Int J Pharm Bio Sci 2013 Apr; 4(2): (P) 522 - 531

Research Article **Analytical Chemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

DEVELOPMENT AND VALIDATION OF RP-HPLC PRE-COLUMN DERIVATISATION FOR THE TRACE LEVEL DETERMINATION OF TERT-BUTYLAMINE IN TIGECYCLINE DRUG SUBSTANCE

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ABSTRACT

A new simple, accurate and sensitive HPLC pre-column derivatisation method for the low level determination of *tert*-butylamine in Tigecycline drug substance has been developed. *tert*-butylamine may be present at a trace level in the Tigecycline drug substance, which is used as a reagent in the manufacturing process of Tigecycline. Chromatographic separations were accompanied under isocratic conditions using a Shimadzu HPLC with UV detection and a simple *o*-phthalaldehyde (OPA) derivatisation technique. The method has been fully validated and the limit of detection & quantification for *tert*-butylamine were 2.8ppm & 8.5ppm respectively. The method was linear over the range (8.5 – 1561µg/mL, n=7, r = 0.99993) of the working sample concentration of 20mg/mL. The precision, accuracy and ruggedness of the method are presented.

KEYWORDS:Pharmaceutical analysis, *tert*-butylamine, Amines, Tigecycline, HPLC pre-column derivatisation, RP-HPLC

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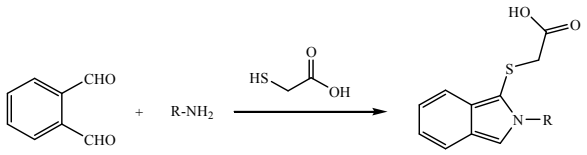
INTRODUCTION

Analyses of aliphatic amines pose special challenges. Lack of an UV response and poor peak shapes on silica based columns make conventional HPLC impractical. Determination of aliphatic amines by GC creates additional challenges due to their high aqueous solubility, volatility, polarity and basic character. The amine group imparts high dipole moment on amine molecules. This dipole moment is responsible for strong interaction with silane groups and siloxane bridges, leading to strong sorption resulting in broad, asymmetrical peaks and poor sensitivity. In addition, amines tend to decompose in the GC column and to sorb to exposed parts of the equipment and instrumentation (sample vials, injector, glass wool, etc,). Primary amines have the strongest sorption tendency, followed by secondary amines and tertiary amines. In short, the GC analyses of underivatised primary amines result in adsorption and decomposition on the column giving tailing peaks[1]. A common method of overcoming these limitations is to convert amines into a derivative and many GC methods involving derivatisation have been reported and several derivatisation reagents have been studied for this purpose. These methods include flame ionization, electron-capture, flame thermionic, mass spectrometric, chemiluminescence and flame photometric detection. However, derivatisation methods are time-consuming and the selectivity of amine derivatives cannot be guaranteed during the analysis of complex mixtures[2-3]. Amines have also proved difficult to handle in liquid chromatography (LC) for several reasons such as low UV detection sensitivities, variable ionization during separation requiring pH suppression and strong interaction with many LC supports. However, these compounds are easily derivatised to products that are strongly UV absorbing, fluorescent, or electrochemically active[4]. The resulting derivatives are usually chromatographed with minimal difficulty and have better detectabilities. One such derivatisation reagent chosen was OPA (*o*-phthalaldehyde), a very common flourogenic agent reacts rapidly, but only with primary amines in an aqueous basic medium (pH 9-11) and in presence of mercaptan to form a fluorescent isoindole derivative[5-8]. The general reactions of primary amines with OPA are shown in the below scheme 1.

Scheme 1
General reactions of primary amines with OPA



R= Methyl, Ethyl, n-butyl, tert-butyl

Ion Chromatography methods of aliphatic amines have been reported by several authors[9-11]. Haddad et al.[9] reported a method using an anion exchange column (Bi*o*-Rad HPX-72-O, 300 x 7.8 mm) with a mobile phase consisting of 10 mM sodium hydroxide. Krol et al.[10] reported the IC of alklyamines and alkanoamines using a poly (butadiene-maleic acid)-coated silica column and a mobile phase of EDTA-nitric acid, containing acetonitrile or methanol. Daigle et al.[11] studied the IC of alkyl amines using a silica-based cation exchange column with oxalic acid, dichloroacetic acid and sulfamic acid as eluents. Jagota et al.[2] reported the assay of amines present as impurities or counter ions in pharmaceuticals using Ion Chromatography technique. However, the already reported *tert*-butylamine in pharmaceuticals was determined at an assay

level rather than at trace levels. Even a recent USP pending monograph recommends quantifying the *tert*-butylamine by non-aqueous potentiometric titration[12].We have attempted to quantify the trace levels of *tert*-butylamine in the drug substance[13] by a simple concept of OPA derivatisation of primary amines. Although HPLC pre-column derivatisation is a well established technique, this proposed HPLC pre-column derivatisation method presents the trace level quantification of *tert*-butylamine in drug substance with more accuracy & precision. Thus, the aim of this paper illustrates a simple, sensitive and rugged procedure for quantifying the trace level *tert*-butylamine in the Pharma sample (drug substance) and so far, this derivatisation method has not been reported especially for the trace level determination and it can be utilized for routine quality assurance.

MATERIALS AND METHODS

1.1 Reagents and materials

Tigecycline Pharma sample was obtained from Gland Pharma limited, Dundigal, Hyderabad & India. *tert*-butylamine (≥99.5%), boric acid (Puriss p.a) and thioglycolic acid (≥98%), were purchased from Sigma-Aldrich (Steinheim, Germany). *o*-phthalaldehyde (≥98.0%) was purchased from Loba chemie (Mumbai, India). Sodium-1-heptane sulfonate (≥98.0%) of HPLC grade from Regis (USA), Methanol (HPLC grade), Potassium hydroxide (GR grade), Glacial acetic acid (Lichrosolv) and Acetonitrile (HPLC grade) were obtained from Merck, India.

1.2 Instrumentation

A Shimadzu LC 2010CHT HPLC equipped with gradient pump, eluent degasser, autosampler, sample cooler, column heating oven and UV detector were used. Data acquisition was performed with a Shimadzu LC solutions chromatography data processor (Shimadzu Corporation, Japan).

1.3 Chromatographic conditions

A 5 µm alltima C18 (250mm length x 4.6mm id) Grace column was used for the determination of residual *tert*-butylamine content. The mobile phase was a mixture of 4.0 g Sodium 1-heptane sulfonate in 510 ml of water, 40 ml of glacial acetic acid and 450 ml of acetonitrile. The flow rate was 1.0 ml/min. The column and the autosampler cooler temperature were maintained at (30±2) and (5±2) °C, respectively. The injection volume was 20 µL and the wavelength of detection at 330 nm.

1.4 Solution preparation

1.4.1 Borate buffer preparation

The borate buffer was prepared by dissolving about 4.94 g of boric acid in 150 ml of distilled water, adjusting the solution pH to 10.4 with 50% w/v solution of potassium hydroxide and finally made up the volume to 200 ml with distilled water.

1.4.2 Derivatizing reagent preparation

1.0 g of *o*-phthalaldehyde was dissolved in 5 ml of methanol and added 95 ml of borate buffer solution. To this, added 2.0 ml of thioglycolic acid (Mercaptoacetic acid) and readjusted the pH to 10.4 with 50% w/v potassium hydroxide solution.

1.4.3 Blank preparation

Accurately pipetted out 4.0 ml of distilled water into a 10 ml volumetric flask, added 2.0 ml of derivatizing reagent and made up the volume with methanol. The solution was stored at room temperature for 1 hour.

1.4.4 tert-butylamine standard preparation

50 mg of *tert*-butylamine standard was weighed into a 100 ml volumetric flask containing about 30 ml of distilled water and diluted to volume with distilled water. Accurately pipetted out 0.4 ml of *tert*-butylamine standard stock solution into a 10 ml volumetric flask, added 3.6 ml of distilled water and 2.0 ml of derivatizing reagent and made up the volume with methanol. The solution was stored at room temperature for 1 hour.

1.4.5 Sample preparation

200 mg of sample was weighed accurately into a 10 ml volumetric flask, added 4.0 ml of distilled water and dissolved the contents (if necessary

sonicated). Added 2.0 ml of derivatizing reagent and made up the volume with methanol. The solution was stored at room temperature for 1 hour.

RESULTS AND DISCUSSION

1.5 Development of chromatographic method

Because of the poor UV absorptivity of *tert*-butylamine, development of HPLC method with UV detection for the direct estimation of *tert*-butylamine in the drug substance was not considered in this study. The possibility of developing a Headspace GC method was investigated first. Since *tert*-butylamine is volatile in nature with a boiling point of about 60°C, a Headspace GC method was developed with a SPB-624 (6% cyanopropylphenyl-94% dimethylpolysiloxane) with *N, N*-Dimethylacetamide as diluent[14]. This technique, as expected, the peak shape and the repeatability of the method were not good. Other diluents such water, Dimethyl sulfoxide and a mixture of water & *N*, *N*-Dimethylformamide also resulted in poor peak shape and no improvement in the signal to noise ratio at low levels. Different types of GC columns, such as ZB-5 column (5%-Phenyl-95%-dimethylpolysiloxane), TR-WAX (polyethylene glycol), Durabond CAM (nonbonded base deactivated polyethylene glycol) and Rtx-5 Amine (Crossbond 5% diphenyl / 95% dimethylpolysiloxane) were studied to improve the peak shape and also the repeatability. However, no improvement in the peak shape or the repeatability was found with any of the columns. Also, the peak observed at lower levels was very broad which resulted in poor signal to noise ratio. Even the Ion chromatography technique was attempted; however it was unsuccessful due to interference of drug matrix with the *tert*-butylamine peak[15]. These observations suggested to look for a robust and sensitive method, and hence the classical HPLC derivatisation with OPA (*o*-phthalaldehyde] was selected as an ideal tool.The Pre-column derivatisation of primary amines with *o*-phthalaldehyde could be detected in their derivatised form directly by UV detection; *tert*-butylamine was treated with *o*-phthalaldehyde and thioglycolic acid in the presence of borate buffer at pH of about 10.4. The solution was kept at room temperature for 1 hour to get derivatised to form a stable derivative, which is analysed by a HPLC using UV detection. A mixture of 4.0 g Sodium 1-heptane sulfonate in 510 ml of water, 40 ml of glacial acetic acid and 450 ml of acetonitrile was selected as the mobile phase and a reverse phase C18 (USP L1) column was utilized for better separation. Using this mobile phase conditions, the retention time of *tert*-butylamine was found to be about 17 min with no interference of peaks from the derivatizing reagent and sample matrix. As a part of method development, various experimental parameters of sample preparations such as derivatisation time and derivatising reagent volume were studied to achieve the ideal condition for derivatisation as indicated in Table 1.

Table 1
Experimental studies for derivatisation of tert-butylamine

Experiment	Derivatisation time (min)	Derivatising reagent volume (ml)	Derivatisation reaction temperature (°C)	Area response
Experiment 1	Immediate preparation	2.0	Ambient	2708799
Experiment 2	30	2.0	Ambient	2688422
Experiment 3	60	2.0	Ambient	2678808
Experiment 4	90	2.0	Ambient	2669706
Experiment 5	60	1.5	Ambient	2872567
Experiment 6	60	2.0	Ambient	2803159
Experiment 7	60	2.5	Ambient	2836409

From the results obtained, optimized derivatisation conditions chosen are 2.0 ml derivatising reagent volume, 60 min derivatisation time and ambient room temperature as derivatisation reaction temperature. Based on the proposed specification limit of 1000 ppm of *tert*-butylamine, the optimum sample concentration of drug substance was arrived at 20 mg/ml. A preliminary accuracy study was conducted at concentration levels of 50%, 100% and 150% of the proposed specification limit of 1000 ppm with respect to the test concentration of 20 mg/ml. As a part of robustness study, flow rate and column oven temperature were investigated, and minor variations in these parameters had not influenced significantly the results.

1.6 Method validation

The optimized RP-HPLC pre-column derivatisation method was validated according to ICH guidelines[16] with respect to specificity, accuracy, precision (repeatability and intermediate precision), linearity, limit of detection & limit of quantification, range and robustness. System suitability features were also assessed.

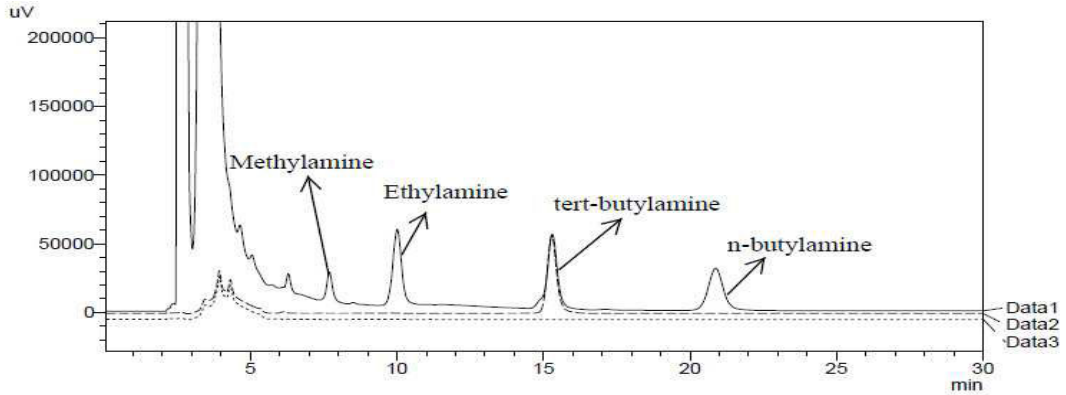
1.6.1 System suitability test

The system suitability test performed according to USP 30[17] and BP 2007[18] indications. The observed RSD values for six replicate *tert*-butylamine standard injections at the specification level (1000ppm) were well within the usually acceptance criteria (≤ 5.0%). The results obtained are within acceptable limits.

1.6.2 Specificity

Specificity of the method was demonstrated by its ability to separate *tert*-butylamine peak from Tigecycline sample matrix, the derivatizing reagent peaks and the other primary amines such as methylamine, ethylamine and *n*-butylamine etc.,. *tert*-butylamine was well resolved from all the above specified primary amines as illustrated in (Fig 1). Moreover, it is significant to note that no interference from derivatising reagent peaks and API sample matrix was found at the retention time of *tert*-butylamine as illustrated in (Figs. 2-6). The peak purity and spectral match of the *tert*-butylamine peak from the derivatised standard and *tert*-butylamine spiked sample solution was demonstrated authenticating the ability of the method to assess unequivocally the analyte of interest in the presence of potential interference. From the above data, it could be concluded that the proposed method is highly specific.

Figure 1
Typical overlaid HPLC chromatogram of tert-butylamine with other primary amines



Data 1: Mixture of primary amines spiked with the Tigecycline sample chromatogram (——)
Data 2: Standard tert-butylamine chromatogram (- - - - -)
Data 3: Blank chromatogram (.....)

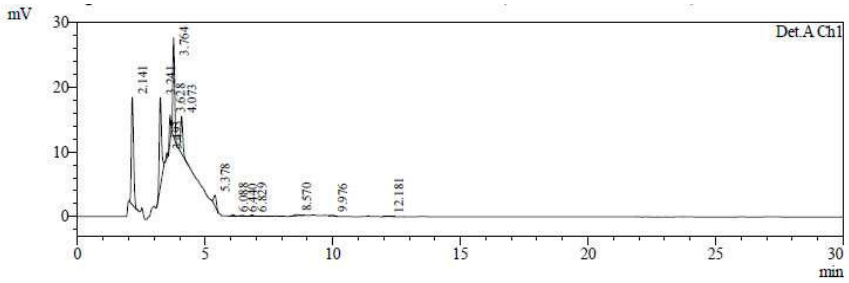


Figure 2
Typical blank (derivatizing reagent) chromatogram

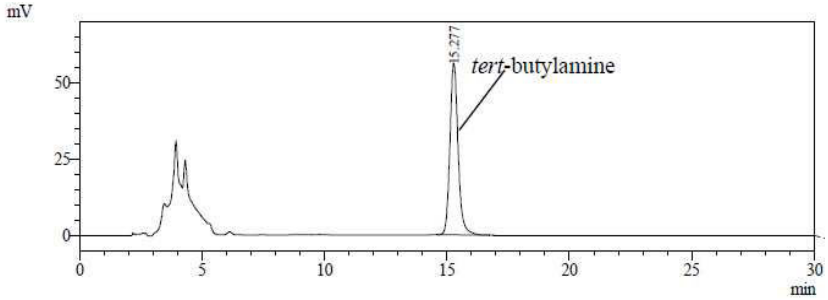


Figure 3
Typical standard chromatogram of tert-butylamine

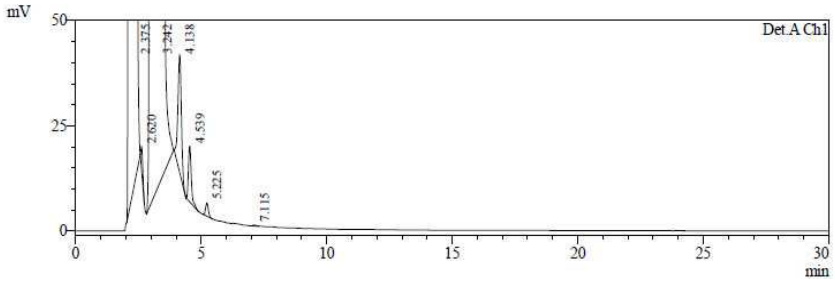


Figure 4
Typical sample chromatogram of Tigecycline drug substance without derivatisation

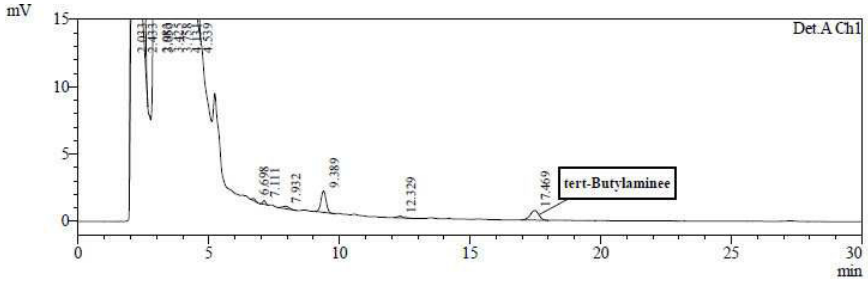


Figure 5
Typical sample chromatogram of Tigecycline drug substance with derivatisation

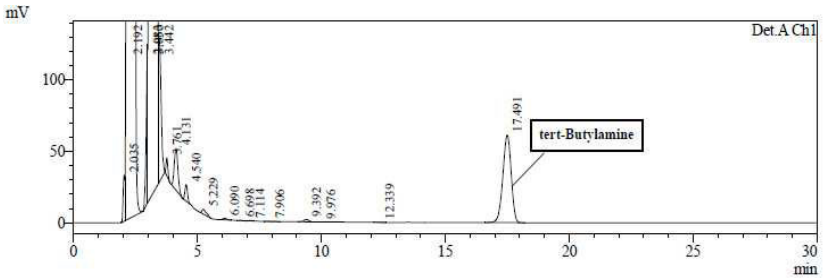


Figure 6
Typical sample chromatogram of Tigecycline drug substance spiked with tert-butylamine

1.6.3 Linearity and range

Linearity was studied in the range from limit of quantification to 150 % of the specification limit of *tert*-butylamine (NMT 1000 ppm) with respect to the test concentration of 20 mg/ml. Seven concentration levels were prepared across the range and injected each solution once. The area response was plotted against the concentration. The coefficient of determination (r^2) obtained was 0.9998. The slope of the regression line, y-intercept and % y-intercept were 62607.832407, 3578 and 0.3%, respectively.

1.6.3.1 Determination of limit of quantification and limit of detection (LOQ and LOD)

The detection limit (DL) and quantitation limit (QL) for *tert*-butylamine were determined by the slope method using a series of linearity solutions ranging from the expected minimum level of detection to 100 ppm with respect to the test concentration of 20 mg/ml of Tigecycline sample. The DL and QL obtained for *tert*-butylamine were 2.8 and 8.5 ppm, respectively. A solution containing *tert*-butylamine was prepared around its QL concentration and injected in six replicates. The RSD (n=6) value obtained for the area of *tert*-butylamine at QL was 2.1%.

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1.6.4 Accuracy

Accuracy of the method was validated through recovery experiments by spiking known amount of *tert*-butylamine at QL, 50%, 100% & 150% of the specification limit with respect to the sample concentration of 20 mg/ml. Each preparation was analysed in six replicates (n=6) and percent recovery was calculated. The experimental results revealed that the recovery was found to be between 93.6 and 99.3% for *tert*-butylamine. Therefore, based on the recovery data (Table 2) the estimation of *tert*-butylamine as prescribed in this report has been demonstrated to be accurate for intended purpose and is adequate for routine analysis

Table 2
Precision and recovery

Recovery level	% Individual recovery		% Mean recovery	% RSD
	Lower	Upper		
QL	97.6	103.4	99.3	2.1
50%	97.8	99.1	98.6	0.5
100%	98.8	99.4	99.0	0.2
150%	92.7	94.8	93.6	0.8

1.6.5 Method precision and ruggedness

ICH (International Conference on Harmonization of technical Requirements for Registration of Pharmaceuticals for Human Use) considers ruggedness as the method reproducibility and intermediate precision.Repeatability was determined by analyzing six different spiked sample preparations (spiked with *tert*-butylamine) at 100% level of the specification of *tert*-butylamine with respect to test concentration of 20mg/mL of Tigecycline. The content of *tert*-butylamine in terms of mean recovery from six preparations was calculated and found to be 99.0% and the % RSD of 0.2% compared with an acceptance limit of 10.0%. The ruggedness of the method was evaluated by performing the spiked sample analysis of *tert*-butylamine in six replicates using two different columns, different analysts on different days and in different laboratory. The overall RSD (n=12) of content of *tert*-butylamine in terms of % recovery obtained was 1.2% against the acceptance limit of 10.0%. The ruggedness as listed in Table 3 reveals that the method has good reproducibility and intermediate precision

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Table 3
Precision and intermediate precision

# Parameter	# Preparations	% Recovery
PRECISION	Prep-1	99.1
	Prep-2	99.1
	Prep-3	99.4
	Prep-4	98.8
	Prep-5	99.0
	Prep-6	98.9
INTERMEDIATE PRECISION	Prep-1	100.8
	Prep-2	101.2
	Prep-3	101.3
	Prep-4	101.6
	Prep-5	101.6
	Prep-6	101.6
AVERAGE		100.2
SD		1.2285
% RSD		1.2

1.6.6 Solution stability

The stability of *tert*-butylamine standard and sample solution spiked with *tert*-butylamine was studied by measuring the area response of the standard preparation and content of *tert*-butylamine in the sample preparation stored at 25±2°C and 2-8°C and injected over a period of 2 days. The evaluated results reveal that the standard and sample solutions were stable for 2 days when stored at 2-8°C and unstable at room temperature beyond 6 hrs duration.

1.6.7 Robustness

In order to demonstrate the robustness of the method, system suitability parameters and the % recovery of *tert*-butylamine were verified by making deliberate change in chromatographic conditions, i.e, change in flow rate by ±0.1 ml min^{-1}, change in buffer concentration by ±10%, change in organic composition of mobile phase by ±2% absolute, change in column oven temperature by ±5°C and change in derivatisation time by 15 minutes. The system suitability criteria (% RSD of standard injections) and the % recovery were monitored in all robust conditions. The method was demonstrated to be robust over an acceptable working range of its HPLC operational conditions.

CONCLUSION

The HPLC pre-column derivatisation method described in this paper was proved to be an ideal tool for the easy estimation of *tert*-butylamine in the drug substance even at trace

levels to comply with the ICH/FDA/EMEA regulatory requirement. Method validation data demonstrated that the developed method is sensitive and accurate for the determination of *tert*-butylamine and robust to minor variations in the chromatographic parameters. The identity and specificity of the *tert*-butylamine peak was well established by the non-interference with other possible primary amines. This method is rapid and simple to adopt. Hence, the proposed HPLC pre-column derivatisation method can be used conveniently for the routine quality control of *tert*-butylamine in Tigecycline drug substance.

ACKNOWLEDGEMENTS

The authors wish to thank the management of Gland Pharma limited for providing facility to perform this study and take this opportunity to acknowledge the synthesis R & D team of Gland Pharma for providing the drug substance.

REFERENCES

1. Namiesnik J, Jastrzebska A, Zygmunt B, Determination of volatile aliphatic amines in air by solid-phase microextraction coupled with gas chromatography with flame ionization detection. J. Chromatogr, 1016 (1): 1-9, (2003)
2. Jagota N K, Chetram A J and Nair J B, Ion chromatography of amylamine and tert.-butylamine in pharmaceuticals. J. Chromatogr A, 739 (1-2): 343-349 (1996)
3. Yilmaz B and Cavus M, Int J pharm. Bio. Sci., Development and validation of gas chromatography-mass spectrometry method for Determination of Metoprolol in Rabbit plasma. 1(1): 1-13, (2010)
4. Tzun-Yu Chou, Colgan S T, Kao D M and Krull S IRA, Pre-Chromatographic derivatisation of primary and secondary amines with a polymeric anhydride for improved high-performance liquid chromatographic detection. J. Chromatogr, 367: 335-344, (1986)
5. Schuster R, Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatisation and high-performance liquid chromatography. J. Chromatogr, 431: 271-284, (1988)
6. Kamisaki Y, Takao Y, Itoh T, Shimomura T, Takahashi K, Uehera N and Yoshino, Determination of γ-aminobutyric acid in human cerebrospinal fluid by isocratic high-performance liquid chromatography. J. Chromatogr, 529 (2): 417-423, (1990)
7. Liu H and Worthen H G, Measurement of free amino acid levels in ultrafiltrates of blood plasma by high-performance liquid chromatography with automatic pre-column derivatisation. J. Chromatogr, 579: 215-224, (1992)
8. Nargish P, Tapas K Mandal, Vijaylaxmi Saxena and Sabyasachi Sarkar, A rapid quantification of serum free methionine by HPLC in Relevance to poultry industry. Int J pharm. Bio. Sci., 1(2): 1-6,(2010)
9. Haddad P R, Hao F and Glod B K, Factors affecting retention of basic solutes in ion-exclusion chromatography using an anion-exchange column. J. Chromatogr A, 671(1-2): 3-9 (1994)
10. Krol J, Alden P G, Morawski J and Jackson P E, Ion chromatography of alkylamines and alkanolamines using conductivity detection. J. Chromatogr, 626 (2): 165-170 (1992)
11. Daigle R, Mehra M C and Mallet V, Single column ion chromatography of ammonium ion, alkali metals and alkyl amines. Chromatographia, 32 (3-4): 143-147 (1991)
12. USP Pending Monograph, Perindopril Erbumine,http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/pendingStandards/m5236_authorized.pdf
13. Chandrasekhar BVVN, Ramesh kumar N, Kalyan Chakravarthy A and Mukkanti K, Synthesis of Montelukast sodium via a nitrile intermediate; Synthesis and characterization of impurities. Int J pharm. Bio. Sci., 2(3): 417-425, (2011)

14. Groman A and Kamienska-Duda A, Acta Poloniae Pharmaceutica, Drug Research, Validation of an analytical procedure – Control of residual solvents in Pharmaceutical substance. 65 (6): 783-787 (2008)
15. IC application work AW IN6-0822-052008, Tertiary butylamine in Pharma sample, Metrohm.
16. ICH, Validation of Analytical Procedures, Q2 (R1), step 5, 2005.
17. The United State Pharmacopeia, 30th edn, United State Pharmacopeia Convention, System Suitability Testing, Rockville, USA, 2007.
18. British Pharmacopeia, Her Majesty's Stationary Office, System Suitability Testing, London, UK, 2007.