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Research Article

DEVELOPMENT AND VALIDATION OF A CHIRAL LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ENANTIOMERIC PURITY OF BENZPHETAMINE

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ABSTRACT

The present work narrates a liquid chromatographic method for the determination of enantiomeric purity of D-benzphetamine which is an anorectic agent and also reduces caloric intake. Resolution between the benzphetamine enantiomers was >3.0 within 10 minutes on Chiralpak AD-H (250 mm × 4.6 mm) column using methanol and diethyl amine (100:0.2 *v/v*) as mobile phase with a flow rate of 1.0 mL min^{-1} at 25 °C. The detection was made at 220 nm using UV detector. The effects of diethyl amine and temperature were evaluated for better enantioselectivity and resolution of enantiomers. The method was validated for L-benzphetamine (as an impurity) in terms of accuracy, precision and linearity, and the correlation coefficient was found to be >0.9994. The recoveries were obtained in the range of 95.5–103.2%. The limits of detection and quantification of L-enantiomer were 0.012 and 0.035 µg mL^{-1}, respectively. The developed method was found to be suitable for quantification of L-enantiomer in D-benzphetamine drug substance.

Keywords: Enantiomeric purity, Benzphetamine hydrochloride, Validation, Liquid chromatography.

INTRODUCTION

Chirality is a major consideration in drug discovery because most of the drugs currently under development are chiral [1,2]. A number of synthetic chiral drugs are still distributed as racemic mixture in spite of the fact that one enantiomer possesses very different and significant pharmacological, toxicological activities from the antipode and may even disturb other biological processes and cause catastrophic side effects[3]. Thus, the enantiomeric separation and analysis of chiral drugs have become essential in the pharmaceutical field[4]. According to the guidelines of the U.S. Food and Drug Administration (FDA), the pharmaceutical companies have to develop therapeutically active enantiomers of chiral drugs. However, the enantiomers of the drugs should be separated and studied their pharmacological and metabolic pathways[5]. Hence enantio-separation and enantio-purity determination of chiral drugs are very important in pharmaceutical investigations such as pharmacological and toxicological studies.

Benzphetamine (BPA) is a chiral compound (Fig. 1), sympathomimetic amine and is classified as an anorectic. The main function of D-isomer of BPA is to reduce appetite, which in turn to reduce caloric intake. D-BPA can cause vivid hallucinations if taken for the wrong purpose. D-BPA is a white crystalline powder readily soluble in water and 95% ethanol. The chemical name of D-BPA is *N*,α-dimethyl–*N*(phenylmethyl)-benzeneethanamine hydrochloride, during its synthesis L-BPA present as a chiral impurity. Enantiomers of racemic drugs often differ in pharmacokinetic behavior or pharmacological action[6]. So, enantioseparation of chiral drugs is important in pharmaceutical investigations and also analytical method development.

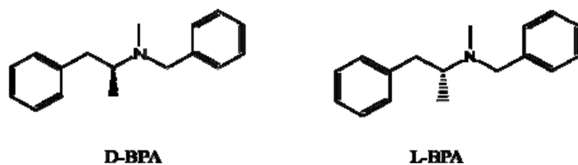

Fig. 1: Structures of BPA enantiomers

The development of analytical methods for the quantitative analysis of chiral drugs and for the assessment of enantiomeric purity is extremely challenging due to the fact that enantiomers possess virtually identical properties. Chiral chromatography is an important analytical tool for separation and determination of inactive enantiomers present in enantiomerically pure drugs and finished products. Among the separation techniques, high resolution liquid chromatographic (LC) methods employing chiral stationary phases (CSP's) are more rapid and suitable for the resolution of racemic mixtures of pharmacologically active chemical entities[7,8,9]. Several CSP's are now available to allow the direct separation and determination of drug enantiomers and racemates. The amylose based stationary phases are one of the commonly employed CSPs for the separation and enantiomeric purity determination[10,11]. The ability of chemically modified cellulose to separate a variety of racemates has recently been reviewed by Okamoto[12]. The enantiomeric inclusion in chiral cavities which might be multiple and competitive in cellulose and amylose based CSPs seems to be responsible for the chiral discrimination[13].

The main intention of the present research work was to develop a sensitive, selective, precise and accurate LC method for the determination of L-BPA in D-BPA drug substance. The separation of BPA enantiomers was carried out on a newly commercialized CSP, amylose tris(3,5-dimethylphenylcarbamate) coated on 5 µm silica-gel (Chiralpak AD-H) column. After successful development, the method was validated as per ICH guidelines[14].

MATERIALS AND METHODS

Chemicals and Reagents

Enantiomeric samples of BPA were received from Virchow Laboratories Limited (Hyderabad, India) with declared purities of 99.5 and 96.8%, respectively. LC grade methanol (MeOH) and diethylamine (DEA) were purchased from Merck (Schuchardt, Germany). High pure water was prepared by using Millipore (Bedford, USA) Milli-Q plus purification system. The chiral column used during the study was Chiralpak AD-H (250 mm x 4.6 mm, 5 µm particle size) from Daicel Chemical Industries, Japan.

Instrumentation

LC experiments were carried out on Waters HPLC system (Milford, USA), consists of tertiary pump assemble, sample manager and a photodiode array detector. The output signal was monitored and processed using Waters Empower software. A Waters Breeze HPLC system used for method validation was consists of 2695 separation module and a 2487 dual wavelength detector. The LC system used for intermediate precision was Waters Breeze with an auto sampler.

The output signal was monitored and processed using the millennium 32 software on Pentium computer.

Chromatographic Conditions

Chromatographic conditions were optimized using the Chiralpak AD-H, 250 mm x 4.6 mm, 5 µm column. The mobile phase was a mixture of MeOH and DEA (100:0.2 *v/v*), kept at a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 25 °C and detection was carried at 220 nm. The injection volume was 10 µL.

Preparation of standard stock solutions

The standard stock solutions of D-BPA (1.0 mg mL^{-1}) and L-BPA (1.0 mg mL^{-1}) were prepared by dissolving the appropriate amount of the substance in the mobile phase. One mL of standard stock solutions of D-BPA and L-BPA were mixed in a 100 mL flask and makeup with mobile phase for system suitability purpose. The test solution was prepared in the mobile phase by dissolving the drug substance equivalent to 1.0 mg mL^{-1}. The standard stock solutions were further diluted as per required concentrations during validation experiments.

Method Validation

Precision

Method precision was determined by measuring the repeatability and intermediate precision was determined for retention times and peak areas of L-BPA. Method precision was carried out by the same analyst in different time intervals over one day and intermediate precision was carried out by different analyst over three days. The repeatability of the method was determined by injecting replicate injections of system suitability solution. The intermediate precision was calculated over three days by performing six consecutive injections per each day. Precision was reported as percentage of relative standard deviation (% RSD).

Method precision of L-BPA impurity was evaluated by carrying out six independent injections of a 1.0 mg mL^{-1} solution containing D-BPA spiked with 0.10% of L-BPA impurity. The % RSD for the peak area of L-BPA impurity was calculated. The intermediate precision of the method was also evaluated. The % RSD for six individual spiked preparations was calculated.

Limit of detection and limit of quantification

The limit of detection (LOD) is defined as the lowest concentration of the analyte that can be clearly detected above the baseline signal, estimated as three times from the signal to noise ratio[15]. The limit of quantitation (LOQ) is defined as the lowest concentration of the analyte that can be quantified with suitable precision and accuracy, estimated at 10 times from the signal to noise ratio[15]. The LOD and LOQ were achieved by injecting a series of dilute solutions of D-BPA and L-BPA.

Linearity

The linearity of the developed method was performed using the standard solutions of D-BPA and L-BPA at different concentration levels ranging from LOQ to 150% of the specification limit. Triplicate injections of each solution were made under the chromatographic conditions as prescribed above, using 10 µL of injection volume. Plotted a graph between the peak response against the corresponding concentration and the linear regression equations were calculated.

Linearity for detector response was assessed by preparing six standard solutions of D-BPA and L-BPA ranging from LOQ to 150% of the specification limit. The peak area versus the concentration data was performed by least-squares linear regression analysis.

Accuracy

The accuracy of the method was evaluated by spiking L-BPA at three different concentration levels of 50%, 100% and 150% to the specification limits in triplicate. The samples were analyzed by the proposed method and the percentage recoveries for L-BPA at each level and each replicate were calculated.

The accuracy of L-BPA impurity was evaluated in triplicate at three concentration levels i.e. 0.5, 1.0 and 1.5 µg mL^{-1} in bulk drug sample. The % recoveries were calculated.

Robustness

Robustness evaluates the influence of a number of method parameters like flow rate, mobile phase composition, column temperature and wavelength changes on the responses prior to a transfer to another laboratory. To determine the robustness of the developed method experimental conditions were purposely altered. The effect of flow rate on enantiomeric resolution was investigated at 0.9 and 1.1 mL min^{-1}. The effect of temperature on resolution between D-BPA and L-BPA was investigated at 25 °C and 35 °C keeping other conditions as indicated in the method.

Solution stability and mobile phase stability

The solution stability of L-BPA impurity was carried out by leaving the test solution in a tightly capped volumetric flask at room temperature for three days. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions for a 6 h interval up to three days. Mobile phase prepared was kept constant during the study period.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The main aim of the present work was to develop a rugged and robust method to separate the BPA enantiomers and a precise quantitative determination of L-BPA. For the method development, different chiral columns and mobile phases were investigated using the racemic mixture of BPA enantiomers.

Various compositions of MeOH and DEA were used as the mobile phase during our initial efforts on Chiralcel OJ-H, Chiralcel OD-H and Chiralpak AD-H columns. No separation on Chiralcel OJ-H column, a partial enatioseparation on Chiralcel OD-H column (Fig. 2a) and a good enatioseparation (with a resolution greater than 3.0) on Chiralpak AD-H column (Fig. 2b) were observed by using a composition of 100:0.2 (*v/v*) (MeOH and DEA).

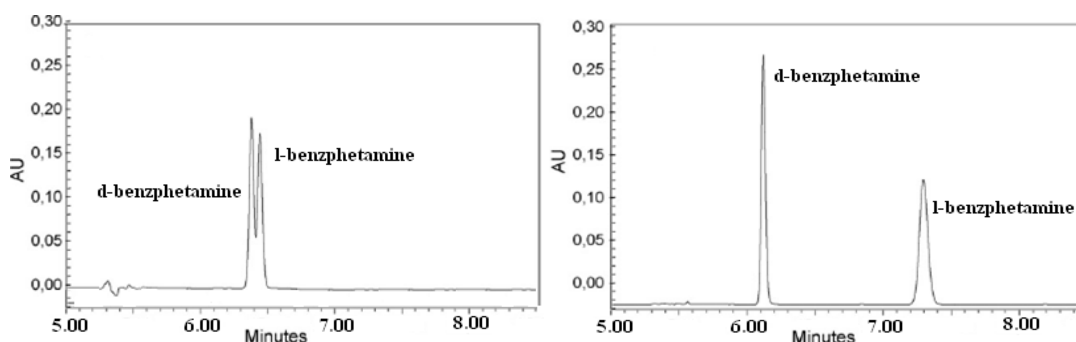

Fig. 2: HPLC chromatogram obtained on Chiralcel OD-H (a) and Chiral Pak AD-H (b) from system suitability solution

The Chiralpak AD-H column having carbamate groups which can interact with chiral solutes. In the present study, the available functional groups on BPA enantiomers can form hydrogen bonds and π–π interaction with amylose based CSP. The solutes which possess aromatic rings can allow extra stabilizing effect to the solute–CSP complex by locating of the aromatic ring into the chiral cavity[16]. In the present study also, this type of stabilization effect can be possible due to the presence of the aromatic functionality on the BPA. Due to these interactions, separation of BPA enantiomers on Chiralpak AD-H column could be achieved. Improved peak shapes and better resolution between D-BPA and L-BPA were observed by addition of DEA.

Different temperatures and flow rates were studied on resolution and retention times of BPA enantiomers. The most optimum conditions were found using a mobile phase consisting of MeOH and DEA (100:0.2 *v/v*) at flow rate of 1.0 mL min^{-1} on Chiralpak AD-H column maintained at 25 °C (Table 1).

Table 1: Chromatographic performance data

Compound	t_R (min)[a]	RRT[b]	Resolution	Tailing factor	Theoretical plates	RRF[c]
D-BPA	6.12	--	--	1.16	3000	-
L-BPA	7.31	1.19	3.15	0.92	2800	0.96

[a]Retention time; [b] Relative retention time; [c] Relative response factor

Validation

To demonstrate the intended use of the developed method, validation was carried out as per ICH guidelines[15]. The method validation parameters include system suitability, precision, LOD, LOQ, linearity and accuracy.

System suitability

System performance was verified by injecting the system suitability solution. Injected the suitability solution for six times and calculated the % RSD. Results were shown in Table 2.

Table 2: LOD, LOQ, linearity and precision data

Parameter	D-BPA	L-BPA
LOD (µg mL^{-1})	0.011	0.012
S/N ratio	2.9	3.1
LOQ (µg mL^{-1})	0.031	0.035
S/N ratio	9.6	10.1
Regression statistics		
Slope	155378	187029
Intercept	2736.2	-3722.5
Correlation coefficient (r^2)	0.9996	0.9995
Repeatability (% RSD)[a]	1.12	1.35
Inter-day precision (% RSD)[b]	0.96	1.06
Precision at LOQ	1.34	1.68

[a] % RSD for six replicate injections of system suitability solution.

[b] average % RSD for six replicate injections of system suitability solution at three different days.

Precision

Repeatability

The results of the repeatability were presented in Table 2. The % RSD for the area count of six replicate injections was below 1.5%. Based on the % RSD values it was demonstrated that method was precise and stable.

Inter-day precision

The average % RSDs for the peak areas of D-BPA and L-BPA obtained from inter-day precision were shown in Table 2. % RSDs for six replicate injections of system suitability solution for 1st day were 0.94 and 1.12, 2nd day were 0.98 and 1.06 and 3rd day were 0.96 and 1.01. It indicates that the solution was stable and method was precise.

LOD and LOQ

The LOD and LOQ for D-BPA and L-BPA were determined by signal to noise ratio method. The LOD and LOQ for both the peaks were shown in Table 2. The % RSDs for D-BPA at LOQ level was 1.34% and for L-BPA was 1.68.

Linearity

Linearity of the proposed method was evaluated by injecting the enantiomers at different concentration levels ranging from LOQ to 150% of the working concentration levels. Six different concentrations includes LOQ, 0.05%, 0.075%, 0.10%, 0.125% and 0.15% were prepared and injected in triplicate at each concentration level. The mean area calculated and plotted a graph between the average area and concentration. The correlation co-efficient (r^2), slope and intercept were shown in Table 2.

Accuracy

The percentage recovery of L-BPA in spiked samples was ranged from 95.5 to 103.2% w/w. The accuracy results were presented in Table 3.

Table 3: Accuracy results

% L-BPA added		% Recovery	Average % recovery	% RSD[a]
50%	1st	97.4	96.5	
	2nd	96.7		1.00
	3rd	95.5		
100%	1st	98.7	99.7	
	2nd	99.2		1.38
	3rd	101.3		
150%	1st	99.3	101.6	
	2nd	102.2		1.99
	3rd	103.2		

[a] Mean of triplicate determination at each level.

Robustness

The results of robustness were presented in Table 4. The resolution between D-BPA and L-BPA was always >2.5, illustrating the robustness of the method in all the deliberate varied chromatographic conditions (flow rate and column temperature).

Table 4: Robustness results

Change in chromatographic	Resolution	% L-BPA
Initial	3.1	0.12
Flow rate (+0.1mL min^{-1})	3.4	0.11
Flow rate (-0.1mL min^{-1})	2.8	0.12
Column oven temp (-5° C)	2.6	0.11
Column oven temp (+5° C)	3.8	0.12
% RSD		4.72

Solution stability and mobile phase stability

The RSD of L-BPA during solution stability and mobile phase stability experiments was within 1.0% RSD. No significant change was observed in L-BPA content during solution stability and mobile phase stability experiments. The experimental data of solution stability and mobile phase stability confirms the sample solution and mobile phase are stable up to three days.

CONCLUSIONS

The developed LC method for quantitative determination of L-BPA is precise, accurate and selective. The validated method shows the

satisfactory data for all the method validation parameters. The developed method can be used for the determination of L-BPA in benzphetamine samples.

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