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Short communication

Development and validation of indirect RP-HPLC method for enantiomeric purity determination of D-cycloserine drug substance

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ABSTRACT

A new chiral purity method was developed for D-cycloserine (D-cys) by reverse phase HPLC and validated. Chiral derivatizing reagents, viz., o-phthalaldehyde and N-acetyl-L-cysteine were utilized in this method. The resultant diastereomers were resolved using Zorbax SB Phenyl HPLC column under isocratic elution. A mobile phase of 95:05 (v/v), 20 mM Na_2HPO_4 (pH 7), and acetonitrile, respectively, was used with the flow rate of 1.0 mL/min and UV detection at 335 nm. The method development with different chiral stationary phases and chiral derivatization reagents were also investigated. The stability of diastereomer derivative and influence of organic modifier and pH of the mobile phase were studied and optimized. The stability-indicating capability of the method was established by performing stress studies under acidic, basic, oxidation, light, humidity and thermal conditions. The detection and quantitation limit of L-cycloserine (L-cys) were 0.015 and 0.05% (w/w), respectively. A linear range from 0.05 to 0.30% (w/w) was obtained with the coefficient of determination (r^2) 0.998. The recovery obtained for L-cys was between 92.9 and 100.2%. This method was applied successfully in pharmaceutical analysis to determine the content of L-cys in D-cys bulk drug.

1. Introduction

D-Cycloserine or D-4-amino-3-isoxazolidinone [1] is a broad-spectrum antibiotic which is used in the treatment of tuberculosis (Fig. 1a). The most important property of D-cycloserine (D-cys) is the inhibition of the growth of *Mycobacterium tuberculosis*. The worldwide resurgence of tuberculosis, the emergence of multiply drug-resistant tuberculosis and the problematic use of available drugs required to treat these infections, have resulted in the application of D-cys as a second line drug for the treatment of tuberculosis [2]. Official monographs available for D-cys in United States Pharmacopoeia and The International Pharmacopoeia refers only assay or impurities determination by LC but chiral purity estimation was not reported [3,4]. Also, there is no method published so far for the enantiomeric purity of D-cys by HPLC except a capillary electrophoresis–mass spectrometry (CE–MS) method utilizing crown ether as a chiral selector [5]. According to International Conference on Hormonization (ICH), Food and Drug Administration (FDA) and European Medicines Agency (EMEA) guidelines, any identified impurity must be controlled to less than 0.15% with respect to (D-cys) drug substance [6–8]. Hence, the present investigation was initiated with the objective to develop a simple, sensitive and stability-indicating method for L-cys enantiomer impurity determination in D-cys drug substance by reverse phase (RP) HPLC.

Amylose, cellulose, crown ether and protein based chiral stationary phases were investigated for the direct estimation of L-cys in D-cys by HPLC but no good separation could be achieved. Hence, indirect [9,10] chiral separation of enantiomers after derivatization with chiral Marfey's reagent, Sanger's reagent and o-phthalaldehyde (OPA) with N-acetyl-L-cysteine (NAC) were evaluated [11–15]. Derivatization with OPA and NAC has been utilized in this study for the determination of L-cys in D-cys drug substance. OPA–NAC has the advantage of rapid and complete derivatization at room temperature [16,17] itself. Selection of reverse phase HPLC column, mobile phase composition and pH of the mobile phase were found to play a vital role in the separation and sensitivity of the developed method. The proposed RP-HPLC method with UV detection has been validated using ICH and USP [18,19] guidelines as references.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile, AR grade Na_2HPO_4 and *ortho*-phosphoric acid (88%) were purchased from Merck (Mumbai, India). The water used was from a Milli-Q purification system,

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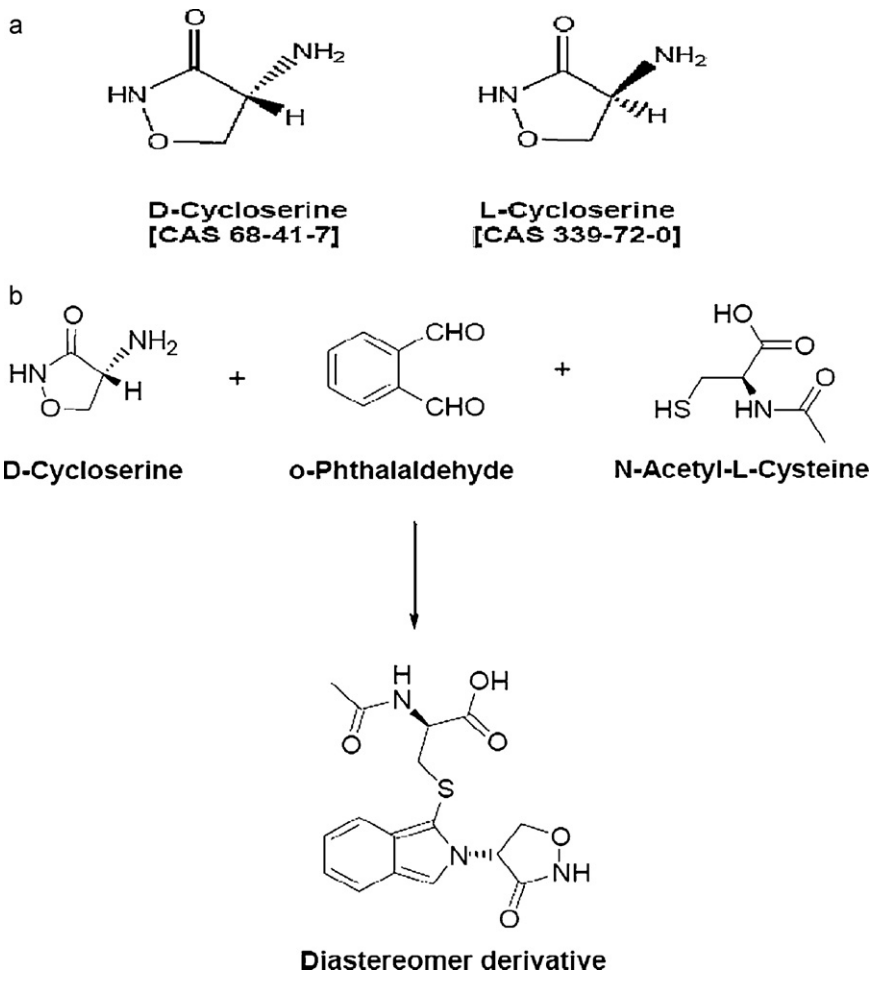

Fig. 1. (a) Chemical structure of D-cycloserine and L-cycloserine. (b) Schematic representation of the formation of diastereomer derivative and its chemical structure.

Millipore (Bedford, MA, USA). Analytical grade boric acid and sodium hydroxide were from Qualigens Fine Chemicals (Mumbai, India) and Ranbaxy Fine Chemicals (New Delhi, India), respectively. Absolute alcohol (AR quality) was from Hayman Limited (Essex, England). o-Phthalaldehyde (purity ≥99.6%) and L-cycloserine (purity ≥95%) were purchased from Sigma–Aldrich (Buchs, Switzerland). N-acetyl-L-cysteine (purity ≥99%) was obtained from Sigma–Aldrich (St. Louis, MO, USA). D-Cycloserine drug substance was obtained from Shasun Chemicals and Drugs Ltd. (Chennai, India).

2.2. Equipments and chromatographic conditions

The HPLC system consisted of a Waters Alliance separation module 2695 equipped with Waters 2487 dual wavelength absorbance detector (Milford, MA, USA). Waters Empower 2 software (Build 2154, Waters) was used for the data acquisition and processing. A Shimadzu LC-2010A HPLC system equipped with UV–VIS detector and having Class-VP software was used for intermediate precision and also for method development purpose (Kyoto, Japan). Peak purity/homogeneity studies were carried out using Waters 2996 photodiode array (PDA) detector (Milford, MA, USA) and Thermo Finnigan Surveyor LC system coupled with LCQ DECA XP Plus ion-trap mass (LC–MS) spectrometer (San Jose, CA, USA). Agilent Zorbax SB phenyl HPLC column of 250 mm length × 4.6 mm id, 5 μm particle size (Palo Alto, CA, USA) was used. The column was kept at (30 ± 2) °C. A mobile phase of 95:05(v/v) 20 mM Na_2HPO_4 (pH 7 using *ortho*-phosphoric acid), and acetonitrile, respectively, was used. Chromatograms were obtained with ultraviolet detection at the wavelength of 335 nm. The injection volume was 15 μL and the flow rate was 1.0 mL/min. The total run time was 20 min.

2.3. Solution preparation

Chiral derivatization reagent (CDR) was prepared by mixing 25.0 mL each of 0.05 M OPA (prepared in ethanol) and 0.05 M NAC (prepared in water) in a 250 mL volumetric flask and diluted to volume with 0.1 M borate buffer (pH 9.5). 0.1 M borate buffer (pH 9.5) was used as diluent in all the preparations. System suitability solution was prepared by dissolving 5.0 mg each of L-cys and D-cys using 5.0 mL diluent in 10 mL volumetric flask and diluted to volume with diluent. 2.0 mL of this solution and 10.0 mL of CDR were added into a 25 mL volumetric flask and diluted to volume using diluent. Resolution between L-cys and D-cys was evaluated as part of system suitability with the acceptance criteria of not less than 1.5. Standard stock was made by dissolving L-cys in diluent to obtain 0.002 mg/mL solution. Standard preparation was made by mixing 3.0 mL of standard stock solution and 10.0 mL of CDR in a 25 mL volumetric flask and diluted to volume using diluent (0.24 μg/mL). Sample preparation was made by dissolving 25 mg of D-cys sample using diluent in 25 mL volumetric flask and diluted to volume. 4.0 mL of this solution and 10.0 mL of CDR were added into a 25 mL volumetric flask and diluted to volume using diluent (0.16 mg/mL).

2.4. Stress conditions

The D-cys bulk drug sample was treated with 0.1 N HCl for acid stress and the solution was heated at (70 ± 2) °C for 10 min. Base hydrolysis was performed in 0.1 N NaOH and the solution was then subjected to heating at (70 ± 2) °C for 10 min. The drug substance was treated with 0.2% hydrogen peroxide solution at (25 ± 2) °C to a period of 10 min for oxidative degradation. D-cys sample was spread on a Petri dish and exposed to 95 ± 5% relative humidity at 25 ± 2 °C in a humidity chamber for 4 h. A thin layer of sample was spread on a Petri glass dish and subjected to thermal stress at (105 ± 2) °C in a dry heat oven for 72 h. Photolytic studies were conducted by exposing the drug to UV and fluorescent light for 72 h.

3. Results and discussion

3.1. Development of chromatographic method

Preliminary experiments for the direct separation of Cys enantiomers were performed on polysaccharide stationery phases viz., CHIRALCEL OD-H, OJ-H, CHIRALPAK AD-H, AS-H, CHIRALPAK AD-RH and CHIRALCEL OD-RH in normal and reverse phase elution mode but no resolution between D-cys and L-cys was obtained. Also, when crown ether (CROWNPAK CR+), ligand-exchange (SUMICHIRAL OA6100) and protein (Chiral-AGP) based chiral stationery phases (CSP) were investigated, no clear separation could be achieved. These results suggested for the indirect chiral separation through derivatization procedures. Considering the structural factors of D-cys, being a primary amine, derivatization with chiral Marfey's, Sanger's and OPA–NAC reagents followed by reverse phase HPLC separation of the derivatized diastereomers were studied and the separation was good but the derivatization reaction progressed very slowly with chiral Marfey's, Sanger's reagents at room temperature (25 ± 2) °C and also the derivatization reaction was not complete even when it was maintained at 40 °C for one hour. However, derivatization with OPA–NAC yielded a very good separation and the reaction was found to be complete at room temperature (25 ± 2) °C itself. Therefore, OPA–NAC was selected as suitable derivatization reagent as it was very economical, easy to operate, rugged and forms stable diastereomer derivatives [16,17]. Fig. 1b represents the derivatization reaction and structure of the diastereomer derivative. Derivatization time was studied from 5 to 60 min and no significant change in the peak area was found, hence, 10 min was considered as adequate and finalized. Initially, separation of diastereomers were attempted using octadecyl silane/octyl silane (Phenomenex Luna C18/C8,) RP stationary phases and no baseline resolution obtained with C18, however the separation was good in C8 but peak response (sensitivity) was found to be poor. Hence, RP cyano (Zorbax SB-CN) and phenyl columns (Zorbax SB-phenyl) were evaluated for the separation. In cyano column, the reproducibility was not satisfactory, however, with phenyl column, clear baseline resolution was obtained with shorter retention time.

The effect of variation in pH and acetonitrile of the mobile phase on retention and resolution were studied. When the pH of the buffer solution was reduced to 6.5, a 3–4 min increase in retention was found with no significant change in the resolution (R) but with poor peak height (broad peak), however, decrease in R with reduced retention was obtained when the pH was increased to 7.5. Since, D-cys is known to degrade in acidic medium [20], lower pH (<6.5) was not considered for mobile phase. Increase of acetonitrile content in mobile phase (+2%) resulted in the reduction of R but decrease in acetonitrile (−2%) enhanced the retention and R but with relatively poor peak shape and height (less sensitive). Methanol was not preferred in the mobile phase due to high retention and poor (broad) peak shape. From all the investigations carried out, the optimal chromatographic conditions finalized for the separation of L-cys and D-cys was 95:05 (v/v), 20 mM Na_2HPO_4 (pH 7 using *ortho*-phosphoric acid), and acetonitrile, respectively. Fig. 2 represents the typical chromatograms of sample preparation and system suitability solution containing D and L cys. It is significant to note that L-cys chiral impurity is well resolved from D-cys drug substance with R of more than 1.5. Typical retention time of L-cys and D-cys obtained with the proposed method were 10.4 and 11.8 min, respectively. Retention factor (k) for L-cys and D-cys were 3.2 and 3.7, respectively.

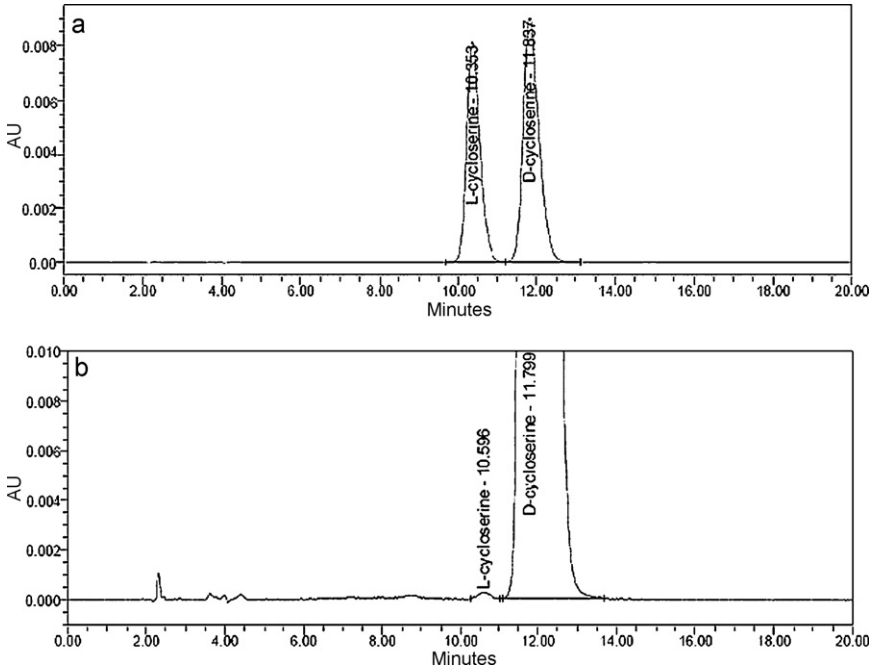

Fig. 2. HPLC chromatograms showing the (a) separation between D-cys and L-cys from system suitability solution and (b) D-cys drug substance containing 0.08% (w/w) of L-cys.

Table 1
Method validation data for L-cycloserine impurity.

Validation parameter	Results L-Cycloserine
DL, QL	
Detection limit (%, w/w)	0.015
Quantitation limit (%, w/w)	0.050
Precision at QL (n = 6, % RSD)	3.4
Linearity	
Calibration range (%, w/w)	0.05–0.30
Calibration points	7
Slope	64,222
Intercept	242
Coefficient of determination (r^2)	0.998

Validation parameter	Results L-Cycloserine				
Accuracy					
Added (%, w/w)	0.050	0.075	0.150	0.180	0.225
Recovered (%, w/w)	0.047	0.070	0.147	0.179	0.225
% Recovery	94.7	92.9	98.2	99.8	100.2
% RSD (n = 3)	1.2	3.0	2.6	3.8	0.3

3.2. Method validation

3.2.1. Specificity

To demonstrate the specificity and stability indicating capability of the proposed method, D-cys sample was subjected to stress by acid, base, hydrogen peroxide, heat, humidity, UV and fluorescent light. The stressed samples were assayed to determine the percentage of degradation. The D-cys showed degradation of about 7%, 8%, 13% and 7% under acid, humidity, hydrogen peroxide and thermal conditions, respectively, whereas no significant degradation was observed in base and photolytic conditions. The homogeneity of D-cys peak in each stressed sample was examined by peak purity testing utilizing PDA detector. Peak purity values were calculated using Waters Empower 2 software by comparing the UV spectrum at the apex of the peak with the spectra generated across the peak and the peak was considered as pure (homogeneous) if the purity angle is less than purity threshold [21]. In all the degraded samples, the purity angle obtained for D-cys peak was less than purity threshold, demonstrating spectral homogeneity. Also, the mass spectrum of the diastereomer derivative was recorded in positive electro spray ionization (+ESI) mode utilizing LC-MS. The degradation products formed during the stress conditions are well resolved from D-cys and L-cys, which proved that the adopted method is specific and stability indicating.

3.2.2. Linearity, accuracy, detection (DL), quantitation (QL) limit and stability of analyte solution

The linearity was evaluated by measuring area response for L-cys over the range of 0.05(QL) to 0.30% (w/w) relative to sample preparation which corresponds to the absolute concentration of 0.08–0.48 μg/mL. Seven concentrations (n = 7) were prepared across the range and injected each in triplicate. The mean (n = 3) area calculated was plotted against the concentration. The coefficient of determination (r^2) obtained for L-cys was 0.998. The slope of regression line and y-intercept was 64222 and 242, respectively (Table 1). Accuracy of the method was validated through recovery experiments by spiking known amount of L-cys at 0.05(QL), 0.075, 0.15 and 0.18% (w/w) with D-cys, relative to sample concentration (0.16 mg/mL). Each preparation was analyzed in triplicate (n = 3) and percent recovery was calculated. The recovery was found (Table 1) to be between 92.9 and 100.2% with the RSD (n = 12) of less than 3.8%. The DL and QL for L-cys were determined by signal-to-noise ratio (S/N) method. The DL and QL obtained for L-cys was 0.015 and 0.05% (w/w) relative to sample preparation which corresponds to the absolute concentration of 0.024 and 0.08 μg/mL, respectively. A solution containing L-cys was prepared around its QL concentration and injected in six replicates. The RSD (n = 6) value obtained for the area of L-cys at QL was 3.4% (Table 1). The stability of L-cys standard preparation and D-cys sample preparation were studied (stored at 25 ± 2 °C) over a period of 24 h. The RSD (n = 13) value obtained for the area response of L-cys was 3.3%. The RSD (n = 13) values obtained for the chemical purity and enantiomeric purity (%ee) of D-cys sample preparation were 0.31% and 0.10%, respectively.

Table 2
Robustness.

Variation	Resolution[a]	L-cys (%, w/w)
No variation	2.0	0.083
Flow rate (+10%)	1.9	0.076
Flow rate (−10%)	2.0	0.074
Column oven temperature (+5 °C)	1.9	0.075
Column oven temperature (−5 °C)	2.1	0.074
Wave length (+5 nm)	2.0	0.074
Wave length (−5 nm)	2.1	0.080
pH of buffer (+0.2)	2.0	0.071
pH of buffer (−0.2)	2.2	0.077
Organic ratio (+1.5%)	1.6	0.083
Organic ratio (−1.5%)	2.3	0.081
RSD (%)		5.27

[a] Resolution between D-cys and L-cys.

3.2.3. Repeatability and intermediate precision

The ruggedness of the method was evaluated by performing D-cys bulk drug sample analysis in six replicates using two different columns, different instruments and different analysts on different days. The RSD of the results (n = 6) obtained with Shimadzu and Waters HPLC instruments were 2.1 and 3.9%, respectively. The overall RSD (n = 12) of this study for L-cys content was 4.9%. Repeatability was performed by injecting L-cys standard preparation (0.15%, w/w) in six replicates and the RSD (n = 6) value obtained for the area response of L-cys was 2.5%.

3.2.4. Robustness

This study was performed by making small but deliberate variations in the method parameters. The effect of variation in flow rate, wavelength of detection, mobile phase composition, pH of buffer in mobile phase and column oven temperature on the L-cys determination was studied. The results including system suitability test are presented in Table 2. Under all the variations, system suitability requirements were found to be well within the specified acceptance criteria and hence the proposed method was concluded as robust.

4. Conclusion

The indirect RP-HPLC method described in this investigation was proved to be an ideal tool for the low level (<0.05%, w/w) detection of L-cys impurity in D-cys bulk drug. Method validation data demonstrated that the developed method is sensitive as well as accurate for the estimation of L-cys and robust to minor variation in the chromatographic parameters. The presently developed method involves conventional and versatile HPLC–UV detection with commonly available reagents and also it is rapid, economical and simple to adopt as it utilizes non-chiral RP-HPLC column with short run time. The specificity and stability-indicating capability of the method was established through forced degradation studies followed by PDA peak purity and MS studies. Hence, the proposed RP-HPLC method with UV detection can be used conveniently in the pharmaceutical analysis for stability monitoring and routine quality control of L-cys chiral impurity in D-cys drug substance.

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References

[1] The Merck Index, 14th ed., Merck & Co., Inc., USA, 2006, pp. 461–461.
[2] S. David, Synergic activity of D-cycloserine and β-chloro-D-alanine against Mycobacterium tuberculosis, J. Antimicrob. Chemother. 47 (2001) 203–206.
[3] US Pharmacopeial Convention, USP32 NF27, 2009, p. 2043.
[4] WHO, The International Pharmacopoeia, WHO, Geneva, 2008.
[5] Y. Tanaka, K. Otsuka, S. Terabe, Separation of enantiomers by capillary electrophoresis–mass spectrometry employing a partial filling technique with a chiral crown ether, J. Chromatogr. A 875 (2000) 323–330.
[6] ICH, Impurities in New Drug Substances, Q3A [R2], Step 5, 2006.
[7] U.S. FDA, Impurities in Drug Substances, 2000.
[8] EMEA, Impurities in New Drug Substances, CPMP/ICH/2737/99, 2006.
[9] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd ed., John Wiley & Sons, New York, 1997, pp. 539–540.
[10] S. Ahuja, Chiral Separations—Applications and Technology, ACS, Washington, 1997, pp. 142–143.
[11] I. Ilisz, R. Berkecz, A. Peter, Application of chiral derivatizing agents in the high-performance liquid chromatographic separation of amino acid enantiomers: a review, J. Pharm. Biomed. Anal. 47 (2008) 1–15.
[12] G. Subramanian, Chiral Separation Techniques—A Practical Approach, Wiley, VCH, NY, 2001, pp. 188–192.
[13] H. Bruckner, C. Gah, High-performance liquid chromatographic separation of DL-amino acids derivatized with chiral variants of Sanger's reagent, J. Chromatogr. A555 (1991) 81–95.
[14] R. Bhushan, V. Kumar, Indirect resolution of baclofen enantiomers from pharmaceutical dosage form by reverse phase liquid chromatography after derivatization with Marfey's reagent and its structural variants, Biomed. Chromatogr. 22 (2008) 906–911.
[15] N. Nimura, T. Kinoshita, o-Phthalaldehyde-N-acetyl-L-cysteine as a chiral derivatization reagent for liquid chromatographic optical resolution of amino acid enantiomers and its application to conventional amino acid analysis, J. Chromatogr. A352 (1986) 169–177.
[16] M.C. Garcia Alvarez-Coque, M.J. Medina Hernandez, R.M. Villanueva Camanas, C. Mongay Fernandez, Formation and instability of o-phthalaldehyde derivatives of amino acids, Anal. Biochem. 178 (1989) 1–7.
[17] M.C. Garcia Alvarez-Coque, M.J. Medina Hernandez, R.M. Villanueva Camanas, C. Mongay Fernandez, Studies on the formation and stability of isoindoles derived from amino acids, o-phthalaldehyde and N-acetyl-L-cysteine, Anal. Biochem. 180 (1989) 172–176.
[18] ICH, Validation of Analytical Procedures, Q2 (R1), Step 5, 2005.
[19] US Pharmacopeial Convention, USP32 NF27, 2009, pp. 733–736.
[20] J.W. Lamb, K. Florey (Eds.), Analytical Profiles of Drug Substances, vol. 1, Academic Press, NY, 1972, pp. 53–64.
[21] I. Krull, M. Swartz, Determining specificity in a regulated environment, LC-GC 19 (2001) 604–614.