation for his constant encouragement. Authors wish to acknowledge the Process research and development group for providing the samples of sibutramine and its related substances for our research. The authors would also like to thank the colleagues in separation science division of Analytical research and development of Dr Reddy's Research Foundation for their co-operation in carrying out this work.

References

[1] A. Kozlik., E.C. Wilm Shurst, J.E. Jeffery (Boots) GB 2098 602
[2] James E. Jeffery, Frank Kerrigan, Thomas K. Miller, Graham J. Smith, Gerald B. Tometzki, J. Chem. Soc., Perkin Trans., 1 (025) (1996) 83–89.
[3] W.R. Buckett, P.C. Thomas, G.P. Luscombe, Prog. Neurophychopharmacol. Biol. Psych. 12 (1998) 575.
[4] James W. Young, (Sepracor) PCT Int. Appl. WO 94 00,047(Cl. A51K31/145)
[5] James W. Young, (Sepracor) PCT Int. Appl. WO 94 00,114(Cl. A61K31/135)